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FOREWORD

The Myanmar Academy of Arts and Science (MAAS) was constituted on August-16, 1999 with four major fields of endeavour, namely:

(a) Introduction to Modern Methods of Teaching and Learning
(b) Promotion of Research Activities through Research Guidelines
(c) Dissemination of Knowledge and Emerging Technologies
(d) Motivating New Generation of Experts and Academics

In pursuance of these endeavours, MAAS has, since the year 2001, held Research Conferences and published research papers in the Journal of the Myanmar Academy of Arts and Science.

At the Research Conference held on 19- 21 December 2022, a total of (223) research papers were read and outstanding papers have been published in volume XXI as follows:

Vol. XXI, No.1	Chemistry, Industrial Chemistry
Vol. XXI, No.2	Physics, Mathematics and Computer Studies
Vol. XXI, No.3	Zoology, Botany, Marine Science
Vol. XXI, No.4	Myanmar, Oriental Studies, Archaeology, Anthropology and Library and Information Studies
Vol. XXI, No.5	Geography, History, International Relation, Geology, Statistics, Management Studies, Law, Journalism
Vol. XXI, No.6	Educational Theory and Management, Curriculum and Methodology
Vol. XXI, No.7	Educational Psychology

The executive committee members of Myanmar Academy of Arts and Science had been reconstituted on 4 August 2022 and again reconstituted on 8 March 2024, by the Ministry of Education with the Approval of the Government of the Union of Myanmar. Accordingly, the Publication Committee along with the Editorial Board have been formed. The primary mission of the academy is to develop and promote Higher Education in preparing future generations to meet the challenges in the 21st century.

The majority of the papers in these issues represent findings of research conducted by aspirants as well as postgraduate candidates in partial or total fulfillment of requirement for these degrees. We, the members of MAAS, do appreciate the editing work done by senior professors and scholars of high standing, these papers would prove useful, and not only for other candidates but also for all those who are interested in the results of systematic research and inquiry. Due to the outbreak of covid-19 pandemic; unfortunately, a delay in the date of publication had occurred.

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GENETIC DIVERSITY OF WHITE-HANDED GIBBON *HYLOBATES LAR* (LINNAEUS, 1771) AT YWAR KAING KAUNG VILLAGE IN DAWNA MOUNTAIN RANGE CORRIDOR*

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Abstract

The white-handed gibbon or *Hylobates lar* is currently classified as Endangered on the IUCN Red List of Threatened Species. Although the total population size is not small, forest fragmentation by human activity has affected the stability of local populations. To uncover the effects of forest fragmentation on the conservation of local white-handed gibbon populations, this study assessed the genetic status of an isolated white-handed gibbon population living in Ywar Kaing Kaung (YKK) village, Kayin State, Myanmar, where 26 individuals were living in nine groups. 631-bp nucleotide sequence consisting of the hypervariable region I (HVR-I) of mitochondrial DNA was analysed. Sequences were determined in nine of 18 samples from adult individuals. Among those, two haplotypes were found. Phylogenetic trees and a haplotype network uncovered that both haplotypes observed in YKK clustered with those of white-handed gibbons living in central Thailand, a subspecies *H. lar entelloides*. Haplotype diversity of the YKK population was low (0.556) compared with those of a white-handed gibbon population in Khao Yai National Park (KYNP), Thailand (0.823) and those of a siamang population in Sumatra, Indonesia (0.886), which suggested the strong bottleneck effect on the YKK population. On the other hand, nucleotide diversity was comparable (0.00357) with that of the KYNP population (0.00238). The low genetic diversity of the YKK population suggested the importance of genetic management at the local population level in white-handed gibbons.

Keywords conservation genetics, *Hylobates lar*, mtDNA, phylogeny

Introduction

Gibbons, or the small apes, belong to the family Hylobatidae within the superfamily Hominoidea. They are found in the various types of forests of Southeast and South Asia. They are arboreal, preferring the upper level of the forest canopy (Hollihn, 1984). In Myanmar, white-handed gibbons (*Hylobates lar*), western hoolock gibbons (*Hoolock hoolock*), eastern hoolock gibbons (*Hoolock leuconedys*) and Gaoligong hoolock gibbons (*Hoolock tianxing* or *Hoolock leuconedys tianxing)* are distributed (Geissmann *et al.*, 2013; Fan *et al.*, 2017). Among those, white-handed gibbons occur only east of the Thanlwin (or Salween) river in the southern part of Myanmar. This includes part of Shan State, Kayah State, Kayin State, Mon State, and Tanintharyi Region (Geissmann *et al.*, 2013). Outside of Myanmar, the species is distributed in Laos (the western side of the Mekong River), a large part of Thailand (except the northeastern region), Peninsular Malaysia, and the northern part of Sumatra, Indonesia (Brandon-Jones *et al.*, 2004; Roos *et al.*, 2014). A small part of Yunnan, China also used to be a distributed zone, but the population has been considered extinct in the last few decades (Grueter *et al.*, 2009). There is no estimate on the total population size of this species; 15,000-20,000 individuals in Thailand (which was based on personal communication, Geissmann, 2007) is the only available estimate so far. Although the exact population size is unknown, their habitat has been decreasing, and thus the species is categorized as Endangered on the IUCN Red List of Threatened Species (IUCN 2022). For many gibbon species, several threats have been recognized, such as habitat loss and fragmentation, habitat degradation, hunting, and illegal trade (Geissmann, 2003; 2007).

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* Best Paper Award Winning Paper in Zoology (2022)

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This is also true for gibbons living in Myanmar. Extensive surveys of the hoolock gibbons in Myanmar have revealed that habitat loss and fragmentation were the most serious threats to hoolock gibbons (Lwin *et al.*, 2011; Geissmann *et al.*, 2013). Although there is no detailed report on the status of white-handed gibbons in Myanmar, a similar situation probably exists. White-handed gibbons are legally protected in Myanmar but most of their habitat ranges outside of limited protected areas (Geissmann *et al.*, 2013). Therefore, to conserve endangered white-handed gibbons, it is important to understand the status of this species especially outside of the protected areas. Ywar Kaing Kaung (YKK) village, Kayin State is located at the foot of the western side of Dawna Mountain Range. About 250 ha of fragmented forests exist in the village and the presence of white-handed gibbons has been known. The forests are surrounded and fragmented by crop fields made by the villagers. In Kayin traditions, gibbons are considered respectful animals representing forests, and gibbons are not a target of hunting (Htoo and Grindley, 2010). Therefore, there is no conflict between the gibbons and the villagers. Rather, the major issue on this white-handed gibbon population is the small capacity of the fragmented forests and the isolation, i.e., about 10 km apart from the nearest forest in Dawna Mountain Range. The isolation of animal populations can result in local extinction by stochastic events because of its small local population size. In addition, isolation limits access to mates, which would induce inbreeding. Inbreeding would cause the loss of genetic diversity and expression of maladaptive characteristics related to recessive alleles, i.e., inbreeding depression (Frankham, 2005). Therefore, maintaining the level of genetic diversity is one important issue in conserving a small population of animals. So far, there has been no evaluation of the genetic diversity of white-handed gibbons at a local population level, the assessment of genetic diversity in the YKK village would provide basic information on the genetic diversity of isolated gibbon populations.

In addition, white-handed gibbons are classified into five subspecies, namely *H. lar lar* (Malaysian white-handed gibbons), *H. lar vestitus* (Sumatran white-handed gibbons), *H. lar entelloides* (Central white-handed gibbons), *H. lar carpenteri* (Carpenter's white-handed gibbons), and *H. lar yunnanensis* (Yunnan white-handed gibbons) (Groves, 2001). Some morphological differences such as in pelage colour and hair length between subspecies have been reported (Groves, 1968; 1972; Marshall and Sugardjito, 1986). However, it is difficult to identify the subspecies in captivity only based on the slight morphological differences (Woodruff *et al.*, 2005). A recent study on the cytb gene of mitochondrial DNA (mtDNA) suggested the genetic difference between the five subspecies (Thinh *et al.*, 2010), but because of the limited number of each subspecies analysed in the study, the result was inconclusive. A study focused on the hypervariable region I (HVRI) of mtDNA suggested that *H. lar entelloides* and *H. lar carpenteri* were not distinguishable by mtDNA (Woodruff *et al.*, 2005). YKK village is located at the reported intergrade zone between *H. lar entelloides* and *H. lar carpenteri*, 15°N-17°N (Groves, 1972), and thus it is interesting to know the genetic characteristic of this population by the means of mitochondrial DNA phylogeny, which may contribute the conservation of white-handed gibbons at the subspecies level. Overall, the present research was focused on the following objectives:

- to examine the genetic diversity of the white-handed gibbon population in Ywar Kaing Kaung Village
- to investigate the phylogenetic position of the white-handed gibbons in Ywar Kaing Kaung village among white-handed gibbons.

Material and Method

Study area

YKK village is located at 16°56'14" N and 97°56'12" E and a 2 km^2 area in Pai Kyu sub-township, Hlaing Bwe Township, Hpa An District, Kayin State. Kayin State is bordered by Mae Hong Son, Tak and Kanchanaburi provinces Thailand to the east; Bago Region; Mon State to the west and south; Mandalay Region and Shan State to the north (Fig.1).

Study period

The survey was conducted from January, 2020 to December, 2021.

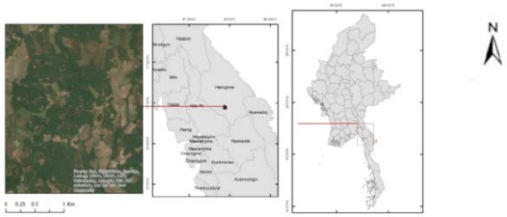

Figure 1 The study area of Ywar Kaing Kaung village

Method and Faecal sample collection

Nine white-handed gibbon groups consisting of 26 individuals, including eight adult males and eight adult females, in the YKK village were the subjects of this study. Faecal samples were immediately collected after observing the defecations from the 18 adult gibbons of the nine groups at YKK village from 2018 to 2019. Each sample was stored in a 50 ml sterile tube containing 30 ml 99.5% ethanol for 24 h, and then transferred into a 50 ml tube filled with 20-30 g silica gel beads (ethanol and silica two-step method, Nsubuga *et al.*, 2004) (Plate 1)

A. White-handed gibbon feces

B. Feces preserved in the ethanol

C. Dried up feces with silica gel beads

Plate 1 Collection of faecal samples in the study area (ethanol-silica two-step method)

DNA Extraction

DNA was extracted with the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany) with the following modifications. In the first step, a faecal sample was scraped by a sterile surgical blade and measured 100-200 mg particles by a digital scale. The faecal sample was put into a 2 ml tube, 1 ml inhibit EX buffer was added and mixed by vortex for 1 min. The sample was incubated overnight at room temperature. After this step, experimental procedures were conducted following the manufacturer's protocol. At the last step, 200 µl Buffer ATE was applied on the membrane and incubated for 20 min in order to retrieve the larger amount of DNA into the buffer.

PCR amplification of mitochondrial control region

The present study focused on HVRI of the displacement loop (D-loop) region of mitochondrial DNA (mtDNA) that has been used extensively in molecular evolutionary studies

(Vigilant *et al.*, 1989) and thus has a well-studied mutation rate (Tamura and Nei, 1993; Parsons *et al.,* 1997). Approximately 700-base pair (bp) fragment consisting of the HVRI was PCR amplified and sequenced using the following gibbon-specific primers: 5'-CTTCACCCTCAGCACCCAAAGC-3' (Andayani *et al.,* 2001) and 5'-AAGACAGATACTGCGACATAGG-3' (Matsudaira *et al.,* 2013). PCR was conducted following Matsudaira *et al.* (2013). PCR products were detected by agarose gel electrophoresis and were purified using the PCR Clean-up Mini Kit (Favorgen, Taiwan) following the manufacturer's protocol of the kit.

Sequencing

The purified PCR products were used as templates for direct sequence reactions. The cycle sequencing reaction was carried out in a final volume of 10 µl, containing 2 µl of purified PCR product, 0.32 µl of 10 pmol/µl of primer (the forward or the reverse primer), 0.4 µl of BigDye^{TM} Terminator v3.1, 1.8 µl of 5x Reaction buffer and 5.48 µl of distilled water. The reaction mix was purified by ethanol precipitation. Then, sequencing was operated in ABI 3500 Genetic Analyzer (Thermofisher Scientific, USA).

Phylogenetic tree and haplotype network construction

The sequences of HVRI were imported to MEGA-X (Kumar *et al.*, 2018) and were aligned using the Muscle Sequence Alignment Program (Edgar, 2004). Phylogenetic trees were constructed by maximum likelihood (ML; Felsenstein, 1981) and neighbour-joining (NJ; Saitou and Nei, 1987) methods. Tamura-Nei model (TN93; Tamura and Nei, 1993) was used for the evolution model. Bootstrap analysis was carried out for 1000 replications (Felsenstein, 1985). In the phylogenetic analysis, we included the sequences of white-handed gibbons with known wild origins, and thus subspecies, from previous studies (Table 1). They were *H. lar entelloides* from Khao Yai National Park (KYNP), Thailand (Matsudaira *et al.*, 2013; 2022; Markviriya *et al.*, 2022) and Kaeng Krachan National Park (KKNP), Thailand (Matsudaira *et al.,* 2022), and *H. lar lar* from National Wildlife Rescue Centre (NWRC), Malaysia (Gani *et al.*, 2021). The sequences from KYNP also included the sequences obtained from some hybrid gibbons between white-handed gibbons and pileated gibbons (*Hylobates pileatus*). We only included typical white-handed gibbon haplotypes. The individuals of NWRC were all captive individuals, but they were originated from wild and all were from Peninsular Malaysia (Gani *et al.*, 2021). In addition to the white-handed gibbon sequences, outgroup sequences of one agile gibbon (*Hylobates agilis*) and one pileated gibbon (Matsudaira and Ishida, 2010) were included. All the sequences were obtained from GenBank (NCBI; National Center Database for Biotechnology Information). To ensure the results of phylogenetic tree analysis, and to look more detailed relationship among the mtDNA sequences of the white-handed gibbons, haplotype network analysis was conducted. A haplotype network based on the minimum-spanning method (Bandelt *et al.*, 1999) was constructed by using PopART (Leigh and Bryant, 2015). In this analysis, only the sequences of white-handed gibbons were used.

Evaluation of genetic diversity

Genetic diversity within each population was estimated by computing haplotype diversity (h; Nei, 1973) and nucleotide diversity (π; Nei, 1987) by using DnaSP (version 6.12.03) (Rozas *et al.*, 2017). The genetic diversity of the gibbon population of YKK was compared with that of other gibbon populations; white-handed gibbons from KYNP (n = 37 adult individuals of 17 groups) (Matsudaira *et al.,* 2018), and siamangs (*Symphalangus syndactylus*) from Bukit Barisan Selatan National Park (BBSNP), Sumatra (n = 15 adult individuals of six groups) (Lappan, 2007). All these sequences were obtained from GenBank, and both haplotype diversity and nucleotide diversity were calculated by referring to the individual haplotypes reported by the

previous studies. In the case of the KYNP population, some individuals showed an mtDNA haplotype introgressed from pileated gibbons (Matsudaira *et al.*, 2013). Because the large sequence difference between white-handed gibbons and pileated gibbons would inflate the nucleotide diversity, these individuals with the pileated gibbon haplotype were excluded from this analysis.

Results

The mtDNA phylogenetic tree and haplotype network

A total of 18 faecal samples were obtained from the 18 adult gibbons (i.e., one sample per individual) in the nine groups. The 631-bp nucleotide sequences of HVRI of the mtDNA were successfully determined in nine of the 18 samples. Two haplotypes (HYKK_1 and HYKK_2) were found among the nine samples. The haplotype HYKK_1 was observed among five individuals (G1F, G2F, G3F, G4F and G4F) and HYKK_2 was observed among four individuals (G5F, G5M, G7M and G8F). Four substitutions were observed between the two haplotypes. The phylogenetic analysis revealed the presence of three distinct clades (Fig. 2). The first clade consisted of all haplotypes from NWRC, Malaysia, which was supported by relatively a high bootstrap value of 81 in the ML tree and 77 in the NJ tree. The second clade consisted of the two haplotypes from YKK, Myanmar, all haplotypes from KYNP, Thailand, and seven haplotypes from KKNP, which was supported by relatively high bootstrap values of 80 in the ML tree and 87 in the NJ tree. The third clade consisted of one haplotype (LC633868: HKK6A) from KKNP. Phylogenetic relationships among the three clades were not well resolved because the position of the third clade was different between ML and NJ trees. While the third clade clustered with the first clade in ML tree, it clustered with the second clade in NJ tree. This ambiguity is consistent with low bootstrap values on the relationships of three clades in both the ML tree (44) and the NJ tree (48). Within the second clade, the two haplotypes from the YKK population did not make their clade. Not only the haplotypes from YKK but also haplotypes from KYNP and KKNP were not distinct from each other in the clade. Similar to the phylogenetic trees, haplotypes from Peninsular Malaysia made one cluster, and haplotypes from YKK, KYNP and KKNP made another cluster (Fig. 2). The haplotype of KKNP which made the third cluster in the phylogenetic trees was located in the middle of the two clusters. The nucleotide difference between this haplotype and the closest haplotype of the Malaysian cluster was nine, and the Myanmar-Thai cluster was 10. This was consistent with the ambiguous position of the third clade in the phylogenetic trees. The haplotype network showed no apparent distinction of the two YKK haplotypes from KYNP and KKNP haplotypes. Only one or three nucleotide differences were observed between YKK haplotypes and the closest KYNP or KKNP haplotype (Fig.3).

Haplotype diversity (h) and nucleotide diversity (π)

In YKK, haplotype diversity was 0.556 and nucleotide diversity was 0.00357. The haplotype diversity of the YKK white-handed gibbon population was lower than that of the KYNP white-handed gibbon population (0.823) and that of the BBSNP siamang population (0.886). On the other hand, the nucleotide diversity was not different from (or slightly larger than) that of KYNP (0.00238), but almost 10 times lower than that of BBSNP (0.03171) (Table 2).

Table 1 The sequences of *Hylobates lar* and outgroup species used in the phylogenetic tree analysis

No.	Species	GenBank Accession No.	Haplotype	Location	Reference
1.	*Hylobates lar*	-	HYKK1	Yawr Kaing Kaung, Myanmar	This study
2.		-	HYKK2		
3.		AB720991	HKY1	Khao Yai National Park, Thailand	Matsudaira *et al.*, 2013
4.		AB720992	HKY2		
5.		AB720993	HKY3		
6.		AB720994	HKY4		
7.		AB720995	HKY5		
8.		AB720996	HKY6		
9.		AB720997	HKY7		
10.		AB720998	HKY8		
11.		AB720999	HKY9		
12.		AB721000	HKY10		
13.		LC633853	HKY12A	Khao Yai National Park, Thailand	Matsudaira *et al.*, 2022
14.		LC633854	HKY13A		
15.		LC633855	HKY14A		
16.		LC633856	HKY15A		
17.		LC633857	HKY16A		
18.		LC633863	HKK1A	Kaeng Krachan National Park, Thailand	Matsudaira *et al.*, 2022
19.		LC633864	HKK2A		
20.		LC633865	HKK3A		
21.		LC633866	HKK4A		
22.		LC633867	HKK5A		
23.		LC633868	HKK6A		
24.		LC633869	HKK7A		
25.		LC633870	HKK8A		
26.		MT302850	A05	Khao Yai National Park, Thailand	Markviriya *et al*., 2022
27.		MT302851	A29		
28.		MT302852	A17		
29.		MT302853	J20		
30.		MZ407482	HLL08	National Wildlife Rescue Center, Malaysia (all individuals were originated from Peninsular Malaysia)	Gani *et al.*, 2021
31.		MZ407483	HLL19		
32.		MZ407484	HLL14		
33.		MZ407485	HLL11		
34.		MZ407486	HLL16		
35.		MZ407487	HLL15		
36.		MZ407488	HLL17		
37.		MZ407489	HLL18		
38.		MZ407490	HLL10		
39.		MZ407491	HLL09		
40.		MZ407492	HLL20		
41.		MZ407493	HLL07		
42.	*Hylobates agilis*	AB504748	-	-	Matsudaira and Ishida, 2010
43.	*Hylobates pileatus*	AB504749	-	-	

Table 2 Comparison of Haplotype diversity (*h*) and Nucleotide diversity (π) with other location

Location	Species	Number of Group	Number of adult individuals analysed	Number of Haplotype	Haplotype diversity (*h*)	Nucleotide diversity (π)	Data source
Ywar Kaing Kaung, Myanmar (YKK)	White-handed gibbons	9	9	2	0.556	0.00357	This study
Khao Yai National Park, Thailand (KYNP)	White-handed gibbons	17	37	10	0.823	0.00238	Matsudaira *et al.*, 2018
Bukit Barisan Selatan National Park, Sumatra (BBSNP)	Siamangs	6	15	8	0.886	0.03171	Lappan, 2007

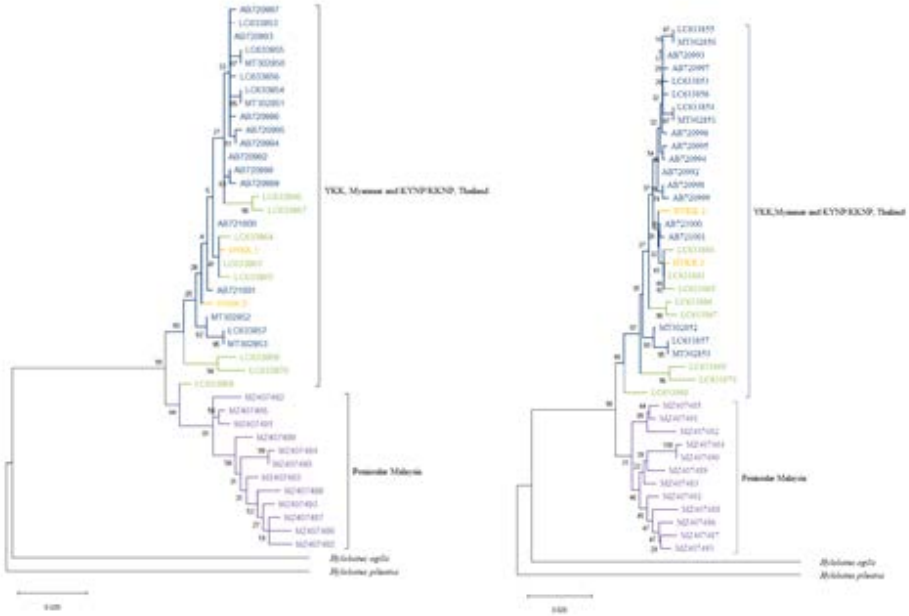

Figure 2 ML and NJ tree constructed from the 631-bp partial mtDNA sequences of *H. lar* in Ywar Kaing Kaung village (orange), Khao Yai National Park(blue), Kaeng Krachan National Park (green), Wildlife Rescue Centre (violet) and two outgroup species. The tree was constructed using Tamura-Nei model (TN93). Bootstrap analysis was carried out 1000 replications.

Figure 3 Minimum joining haplotype network of the 631-bp partial mtDNA sequences of *Hylobates lar* in Ywar Kaing Kaung village (orange), Khao Yai National Park (blue), Kaeng Krachan National Park (green), Wildlife Rescue Centre (violet)

Discussion

Phylogenetic position of the study population within white-handed gibbon

In this study, a total of nine partial mtDNA sequences were successfully obtained. The nine sequences showed two haplotypes in YKK population. Both phylogenetic tree and haplotype network showed that YKK, KYNP and KKNP white-handed gibbons were genetically

not distinguishable at the population level. White-handed gibbons living in KYNP and KKNP have been recognized as subspecies *H. lar entelloides* (Groves, 2001). Therefore, the most plausible subspecies status of YKK population is *H. lar entelloides*. Alternatively, YKK population might be *H. lar carpenteri*, which has been reported to be distributed to northern Thailand and a part of Myanmar. We could not include reference haplotypes from *H. lar carpenteri*, and thus it was not possible to completely deny this alternative hypothesis. However, if YKK population is *H. lar carpenteri* and not *H. lar entelloides*, our results rather oppose this classification, and *H. lar carpenteri* may better to be integrated into subspecies *H. lar entelloides*. The future studies including samples from the northern side such as Shan State, Myanmar, and the northern part of Thailand would solve this question. Regardless of the subspecies status, the genetic similarity between YKK population and KYNP/KKNP populations suggested that this group of white-handed gibbons are widely distributed and originally shared a maternal gene pool.

Genetic diversity among white-handed gibbon in Ywar Kaing Kaung village

The low haplotype diversity of mtDNA in YKK population suggested that this population has experienced a strong bottleneck. This is consistent with the known history that this population was separated from the Dawna Mountain Range population because of the fragmentation of the forests due to anthropogenic activities. Different from the haplotype diversity, the nucleotide diversity was not largely different between YKK and KYNP populations. This is probably because of the small sample size of YKK population and the stochastic process that randomly inherited the mtDNA haplotypes from the gene pool of the source population in Dawna Mountain Range. Nucleotide diversity of mtDNA was largely different between the white-handed gibbon populations and the siamang population. This was probably derived from the species difference. In the case of gibbons, the comparison of nucleotide diversity of mtDNA may be valuable only when it is performed for the same species.
One limitation of this study was that the sequences of only nine of the 18 adult individuals (50%) were determined. Although nine sequences seemed to reflect well the genetic status of the population, the future study should include all adult individuals of the population to precisely evaluate the effect of the forest fragmentation and the isolation on the genetic diversity. This study revealed the quite low genetic diversity in the isolated small population of white-handed gibbons in YKK village. Because of its small population size and the isolation from the neighboring Dawna Mountain Range population, the genetic diversity of the YKK population will decrease rapidly. To maintain the population healthier, the level of genetic diversity should be maintained at a certain level. To accomplish this, the translocation of some animals from the nearby population, or connecting the forest habitat to the source population might be better to planning before this population would locally extinct. The continuous monitoring of the population is important.

Conclusion

The phylogenetic analysis confirmed that the genetic affinity of gibbons in the YKK population does not deviate from other white-handed gibbon populations, and suggested the plausible subspecies status as *H. lar entelloides*. The genetic diversity of the study population was lower than the genetic diversity of the populations in other protected areas. To conserve the YKK white-handed gibbon population, maintaining their genetic diversity is important. For this purpose, translocation of some animals to the population might be required in the future. Keeping monitoring the both ecological and genetic status of the population is inevitable.

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PHYLOGEOGRAPHY OF LONG-TAILED MACAQUE, *MACACA FASCICULARIS AUREA* I. GEOFFROY [1831] FROM MON AND KAYIN STATES, MYANMAR

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Abstract

Macaca fascicularis aurea (Burmese long-tailed macaque) is one of the ten subspecies of the long-tailed macaques and is distributed along the Andaman seacoast. In Mon and Kayin States, Myanmar, the macaques inhabit some isolated limestone mountains. To uncover the phylogeography of *M. fascicularis aurea* in Mon and Kayin States and its relationship with those of other areas, the hypervariable segment 1 of mitochondrial DNA (mtDNA) was analysed using non-invasive faecal samples. Thirty-one sequences of *M. fascicularis aurea* were analysed; eight sequences from six populations in Mon State, seven sequences from three populations in Kayin State, four sequences from three populations at the Mergui Archipelago, and eleven sequences from three populations in the Thai Andaman seacoast. Both phylogenetic tree and haplotype network analyses revealed the presence of two mtDNA groups representing geographical differences: one group consisted of the populations from Mon and Kayin States, namely the mainland clade, and the other group consisted of the populations from the Mergui Archipelago and Thai Andaman seacoast, namely the coastal-island clade. Divergence time estimations suggested that *M. fascicularis aurea* initially diverged into the two clades one million years ago. Among the mainland clade, the divergence time of the most recent common ancestor was estimated to be four hundred thousand years ago. Three populations of Kayin State showed the same mtDNA haplotype and suggested a close maternal relationship. No spatial tendency was observed among the populations of Mon State. This study confirmed the close maternal genetic relationship among *M. fascicularis aurea* in Mon and Kayin States.

Keywords mitochondrial DNA, hypervariable segment 1, phylogenetic relationships

Introduction

Macaca fascicularis is one of the most geographically widespread and abundant non-human primate species in Southeast Asia, including Thailand, Indonesia, Malaysia, the Philippines, etc. The species is found in a wide geographic area that encompasses continental and insular populations (Fooden, 1995). *M. fascicularis aurea* (Burmese long-tailed macaque) is one of ten *M. fascicularis* subspecies and is distributed along the Bay of Bengal and the Andaman seacoast, including the Mergui Archipelago (Fooden, 1995; San and Hamada, 2011, Bunlungsup *et al*., 2016). This subspecies has been studied intensively in their use of stone tools for foraging, which is unique among Old World monkeys (Malaivijitnond *et al*., 2007; Gumert *et al*., 2009).

The genetic uniqueness of this subspecies among *M. fascicularis* has been revealed (Bunlungsup *et al*., 2016; Matsudaira *et al*., 2018; Osada *et al*., 2021; Padphone *et al*., 2021). The first phylogenetic study of *M. fascicularis aurea* revealed that *M. fascicularis aurea* was genetically distinct from *M. fascicularis fascicularis* both in the mitochondrial and Y chromosome phylogeny (Bunlunsup *et al*., 2016). Subsequently, a whole mitochondrial DNA (mtDNA) sequence analysis suggested ancient mtDNA introgression from a sinica- species group member to *M. fascicularis aurea* (Matsudaira *et al*., 2018). Whole- genome sequence analysis confirmed ancient introgression from the sinica-species group to *M. fascicularis aurea* but not to *M. fascicularis fascicularis* in the nuclear genome (Osada *et al*., 2021). Restriction Site Associated DNA Sequencing (RADseq) focusing on four *M. fascicularis aurea*

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populations together with *M. fascicularis fascicularis* and *M. mulatta* populations revealed some extent of introgression, or gene flow, from *M. fascicularis aurea* to *M. fascicularis fascicularis* populations near the subspecies contact zone, but less or negligible amount of introgression from *M. fascicularis fascicularis* to *M. fascicularis aurea* (Padphone et al., 2021).

It is interesting how this genetic uniqueness has been formed. For example, when and where did *M. fascicularis aurea* diverge from *M. fascicularis fascicularis*? When and where did *M. fascicularis aurea* meet the sinica-species group and hybridise? Many aspects of the evolutionary history of *M. fascicularis aurea* were still not clear. This was mainly due to the limited number of populations analysed in the previous studies. Most of the *M. fascicularis aurea* populations previously analysed were those of the islands of the Mergui Archipelago and Thai Andaman seacoast. Only one population of Bayin Nyi Naung, in Kayin State was analysed as a representative of the "mainland" area, and not near the coast. In this study, to improve our knowledge of the evolution of *M. fascicularis aurea*, the phylogeography of the nine populations of *M. fascicularis aurea* from the mainland area, Mon and Kayin States, were studied for the following objectives: to investigate the phylogeographic relationship of *M. fascicularis aurea* in Mon and Kayin States and to uncover the divergence times of *M. fascicularis aurea* living in mainland Myanmar.

Materials and Methods

Study sites

Six populations in Mon State (Kyauk Taung [KT], Mein Ma Hlein [MMH], Eaint Phet Taung [EPT], Kyauk Tha Lone Taung [KTL], Kaylar Tha [KLT] and Kha Yone Gu [KYG]) and three populations in Kayin State (Kaw Goon [KG], Ya Thae Pyan [YTP] and Bayin Nyi Naung [BYNN]) were investigated. (Table 1 and Figure 1)

Study period

The present study was conducted from 2018 to 2020.

Sample collection and DNA Extraction

Faecal samples were collected from the nine study populations. The epithelial cells of the digestive tract were collected by the tip of a cotton bud from faecal samples. The cotton bud was rolled on the surface of faecal samples and then dipped in the 1 ml of lysis solution in a microcentrifuge tube (Hayaishi and Kawamoto, 2006). This process was conducted three or more times until the lysis solution in the tube converted to dingy colour. The tubes were kept in a zip bag and stored at room temperature until DNA extraction. DNA extraction from the faeces was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Germany). DNA extraction was carried out according to the manufacturer's protocol with some modifications. At first, the supernatant of the lysis solution with the faecal sample was transferred to a 2 ml microcentrifuge tube. The total volume of faecal lysis solution was adjusted to 1.4 ml by adding buffer ASL and vortex mixed well, which was different from the manufacturer's protocol. At the final step, 200 µl of Buffer AE was incubated on the QIAamp membrane for 20 min to maximize the amount of retrieved DNA. The remaining steps of the extraction were carried out according to the manufacturer's procedures.

PCR and sequencing

The fragment of mtDNA, hypervariable segment 1 (HVS1) was PCR amplified using the primers MFA-DF2: AGCATGATATTCCGTCCACTCAG and MFA-DR2: GGTGATAGACC TGTGATCCATCG. PCR amplifications were carried out in a 25 µl mixture, consisting of 13.85 µl of deionized water, 2.5 µl of 10× PCR Buffer II for AmpliTag Gold, 2.5 µl of 2mM each dNTP, 2.0 µl of 25mM $MgCL_2$, 1.0 µl of 100mg/ml BSA (Sigma Aldrich, USA), 0.5 µl each of forward and reverse primers, 0.15 µl of AmpliTaq Gold (Thermo Scientific, USA), and 2 µl of DNA template. The PCR cycle conditions were as follows; the initial denaturation at 94°C for 9 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 min, and extension at 72°C for 1 min and the final extension at 72ºC for 10 min. PCR products were separated by 1% agarose-TAE gel electrophoresis and visualized by ethidium bromide staining and UV transillumination. The purification of the PCR products was conducted by using FavorPrep^{TM} PCR Purification Kit (Favorgen, Taiwan). The nucleic acid concentration and purity of each purified PCR product were measured by using a Nanodrop Spectrophotometric machine (Thermo Scientific, USA).

PCR direct sequencing was conducted by using, Big-Dye Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific, USA) with the same PCR primers. The electrophoresis was conducted by an ABI 3500 Genetic Analyzer (Thermo Scientific, USA).

Data analysis and phylogenetic tree construction

The mtDNA sequences of *M. fascicularis aurea* determined in this study (14 individuals) were analysed together with sequences determined in previous studies: 16 mtDNA sequences of *M. fascicularis aurea* (Accession number: LC093210 to LC093225) from the southernmost part of Myanmar and the Southwestern part of Thailand (Bunlungsup *et al.*, 2016) and one mtDNA sequence of *M. sylvanus* (Barbary macaque) (Accession number: NC_002764) as the outgroup. These sequences were downloaded from GenBank. The sequences were aligned by MUSCLE implemented in MEGA X (Kumar *et al*., 2018), and the insertion and deletion sites were removed, which resulted in the dataset of 31 sequences consisting of 675 nucleotide sites.

Phylogenetic trees were constructed using a distance-based method, the Neighbour-Joining (NJ; Saitou and Nei, 1987), and the maximum likelihood method (ML; Felsenstein, 1981) using MEGA X. In both the analyses, the Tamura-Nei model (Tamura and Nei, 1993) was used as the substitution model. The reliability of the phylogenetic relationship was assessed by 1,000 bootstrap replicates.

Haplotype network analysis of mtDNA

A total of 25 mtDNA sequences, nine sequences from the nine present study populations and the rest of those from the previous study populations were analysed by haplotype network analysis to uncover the geographical relationship between the haplotypes of Mon and Kayin States. A minimum-spanning network (Chen and Morris, 2014) of the mtDNA sequences was constructed by using PopART 1.7 (Leigh and Bryant, 2015).

Divergence time estimation

The divergence times of mtDNA were estimated by the Bayesian framework using BEAST2 (v2.6.3) (Boukaert *et al*., 2014). For this analysis, the gamma site model and Hasegawa-Kishino-Yano (HKY) substitution model were used. The relaxed clock log normal model was used as the clock model and the Coalescent Bayesian Skyline model was used for the tree prior. Markov Chain Monte Carlo (MCMC) runs were conducted by using the chain length of 30,000,000 generations and logging every 1000 generations. Sample distributions of multiple independent replicated runs were combined with LogCombiner 2.6.3 and summarized

(25% burn-in) by TreeAnnotator 2.6.3 (both programs are part of the BEAST2 package). For calibration priors of divergence times, the divergence between *M. sylvanus* and *M. fascicularis aurea* was assumed as the normal distribution divergence of 5.0 million years ago (MYA) (95% lower and upper limits: 5.5 - 6.5 MYA) (Alba *et al.*, 2014; Roos *et al.*, 2019). The phylogenetic tree with the divergence times was visualized in FigTree v1.4.4.

(https://github.com/rambaut/figtree/releases).

Table 1 Study sites

No.	State	Name of location	Coordinate
1.	Mon	Kyauk Taung (KT)	16º 49' N, 97º 35' E
2.		Eaint Phet Taung (EPT)	16º 30' N, 97º 76' E
3.		Mein Ma Hlein Taung (MMH)	16º 30' N, 97º 49' E
4.		Ka Yone cave (KY)	16º 31' N, 97º 42' E
5.		Kay Lar Tha (KLT)	17º 13' N, 97º 05' E
6.		Kalar Kyauk Tha Lone (KTL)	16º 19' N, 97º 42' E
7.	Kayin	Kaw Goon cave (KG)	16º 49' N, 97º 35' E
8.		Ya Thae Pyan cave (YTP)	16º 44' N, 97º 29' E
9.		Bayin Nyi Naung mountain (BYNN)	16º 58' N, 97º 29' E

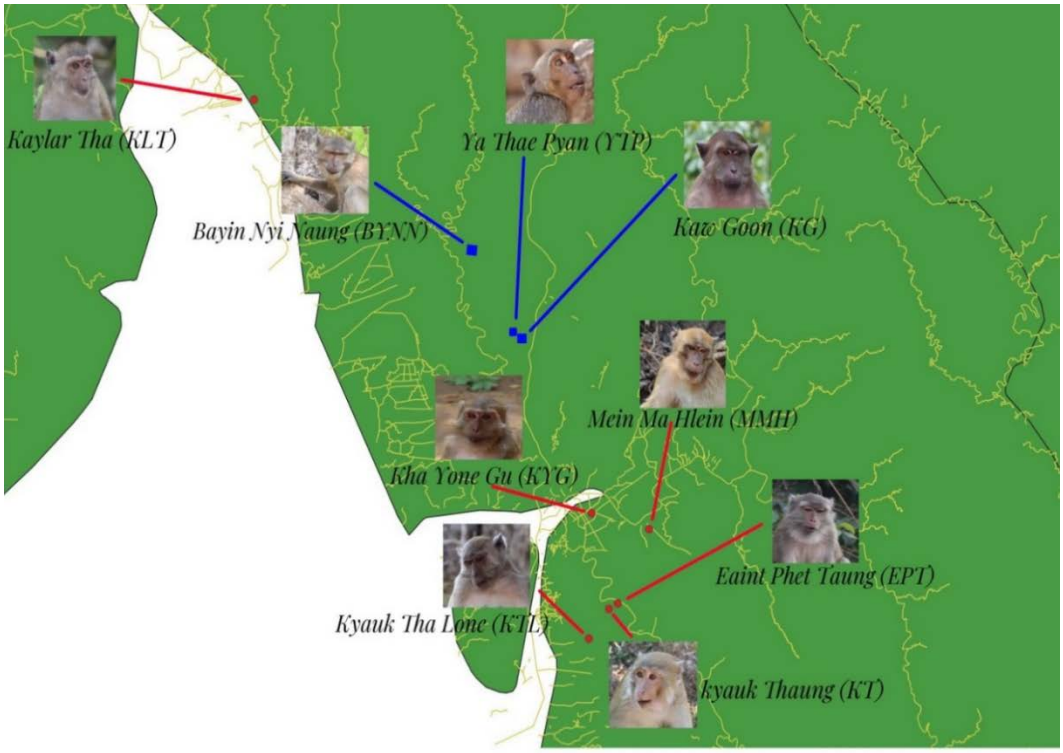

Figure 1 Locations of the study sites of *M. fascicularis aurea* from Mon and Kayin States.

Red cycles indicate Mon State populations (n= 6) and blue quadrangles indicate Kayin State populations (n= 3). Yellow lines indicate river and canals.

Results

Phylogenetic relationship of *M. fascicularis aurea* from Mon and Kayin States

In this study, a total of 14 sequences were determined for the *M. fascicularis aurea* samples collected in the nine populations (one to two samples per population). By combining these sequences with the downloaded sequences of *M. fascicularis aurea* (n = 16) and *M. sylvanus* (n = 1), a total of 31 sequences were analysed. After the alignment, some poorly aligned positions were removed and the final alignment comprised a total of 675 bp.

Both ML and NJ trees showed the same tree topology (Fig 2 and 3). The mtDNA sequences of *M. fascicularis aurea* populations were separated into two main clades, namely the coastal-island clade and the mainland clade. The coastal-island clade comprised Thai Andaman seacoast populations (Piak Nam Yai Island [PNY], Mangrove Forest Research Center [MFRC] and Wat Paknam Pracharangsarith [WPN]) and Mergui Archipelago populations (Lampi Island [LPI], Zadetkyi [ZDK] and Jarlan Island [JLI]). The mainland clade, on the other hand, was the assemblage of the populations in Mon and Kayin States. The three study populations of Kayin State (YTP, KG and BYNN) clustered tightly and separated from the other populations of Mon State, which was supported by moderate and high bootstrap values in the ML (68) and NJ (89) trees.

Among the Mon State populations, MMH was tightly clustered with the Kayin State populations with high bootstrap values both in ML (87) and in NJ (95) trees. In addition, the three populations, KTL, KT and KLT, clustered each other with high supporting values in the ML (97) and NJ (96) trees.

In the coastal-island clade, the three populations, JLI, LPI and ZDK from the Mergui Archipelago, were clustered with the Thai populations (PNY, MFRC and WPN).

Haplotype Network of *M. fascicularis aurea*

The haplotype network is shown in Fig 4. The 10 sequences of the mainland region showed seven haplotypes. The remaining 15 sequences were from the previous study (Bunlungsup *et al.*, 2016) and consisted of seven sequences from Mergui Archipelago populations and eight sequences from Thai Andaman seacoast populations. These 15 sequences showed seven haplotypes and each haplotype consisted of one to four sequences.

In the haplotype network, relatively large nucleotide differences between the haplotypes from the mainland clade and the coastal-island clade were observed. 33 substitutions were observed between BYNN and WPN1298, and 38 substitutions were observed between EPT and PNY2005. On the other hand, only one to 21 substitutions were observed between the mainland haplotypes, and five to 12 substitutions were observed between the coastal-island haplotypes. This large difference between the two regions was consistent with the phylogenetic trees that showed the two clades among *M. fascicularis aurea*.

Among the mainland region, the sequences from the three populations of Kayin State (BYNN, KG, YTP) showed the same haplotype. In addition, one sequence from the previous study (Mfa_BNT 1447, Bunlungsup *et al*., 2016), which was obtained from a sample that had been collected at Bayin Nyi Naung Mountain in 2007 (Aye Mi San, unpublished data), showed only one nucleotide difference from the BYNN sequence collected at the same location in 2019. Furthermore, the haplotype of MMH was the closest to the haplotype from the Kayin State populations.

Among the mainland region, no clear relationship between the haplotype network and the distribution of populations was observed, except that Kayin State populations showed the same haplotype.

Among the coastal-island region, no tendency was observed. The number of substitutions between haplotypes was similar to that observed among the mainland populations. The haplotypes of island populations relatively diverged from those of the coastal populations, and were more largely different from those of the mainland populations.

Divergence times of *Macaca fascicularis aurea*

The divergence time of the mtDNA of *M. fascicularis aurea* and that of *M. sylvanus* was estimated to be 4.74 MYA (95% high posterior density credibility interval [HPD CI] = 3.27–6.37). Among *M. fascicularis aurea*, the divergence between the two clades, *i.e.*, the mainland clade and coastal island clade was estimated to have occurred 1.0 MYA (95% HPD CI= 0.40 – 1.85). Furthermore, the divergence within the mainland clade was estimated to have started about 0.40 MYA (95% HPD CI = 0.14 – 0.76). On the other hand, the divergence within the coastal-island clade was estimated to have started around 0.31MYA (95% HPD CI = 0.10 – 0.59). Within the mainland clade, EPT and KYG formed a cluster and the cluster was estimated to have split from the other cluster at 0.3 MYA (95% HPD CI = 0.14 – 0.76). KLT, KT and KTL populations of Mon State separated from the remaining cluster at 0.25 MYA (95% HPD CI = 0.09 – 0.5). The divergence time between the three populations of Kayin State and MMH population of Mon State was estimated to be 0.09 MYA (95% HPD CI = 0.02 – 0.22) (Table 2 and Fig 5).

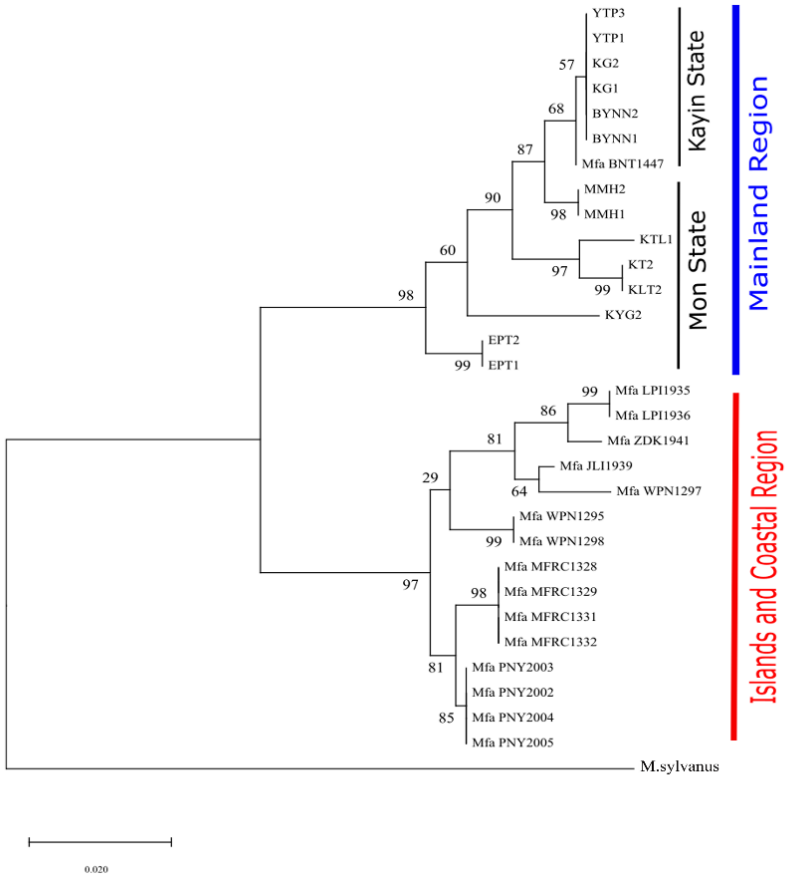

Figure 2 Maximum-likelihood phylogenetic tree of *M. fascicularis aurea* on mtDNA (675 bp) sequences

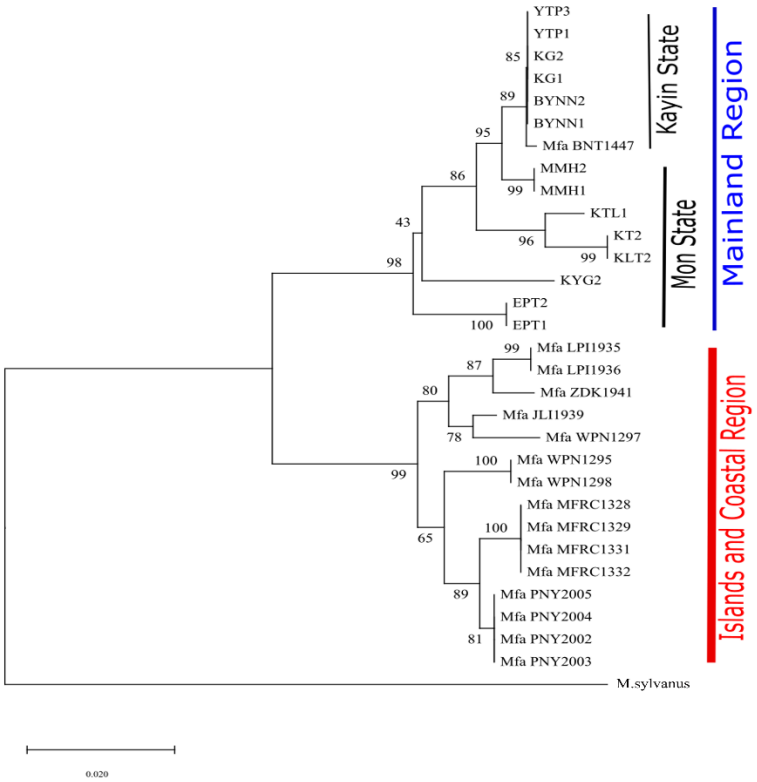

Figure 3 Neighbour-joining phylogenetic tree of *M. fascicularis aurea* on mtDNA (675 bp) sequences

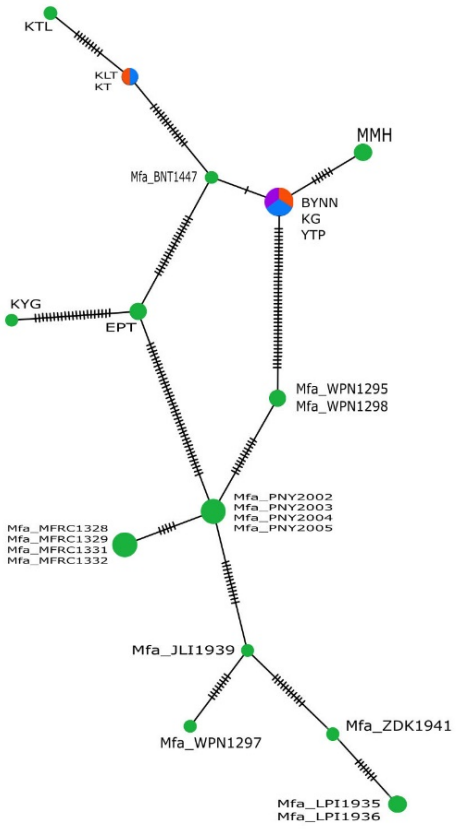

Figure 4 The Minimum-spanning network of mtDNA (675 bp). The short bars on each edge indicate substitutions

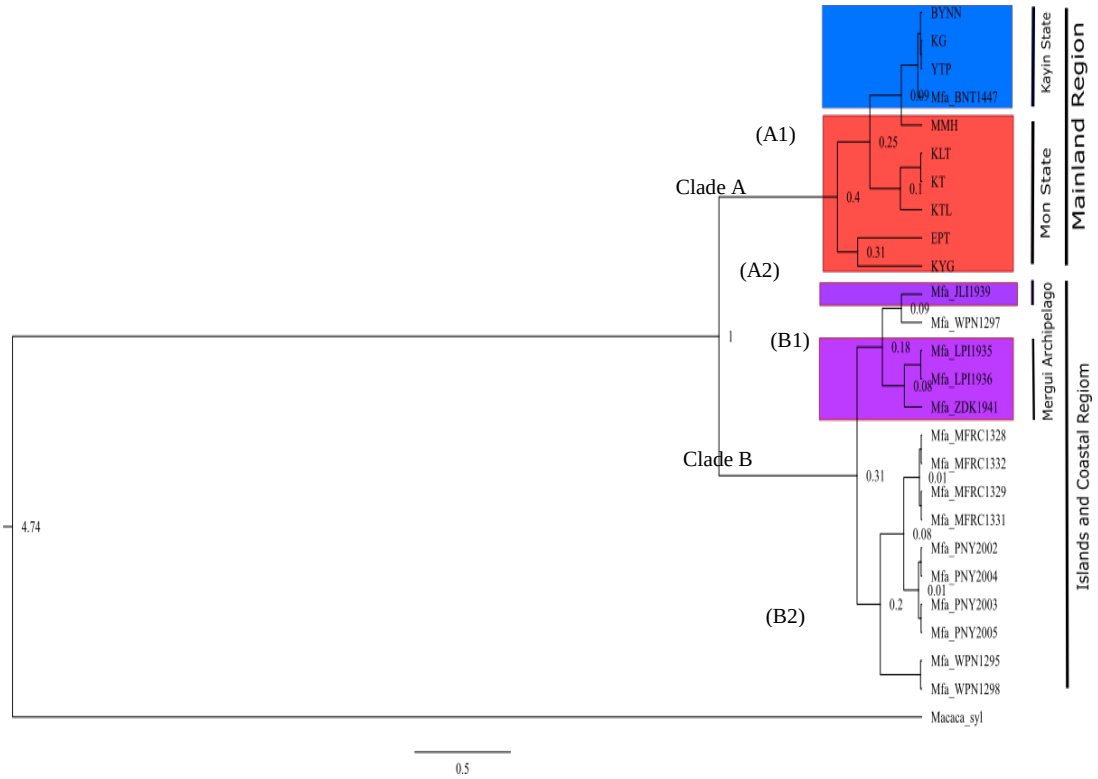

Figure 5 Phylogenetic tree with the estimated divergence times of partial mtDNA (675 bp)

Table 2 Divergence time of the mtDNA (in million years ago)

Divergence	Height median (MYA)	95% Credibility interval
Sylvanus- Asian macaques	4.74	3.27 - 6.37
Mainland-coastal region	1.00	0.40 – 1.85
The mainland clade (A1 and A2)	0.40	0.14 – 0.76
The mainland clade (Mon-Kayin State)	0.09	0.02 – 0.22
The coastal-island clade (B1 and B2)	0.31	0.10 – 0.59

Discussion

This study confirmed the presence of two clades among the mtDNA phylogeny of *M. fascicularis aurea*, one representing the populations from the mainland region and the other representing the populations from the coastal-island region, which was consistent with the mtDNA phylogeny shown by Bunlungsup *et al.*, 2016. In the previous study, only one sequence from the mainland region (BYNN) was implemented in the analysis, and thus the presence of the mainland clade and its geographical extent were not clear. In this study, all nine populations from Mon and Kayin States formed one clade, and thus maternally close relationship among the Mon and Kayin populations was uncovered.

Among the mainland clade, samples from the three populations in Kayin State (BYNN, KG, YTP) showed the same mtDNA haplotype (except that one previous published sequence

showed one nucleotide difference from the common haplotype) and thus genetically distinct from the populations of Mon State. These three populations are located between two major rivers, Donthami and Thanlwin, which might prevent the migration of monkeys between Mon and Kayin States as natural barriers.

Among the populations from Mon State, no clear relationship between the geographic distributions and the phylogenetic relationships of mtDNA was observed. This might be because of the absence of major geographic barriers among the Mon State populations and/or low resolution in the use of HVS1 of mtDNA.

The estimated divergence time of mtDNA between the mainland clade, and the coastal-island clade was about 1.00 MYA which was consistent with the age estimated by the whole mitochondrial genomes (Matsudaira *et al*., 2018). The divergence time estimates of the mtDNA sequence analysis indicated that the divergence of the mainland clade started earlier (0.40 MYA) than the coastal-island region (0.31 MYA). Further study including more populations from both areas is required to confirm this observation.

Still, there is a gap of unsampled populations between the northern part of Mon State sampled in the present study and the southern part of Mergui Archipelago sampled in the previous study. Further sampling of populations located between the two areas will uncover the border of the distribution of the two mtDNA clades. The border may reflect the maternal origin of *M. fascicularis aurea* where the mtDNA introgression from the sinica-species group occurred. In addition, there are some populations distributed along the Bay of Bengal which has not been studied. Further investigation of *M. fascicularis aurea* in the area should also be studied to delineate the scenario of the origin of *M. fascicularis aurea.*

Conclusion

The phylogenetic study of *M. fascicularis aurea* was conducted for the populations of Mon and Kayin States based on mtDNA. The phylogenetic analyses confirmed that the populations of Mon and Kayin States are maternally related to each other and form the mitochondrial clade, the mainland clade, which is distinct from that of the coastal-island populations. Furthermore, the estimated divergence time suggested that the divergence among the mainland populations may have started earlier than that of the coastal-island populations. Still, the exact origin of the *M. fascicularis aurea* is not clear. Further studies focusing on the unsampled region, i.e., the gap between the mainland and island-coastal regions, and the Bay of Bengal are expected to uncover more details of the evolutionary history of *M. fascicularis aurea*.

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DETECTION AND ENUMERATION OF COLIFORMS IN TUBE WELL WATER COLLECTED FROM UNIVERSITY OF YANGON CAMPUS

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Abstract

The aim of this study was to determine the hygienic status of the tube well water supply from Yangon University campus. A total of ten water samples were collected from the tube wells in the campus. Microbiological analysis was carried out at the Microbiology laboratory, Department of Zoology, University of Yangon and water quality parameters were examined at the Water and Soil Examination Laboratory, Thaketa Township. Water samples were analyzed bacteriologically for total coliform and fecal coliform counts by the MPN method. The highest total coliform counts of >1100 MPN/100mL were observed in the water samples of V, VI, VII and X. The highest fecal coliform counts of >1100 MPN/100mL were found in the water samples of V, VI and VII. Water samples were then treated with calcium hypochlorite to reduce the contamination of bacteria. After chlorination, the highest total coliform counts of 4 MPN/100mL were detected in water samples I and II. But the fecal coliform counts could not be found in all the water samples after the treatment. Regarding the identification of bacteria, five groups of bacterial species were isolated, namely *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., *Enterobacter* sp. and *Pseudomonas* sp. After chlorination, these genera of bacteria could not be isolated from all water samples. Analysis of water physico-chemical parameters revealed that pH ranged from 5.0 to 7.3; temperature ranged from 25˚C to 28.1˚C; total dissolved solids (TDS) ranged from 8mg/L to 12mg/L. Temperature and TDS values were normal according to WHO (2011), but pH values were out of the WHO guideline values for drinking water. According to the present study, tube well water in University of Yangon campus should be chlorinated to kill and reduce bacteria levels and to get safe water for public utilization.

Keywords Total coliform counts, fecal coliform counts, chlorination, physico-chemical parameters

Introduction

Water is very important for survival and growth of all living organisms. Actually, 70 percent of the human body is made up of water. The body is helped to metabolize fat by water that also helps us maintain our body temperature through perspiration. Dehydration is tended to give rise by lack of water in the body, thereby posing hurdles for the blood to circulate (Skinner and Carr, 1976).

Nowadays, increased human population, industrialization, use of fertilizers in the agriculture and man-made activities make natural water highly polluted with different harmful contaminants. So, water pollution caused by harmful microorganisms, is a global problem. It is difficult and expensive to test water for each of these germs. Instead, public health workers measure coliform bacteria levels in order to know water quality. When coliforms are present in drinking water, it is suggested that there may be feces and disease-causing agents in the water. The most commonly used indicators of fecal pollution in water and food are fecal coliform bacteria. Direct person to person contact, contaminated food, and contaminated water transmit the diseases (Pleczar *et al*., 1974).

Tube well water is commonly used and very useful in our daily life. But, adequate construction and well protection is vital to get clean tube well water. Tube well water may have several pathogenic bacteria. Some of these may be *Escherichia coli*, *Klebsiella* species, *Salmonella* species, *Enterobacter* species, and *Pseudomonas* species, etc. Most of these bacteria are harmful to humans.

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One of the most commonly used water purifying methods is chlorination. Chlorine is added to water either as elemental chlorine (chlorine gas), or chlorinating chemicals such as calcium hypochlorite (tablets or granules) or solutions of sodium hypochlorite (liquid bleach). In chlorination of a piped distribution system, it is desirable to maintain free chlorine residues of concentration 0.2-0.5mg/liter throughout, to reduce the risk of microbial regrowth and the health risk of recontamination WHO (1997). For domestic water use, the WHO safe water system for household disinfection in developing countries allows a dosage of 1.875 or 3.75 mg/L of sodium hypochlorite with a contact time of 30 minutes.

In University of Yangon, tube well water is used primarily as a source for drinking, food preparation, showering, washing and gardening. People in University of Yangon campus who use tube wells assumed them as safe water source. Since there is paucity of information concerning microorganisms in the tube well water, the present research was conducted to assess the coliform levels in the tube well water, to isolate and identify the bacteria from the tube well water before and after treatment with calcium hypochlorite and to examine the effectiveness of calcium hypochlorite for control of pathogenic bacteria contaminated in the tube well water.

Materials and Methods

Study areas and sites

University of Yangon campus (16°49'44"N and 96°08'15"E) and Fisheries and Aquaculture compound (16°50'05"N and 96°08'13"E), University of Yangon were selected as study areas (Fig.1). A total of ten water samples were collected from tube wells (110-200 ft) near the departments and hostels at the University of Yangon. Samples from the sites designated as I, II, III, V, VI, VII, VIII, IX and X were collected from water taps of Zoology Department, Inya Hostel, Shwebo Hostel, University Laboratory Building, Professors' Houses, International Cooperation Office, Thiri Hostel, University of Yangon Research Center and Fisheries and Aquaculture building respectively. Sample-IV was directly collected from the tube well near Staffs' Housing.

Study period

The study period was from February to August, 2022.

Sampling methods

Water samples were collected in three sterile glass bottles with caps per sample with the amount of approximately 1000 ml per bottle and packed inside black plastic bags to prevent from light. Two glass bottles were carried to the Microbiology Laboratory, Department of Zoology, University of Yangon. After arrival at the laboratory, one of the collected two glass bottles was treated with calcium hypochlorite (2mg/L). And these two glass bottles (one with calcium hypochlorite and another one without calcium hypochlorite) were studied in two steps: first step for MPN (Most Probable Number) after Brown (2007) and second step for isolation and identification of bacteria, which was done after Atlas (1995) and, Dubey and Maheshwari (2002). And then, the third collected glass bottle of water was sent to the Water and Soil Examination Laboratory, Freshwater Aquaculture Research, Aquaculture Division, Department of Fisheries, Ministry of Agriculture, Livestock and Irrigation, Thaketa Township for measurement of the physico-chemical parameters (except temperature) according to WHO (1993). Temperature was measured at the sampling sites by using a mercury thermometer (Plate 1).

Characterization of the isolated bacteria

Isolated bacteria were characterized by means of colonial morphology, Gram-staining reaction, motility test, and biochemical reactions (Hucker and Conn, 1923).

Colonial morphology

The characteristic features of colony morphology such as colour, shape, surface, elevation and edges of colonies were determined after methods of Bisen and Verma (1998).

Gram-staining

The most widely used bacteriological stain, the differential Gram-stain, was used to observe the Gram-staining nature and shapes of the bacterial cells.

Biochemical tests

Confirmation of bacteria by biochemical reactions was based on the methods as described by Collins and Lyne (1995), Bisen and Verma (1998) and HiMedia (1998).

Identification of bacterial isolates

To determine the identification of the isolates, colonial and cell morphology, Gram-nature and biochemical properties recorded during the work were compared to those described in Bergey's Manual of Determinative Bacteriology by Breed, Murry and Smith (1994) and Cowan and Steel's Manual for the Identification of Medical Bacteria (Cowan, 2009).

A. University of Yangon campus

B. Fisheries and Aquaculture compound, University of Yangon

Figure 1 Map showing locations of University of Yangon campus and Fisheries and Aquaculture compound (Source: from Google Earth, 2021)

A. House near tube well (Department of Zoology)

B. Decaying leaves on storage reservoir (Inya Hostel)

C. Toilet near tube well
(University Laboratory Building)

D. Toilet near storage reservoir
(University Laboratory Building)

Plate 1 Surrounding conditions of some studied tube wells and water storage reservoirs

Results

Most Probable Number of total and fecal coliforms in the water samples before treatment with calcium hypochlorite

Total coliforms and fecal coliforms were detected using MPN method for the water samples of ten sampling sites. Sample-I (near Department of Zoology) had the total coliform count of 150 MPN/100mL and the fecal coliform count of 93 MPN/100mL. Sample-II (near Innya Hostel) had higher total coliform count of 460 MPN/100mL and the fecal coliform count of 460 MPN/100mL. Sample-III (near Shwe-bo Hostel) also had higher total coliform count of 460 MPN/100mL and the fecal coliform count of 460 MPN/100mL. Sample-IV (near Staffs' Housing) had the lowest total coliform count of 23 MPN/100mL and the lowest fecal coliform count of 23 MPN/100mL. Sample-V (near University Laboratory Building) had the highest total coliform count of >1100 MPN/100mL and the highest fecal coliform count of >1100 MPN/100mL. Sample-VI (near Professors' Housing) also had the highest total coliform count of >1100 MPN/100mL and the highest fecal coliform count of >1100 MPN/100mL. Sample-VII (near International Cooperation Office) also had the highest total coliform count of >1100 MPN/100mL and the highest fecal coliform count of >1100 MPN/100mL. Sample-VIII (near Thiri Hostel) had the low total coliform count of 43 MPN/100mL and the low fecal coliform count of 43 MPN/100mL. Sample-IX (near University of Yangon Research Center) had the total coliform count of 210 MPN/100mL and the fecal coliform count of 93 MPN/100mL. Sample-X (Fisheries and Aquaculture building compound) had the highest total coliform count of >1100 MPN/100mL and the fecal coliform count of 1100 MPN/100mL (Fig. 2).

Most Probable Number of total and fecal coliforms in the water samples after treatment with calcium hypochlorite

After treatment with 2mg/L calcium hypochlorite with a contact time of 30 minutes, total coliform counts and fecal coliform counts were considerably lowered in all water samples. After treatment, samples-III, IV, V, VI, VII and VIII had no total coliform and fecal coliform counts. All samples had no fecal coliform counts. Sample-I and II had very low total coliform counts of 4 MPN/100mL respectively. Sample-IX and X had total coliform counts of 3 MPN/100mL (Fig. 3).

Characterization and identification of bacteria isolates

A total of five different colony types were detected from the culture media (Table 1). Morphology of the colonies of the isolates were found as follow:

Colonial morphology of identified genera of bacteria

Colony shape, edge, elevation, texture, and colour were observed in the following isolates.

Escherichia coli

Escherichia coli colonies on selective agar were detected as circular in shape with smooth edge, flat and glistening surface and a metallic sheen.

Klebsiella sp.

Klebsiella sp. as pure isolate had pink to purple colonies, circular in shape with entire edge, convex and smooth.

Salmonella sp.

Salmonella sp. as pure culture on EMB agar gave colonies pink in colour, circular in shape with entire margin, convex, smooth and gummy.

Enterobacter sp.

Enterobacter sp. as pure isolate had colonies circular in shape with entire margin, convex and pink in colour on EMB agar.

Pseudomonas sp.

Pseudomonas sp. as pure colony was purple in colour, circular in shape, entire margin, convex and smooth on EMB agar (Table 1).

Gram staining reactions and cell shapes of identified genera of bacteria

After recording the colonial morphology of the isolates, gram-staining properties and cell shapes were observed using Gram-staining method. All isolates were Gram-negative rod shape bacteria that showed pink to purple colour under oil immersion magnification (Table 1).

Biochemical tests

Indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Carbohydrate formation test (TSI) and H_2S production test were performed to identify the isolated bacteria. Detailed results of the biochemical tests of the five identified genera of the isolated bacteria are described and shown in (Table 1 and Plate 2).

Motility test for identified genera of bacteria

Escherichia coli, *Salmonella* sp., *Enterobacter* sp. and *Pseudomonads* sp. showed motility by spreading growth in the Sulphide Indole Motility (SIM) semisolid medium. But *Klebsiella* sp. did not show any motility in the SIM semisolid medium (Table 1 and Plate 2).

Occurrence of genera of bacteria isolates in the water samples before treatment with calcium hypochlorite (2mg/L)

Five genera of bacteria, *Escherichia coli*, *Klebsiella* sp., *Salmonella* sp., *Enterobacter* sp. and *Pseudomonads* sp. were discovered in the water samples. *Escherichia coli* was found in the samples I, IV, VI and VIII. *Klebsiella* sp. was found in the samples V, VI, VII and X. *Salmonella* sp. was found in the samples II and VII. *Enterobacter* sp. was found in the samples I and X. *Pesudomonads* sp. was found in all samples I, II, III, IV, V, VI, VII, VIII, IX and X. Unidentified spp. were found in the water samples IV, VI and VII. After treatment with 2 mg/L calcium hypochlorite, the five genera of bacteria isolates were no longer detected in all water samples.

The physico-chemical parameters of the water samples

The pH of water samples ranged from 5.0 to 7.3. Temperature value ranged from 25˚C to 28.1˚C. The total dissolved solids ranged from 8mg/L to 12mg/L. The dissolved oxygen ranged

from 4.5mg/L – 7.3mg/L. The biochemical oxygen demand (BOD) ranged from 0mg/L to 2.5mg/L. The chemical oxygen demand (COD) ranged from 0.73mg/L to 11.04mg/L. (Table 2).

Table 1 Morphological characters and biochemical properties of the identified bacteria isolates from the water samples

Sr no.	Morphological Tests			Biochemical Tests									Identified bacteria isolates
	Colony Morphology	Gram Stain	Shape	TSI			Cit	MR	VP	SIM			
				butt	slant	gas				H_2S	I	M	
1.	Metallic sheen, circular, entire, flat and glistening on EMB agar	-	Rods	A	A	+	-	+	-	-	+	+	*Escherichia coli*
2.	Pink, circular, entire, convex, smooth on EMB agar	-	Rods	V	V	-	+	V	V	-	V	-	*Klebsiella* sp.
3.	Pink, circular, entire, convex, smooth and gummy on EMB agar	-	Rods	A	K	-	+	+	-	V	-	+	*Salmonella* sp.
4.	Pink, circular, entire, convex, smooth on EMB agar	-	Rods	A	A	-	+	V	+	-	-	+	*Enterobacter* sp.
5.	Purple, circular, entire, convex, smooth on EMB agar	-	Rods	V	K	+	+	V	-	-	-	+	*Pseudomonas* sp.

TSI = Triple Sugar Iron
Cit = Simmon's citrate
MR = Methyl Red
VP = Voges-Proskaurer
SIM = Sulphide Indole Motility
I = Indole

(+) = acid formation or positive reaction
(-) = no change
A = acid
K = alkaline
V = variable (positive or negative)
M = motility

Table 2 Physico-chemical parameters of the water samples collected from ten sampling sites

Parameter	I	II	III	IV	V	VI	VII	VIII	IX	X	WHO Drinking Water Standard*
pH	6.0	5.0	5.5	5.0	6.0	5.5	5.2	5.5	7.3	6.5	6.5-8.5
Temperature (˚C)	25.6	27.5	26	27.4	26.1	28.1	26.4	25	27	27	20-30
TDS (mg/L)	8	10	10	8	12	10	8	10	10	8	< 600
DO (mg/L)	4.5	5.0	5.5	5.0	6.0	5.5	5.2	5.5	7.3	6.5	NG
BOD_5 (mg/L)	0.5	0	0.75	2.5	1.5	1.75	2.5	0.5	0.5	0.75	NG
COD (mg/L)	1.84	5.88	4.78	3.68	3.68	3.68	6.25	5.88	0.73	11.04	NG

*Guidelines for Drinking Water Quality, 4th ed. World Health Organization, 2011

TDS = Total Dissolved Solids
DO = Dissolved Oxygen
BOD_5 = Biochemical Oxygen Demand
COD = Chemical Oxygen Demand
NG = No Guideline

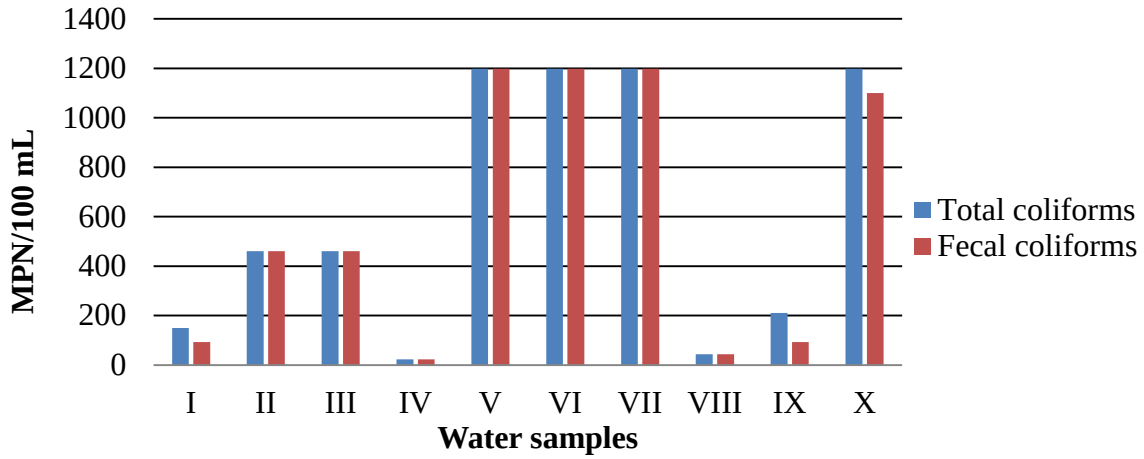


Figure 2 Total coliform and fecal coliform counts of the water samples before treatment with calcium hypochlorite

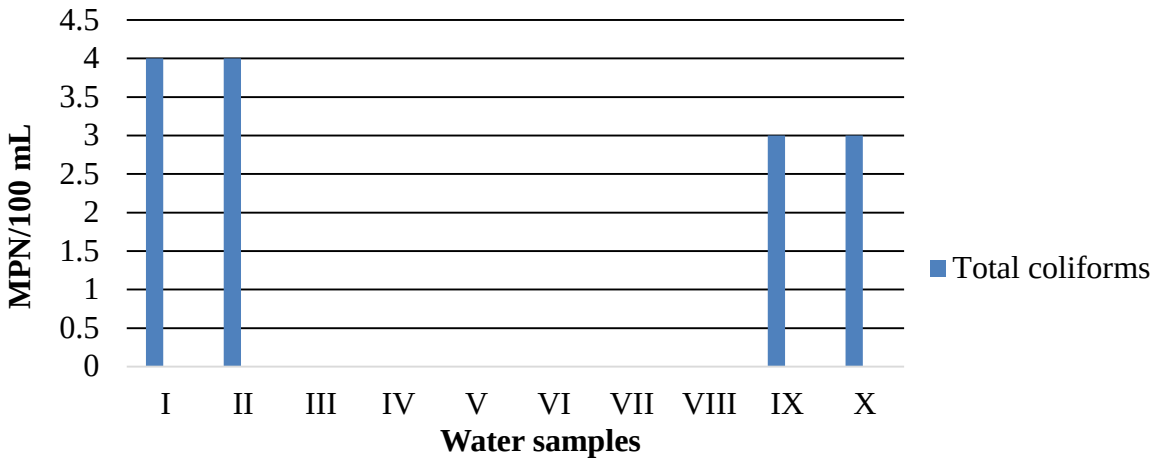
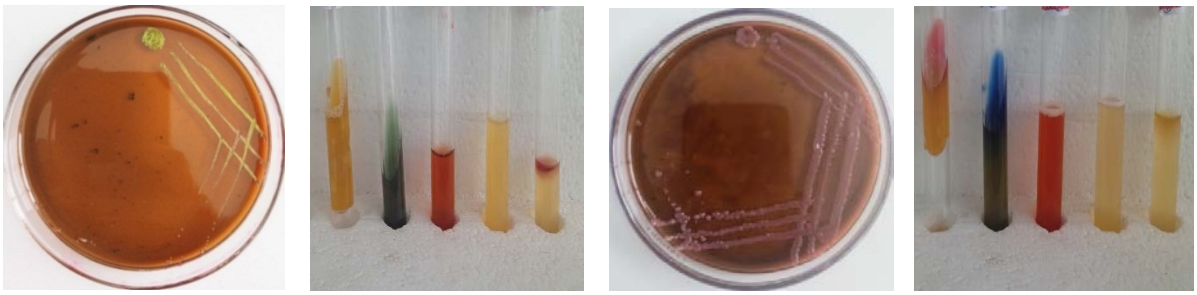


Figure 3 Total coliform counts of the water samples after treatment with calcium hypochlorite (2mg/L)



A. *Escherichia coli* colonies on EMB agar

B. Biochemical tests for *E. coli*

C. *Pseudomonas* sp. colonies on EMB agar

D. Biochemical tests for *Pseudomonas* sp.

1. TSI = Triple Sugar Iron
2. Simmon's Citrate
3. MR = Methyl Red
4. VP = Voges-Proskaurer
5. SIM = Sulphide Indole Motility

Plate 2 Some morphological and biochemical characteristics of some bacteria isolates

Discussion

In the present study, a total of 10 water samples from different tube wells in University of Yangon campus were collected and analyzed to assess the levels of total coliform and fecal coliform contamination. The water samples were then treated with 2 mg/L calcium hypochlorite with a contact time of 30 minutes. Before treatment with calcium hypochlorite, the total coliform counts of samples I, II, III, IV, V, VI, VII, VIII, IX and X are 150 MPN/100mL, 460 MPN/100mL, 460 MPN/100mL, 23 MPN/100mL, >1100 MPN/100mL, >1100 MPN/100mL, >1100 MPN/100mL, 43 MPN/100mL, 210 MPN/100mL and >1100 MPN/100mL respectively. And the fecal coliform counts of samples I, II, III, IV, V, VI, VII, VIII, IX and X are 93 MPN/100mL, 460 MPN/100mL, 460 MPN/100mL, 23 MPN/100mL, >1100 MPN/100mL, >1100 MPN/100mL, >1100 MPN/100mL, 43 MPN/100mL, 93 MPN/100mL and 1100 MPN/100mL respectively. The highest total coliform counts and fecal coliform counts were found in the water samples of S-V (University Laboratory Building), S-VI (Professors' Housing), S-VII (International Cooperation Office) and S-X (Fisheries and Aquaculture Building). The tube well and storage reservoir of S-V is very near to the toilet. There are trees and shrubs around the tube well of S-VI and it seems people also take bath near the tube well because of the existence of a shower facility. And the tube well of S-VII is near to a house and there are some rubbish around the tube well. Furthermore, the storage reservoir of S-VII tube well is very near to the house. Moreover, the tube well of S-X is near a garbage dump.

The second highest total coliform and fecal coliform counts were found in the water samples of S-II (Inya Hostel) and S-III (Shwebo Hostel). It might be due to the presence of decaying leaves on the storage tank of S-II or due to contamination of water pipe and water tap. And, in S-III, the tube well is near to a house. The third highest total coliform and fecal coliform counts were found in the water sample of S-IX (University of Yangon Research Center) because the tube well of S-IX is located between two houses. The fourth highest total coliform and fecal coliform counts were found in the water sample of S-I (Department of Zoology) because the tube well of S-I is near a house.

After treatment with calcium hypochlorite (2mg/L), the highest total coliform counts were recorded in the water samples of S-I (Department of Zoology) and S-II (Inya Hostel) which were 4 MPN/100mL but fecal coliform counts were not observed. Fecal coliform bacteria in the drinking water samples should be 0 counts MPN/100 mL according to WHO (2004). After treatment with chlorine (5mg/L), the highest coliform count recorded was 210 MPN/100mL but fecal coliform counts were not observed (San Thaw Tar Aung, 2017). Total coliforms and fecal coliforms were highest in untreated pond water samples from a Thaketa pond (Su Mon Win, 2014). The global standard for *E. coli* count was 0-1 MPN/100mL in drinking water (Altas, 1993).

In the present study, five groups of bacteria were identified in all water samples before treatment such as *Escherichia coli, Klebsiella* sp., *Salmonella* sp., *Enterobacter* sp., and *Pseudomonas* sp. Among all bacteria isolates, *Pseudomonas* sp. had highest frequency of occurrence in the water samples and it causes from mild symptoms such as ear and eye pain to severe symptoms such as diarrhea, pneumonia and urinary tract infections. *Escherichia coli* causes diarrhea, bloody diarrhea and hemolytic uremic syndrome. *Klebsiella* sp. causes meningitis, endophthalmitis, liver and splenic abscesses and bacteremia. *Salmonella* sp. causes typhoid fever and *Enterobacter* sp. causes respiratory infections, soft tissue infections, osteomyelitis and endocarditis (Ramirez and Giron, 2022). So, all groups of the identified bacteria in the present work are harmful. The isolates of *Escherichia coli* had highest frequency of occurrence in the water samples from Hlawga Reservior (San Thaw Tar Aung, 2017).

The isolates of *Escherichia coli*, *Salmonella* sp., *Enterobacter* sp., *Bacillus* sp., and *Pseudomonas* sp., were found in pond water of all sites in all seasons (Su Mon Win, 2014).

In this research, pH values of the tube well water samples were observed from 5.0 to 7.3 in which some were quite acidic. The WHO drinking water standard of pH was between 6.5 to 8.5 (WHO, 2011). The pH can also fluctuate because of precipitation, rain water and wastewater (Fondriest Environmental Learning Center, 2013). Temperature value was found between 25˚C-28.1˚C in all study sites which was within the WHO limit of 20-30 ˚C. Total dissolved solids (TDS) was found at value of 8-12mg/L. Standard TDS value was <600mg/L according to WHO (2011).

Although there are many harmless *Escherichia coli* strains, the presence of *E. coli* in water indicates that the water is unsafe and may contain other fecal coliforms bacteria. In the present study, fecal coliforms counts before treatment were unacceptable according to WHO (1993) guideline. The presence of fecal coliforms may be because of dirty environment, pipes and tanks. Temperature and total dissolved solids in all water samples were normal but some of the pH values were under the limits. So, these water supplies from the tube wells were not suitable to drink without chlorination or boiling. A regular chlorine treatment should be performed before consumption.

Conclusion

In the present study, total coliform and fecal coliform counts were high in all the water samples tested. Indication of the presence of bacteria in the water samples suggested that the water was not fit for drinking without proper processing. The physico-chemical parameters of temperature and total dissolved solids in all water samples were normal according to WHO guidelines. But some of the pH values were lower than the WHO standard limits. It was suggested that some pH levels were acidic or declining because of precipitation of rain water and contamination by wastewater. After treatment with 2mg/L calcium hypochlorite, total coliform counts were considerably lowered and fecal coliform counts were not observed. But the fecal coliform counts before treatment was unacceptable according to WHO (1993) guideline and they were harmful to humans. It might be due to some tube wells being very near to the toilets, houses and garbage dump. In addition, trees, shrubs and decaying leaves were also not far enough from the tube wells and the storage tanks. It might also be due to dirty water pipes and contaminated water taps. So, these water supplies are unsafe to drink without chlorination or boiling or other suitable processing.

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MOLECULAR GENETIC VARIATION IN *ANABAS TESTUDINEUS* (BLOCH, 1792) FROM SOME RIVERS OF MYANMAR

Pann Myat Shwe Yee[1]

Abstract

A total of thirteen fish samples of *Anabas testudineus* were collected from four different geographical sites, Maubin, Sittwe, Dawei and Kalay of Myanmar, to examine the morphology and molecular genetics during the study period of December 2019 to August 2020. In the present study, the 15 morphometric variable characteristics were recorded for all *Anabas* specimens. The largest total length of 17cm was observed for the species of Maubin and the smallest of 8.5cm for that of Sittwe. The dorsal fin had 16 to 18 strong spines and 8 to 10 soft rays inserted over or slightly in advance of the pectoral fins; the anal fin was with 8 to 11 spines and 9 to 11 soft rays. The body weight was the highest, 82.10g, in Maubin and the lowest is 11.26g in Sittwe. Extraction of high-quality and quantity DNA was conducted, that was a fundamental requirement for genetic research. The highest quality of DNA from fish samples (value of A260/A280 was 2.02) from Sittwe, and the lowest quality (value of A260/A280 was 1.74) from Maubin were obtained. The mitochondrial cytochrome oxidase subunit I (COI) genes were amplified using PCR reaction. A product ~700 bp length was obtained. After the sequencing, a total 4 haplotypes with 2 variable sites, haplotype diversity of 0.67 and nucleotide diversity of 0.32%, were noted for CO1 gene (615 bp, n=4) dataset of the studied species. Genetic distance ranged from 0.0016 to 0.0032% among four samples of *Anabas testudineus* for CO1 gene. In Neighbor-joinging (NJ) and Maximum likelihood (ML) phylogenetic tree analyses, all four samples of native Myanmar *Anabas testudineus* clustered into a strong single cluster for all datasets. Inferred ancestral sequences tree was constructed using CO1: AS, AD, AM1 and AK among sites treated as being uniform G nucleotide.

Keywords *Anabas testudineus,* CO1 gene, haplotype, nucleotide diversity, genetic distance, phylogenetic tree.

Introduction

Perciformes is the largest and most diverse order of teleosts in the world, containing about 41 % of all bony fish comprising greater than 10,000 species and about 160 families (Nelson, 2006). The family Anabantidae belonged to Perciformes, with thirty-four species in it. There are two identified species in the genus *Anabas*: *Anabas cobojius* (Hamilton, 1822) and *Anabas testudineus* (Bloch, 1792 Froese and Pauly, 2012). The climbing perch, *A. testudineus* (Bloch, 1792) is an associate of this family, being considerable in several components of Asia: Bangladesh, China, India, Malaysia, Myanmar, Pakistan, Philippines, Sri Lanka, and Thailand (Rahman, 2005). This is a very hardy fish and plays a significant role in fisheries and aquaculture practices (Froese and Pauly, 2014). Therefore, it will bear extremely adverse water conditions such as low oxygen, polluted water, and so on (Pethiyagoda, 1991) through an accessory air respiration organ referred to as the labyrinth organ (Rahman, 1989).

In Myanmar, the climbing perch is known by the local name of Nga bye ma and well known as a delicacy for its great taste. This species is considered as a valuable item of diet for sick and convalescents (Saha, 1971).

The application of DNA barcoding in the form of sequence data of cytochrome c oxidase subunit I mitochondrial gene (mtDNA-COI) has been appreciably used for taxonomy study and organism identification (Yudhistira and Arisuryanti, 2019). It is now considered highly desirable to include sequences from mitochondrial COI gene to identify freshwater fish species accurately

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(Dahruddin *et al*., 2017). In fish, DNA barcodes were advanced as a rapid and correct device for species identity through the use of universal primers (Ivanova *et al.*, 2007).

Generally, all the freshwater fish were identified based on their morphology. Nevertheless, the morphological identification, sometimes, wasn't generally inaccurate and incorrect e.g. climbing perch and gourami (RIPED, 2008). Molecular identity the usage of the COI gene as a DNA barcoding marker is required to investigate the correct species name of the freshwater fish COI gene as a DNA barcoding marker has many advantages such as it can be used for small amount samples of the life stages, and differentiation between similar phenotypes of fish (Dudu *et al*., 2016).

Traditional morphology-based taxonomic procedures are time-consuming and now no constantly sufficient for identity to the species level, and therefore a multidisciplinary technique to taxonomy that consists of morphological, molecular, and distributional data is vital (Krzywinski and Besansky, 2003). Hebert *et al.* (2003a, b) have proven that the analysis of short, standardized genomic regions (DNA barcodes) can discriminate morphologically recognized animal species. In fact, it has already been established that only those COI sequences that meet these strict standards might be precise as DNA barcodes by the National Center for Biotechnology Information's GenBank (NCBI, GenBank; www.ncbi.nlm.nih. gov/Genbank), the European Molecular Biology Laboratory (EMBL; www.embl.org), and the DNA Data Bank of Japan (DDBJ; www.ddbj.nig.ac.jp).

According to Hubert *et al*., (2008) sequencing the fish cytochrome oxidase subunit 1 (COI) gene is an efficient DNA barcoding technique for identifying freshwater fish species and creating a phylogenetic tree. It can aid in our understanding of the evolutionary history of morphological and ecological traits in marine invertebrates and other organisms (Arrigoni *et al.*, 2014).

Hence, in the present study, an attempt was made to analyze the genetic diversity of native Myanmar *Anabas testudineus* (Nga bye ma) based on morphology and COI sequence data to investigate the genetic variation between populations.

Materials and Methods

Study areas

The present study areas for sample collection were located in Ayeyarwady Region, Tanintharyi Region, Rakhine State, and Sagaing Region (Fig.1). Molecular genetics laboratory works were carried out at the Department of Zoology, Dagon University.

Study period

The study was carried out from December 2019 to August 2020.

Specimen collection

A total of 13 fish specimens of native climbing perch *Anabas testudineus* (Bloch, 1792) were randomly collected from local fishermen at four geographical sites; Maubin, Ayeyarwady Region (n=10), Dawei, Tanintharyi Region (n=1), Sittwe, Rakhine State (n=1), and Kalay, Sagaing Region (n=1). The sample codes were named as Maubin (AM), Dawei (AD), Sittwe (AS) and Kalay (AK).

Morphometric examination

Initially, the external characteristics including color, body shape, head, and tail of collected fish individuals were examined according to the methods of Talwar and

Jhingran (1991) and Barman *et al*. (2014). Then, the morphometric measurements such as body weight, total length, standard length, and 12 other parameters including dorsal fins, anal fins, pectoral fins, and so on were taken. After the examination, the fins (dorsal, anal, and pectoral) were clipped and immediately preserved in the 1.5 ml microcentrifuge tube containing 70% Analytical grade ethanol and kept in a freezer (-20°C) until the DNA Extraction.

Genetic analysis

Genomic DNA extraction

For the genomic DNA extraction from the stored fin tissues, approximately ≤25 mg of the stored fin tissues were taken and put into 1.5 ml microcentrifuge tube. Then, genomic DNA was extracted following the protocol provided by the PureLink Genomic Kit (Invitrogen).

Quantification of extracted genomic DNA

The quality and quantity of extracted genomic DNA were measured by using NanoDrop^{TM} spectrophotometer. The amount and purity of extracted DNA (1µl) were measured and observed for A260/280 ratio and A260/230.

PCR amplification using COI gene primers

The amplification of DNA was carried out in thermal-cycler (Simpli Amp PCR System). The COI gene approximately ~700 bp fragment length located in the mitochondrial genome of the climbing perch was successfully amplified using the universal fish primer named Fish F1 and Fish R1 (Ward *et al*., 2005). The sequences of the primers are: Fish forward F1 (5´TCA ACC AAC CAC AAA GAC ATT GGC AC 3´) and Fish reversed R1 (5´TAG ACT TCT GGG TGG CCA AAG AAT CA 3´). Each 25 µL PCR reaction comprises as: 2 µL of extracted DNA template, 12.5 µL of Gold Taq® G2 Master mix (Ref. M743A, Promega, Madison, WI USA), 1 µL (0.01 mM) of forward primer, 1 µL (0.01 mM) of reverse primer, and 8.5 µl of nuclease free water (Ref. P119A, Promega, Madison, WI USA). The PCR thermal cycling conditions involved an initial denaturation at 94°C for 3 min, denaturation at 94°C for 45 s, annealing temperature of 50°C for 45 s and elongation temperature of 72°C for 1 min for 30 cycles, and concluded with a final elongation step at 72°C for 7 min followed by a hold at 4°C.

Agarose gel electrophoresis

The amplified PCR products were checked in 1% agarose gel at 135 VDC for 25 mins. Gel was then stained using ethidium bromide (EtBr) solution. PCR bands were visualized under the UV transilluminator and photographed. The 100 bp ladder (Thermo Fisher Scientific) was used to estimate PCR band size in bp (base pairs).

Purification of PCR product

The successful PCR products were purified by using Exosap Kit (Thermo Fisher Scientific BallicsUAB). Firstly, 5 µl of PCR product was mixed with 2 µl of Exosap. And then, thermal cycling was done at 37ºC for 15 min and 80 ºC for 15 min.

The purified PCR product was done before sequencing. For this reaction, the same primer set (Forward and Reversed) was separately used for each purified PCR product of the sample. For the control, pGEM -3zf (+) as the template sample and -20M13 as control primer were used. Total reaction volume was 20 µl and 30 cycle sequencing. Each 20 µl of sequencing components were prepared as follows: 4 µl of Big Dye Mix, 2 µl of 5X buffer, 11 µl of Nuclease free water, 1 µl of primer and 2 µl of 1st purification product. The running conditions for the sequencing were as follows: hold duration at 96 ºC for 2min, denaturation at 96 ºC for 30 sec, annealing temperature of 55 ºC for 15 sec, and extension temperature of 60 ºC for 4min followed by a hold at 4 ºC.

For the preparation of 2nd purification using Centri-sep (Thermo Fisher U.S.A), the cap of the tube was opened and added 800 µl nuclease-free water. After that the bottom cap of the tube was removed and placed into a new 2 µl collection tube and checked to drain and incubated 30 min at room temperature. The reaction vessels can be successfully precipitated and become visible and measure at least 200-250 µl removed by pipetting. Totally the removed solvent had to retain 500 µl. Finally, the collection tube was discarded and placed it in the new sterile 1.5 ml microcentrifuge tube.

Next step, 20 µl of cycle sequencing product was slowly added at the center of the centri-sep by pipetting. After that, the tube was centrifuged for 2 min at 1800 rpm and it would be resuspended in a 1.5 ml sterile tube and obtained a second purification product. It was kept into a laminar flow and inverted it for overnight. If the dry sample was added 20 µl Hidi formamide (Thermo Fisher Scientific) into the tube and mixed well by vortex to obtain a homogeneous solution and spring down by mini centrifuge. Eventually, this tube was incubated in 95 ºC for 2 min and immediately transferred into the -20 ºC and left for 2 min. This prepared sample can be ready to use in the 96 well plate by pipetting.

Sequencing in ABI 3500 Genetic Analyzer

DNA sequencing was done in ABI 3500 Genetic Analyzer auto sequencer (Applied Biosystems). The obtained sequence data were downloaded from ABI 3500 Computer onto CD discs and transferred to the laptop computer. And then, the sequence was edited by using MEGA-X software. After alignment, the sequencing data from the chromatogram was converted into STARDANT format and identified the sequence using BLAST at the nucleotide database of the National Center for Biotechnology Information (NCBI) to determine the best match homology.

Genetic analysis

Haplotype number, haplotype diversity, nucleotide diversity, polymorphic sites, genetic distances, and phylogenetic analyses of the aligned sequences were conducted by using MEGA X software, BioEdit V.7, and DNA Sequence Polymorphism V6.12.03.

The reference sequences for *Anabas* species were obtained from GenBank/ EMBL database.

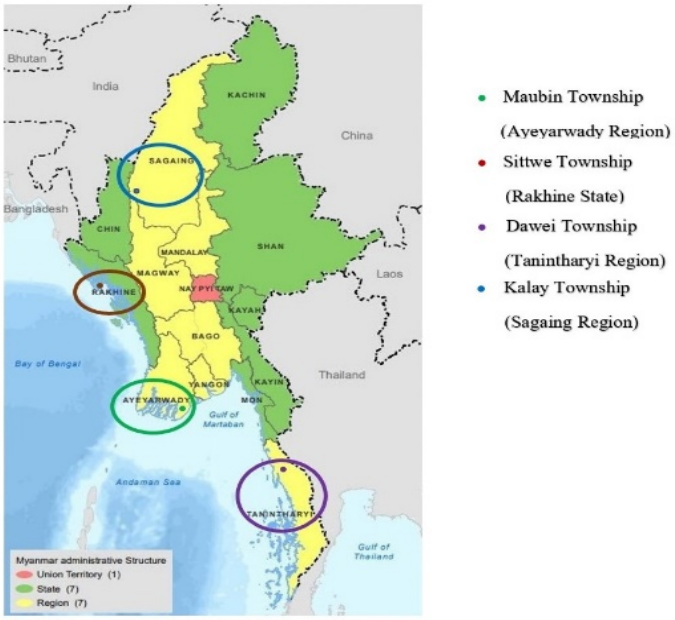


Figure 1 Location map of fish sampling sites in Myanmar

Results

External appearance of studied *Anabas testudineus* species

External characteristics including color, body shape, head and tail of fish individuals were observed in the present study. Some remarkable change in color for *A. testudineus* was observed among the collected specimens from different study sites. The color of the dorsal surface is dark to pale greenish, very pale below, back dusky to olive found in Maubin and Dawei. Ventrally longitudinal stripes were observed on the head of all examined *Anabas* species. The iris is golden reddish color in the fish from Maubin.

Morphometric measurements of studied *Anabas testudineus* species

According to the data obtained, four *Anabas* species are similar in eye diameter (0.8cm). Dorsal spine rays (17), dorsal soft rays (8), and anal soft rays (8) were recorded at the same number. The variation of total length (17cm) and body weight (82.10g) was highest in the fish of Maubin *Anabas* whereas in the smallest of total length (8.5cm) and body weight (11.26g) in the fish of Sittwe. All studied species have dorsal fin (16-18) strong spines and the inserted soft rays (8-10) over or slightly in advance of pectoral fins; anal fin was (8-11) spines and soft rays (9-11) (Plate 1).

Concentration and purity of extracted genomic DNA

The different total concentrations of genomic DNA were observed as Maubin (516.3 ng/µl), Sittwe (1248.5 ng/µl), Dawei (212.9 ng/µl), and Kalay (1353.3 ng/µl). The results of calculated (A260/280nm) ratio by the measurement of the NanoDrop spectrophotometer showed the values of Maubin (1.74), Sittwe (2.02), Dawei (1.80), and Kalay (2.00) indicating a high yield of genomic DNA quality. Similarly, absorbance at 260/230 nm ratio had acceptable contamination showing the value of 1.78, 2.36, 1.95, and 2.16 respectively. It indicated continuing PCR amplification process (Table 1).

PCR amplification using CO1 (Cytochrome oxidase subunit 1) marker gene

All extracted DNA samples were successfully amplified using CO1 primers by PCR. The product was visualized under UV light with 1% agarose gel after electrophoresis checking. The length of mtDNA fragments was ~700 bp by comparing the length of 100 bp Ladder (Plate 2). All PCR products were used in the next step of DNA sequencing.

Genetic Diversity

After the sequencing on ABI 3500 Genetic Analyzer, four CO1 gene sequences (615 bp) were obtained.

Haplotype and nucleotide diversity

Out of four individuals analyzed two different haplotypes were yielded from two variable sites. Haplotype 2 was found in fish individuals from Sittwe and Dawei. The transitional mutation points from C to T were found at the point of 6 bp and 594 bp of the sequence. The haplotype diversity (HD) was 0.67. The nucleotide diversity (π, %) was 0.32% (Table 2).

Genetic distances

Table 3 showed the pairwise genetic distance and evolutionary divergence among the species collected from different study sites.

Phylogenetic relationship

The phylogenetic relationships of *Anabas* species were investigated based on the CO1 gene (615 bp) using the three phylogenetic reconstruction methods.

The obtained four sequence datasets were analyzed by aligning with a total of 16 reference sequences obtained from GenBank. There were six sequences from India, four sequences from Indonesia, one sequence from Korea and Vietnam, and four sequences from Bangladesh (Table 4).

The pairwise genetic distance and evolutionary divergence of the studied sequence with the reference dataset were shown in (Table 5) and sequence alignment stated.

The nucleotide BLAST analysis result and identification of all twenty data of COI mitochondrial gene sequence of the fishes from different regions were obtained. The sequences of the COI mitochondrial gene of sample AK, AM 1, AD, and AS have a similarity of 90-93% if compared to *A. testudineus* recorded in the GenBank database by applying BLAST analysis and identification. The specimen had a similarity score between 92-95% with the species which had been recorded at GenBank. Again, *A. cobojius* as an outgroup database had a similarity of 76-95%.

Multiple phylogenetic analyses showed nearly identical topologies. All examined species belonged to one clade.

Neighbor-joining (NJ) tree analysis and maximum likelihood (ML) tree analysis

This was strongly supported by the neighbor-joining phylogenetic tree, which divided the haplotypes into four discrete allopatric clusters, corresponding to the individual drainage system. However, as noted in the nucleotide divergence values, the differentiation was correlated with geographical distance. The examined haplotypes were located in one cluster with a high supporting bootstrap value.

All other references from GenBank, consisting *Anabas* species formed distinct species-specific clades in the NJ tree. Outgroup is *A. cobojius* references from India and Bangladesh (Fig. 1).

Also, the examined haplotype was located within the same clade in maximum likelihood (ML) tree analysis (Fig. 2).

Inferred ancestral sequences tree

The tree showed a set of possible nucleotides (states) at each ancestral node based on their inferred likelihood at sites A, T, C, and G. For each node only the most probable sites are shown in Fig 3. The initial tree was inferred using the method, AS, AD, AM1, and AK among sites were treated as being uniform G nucleotide. T nucleotide among sites in India, A nucleotide sites in Indonesia, and C nucleotide among sites in Bangladesh.

Molecular genetic characteristics among Myanmar *Anabas testudineus*

Table 6 shows a summary of data analysis of CO1 615bp (n=4) sequence analysis. Out of four sequences, two haplotypes were noted (two in AS and AD with two variable sites: the other identical in AM1 and AK) with a total number of mutations (n=2). In four sequences of study Myanmar *Anabas* species, the nucleotide diversity (π, *%)* was 0.32% and haplotype diversity (HD) was 0.67.

Table 1 Concentration and purity of extracted genomic DNA from *A. testudineus*

Sr. No.	Specimen code	Amount ng/µL	A260/A280	A260/A230
1.	AM 1	516.3	1.74	1.78
2.	AS	1248.5	2.02	2.36
3.	AD	212.9	1.80	1.95
4.	AK	1353.3	2.00	2.16

Table 2 Polymorphic sites, haplotype diversity, and nucleotide diversity

No. of Sequences (n)	No. of haplotype	No. of polymorphic sites	Haplotype diversity (HD)	Nucleotide diversity (π, %)
4	2	2	0.67	0.32

Table 3 Net nucleotide diversities between the haplotypes as a pairwise distance measured based on 615 bp of CO1 gene

Lineage	AK	AM 1	AS	AD
AK	-			
AM 1	0.0016	-		
AS	0.0032	0.0032	-	
AD	0.0032	0.0032	0.0000	-

Table 4 Haplotypes and accession numbers of fish CO1 mtDNA sequences

Haplotype	GenBank Accession No.	Location
AM 1 AK AS AD		This study
Anabas testudineus, India (2019)	MK213550.1	GenBank
Anabas testudineus, India (2019)	MK213553.1	
Anabas testudineus, Indonesia (2020)	MN640070.1	
Anabas testudineus, Indonesia (2020)	MN640071.1	
Anabas testudineus, Indonesia (2020)	MN640072.1	
Anabas testudineus, Indonesia (2020)	KU692243.1	
Anabas testudineus, India (2019)	JX983214.1	
Anabas testudineus, India (2019)	MK213554.1	
Anabas testudineus, Korea (2019)	MK359929.1	
Anabas testudineus, Bangladesh (2019)	MG552721.1	
Anabas testudineus, Bangladesh (2019)	MN083164.1	
Anabas testudineus Vietnam (2019)	MH721200.1	
Anabas cobojius India (2019)	MK213553.1	Outgroup GenBank
Anabas cobojius India (2014)	KC774636.1	
Anabas cobojius Bangladesh (2019)	MK572025.1	
Anabas cobojius Bangladesh (2019)	MK572024.1	

Dataset: 20 sequences of CO1 mtDNA

Table 5 Estimates of evolutionary divergence between studied sequences and references from GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AK_(Kalay)																				
AM_1_(Maubin)	0.0016																			
AS_(Sittwe)	0.0032	0.0032																		
AD (Dawei)	0.0032	0.0032	0.0000																	
A.testudineus_MK213550.1-India	0.0000	0.0016	0.0032	0.0032																
A.testudineus_MK213553.1-India	5.1291	5.2133	5.1858	5.1858	5.1291															
A.testudineus_MK213554.1-India	5.0565	5.1389	5.1100	5.1100	5.0565	0.0031														
A.testudineus_MN640070.1-Indonesia	5.8462	5.8462	5.9582	5.9582	5.8462	6.9555	7.1047													
A.testudineus_MN640071.1-Indonesia	7.3007	7.1484	7.2856	7.2856	7.3007	5.9100	6.0015	6.3644												
A.testudineus_MN640072.1-Indonesia	6.3631	6.3631	6.3871	6.3871	6.3631	6.5165	6.5165	8.0287	6.8593											
A.testudineus_KU692243.1-Indonesia	10.5546	10.4321	10.3902	10.3902	10.5546	11.1892	11.2749	6.5597	8.1281	6.3094										
A.testudineus_JX983214.1-India	10.7766	10.6623	10.6321	10.6321	10.7766	11.1795	11.2647	6.4342	8.0095	6.3650	0.0785									
A.cobojius_MK213553.1-India	5.1291	5.2133	5.1858	5.1858	5.1291	0.0000	0.0031	6.9555	5.9100	6.5165	11.1892	11.1795								
A.cobojius_MK572025.1-Bangladesh	10.7766	10.6623	10.6321	10.6321	10.7766	11.1795	11.2647	6.4596	8.0095	6.3464	0.0768	0.0015	11.1795							
A.cobojius_MK572024.1-Bangladesh	10.7766	10.6623	10.6321	10.6321	10.7766	11.1795	11.2647	6.4596	8.0095	6.3464	0.0768	0.0015	11.1795	0.0000						
A.cobojius_KC774636.1-India	5.1181	5.2019	5.1974	5.1974	5.1181	4.7117	4.6409	5.4906	6.1860	5.2928	11.4381	11.5990	4.7117	11.5990	11.5990					
A.testudineus_MG552721.1-Bangladesh	10.4147	10.4147	10.4910	10.4910	10.4147	11.8105	11.8856	4.3640	11.1522	7.3899	8.1293	8.0265	11.8105	8.0017	8.0017	11.2445				
A.testudineus_MK359929.1-Korea	8.0786	8.0786	8.0786	8.0786	8.0786	8.0322	7.9273	7.8321	8.5152	11.3792	6.2799	6.3089	8.0322	6.2848	6.2848	10.9055	4.5419			
A.testudineus_MN083164.1-Bangladesh	9.2751	9.2751	11.3476	11.3476	9.2751	8.2493	8.1749	8.2635	11.7704	11.3964	6.6303	6.7165	8.2493	6.6914	6.6914	7.9281	7.9456	4.8519		
A.testudineus_MH721200.1-Vietnam	5.0983	5.0983	5.0039	5.0039	5.0983	5.4063	5.3094	4.7327	6.5584	7.1619	10.7367	10.8228	5.4063	10.7969	10.7969	4.7531	8.1605	8.0850	11.0477	

Table 6 COI gene sequence with variable sites of fish *Anabas* species from different sites

n = 4; variable sites = 2; haplotype = 2
615 bp alignment dataset

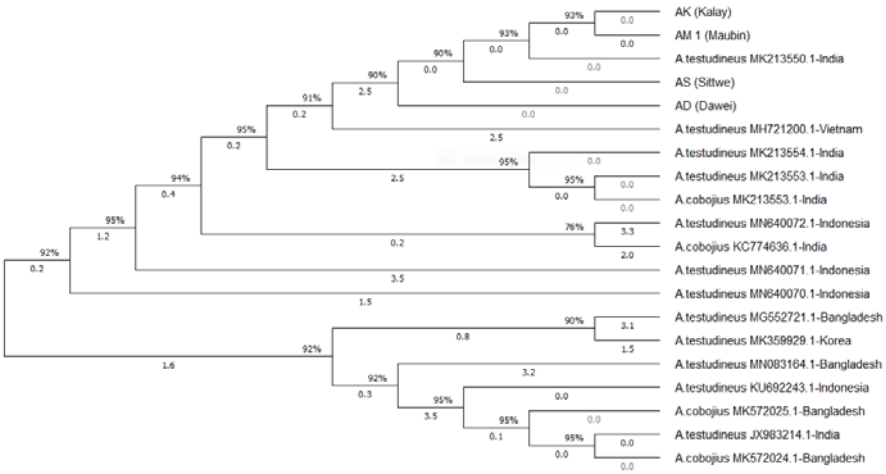


Figure 1 Neighbor joining phylogenetic tree constructed by using CO1 (615 bp) dataset of the present studied *Anabas testudineus* (n=4).

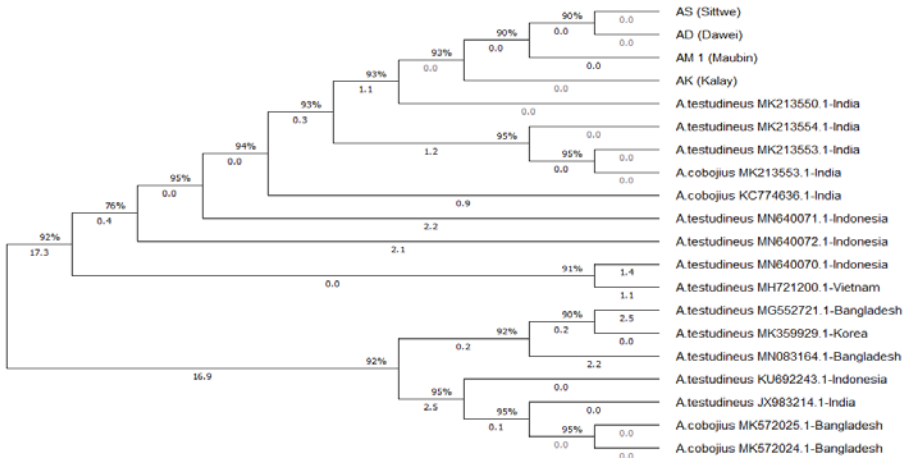


Figure 2 Maximum Likelihood phylogenetic tree constructed by using CO1 (615 bp) dataset of the studied *Anabas testudineus* (n=4).

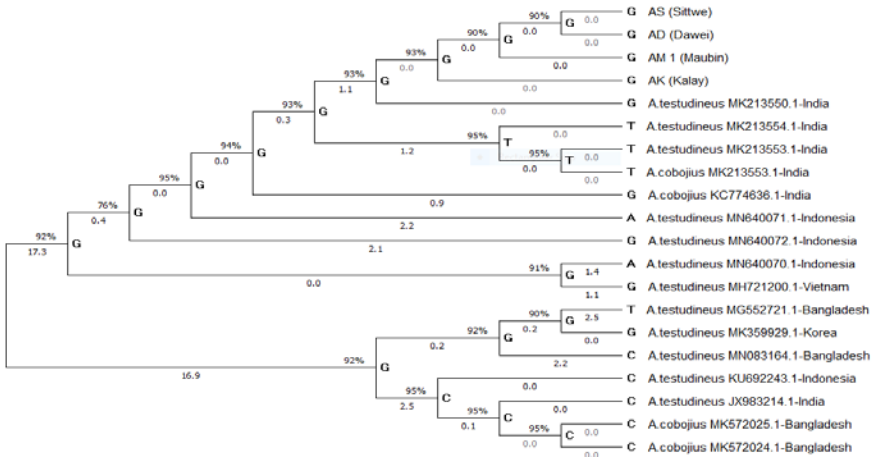
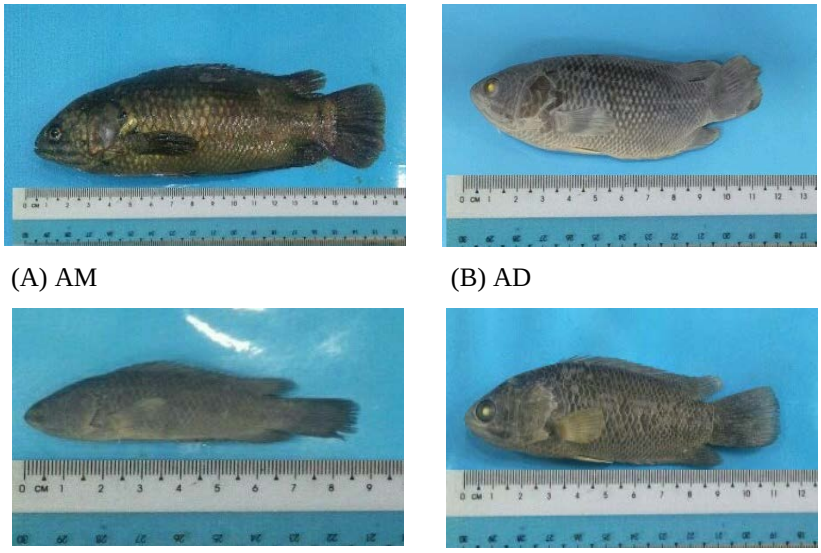


Figure 3 Inferred ancestral sequences tree constructed by using CO1 (615 bp) dataset of the studied *Anabas testudineus* (n=4).



(A) AM (B) AD

(C) AS (D) AK

Plate 1 Some studied fish *Anabas testudineus* from different collection sites
AM=Maubin, AS=Sittwe, AD= Dawei, AK= Kalay

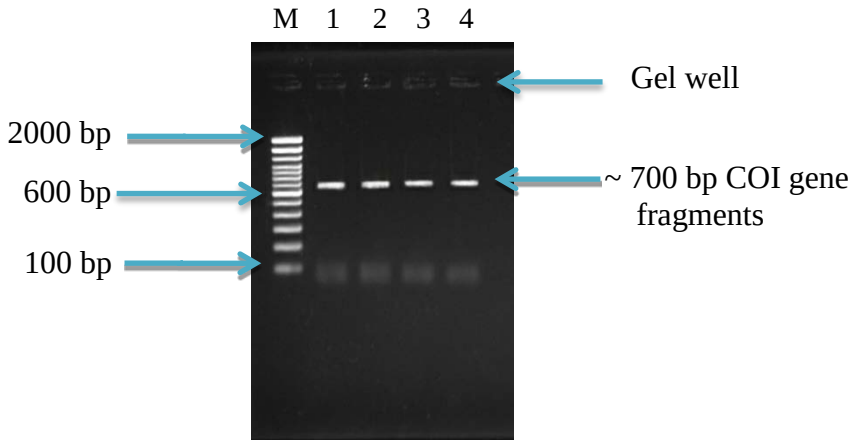


Plate 2 Gel Electrophoresis of PCR product of 1% agarose gel, Lanes 1: AM 1, 2: AS, 3: AD, 4: AK; Lanes M: CSL- MDNA 100 bp Makers; bp: base pair

Discussion

Anabas provides a significant contribution to the lake fisheries for as long as fifty years and as a native fish holds great ecological and economic significance with its exceptional export value and species abundance. *Anabas testudineus* (Bloch, 1792) is an economically critical freshwater species in Southeast Asia countries.

Traditionally, fish species identity has relied on morphological traits, such as body shape, number of scales or fin rays, and coloration patterns. Morphometric and meristic characters are utilized generally to distinguish fish stocks (Turan *et al*., 2004) and it has often been utilized in discrimination and classification studies by statistical techniques (Avsar, 1994). Variety studies based on morphological and molecular approaches can provide valuable information that helps in cataloguing the bioresources, their sustainable use and designing effective conservational techniques (Padmavathi and Gatreddi, 2017). In the present study, the fishes *Anabas* collected

from different geographical locations were separately distinguished and measured for total length (TL), body depth (BD), body weight (Gram) and so on. The result revealed that the variation of the highest length (total length) of the species ranged as 17cm in Maubin, 11.7cm in Kalay, 11.2 in Dawei and 8.5cm in Sittwe region. The body weight is highest in Maubin (82.10g) and lowest in Sittwe (11.26g).

According to the present genetics study, the result was efficient in extracting genomic DNA from the dorsal fins of all fishes. Then Nanodrop spectrophotometer measurements indicated variation in concentration and purity of DNA extracts. The total concentrations ng/µl of the sample were found as AM 516.3, AS 1248.5, AD 212.9, and AK 1353.3. The accurate DNA extraction results at A260/280nm were calculated by visible spectrophotometer with the values AM 1.74, AS 2.02, AD 1.80, and AK 2.00 indicating high yield of genomic DNA quality. Also, the results of absorbance at 260/230 nm calculating with the values were recorded as AM 1.78, AS 2.36, AD 1.95, and AK 2.16. The results showed that the DNA of fish sample from Sittwe and Kalay were much better than other samples in DNA concentration.

As a promising alternative to the traditional species identity based on morphological characters, partial Cytochrome C oxidase subunit I (COI) sequences (DNA barcodes) had been recommended for standardized and routine species identification (Hebert *et al*., 2003b). The present work was concluded as the first attempt to assess the molecular genetic status and phylogenetics of the Myanmar Climbing perch *Anabas testudineus* (Bloch, 1792) (Nga bye ma) as a native species. By using COI barcoding and identification technique, the sequence was aligned on 615 bp length for comparison between different sequences. The sequence was checked for the mutation points before analysis. The COI data combined with the morphometric analysis enabled us to ascertain the fish species. Phylogenetic and molecular evolutionary analyses were conducted using the genetics software MEGA X (Molecular Evolutionary Genetics Analysis) (Tamura and Nei, 1993), by NJ (Neighbour-Joining) and Maximum Likelihood (ML) methods and Inferred ancestral sequences (Kumar, 2018) analyses.

The results revealed that the Nga bye ma specimens from four geographical sites (i.e Maubin, Sittwe, Dawei, and Kalay) clustered in a single clade with strong supporting confidence values in the Neighbor–joining (NJ) and Maximum likelihood (ML) phylogenetic tree analyses. This means that the Myanmar Nga bye ma (*Anabas testudineus*) is distinct species different from other related species referenced from GenBank such as *Anabas testudineus* and the same genus *Anabas cobojius.* Regarding haplotypes, CO1 analysis, it reveals only two haplotypes among the four Nga bye ma specimens from four geographical sites, with only two variable nucleotide sites found in only AS (Sittwe) and AD (Dawei) whereas AK (Kalay) and AM1 (Maubin) showing identical haplotype.

It indicated mitochondrial DNA lineage with two variable sites indicating genetic diversity among the populations of *Anabas testudineus* distributed in the coastal region, Ayeyarwady Delta, and Chindwin basin of Myanmar.

In the obtained data of the present study, evolutionary divergence composed of different CO1 haplotype sequences has no remarkable genetic distance ranged from 0.0016 to 0.0032%. If nucleotide G among sites AS, AD, AM1, and AK in native Nga bye ma in Myanmar is compared with the same G sites of India, Indonesia, Korea, and Vietnam, it showed similarity (90-95%) whereas nucleotide T sites of India and Bangladesh had (90-95%) and then outgrowth *A. cobojius* species of nucleotide C sites had (92-95%).

Conclusion

The results of the study provided inclusive records. Numerous studies had been devoted to investigate *A. testudineus* in Myanmar including morphological and molecular genetics analysis. The present study revealed remarkably low genetic variation of the fish *A. testudienus* from different regions. It was possible to produce the molecular database that assembles the molecular characteristics of the species distributed in Myanmar. These sequences can also be used in further studies to determine the genetic divergence of *Anabas* species distributed in different geographic locations. The present information assists the management of the fish population in the wild to prevent them from being extinct, and increase awareness of fishermen and local people.

Acknowledgments

I am greatly indebted to Dr Tin Maung Tun, Rector, and Dr Khin Chit Chit, Dr Cho Cho and Dr Thidar Aye, Pro-Rectors, University of Yangon for their constant encouragement. I am greatly indebted to Dr Moe Moe Dwe, Professor and Head (Retired), Zoology Department, Dagon University for his encouragement and valuable advice. Special thanks go to Dr Kay Lwin Tun, Professor and Head, Dr Sandar Win, Dr Aye Aye Khaing, and Dr Khin Wai Hlaing, Professors, Department of Zoology, University of Yangon for giving the opportunity to publish this research paper.

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BIOACCUMULATION OF HEAVY METALS IN TILAPIA FISH (*OREOCHROMIS NILOTICUS*) FROM WATER AND SEDIMENT OF KYET MAUK TAUNG DAM, MANDALAY REGION

Cho Cho Thin[1], Nan Sandar Myint[2], May Thu Aung[3], May Thu Rein Oo[4] Khin Thu Zar Win[5], July Maung Maung[6], Kay Lwin Tun[7]

Abstract

Bioaccumulation of heavy metals in fish causes serious threats to human when they are consumed. Thus, detection of toxic metals concentration levels in aquatic component is important. In this study, bioaccumulation of five metals (Fe, Zn, Cd, Pb, As) in meat, gills and liver of Nine Tilapia fish species (*Oreochromis niloticus*), and their environs (water and sediments) of Kyet Mauk Taung Dam, Mandalay Region were analyzed by Flame Atomic Absorption Spectrometer (FAAS) (Perkin Elmer AAanalyst 800 and Winlab-32 software) in Universities' Research Centre (URC). The study was conducted from July 2019 to May 2020. The levels of heavy metal concentration varied as it depended on various tissues of studied fish species. The highest concentrations of Fe and Cd were found in the liver and those of Zn, As and Pb in the gill. The lowest concentrations of all metals were found in meat. According to the results of Transfer Factors, all tested metals accumulated in different tissues of studied fish species came from water and sediment except Fe which came only from sediment. The values observed for all tested metals concentrations in different tissues of studied fish species and their environs except water were lower than the maximum permissible limits. The concentrations of the heavy metals in different tissues from studied fish species did not exceed the dangerous limits given by WHO/FAO and there was no risk for public by eating this species.

Keyword Meat, Gill, Liver, Water, Sediment, Metal Concentration

Introduction

Dams and reservoirs are mainly constructed for irrigation, power generation, flood control, and water supply. They can serve as a sink for accumulation of heavy metals. Their mobility and availability in aquatic environments are primarily controlled by water quality parameters including pH, dissolved oxygen and organic matter content (Ashby, 2011). Dams and reservoirs also play an important role in facilitating the transportation of heavy metals. When water is released from a dam, resuspension of deposited sediments under high flow rate tends to carry heavy metals downstream (Rodbell, 2014).

Anthropogenic activities continuously uninterruptedly increase the quantity of heavy metals in the environment, especially in aquatic ecosystem. Pollution of heavy metals in the aquatic ecosystem is increasing at an alarming rate and has become an important worldwide problem (Malik *et al.,* 2010). Increase in population, urbanization and agriculture activities have further aggravated the situation (Giguere *et al*., 2004). Heavy metals cannot be reduced and they are deposited, assimilated or incorporated in water, sediment and aquatic animals (Linnik and Zubenko, 2000) and thus, causing heavy metals contamination in water bodies (Malik *et al.*,2010). Therefore, heavy metals can be bioaccumulated and biomagnified via the food chain and finally assimilated by human consumption resulting in health risks (Agah *et al.,* 2009).

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Heavy metals enter the aquatic environment from both natural pathways and a variety of anthropogenic sources (Youn-Joo, 2003), and they can have a negative impact on aquatic ecosystems, the food chain, and human health. The concentration of heavy metals in biological compartments, such as fish muscle, is a complex combination of biological and ecological variables (Barletta, *et al*., 2012). In fish, these elements can cause disturbances in growth and reproduction, as well as histopathological alterations in the skin, gills, liver, spleen, and kidneys (Vitek, *et al*., 2007). In humans, heavy metals accumulation has hazardous effects on the brain, liver, kidneys, lungs, and muscles (Petera and Viraraghavanb, 2005).

The wide ranges of contaminants are continuously introduced into the aquatic environments and fish from polluted waters seriously threaten human health due to the bioaccumulation of toxic substances in muscle and other tissues (Sekhar, *et al*., 2003). Furthermore, these contaminants also accumulate in different organs of fish and can cause lethal and a variety of sub lethal effects. Among these toxic substances, heavy metals include one of the main dangerous groups, because they are toxic, persistent and difficult biodegradable. The pollution and the contamination of many ecosystems with heavy metals result from both anthropogenic and geologic sources (Ozmen, *et al*., 2006).

In current century, it is stated that many priorities heavy metals had made their way into the aquatic ecosystem and that their concentrations constantly increased (Topcuoglu, *et al.*, 2002; Barlas, *et al*., 2006). Various fish species have been found to be good indicators of the heavy metal contamination levels in aquatic systems because they occupy different trophic levels (Burger, *et al*., 2002; Svobodova, *et al.*, 2004; Karadede-Akin and Unlu, 2007). The contamination of heavy metals in organs of fish showed that the aquatic environment is polluted (Farkas, *et al.*, 2000).

Therefore, the present study was to determine the concentrations and bioaccumulation of heavy metals (Fe, Zn, As, Pb and Cd) on different tissues of tilapia fish species that are commercially important and this fish species have been consumed as food by local people. Those of water and sediments from Kyet Mauk Taung Dam were also investigated.

Materials and Methods

Study Area

Kyet Mauk Taung Dam, Mandalay Region situated at 20° 48′ 28.3" N to 20° 50′ N and 95° 15′ 04.4" E to 95° 17′ E was chosen as the study area to analyze element concentrations in tilapia fish species and their environs (water and sediment). The dam is used for irrigation, drinking water and fisheries. The quality of this ecosystem has been degrading due to agriculture and human activities. Therefore, the dam was selected as a study area to investigate the heavy metal concentrations.

Study Period

The study was conducted from July 2019 to May 2020.

Collection of Samples

From the study area, 25 specimens of Tilapia fish species were collected once every two months from local fishermen. Identification of studied fish was carried out followed after Talwar and Jhingran (1991). Collected specimens were washed by tap water until the contamination on the body surface was runoff. Total length (cm) and body weights (g) of specimens were measured. After that, they were dissected using stainless steel scalpels and forceps. A part of each tissue (muscles, gill and liver) was removed and weighed. Samples were put into an oven to dry at 90°C and until reached constant weights. After that they were stored at low temperature

until digestion. Digestion of the samples was carried out according to dry method by using a furnace (Model-L-3383). Water and sediment samples were also collected once every two months at study site during the study period.

Sample preparation

Preparation of water and sediment

Each water sample was filtered through a 0.45 μm Whatman filter. The sample was analyzed directly.

The sediment sample was sun dried, grounded and sieved with 200 mm sieve to obtain a find powder. 1.0 g of dried sediment sample in a crucible was placed in a furnace at 200°-250° C for 30 min, and then made ash for 4 hours at 480° C. Then the sample was removed from the furnace and cool down, 2mL of nitric acid was added and evaporated to dryness on sand bath. Then, 2 mL of concentrated HCl was added and transferred to furnace in which the temperature was raised slowly to 450° C and hold at this temperature for 1 hour. The crucible was then removed, cooled and 50mL of deionized water was added. The solution was filtered through 0.45μm Millipore filter paper and then transferred to 25 mL volumetric flask by adding distilled water (Issac and Kerber, 1971). The digested sediment sample was analyzed for heavy metals using the Flame Atomic Absorption Spectrometer (FAAS) (Perkin Elmer AAanalyst 800 and Winlab-32 software) at Universities' Research Centre (URC).

Preparation of fish

Meat, gill and liver samples were dried to constant weight in an oven and dried samples were weight and stored in airtight containers. Digestion was conducted according to dry method. Five grams of dry sample was placed into crucible. And then transfer to a furnace (Model-L3383) and slowly raise temperature to 500° C for 2 hours. Samples were allowed to ash overnight. Once removed, samples were allowed to cool in room temperature and 5 mL nitric acid were added and swirl. After that 10mL HCl were added. The digestion was transferred to furnace and slowly raised temperature to 500° C and hold at this temperature for 1 hour. The crucible was removed, cooled and added 50mL deionized water and transferred to volumetric flask.

Transfer Factor

Transfer factor (TF) in fish tissues from the aquatic ecosystem, which include water and sediments, was calculated according to Kalfakakour and Akrida-Demertzi (2000) and Rashed (2001) as follows:

$$TF = \frac{\text{concentration of metal in fish tissue}}{\text{concentration of metal in environ (water or sediment)}}$$

TF greater than 1 indicates bioaccumulation of metals in fish tissue.

Chemical Analysis

The concentration of elements (Iron, Zinc, Arsenic, lead and cadmium) in different tissues (meat, gill and liver) of studied fish species and aquatic environs of the study area were analyzed tri-replicates by Flame Atomic Absorption Spectrometer (FAAS) (Perkin Elmer AAanalyst 800 and Winlab-32 software) in Universities' Research Centre (URC) at University of Yangon. The results were compared with WHO/FAO maximum permissible limits.

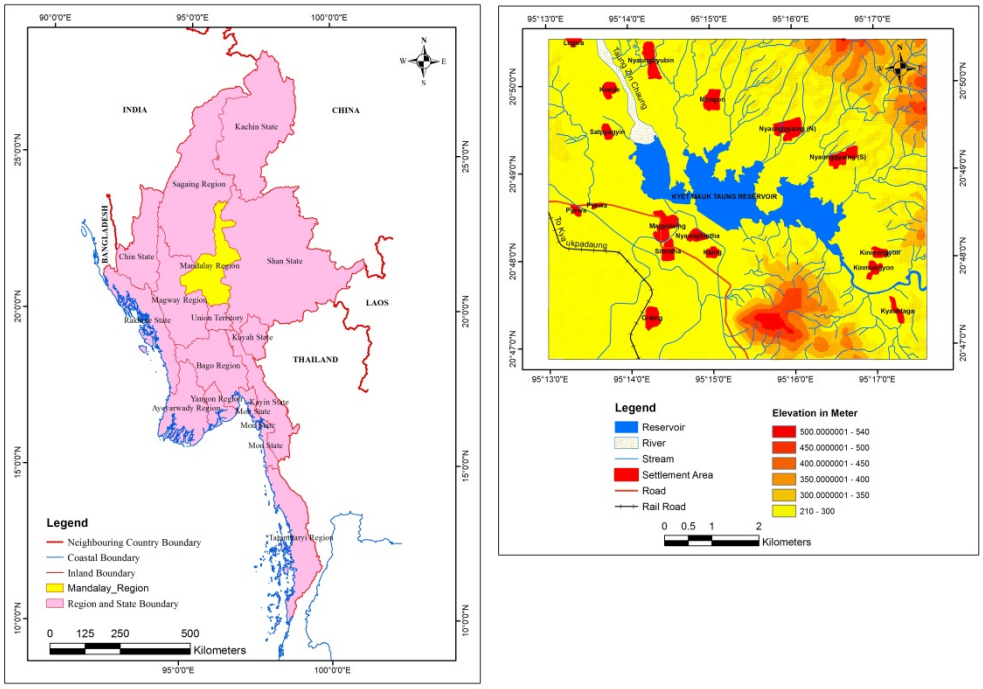

Figure 1 Map of the study area and study sites

Results

A total of 25 individuals of Tilapia fish (*Oreochromis* sp.) were collected from Kyet Mauk Taung Dam, Mandalay Region during the study period (Fig. 1). The size and total weight of collected fish species were presented in Table 1.

The concentrations of heavy metals (iron, zinc, arsenic, lead and cadmium) in different tissues (meat, gill and liver) of studied fish species were presented in Fig. 2, 3, 4, 5, and 6 respectively. Mean concentrations of heavy metals in different tissues of studied fish species were shown in Fig. 7.

The concentrations of iron in liver of studied fish species were found to be higher than those of other organs (meat and gill) during the study period except in September. In September, gill accumulated the highest iron concentrations.

The result showed that, gill accumulated the highest concentrations of zinc in November while lowest concentration in March. Iron and zinc concentrations in different tissues of studied fish species were found to be lower than those of maximum permissible limits recognized by WHO/ FAO (1989, 1990, 2008) (Table 2).

Arsenic concentrations were only found in gill in March and were found to be higher than those of maximum permissible limits.

In the results of present study, gill accumulated the highest concentrations of lead in September whereas muscle accumulated lowest concentrations in November. In July and September, lead concentrations of gill were found to be higher than those of maximum permissible limits recognized by WHO/ FAO.

In cadmium concentrations, liver accumulated the highest concentrations in November and the mean concentrations of Cd in liver were found within the maximum permissible limits recognized by WHO/ FAO.

The mean values of heavy metals (Fe, Zn, As, Pb and Cd) concentrations in different tissues of studied fish species were shown in Fig. (7). The highest concentrations of Fe and Cd were found in liver and those of Zn, As and Pb in gill. The lowest concentrations of all metals were found in meat. The mean values of metal concentrations in different tissues were found to be lower than the maximum permissible limits recognized by WHO/ FAO.

Iron and Zinc concentrations of water were found to be lower than those of maximum permissible limits recognized by WHO/ FAO. Cadmium (0.1 mg/L, 0.089 mg/L, and 0.083 mg/L) concentrations of water in November, January and March were higher than the MPL. Arsenic and lead concentrations of water during the study period were higher than the MPL.

Arsenic, lead and cadmium concentrations of sediment during the study period were observed to be lower than the "threshold effect concentration"(TEC)," midpoint effect concentration"(MEC), and "probable effect concentration" (PEC) (MacDonald *et al.*, 2000) except the arsenic concentration in January. In January, As concentrations of sediment (24.35 mg/L) were higher than MEC permissible limit (21.4 mg/L).

In addition, transferred factor of heavy metals in different organs (meat, liver and gill) of studied fish species from water and sediment were also determined (Table 3). It was found that the transfer factor of Fe in different organs (meat, gill and liver) from water were observed to be greater than that of the limitation value of 1. The transfer factors of Zn, Pb and Cd in different organs from water and sediment were found beyond the limited, which meant that above mentioned fish organs accumulated metal from water and sediment.

Table 1 Various sizes and weights of studied fish species (Mean ± Sd)

Sr. No.	Month	Number	Mean Total Length (Cm)			Mean Body Weight (G)		
1.	July	5	18.8	±	4.75	143.7	±	152.51
2.	September	5	20.7	±	5.25	167.72	±	105.9
3.	November	5	25.1	±	5.92	277.9	±	128.39
4.	January	5	20.4	±	4.04	139.1	±	96.68
5.	March	5	20.9	±	5.27	167.6	±	108.76
6.	May	5	25.1	±	5.24	226.5	±	131.9

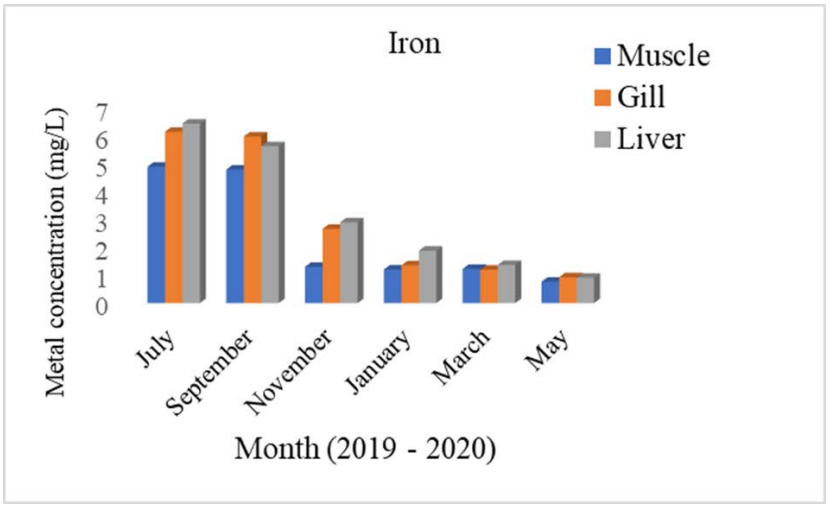

Figure 2 Iron concentrations in meat, gill and liver of studied fish species

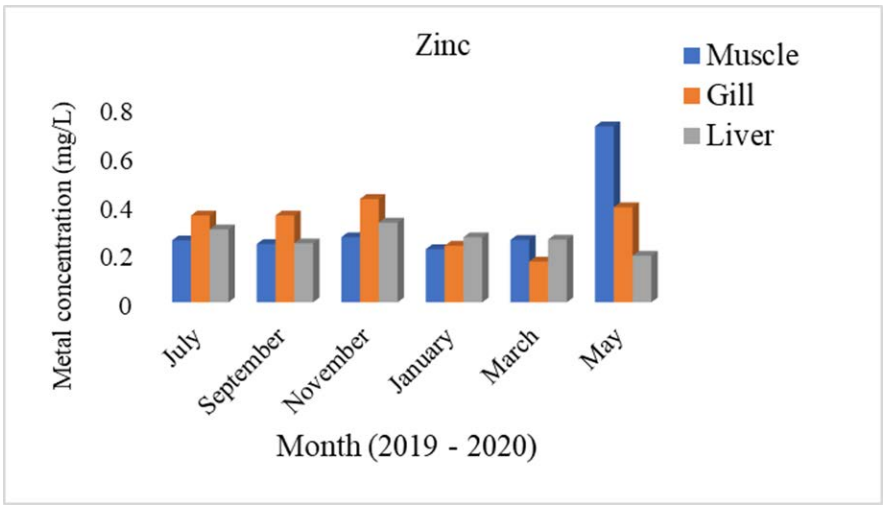

Figure 3 Zinc concentrations in meat, gill and liver of studied fish species

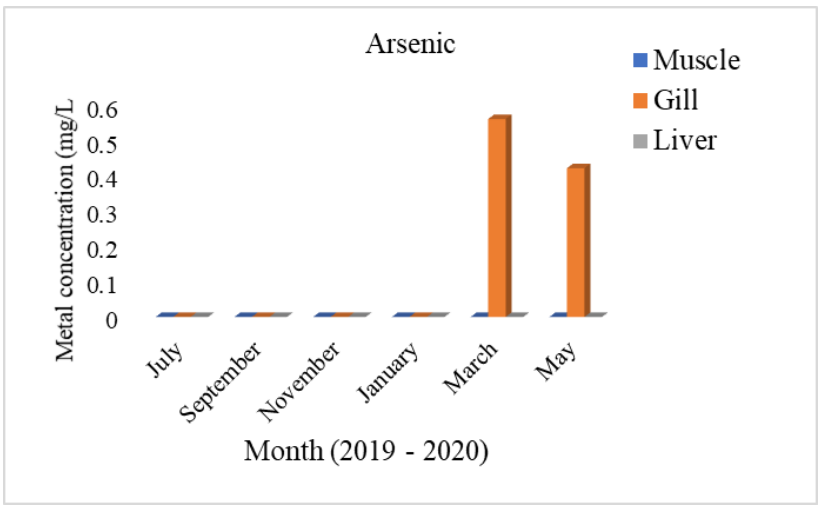

Figure 4 Arsenic concentrations in meat, gill and liver of studied fish species

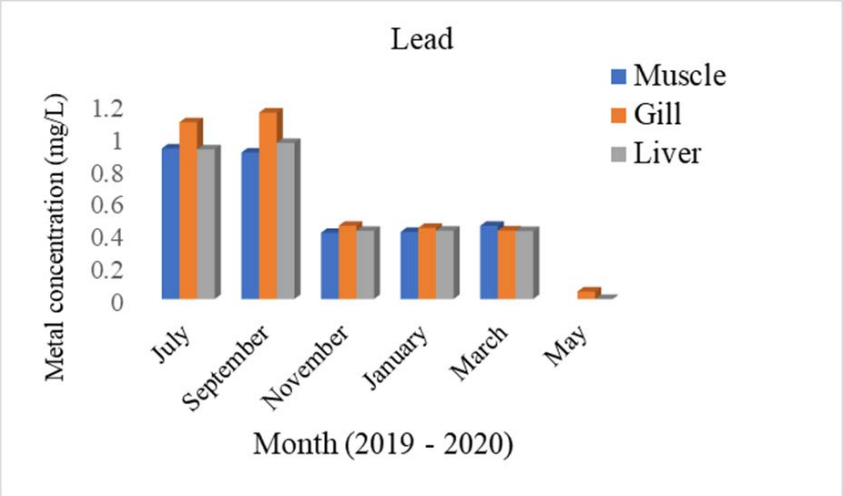

Figure 5 Lead concentrations in meat, gill and liver of studied fish species

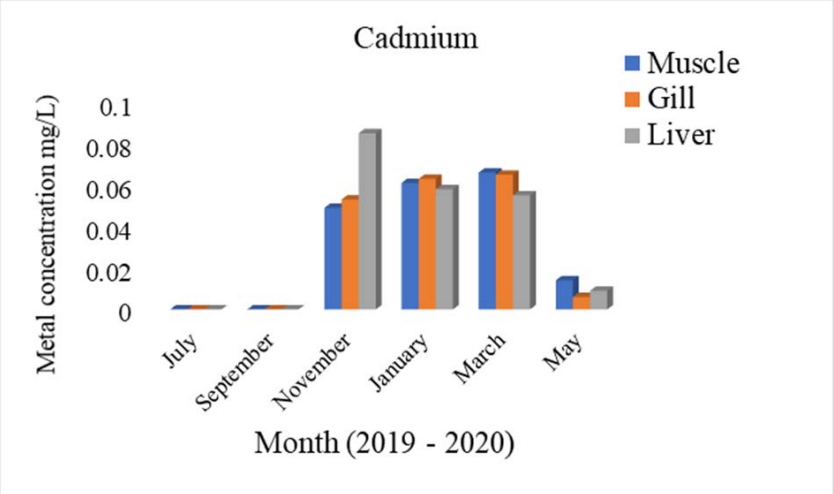

Figure 6 Cadmium concentrations in meat, gill and liver of studied fish species

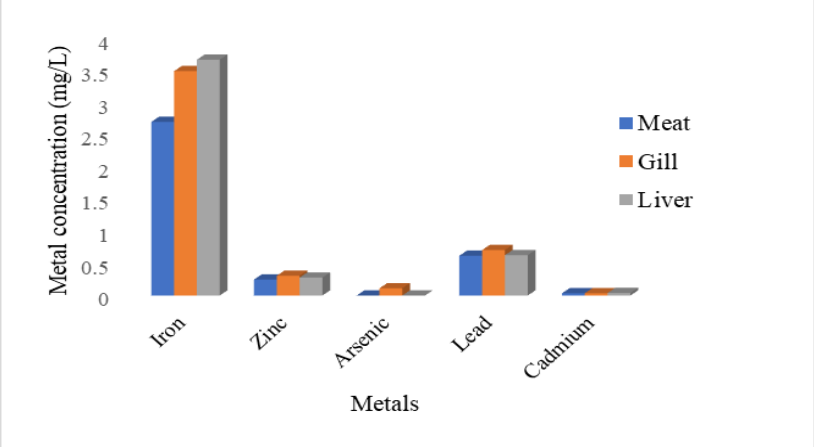

Figure 7 Mean values of metal concentrations in meat, gill and liver of studied fish species

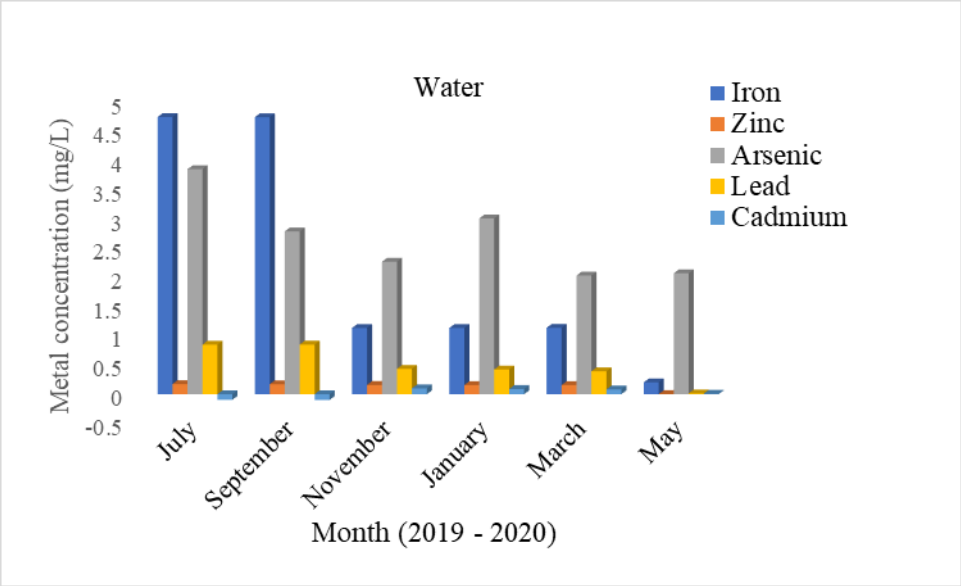

Figure 8 Metal concentrations in water during studied period

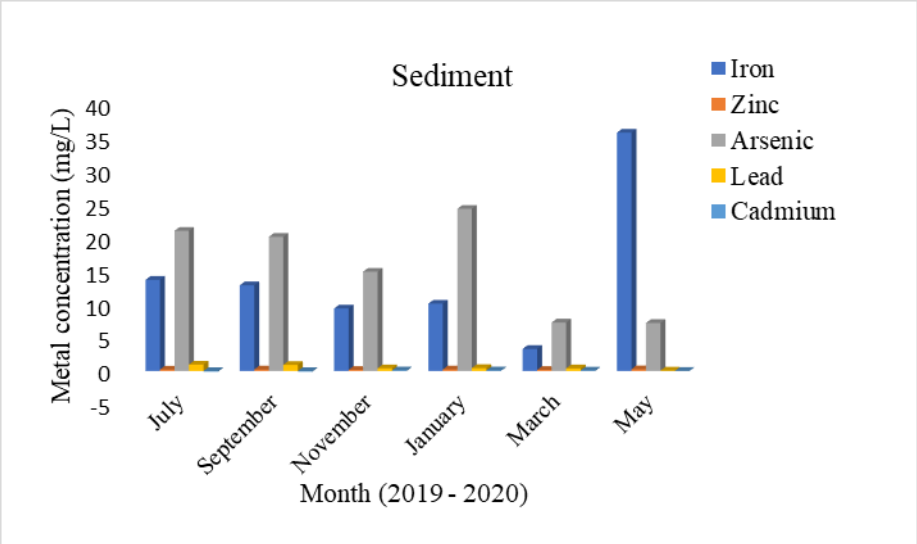

Figure 9 Metal concentrations in sediment during studied period

Table 2 Maximum permissible limits of metal concentrations (mg/L) stated in WHO and FAO guidelines

Sr. No.	Metal	WHO/FAO limit	WHO limit	Sediment		
		Muscle	Water	TEC	MEC	PEC
1.	Fe	100	5	20000	30000	40000
2.	Zn	40	3	120	290	460
3.	As	0.26	0.01	9.8	21.4	33
4.	Pb	1	0.05	36	83	130
5.	Cd	0.2	0.01	0.99	3	5

TEC = Threshold effect concentration

MEC = Midpoint effect concentration

PEC = Portable effect concentration

Table 3 The TF of heavy metals from water and sediment in meat, gill and liver of studied fish species

Metal	TF from water			TF from sediment		
	Meat	Gill	Liver	Meat	Gill	Liver
Iron	1.1	1.4	1.47	0.173	0.22	0.22
Zinc	2.397	2.35	1.94	1.531	1.5	1.24
Arsenic	0	0.061	0	0	0.01	0
Lead	1.08	1.21	1.1	1	1.11	1
Cadmium	2.27	2.21	2.5	2.9	3	3.5

Discussion

Fish are an important food source and represent a major part of many natural food chains. Therefore, the levels of contaminants in fish are of particular interest because of the potential effects of these polluting substances on the fish themselves and on the organisms that consume them, including humans (Burger and Gochfeld 2005). This study was undertaken to investigate the concentrations of Fe, Zn, As, Pb and Cd in different tissues (muscle, gill and liver) of Tilapia fish species and their environs (water and sediment) collected from the Kyet Mauk Taung Dam, Mandalay Region. Moreover, the values of transfer factor in different tissues from aquatic environs (water and sediment) were evaluated. The levels of heavy metal were determined in this species because of its importance for human consumption.

This investigation showed that Fe and Cd concentrations (mean total) were highest in the liver and lowest in the muscle. Zhao, *et al.,* (2012) stated that the accumulation of Fe in the liver is likely linked to its role in metabolism. Fe tends to accumulate in hepatic tissues due to physiological role of the liver in the blood cells and hemoglobin synthesis (Gorur, *et al*., 2012). On the other hand, the liver also showed high level toxic metals such as Cd to displace the normally metallothionein (MT) associated essential metals in hepatic tissues (Amiard, *et al.,* 2006). Similar results of high Fe and Cd in the liver were observed in many field studies according to (Cho Cho Thin, 2017, Zhao, *et al.,* 2012, Amundsen, *et al*., 1997, Dural, *et al*., 2007).

In the present study, the concentrations of Zn, As, and Pb were found to be highest in the gill and lowest in the muscles. Many volcanic complexes are all characterized by geogenic enrichment of Zn (Petrik, *et al*., 2018). The high concentrations of Zn, As and Pb may be from volcanic soil and volcanic ash and anthropogenic sources in the study area.

Gills are the main route of metal iron exchange from water (Qadir and Malik, 2011). They have large surface areas that facilitate rapid diffusion of toxic metals (Dhaneesh, 2012). Therefore, it is suggested that metals accumulated in gills are mainly concentrated from water. This is an agreement with the finding of Cho Cho Thin (2017). Similar result for high Zn, As and Pb concentrations in gills were recorded by Avenant-Oldewage and Marx (2000) and Abu-Hilal, and Ismail, 2008.

In the present study, the studied fish species always showed the lowest concentrations of tested metals (Fe, Zn, As, Pb and Cd) in the muscles. Fe and Cd were accumulated mainly in the liver while Zn, As and Pb revealed their highest concentrations in the gills. Thus, the differences noticed in the levels of accumulation in different organs studied fish species can be attributed to the differences in their physiological roles toward maintaining homeostasis, feeding habits, regulatory ability and behaviors of the species (Cross, *et al*., 1973). However, the majorly of the muscle (meat) had the least concentrations of heavy metals compared with other muscles (gill and liver) in the studied fish species. This is in agreement with the previous finding by Cho

Cho Thin (2017) and Ishaq *et al*., (2011) which showed that meat is not an active organ in the accumulation of heavy metals. Gills, on the other hand, has been reported as metabolically active site and can accumulate heavy metals in higher level. This is evidenced by the position that the gills occupied in the accumulation pattern for the heavy metals (Olaifa *et al*., 2004). The mean values of metal concentrations in different tissues were found to be lower than the maximum permissible limits recognized by WHO/ FAO.

The concentrations of Fe and Zn of water were found to be lower than those of maximum permissible limits recognized by WHO/ FAO. As, Pb and Cd concentrations of water during the study period were higher than the MPL. The high levels of As, Pb and Cd in water can be related (attributed) to industrial and agricultural discharge (Mason, 2002). In the present study, the high levels of heavy metal came from agricultural activities because more than 2515 hectares of agricultural lands are located near the Dam.

The obtained results of all metal concentrations (Fe, Zn, As, Pb and Cd) from sediment during the study period were observed to be lower than the "threshold effect concentration"(TEC)," midpoint effect concentration"(MEC), and "probable effect concentration" (PEC) (MacDonald *et al.,* 2000).

The results showed that the transfer factors of all elements in different tissues of studied fish species from water and sediment were greater than 1 except for Fe from sediment and As from water and sediment. This means that the fish undergo bioaccumulation of these tested metals from the aquatic environs (water and/or sediment) (Kalfakakour and Akrida-Demertzi, 2000; Rashed, 2001). This study indicates that, this species is safe for human consumption. However, it was found that there was high bioaccumulation of heavy metals in fish tissues. Therefore, a regular monitoring of heavy metal levels in fishes is necessary.

Conclusion

In the present study, heavy metals concentrations in studied fish species were found to be lower than the maximum permissible limits. Based on the results, it is concluded that it seems to be appropriate for eating the studied fish species. According to the results of Transfer Factors, heavy metals accumulated in different tissues of the studied fish species came from water and sediment. Therefore, regular monitoring of heavy metal levels in fish tissues is necessary.

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MOLECULAR AND HISTOLOGICAL IDENTIFICATION OF COMMERCIALLY IMPORTANT BAMBOO CLAM, *CULTELLUS SP*. IN KYAUKPYU, RAKHINE STATE

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Abstract

The razor or bamboo clam *Cultellus* is widely distributed in the intertidal zones and estuarine waters along the coast of western Pacific Ocean and is extensively cultured. In the present study, commercially important bamboo clams, *Cultellus* sp., were collected from the deep sea of Kyaukphyu, Rakhine coastal area from October 2020 through September 2021 to investigate the histological features of the bivalve species and the sex ratio of the population. The relation between shell length (mm), wet tissue weight (g), dry tissue weight (g) and sex of the clams was examined. Histological analysis of the gonad was conducted to reveal the gonad development of the clam. Except the undifferentiated stage, four different gonad development stages (developing, ripe, spawning and spent) were found in both male and female clams. In addition, the taxonomic position of the bamboo clam was studied by morphological characters and mitochondrial DNA sequences. Regarding sexual differentiation, higher numbers of male bamboo-clams were recorded than female bamboo clams. DNA sequence of a 1395 bp was obtained from the collected species and the species was identified as *Cultellus maximus*.

Keywords reproductive biology, histological, *Cultellus* sp., bamboo clam

Introduction

The razor clam, *Cultellus* sp., is a near shore marine bivalve known locally in Myanmar as bamboo clam. This razor clam species occurs in Asian pacific coastal waters and lives buried in the relatively flat sandy bottom between depths of 2.5 and 10 m (Nguyen, 2016). There are several known species of allied genus *Solen* Lin-naeus, 1758, but to date, *Cultellus* which is often misidentified as *Sinonovacula* sp. (Nguyen, 2016). *Cultellus* sp. is among the most important commercial bivalves to be considered for aquaculture recently (Nguyen, 1996) due to its potential economic value but its natural production continues to decline (Vu, 2021). However, little information is known about the genetic population structure and reproductive biology of this species within Kyaukphyu, precluding an informed and sustainable harvest program.

Bamboo clam trading has been started in Kyaukphyu area but knowledge of that species is limited. Differentiating between bivalve species based on their morphology is a challenging task due to the wide range of forms and sizes observed in their shells. The unique characteristics of their habitats greatly influence the tremendous variation in shell shapes, even within the same species, and this makes it very difficult to identify the species of any wild individual (Comesana *et. al*., 2001). In order to overcome the confusion stemming from the morphological characteristics of bivalves, recent years have witnessed the utilization of molecular biological techniques. These methods aim to identify individuals by examining a variety of genetic markers. By employing such techniques, scientists strive to enhance accuracy and precision in species identification. In this study, the suitability of mitochondrial DNA sequencing for the identification of clam was evaluated which is a fundamental requirement to extend the research work on that clam.

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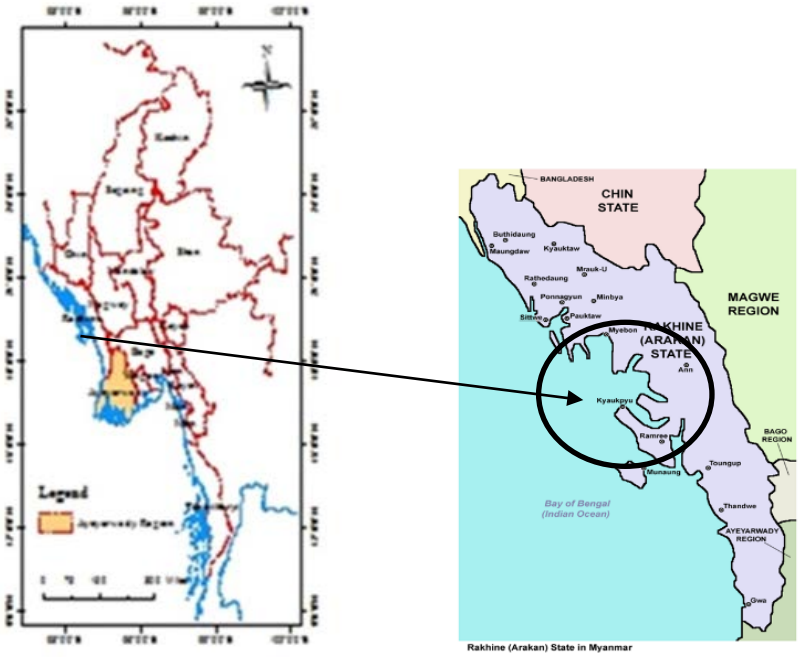
Understanding the reproductive cycle and spawning period of this species will provide information needed for the planning of a better conservation strategy for *Cultellus sp.* and recruitment period of this population. In Myanmar, the reproductive biology of this species is not well understood, and characterization of its gonad development is necessary step that would contribute to a better understanding of its reproductive biology. The objective of the present study was to investigate the DNA sequencing identification and reproductive biology of the bamboo clam *Cultellus* sp. in Kyaukphyu, Rakhine State, Myanmar.

Materials and Methods

Sampling site and the study period

The bamboo clam samples were collected from the intertidal zone of in Kyaukphyu Township of Rakhine State, Myanmar. This study area is located on the north western corner of Yanbye Island on Combermere Bay. It is situated at Latitude 19° 12’ 58” North and Longitude 93° 43’ 56” East (Fig. 1). The study was conducted from October 2020 to September 2021.

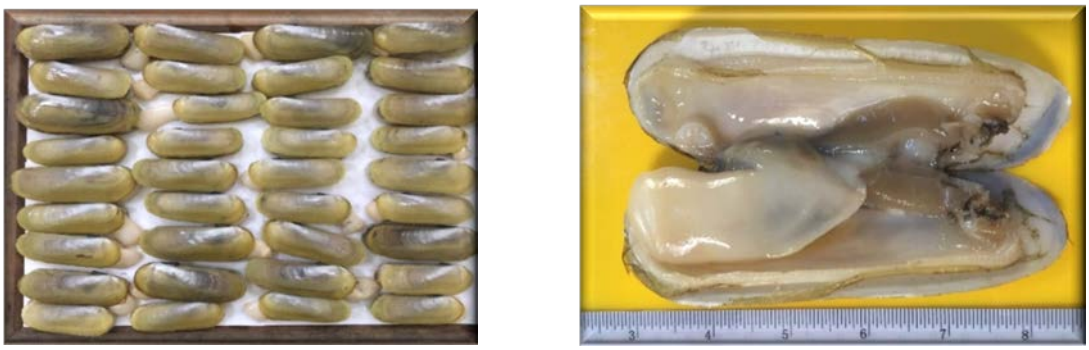


Myanmar Kyaukphyu Township, Rakhine State

Figure 1 Map showing location of Kyaukphyu Township of Rakhine State, Myanmar

Sample collection

Samples were collected during the low tide period. Low tide is the most favorable time for collecting bamboo clams that inhabits the intertidal zone. All samples were transported to the Laboratory of Aquatic Bioscience, University of Yangon immediately after harvest.



(A) (B)

Figure 2 (A) *Cultellus* spp. (B) Internal shell of the studied bamboo clam

Morphological analysis

A total of 100 clams were collected during the study period. In the laboratory, each clam was measured along its shell length to the nearest millimeter with vernier caliper. Immediately, the clams were opened and wet tissue weight was determined after removing excessive water using an absorbent tissue paper. Sexes were examined under the light microscope to determine the gender. Clams were identified by morphometric characters according to Morton (1984) (Fig. 3).

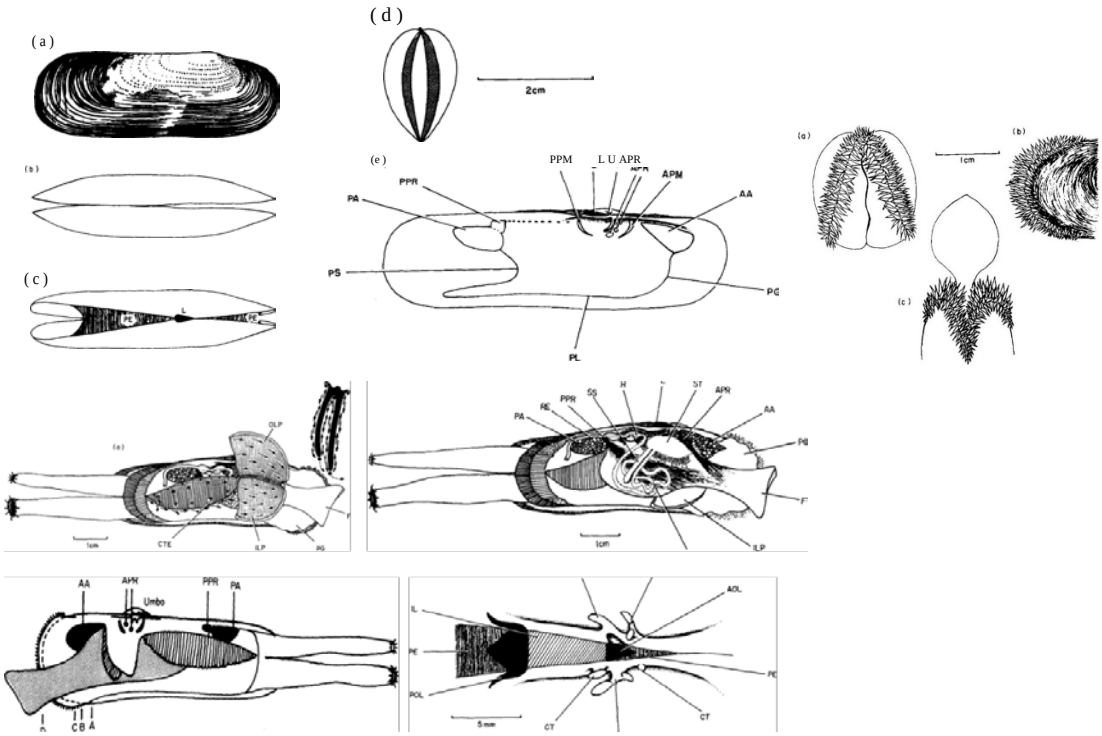


Figure 3 External and internal characters (a) The right; (b) ventral; (c) dorsal and (d) posterior aspects. (e) An internal view of the left shell valve; PPM-Posterior pedal protractor muscle or scar), PPR- Posterior pedal retractor muscle, PPM- Pallial retractor muscles, PS- Pallial sinus, PA- Posterior adductor muscle or scar, L-Ligament., U-Umbo, APR-Anterior pedal retractor muscle or scar, APM-Anterior pedal protractor muscle or scar, AA- Anterior adductor muscle or scar, (f) Pedal gape (i) anterior; (ii) lateral and (iii) dorsal aspects, OLP-Outer labial palp, CTE- Ctenidium, ILP-Inner labial palp. , F- Foot, ES-Exhalant siphon, IS-Inhalant siphon, RE-Rectum, SS-Style sac, H-Heart, ST-Stomach, CT-Cardinal tooth (Morton, 1984).

Histological preparation

A small piece of gonad (3 mm) under the mid region of the mantle lobe was removed, fixed individual pieces with Davidson's solution for 48 h. Samples were then dehydrated in a graded ethanol series, cleared in xylene and blocked-in paraffin wax. All sections, 5 μm in thickness, were cut using a rotary microtome and stained with Harris hematoxylin and counterstained with eosin. The histologically prepared slides were checked under a compound microscope.

Reproductive development analysis

The gonad development of bamboo clam was categorized into 5 stages and assigned stages 1 to 5 for undifferentiated, developing, ripe, spawning and spent, respectively, based on the microscopic appearance of the gonad tissue in histological sections [modified after Heffernan *et al*. (1989)]. Gonad development stages proposed for *Cultellus* sp. are described in Table (1).

Table 1 Gonad development stages proposed for *Cultellus* sp.

Sexual phase	Sex	Description
(1) Undifferentiated		Gonadal area replaced with connective tissue, but no trace of gonadal material
(2) Developing	Male	Spermatogonia and spermatocyte abundant, few spermatids and sperm present
	Female	Mostly oogonia, and attached oocytes and occasional free oocytes present
(3) Ripe	Male	Mostly spermatide, few spermatocyte present
	Female	Free oocytes with distinct nucleus and nucleoi present
(4) Spawning	Male	Follicles partly empty, few sperm present
	Female	Follicles partly empty, few free oocytes present
(5) Spent	Male	Follicles contracted, few in number, occasionally residual sperm present, connective tissue abundant
	Female	Follicles contracted, few in number, occasionally residual oocytes present, connective tissue abundant

DNA extraction

Only ten clams were used for DNA extraction and gene sequencing. The clams were fixed in 70% ethanol before the preparation of DNA extraction (Fig 4 A). PetNAD nucleic acids co-prep kit was used to extract DNA from the gill tissue of the clam (Fig. 4 B). DNA extraction was conducted according to the instructions in PetNAD nucleic acids co-prep kit.

(A) (B)

Figure 4 (A) Fixation of *Cultellus* spp. (B) PetNAD Nucleic Acid Co-prep Kit

PCR amplification and gene sequencing

In order to amplify mitochondrial cytochrome gene, universal primers for bivalve COI region, 28S- LCO1490F (5'- ggtcaacaaatcataaagatattgg -3') and 28S- HCO2198R (5'- taaacttcagggtgaccaaaaaatca -3') were designed (Table 2). For PCR, 5 µL of the extracted DNA in 75.5 µL of water was mixed with 3.0 µL of 25 µM of each primer, 0.5 µL of HS Taq DNA polymerase, 10 µL l of 10 × PCR buffer and 8.0 µL of dNTPs (WizPure, Seongnam, South Korea). The PCR conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72°C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were separated and visualized by electrophoresis on 1.5 % agarose gel containing SYBR Safe DNA gel stain ((WizPure, Seongnam, South Korea) and the DNA was extracted and purified from the distinct bands (around 680 bp) and using FastGene® Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Bunkyo, Tokyo). Purified DNA products were quantified and used for direct DNA sequencing by the PCR primers with an ABI gene sequencer. Genes sequence of the clam was compared with that of other bivalve species using the BLAST search located in the National Center for Biotechnology Information (NCBI) (http//www.ncbi.nih.gov).

Table 2 Primer details for the PCR detection of the studied clam

Primers	Direction	Sequences	Tm
LCO1490	Forward	5’-ggtcaacaaatcataaagatattgg-3’	51°C
HCO2198	Reverse	5’- taaacttcagggtgaccaaaaaatca-3’	44°C

Tm = melting temperature

Results

Morphological description

The clam had two thin, equal-sized valves that were elliptical in shape and marked with conspicuous concentric rings. The valves were joined dorsally by a dark brown ligament which was external and triangular. Opposite the hinge was a distinct heart shaped lunule. The external shell was generally pale yellow-colored or off-white. The internal shell was white or pale yellow margin. Three cardinal teeth were found in left valve, two in right valve. No lateral teeth found in this calm. Right anterior and posterior cardinal teeth were smooth while remaining cardinal tooth was usually bifid. Inner margin was smooth and located at the posterior third.

Posterior pedal retractor muscle and PRM- pallial retractor muscles was present. Pallial sinus was internally deep. Anterior and posterior adductor muscle or scar was generally equal in size. Umbo was posteriorly elongate and Pedal gape was present. Anterior pedal protractor muscle or scar was present where anterior pedal retractor muscle or scar was smaller in size. Periostracum was thickened in this species. Pedal gape, (i) anterior; (ii) lateral and (iii) dorsal aspects, was wide. OLP-outer labial palp, was unridged and CTE- ctenidium was large.

Inner labial palp was ridged where foot was elongate. The body of the clam was surrounded by a mantle. A pair of siphons fused at the bases. Exhalant siphon and inhalant siphon were separate. The siphons were generally creamy white, but the margins were colored in a range of yellows or browns. Rectum and style sac were present in this clam. Stomach was small. According to morphological characters, the clam was tentatively identified as belonging to the families Pharidae or Solenidae or Tellinidae.

DNA sequencing

DNA sequences of a 1395 bp fragment were obtained from the clam (Fig. 5). The BLAST search of database located in the NCBI revealed that the clam DNA sequence was highly homologous to the genus *Cultellus.* Sequence identities are 99.85 % to *Cultellus maximus* (MN119650), 89.74 % to *C. attenuatus* (OQ434723), 88.17 % to *Novaculina myanmarensis* (MW549330) and 87.58 % to *N. gangetica* (MF958991). Identities with the genus *Novaculina* are much lower than to the genus *Cultellus*; 88.17% to *N. myanmarensis* (MW549330) and *N. gangetica* (MF958991) and 86.77% to *Pharella acutidens* (MW311106) (Table. 3).

CCAATAAAATATGATTTTGGCGTTTTGATCTGGATTAGTTGGAACTAGGTTGAGGC……..56
TATTGATTCGTTTGGAGTTGGCTCGGCCTGGTGGTTTTATGGGGGATGAACATTTA…… 112
TATAATGTTATTGTTACTGCTCATGCTTTTGTTATGATTTTTTTTTTGGTTATGCC……..150
AATAATGGTTGGTGGGTTTGGTAATTGATTATTACCTTTAATGTTAACTTCTCCTG……200
ATATGTCTTTTCCTCGAATAAATAATTTGAGGTTTTGGTTGCTTCCTTTTGCGTTG……250
TTTTTTTTGTTATGGTCTGGGTTTGTTGGGAGGGGTATTGGTGCTGGTTG……300
AACTATTTATCCTCCTTTATCTGGAAATTTGGCCCATAGGAGTCCTTCAATGGATT…… 350
ATGGAATTTTTTCTATACATTTGGCGGGTGCTTCTTCTATTTTAGGTGCAGCTAAT…… 400
TTTGTTACAACAATGATTAATATGCGTCCTGGGGTTATGGAGTTGAAGCGTGTTAG…… 450
GT TGTTTGTTTGGTCAGTAGTTATTACTGCTTTTTTATTGGTTTTGGCTATATGTTTG ……..500
TTTGGTCAGTAGTTATTACTGCTTTTTTATTGGTTTTGGCTATACCTGTTTTAGCT…… 550
GGGGCTCTAACAATACTTTTGACAGATCGTCATTTTAATACTTCTTTTTTTGATCC……..600
GAGGGGTGGTGGTGACCCTATTTTGTTTGTTCATTTGTTTTGATTTTTTGGTCACC……..650
TGGAAAGTTTAAGGGGGGGTGGTGCTGATTGCGAGGGGGCGCGGGGGTGG…… 700
GCCGCGCGGCTGACATACACAGCCTGGTCGCCCACGCCGACGACGGTGCA…… 750
AGGTAGAGGGTGGGGTTCCATGTAGGACGTGCCAGCCCTCATCATACTAAGTGCGC……. 80
CGCCCGGCGAGACCAGGACGGACGGCAAGAATACAACGGAAATGGAATGGAGCGGG……850
CTCAAGCATAAGCGTCCGAGCCTTCGAATCAATACGATGCGGAAGAGCCGAAACCT……900
CGCCTGGGCTTGCACTGCAGGGCAACCCGGGACGAGACGCCCCCTTCCGC……950
CCAGGGATCCAGCAGGGGGGGGGCCTGACCCTACCCCCACGGTCGTGGTAAT……1000
GAGAGGTGAGGGTCCACCACCCAGCAGCGAGCCATACCCTTGTTTGGCTG……1050
GTTGCCACAGGCCCGGGGGGGCGGGGCGCCCAGTATACCCCCCCCGGGCG……1100
CCGCGTACCAAAGAAGGGGAGGGGGGGGGGCGGACAAAAAAAAACAAACC……1150
CCCCCCTTCCTTTTATGGGGGGGGTGGCCAACTAGATAAACACTTAGAAA……1200
GGGTTATTTAAAAAACATTTTGCCAGAAAAAAGCGGCGGGGGGGGTCCA……1250
AACAAAAAACAAAAATCAAGGGGCTGTTTTTTTGGGAGAGGGGTTGGCGGC……1300
GAACCC CCTACCCCGCCCCGAAGTAGTGAGAATGAATACAAAAAAAAAAAAA ……1395

Figure 5 Partial sequences of mitochondrial cytochrome gene of the studied clam showing designed primer annealing sites

Table 3 Mitochondrial cytochrome gene partial sequence identities of the studied clam to other species in the NCBI data base

Accession	Description	%
MN119650	*Cultellus maximus* (Gmelin, 1791)	99.85 %
OQ434723	*C. attenatus* Dunker, 1862	89.74 %
MW549330	*Novaculina myanmarensis* (Lamarck, 1818)	88.17 %
MF958991	*N. gangetica* Benson, 1830	87.58 %
MW311106	*Pharella acutidens* (Broderip & Sowerby, 1853)	86.77 %

Growth monitoring and sex ratio of *Cultellus maximus* in natural population

The soft tissue weight of male was 80.4 ± 1.2 g while that of female was 110 ± 1.1g. The mean length of male and female were 10.1 ± 0.8 cm and 13.5 ± 1.5 cm, respectively. The soft tissue weight and the length were not significantly different in males and females clam (t test, P>0.05).

The numbers of females and males *Cultellus maximus* collected from Kyaukphyu were shown in (Table 4). Samples contained a relatively larger number of male clams than females throughout the study period. Significant differences in the sex ratio of male and female were not found in this study.

Table 4 The sex ratio, mean shell length, wet weight and the dry weight of studied clams

Gender	Mean Length	Mean Wet Weight	Sex Ratio
Male	10.1 ± 0.8 cm	80.4 ± 1.2 g	54
Female	13.5 ± 1.5 cm	110 ± 1.1g	46

Gonad development stages of *Cultellus maximus*

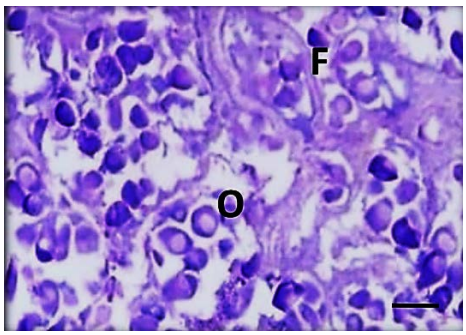
The reproductive development stages of *Cultellus maximus* males and females were examined. Surprisingly, undifferentiated stage was not found. Gonad development of female clams was reasonably synchronized with that of males.

Developing (Plate 1A and 2E): In developing stage, the follicular wall was lined with small oogonia in females or darkly stained spermatogonia in males but most of the follicles were empty.

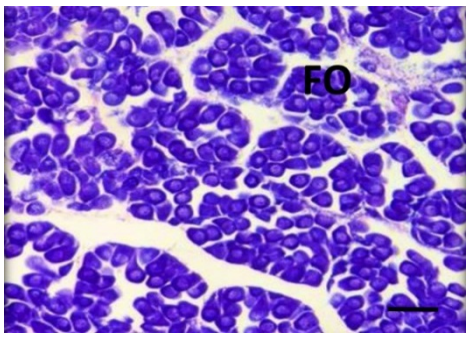
Ripe (Plate 1B and 2F): In ripe stage, oogonia attached to the follicle while oocytes in female and sperm in male escape to the lumen showing that clam was near to spawning. Follicles filled with mature oocytes with distinct nucleus in females and spermatozoa with tails in males, follicles wall was usually very thin and distended. Little or no connective tissue could be observed in this stage. Interestingly, ripe stage in male and female clams was abundantly found.

Spawning (Plate 1C and 2G): In spawning stage, some follicles were empty due to the releasing of oocytes/sperm and some collapse follicles were found. Residual and abnormal oocytes were found in female while small amount and pale color of sperm were found in male. Therefore, the most dominant reproductive stage in this population was the spawning stage followed by the ripe stage.

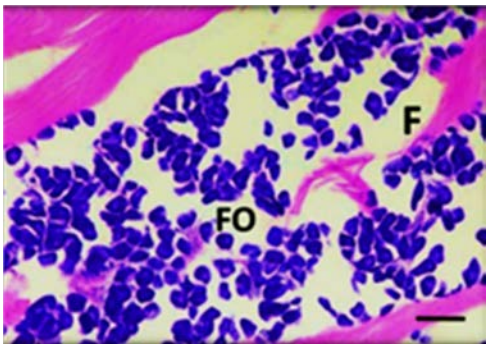
Spent (Plate 1D and 2H): In spent stage, small gametes were present near the follicle periphery during and immediately after spawning indicating that in clam's gonads did not have a resting period between gametogenic stages. Follicles containing degenerating oocytes, often elongated in shape and phagocytes were also present. Connective tissue began to reappear in this stage. The majority of *Cultellus maximus* individuals with spent gonads were seen.



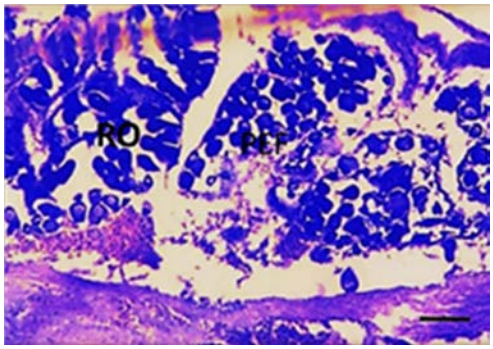
(A) Developing



(B) Ripe

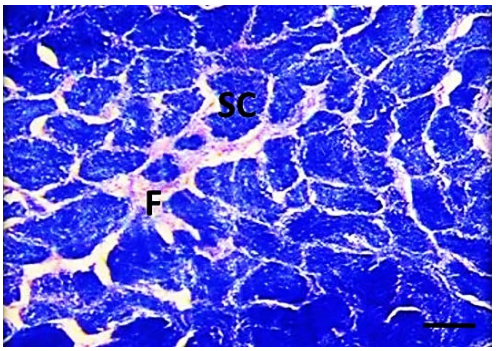


(C) Spawning

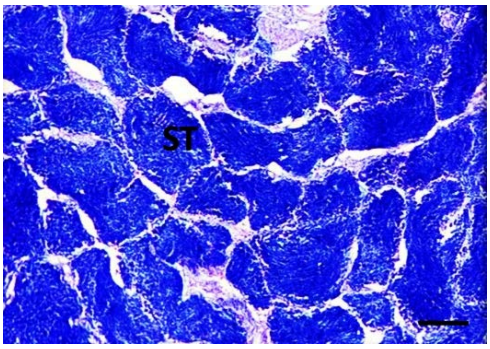


(D) Spent

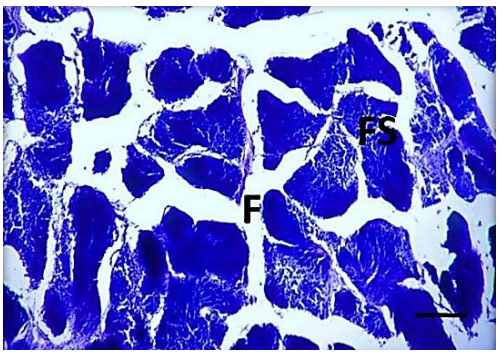
Plate 1 Gonad developmental stages observed in female (A-D) *Cultellus maximus*. All photographs are to the scale /70 µm. O= Oogonia, F = Follicle, FO = Free oocytes with distinct nucleus, PEF = partly empty follicle, RO = Residual oocytes; CT = Connective tissue



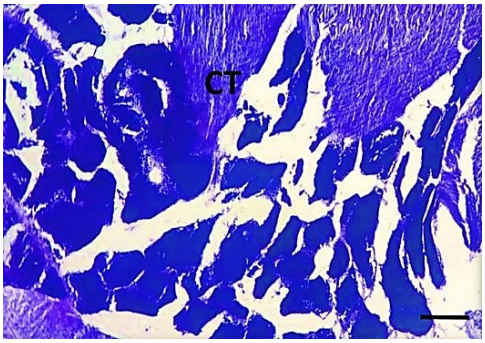
(E) Developing



(F) Ripe



(G) Spawming



(H) Spent

Plate 2 Gonad developmental stages observed in male (E-H) *Cultellus maximus*. All photographs are to the scale /70 µm. F = Follicle, PEF = partly empty follicle, SC = Spermatocysts, ST= Spermatids, FS = Few sperms in follicle, CT = Connective tissue

Discussion

In the present study, mitochondrial cytochrome gene sequencing for the identification of clam was conducted. It revealed that sequence identities of studied clam species indicated to be *Cultellus* sp. with 99.85%. The bamboo clam *Cultellus* species also known as the razor clam, belongs to the family Pharidae (Morton, 1984). Morphological characters revealed that the studied clam could belong to the family Pharidae or Solenidae or Tellinidae. The different

characters among those families were: (1) shell layers (2) hinge teeth (3) pedal gape (4) siphons (5) stomach and (6) style sac. Shell layer of *Cultellus* and *Pharella* has two layers and three in *Novaculina*. (Morton, 1984). In hinge teeth of *Cultellus*, three cardinal teeth in left valve, two in right and no lateral teeth where *Pharella* has two cardinal teeth in each valve and lateral teeth well developed and one to three cardinals in each valve and lateral teeth absent or weak in *Novaculina*. In addition, those characters were insufficient to identify the clam down to the species.

To confirm the genus, DNA analysis was conducted. Although, there was no matched sequence to the mitochondrial cytochrome gene sequence of the studied clam in the NCBI database, the most similar sequence indicated to be *Cultellus maximus* with 99.85% homology. Identities with the genus *Novaculina* are much lower than that of the genus *Cultellus*; 88.17% to *N. myanmarensis* (MW549330) and *N. gangetica* (MF958991) and 86.77% to *Pharella acutidens* (MW311106). Therefore, it is highly possible that the studied clam could not belong to genus *Novaculina*. The clam is therefore identity as *Cultellus maximus*. There are only 2 to 3 base pairs of *Cultellus maximus* and differ from those of clam reported in Korea.

The reproductive cycle with different reproductive stages and sex ratio of the *Cultellus maximus.* were revealed in the present study. They are generally divided into five stages: undifferentiated, developing, ripe, spawning and spent (Choi and Chang, 2003). Four reproductive stages were recorded in the studied clams: developing, ripe, spawning and spent. This classification was based on the histological similarity between the bivalves mentioned above. However, immature-undifferentiated stage was not observed in this species. Lai (2022) studied the reproductive development and conditioning of the clam *Cultellus sp.* in Vietnam and undifferentiated/primordial stage clams were recorded in February and March when salinity dropped less than 5 ppm. However, developing individuals increased from January to March when water temperature decreased < 20º C. Spawning individuals increased after salinity fluctuations in August and September. It is assumed that reproductive development of clam in Kyaukphyu is related to water temperature and salinity.

The reproductive cycle and gonad development of *Cultellus maximus* in Kyaukphyu were investigated. Although, there was no statistically significant difference in the shell length distribution, males were generally smaller in size than the females. The length at first maturity in this study (10.12 cm, 9.65–10.49 cm) is longer than the *Solen thachi* population in the central coast of Vietnam (i.e., 7 cm; Hoang and Tuyen 2016). Kim and Lee (2008) reported that the length at first maturity of *Sinonovacula constricta* (Lamarck, 1818) in western South Korea was 5–6.1 cm.

In *Cultellus maximus*, spawning and ripe stages were abundantly found. Clemente and Ingole (2009) studied the gametogenic development of the clam, *Cultellus sp.* at Chorao Island, Goa. They discussed that clam reached sex maturity when the shell length was over 3.4 cm. Shell length of clams collected in the present study was > 6 cm. All clams collected from Kyaukphyu area were assumed that they were in maturity stage. Various studies on the reproductive biology of bivalves have demonstrated that temperature and food are the most important exogenous factors influencing the reproductive cycle (Beer, 2000; Morriconi *et al*., 2002; Dalai and Goswami, 2001). Devassy and Goes (1989) reported an excess stock of phytoplankton available in the Mangrove areas. Since the role of food supply is vital in the development of gonad (Williams & Babcock 2004), the rapid metabolic reserves in *Cultellus sp.* coincided closely with the periods of high food abundance. Accordingly, the availability of food can be considered as a major determining factor of the seasonal gonadal cycle.

Bivalves often exhibit roughly equal numbers of males and females within a population (Gosling, 2015). However, the sex ratio imbalance observed here is indicative of the unique

geophysical location and environmental conditions such as temperature and food availability, or sampling bias (Rinyod and Rahim, 2011; Hoang and Tuyen, 2016; Trisyani et al., 2019). Individuals' sex at birth is determined by environmental conditions and genetic variation, or the combination thereof (Yusa, 2007). The temporal sex ratio variation has been observed in razor clam populations such as in a *Solen regularis* Dunker, 1862 population in Malaysia (Rinyod and Rahim 2011), in a *Solen* sp. population in Indonesia (Trisyani et al. 2019), and in a *Solen thachi* Cosel, 2002 population in the central coast of Vietnam (Hoang and Tuyen, 2016). Trisyani et al. (2019) hypothesized that the sex imbalance could result from the impact of excessive exploitation on one of the sexes of the *Solen* sp. populations in Indonesia. Bivalve species collected in the present study were from natural population. They might have undergone a greater temperature stresses and presumably had lower food availability (due to short periods of inundation during high tight). These environmental factors may have caused a higher male ratio in the bivalve species.

Conclusion

Morphology and molecular identification of bamboo clam from Kyaukphyu, Rakhine State was conducted. The BLAST search of database located in the NCBI revealed that the clam DNA sequence was highly homologous to mitochondrial cytochrome gene sequence of the species *Cultellus maximus*. The bamboo clam that had been traded in Rakhine state was confirmed as *Cultellus maximus*.

The reproductive cycle and gonad development of *Cultellus maximus* in Kyaukphyu were investigated. It was concluded that *Cultellus maximus* is a species with a continuous development of ganads throughout the year in Kyaukphyu area. The data presented here provided valuable information on the timing of spawning events for *Cultellus maximus* which had utmost importance for the development of sustainable management regimes for bivalve community.

Acknowledgements

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MOLECULAR PHYLOGENETIC STATUS OF SOME SMALL MAMMALS (MURIDAE AND SORICIDAE) OF NATMA TAUNG NATIONAL PARK IN THE NORTHERN PART OF MYANMAR

Khin Myat Myat Zaw[1], San Maung Maung Theint[2]

Abstract

Natma Taung (Mount Victoria) is the highest mountain in the Chin State of the northern part of Myanmar and its elevation is about 10,500 feet. The small mammals are more diverse at Natma Taung National Park. In the present study, seven newly recorded species were selected from Natma Taung National Park in Northern Myanmar. The two species, *Apodemus ilex* (wood mouse) and *Ethenomys eleusis* (oriental-vole), were sequenced to investigate the phylogeographic distribution of mouse species at different study sites of Natma Taung National Park using the mitochondrial cytochrome b gene. One species *Episoriculus caudatus* (brown-toothed shrew) was studied based on phylogenetic analysis of the mitochondrial *Cytb* gene partial (669 bp). The newly recorded species *Apodemus ilex* had 0.02% genetic distances with AY389018 China Gen bank references. *Ethenomys eleusis* was 0.03% with KT899700 and *Episoriculus caudatus* was 0.04% with Gen bank of MK962210 from China. The mt *Cytb* gene sequences analysis showed that Natma Taung National Park had high species diversity and genetic variation. The study of genetic distances and the phylogenetic relationships of the species will support a better understanding of the ecology, species diversity, and geographic distribution of the species.

Keywords cytochrome b (*cytb*), Genetic distances *Apodemus ilex*, *Ethenomys Eleusis*, *Episoriculus caudatus*

Introduction

Natma Taung is the highest mountain in the Chin State of the northern part of Myanmar. The park is administered by the Department of Forestry and Environment. It has 3,053 meters (10,500 ft) above sea level in height and a prominence of 2,231 meters (7,320 ft). Natma Taung, the Chin Hill, is one of the ultra-prominent peaks of Southeast Asia. Natma Taung natural habitat consists of trees, bushes, and grass, which have adapted to the environment. It has research and recreational opportunities of (35) mammal spp., (345) birds spp., (105) amphibian and Reptile spp., (99) butterfly spp., (35) beetles spp., (1024) Plant spp., (99) Orchid spp., and (71) medical plant spp. Among the species recorded at Natma Taung National Park, there are 2 species of the family Muridae, *Apodemus ilex* and *Ethenomys Eleusis*, and one species of the family Soricidae, *Episoriculus caudatus*.

The family Muridae comprised of 730 species from 150 genera of rodent groups, exhibiting the highest percentage (60%) of species within the order Rodentia (Musser and Carleton, 1993). The Muridae, or murids, are the largest family of rodents and mammals, containing approximately 1,383 species, including many species of mice, rats, and gerbils found naturally throughout Eurasia, Africa, and Australia (ADW,2022 https://animaldiversity.org).

Murinae, the Old-World rats and mice, is the largest subfamily of muroid rodents. There are an astonishingly diverse 561 species in this subfamily, which are divided among 126 genera and 29 divisions (Musser and Carleton, 2005 cited in ADW, 2022 https://animaldiversity.org) with over 300 species in 23 genera. Soricidae is by far the most species rich family in the order Insectivora. Its members can be found throughout the world, with the exceptions of the Polar Regions, Australia, and southern South America (ADW, 2022). *Ethenomys* (Muridae:

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Clethrionomyini) inhabits in the Trans-Himalayan Ranges of Southwest China, small parts of Northeast Burma, and the Assam province in India (Suzuki *et al*.,2004).

Asian red-tooth shrews of the *Soriculus* group are among of poorly studied taxa of the tribe Nectogalini (Lipotyphla, Soricidae) (Anderson,1879). Shrews of this group are widely distributed in Asia from northern China, southward to northern Vietnam and Myanmar, and from Kashmir to Taiwan (Hoffmann, 1986). Earlier, *Soriculus* Blyth, 1854 was treated in a broad sense and also included *Episoriculus* Ellerman et Morrison-Scott, 1966 and Chodsigoa Kastchenko, 1907 as the subgenera/synonyms (Hoffmann, 1986; Corbet and Hill, 1992; Motokawa and Lin, 2005). Only recently these taxa were given a full generic rank (Hutterer, 2005; Motokawa *et al*., 2008, 2009; He *et al*., 2010).

Regarding the use of the cytochrome b (*Cytb*) gene, (Khin Myat Myat Zaw *et al*., 2019) suggested that mitochondrial sequences performed to elucidate the genetic structure of the studied species and provided insight into the factors shaping their genetic structure.

The present study was conducted with the aim to explore the biodiversity richness of Myanmar and find the small mammal diversity of Natma Taung National Park which is a famous tourist attraction site and also a precious area of the Chin ethnic group.

Materials and Methods

The specimen collection was conducted at Natma Taung National Park, Northern Chin State, Myanmar. The specimens were collected by trapping under the permission of the Forest Department, Ministry of Natural Resources and Environmental Conservation.

Sampling

Three sampling sites were categorized as Site 1 (5290 ft elevation), Site 2 (9750 ft elevation), and Site 3 (8820 ft elevation) (Figure 1, Table 1). Six specimens of small mammals were collected using local snap traps and Sherman's traps (Plate 2). Traps were placed at the sampling sites from 6:00 – 9:00 pm and harvested at 5:00-8:00 am the next day.

Morphological identification and tissue sample preparation

After checking morphologically and taking the live photos, the live specimen was anesthetized using chloroform to extract liver tissue samples to carry out the molecular phylogenetic study. The entire specimens were labeled with the specimen code, date, and sample site. The morphometric measurements of total length (TL), tail length (T), body weight (W), hind foot length with nail (Hf-cu), hind foot length without nail (Hf-su), ear length (E), Head and Body weight (HB) were also taken to estimate the species tentatively (Table 2). Twenty- five grams (g) of a liver tissue sample of each specimen was preserved in 70% ethanol in a tissue sample collection tube to extract genomic DNA according to the protocol of the manufacturer of the genomic DNA extraction kit (Plate 3). The methods of sampling, sample preparation, taking measurements, skin taxidermy preparation and specimen preservation followed Lundrigan *et al*., (2002), Aplin *et al*., (2003), Stephan *et al*., (2005), Shimada *et al*., (2009), and Suzuki *et al*., (2013). The skin taxidermy of each specimen (Plate 5) was kept as the specimen voucher at the Molecular Biology Laboratory in the Zoology Department, University of Yangon.

DNA extraction, mt *Cytb* gene amplification, and sequencing

Genomic DNA from mouse liver tissue samples was extracted and purified by using QI Amp genomic DNA mini kit from Qiagen Germany. Partial sequences of mitochondrial *Cytb* gene (669 bp) from six individuals were used in molecular analysis and amplified by polymerase chain reaction (PCR), (Shimada *et al*., 2001). The universal primer pairs used for PCR amplification are L14724 and H15915 (Irwin *et al*.,1991). The amplification reactions were carried out for thirty cycles, each cycle consisting of 30 sec at 96˚ C for denaturation 30 sec at 50 ˚ C for primer annealing, and 30 sec at 60 ˚ C for an extension. The amplified mt *Cytb* gene (669 bp) was utilized separately for sequencing reactions. PCR products were purified using polyethylene glycol (PEG) precipitation (Shimada *et al*.,2001). Purified PCR products were cycle sequenced using the terminator cycle sequencing kit and the Big Dye terminator cycle sequencing kit (v.3.1) (Applied Biosystems). Automated sequencing of both heavy and light strands was conducted using the Applied Biosystems 3500 genetic Analyzer. DNA extraction and sequencing were conducted at Molecular Biology Laboratory in Zoology Department, Yangon University.

Mitochondria *Cytb* sequence alignment

All mt *Cytb* sequence outputs were compared with those in the GenBank database by the BLAST program (http:// blast. Ncbi.nlm.nih.gov/blast.gi) for making sure those were corrected sequences. Using BLAST closely related *Cytb* sequences from the public database were retrieved and added to the alignment. After checking for corrected sequences, each base character of all sequences was edited by visual inspection, and then edited sequences from each primer were assembled for each complete sequence by using MEGA X (Tamura *et al*.,1993) to calculate genetic distances.

Phylogenetic Analysis

Phylogenetic analysis was carried out to investigate the evolutionary relationships of the studied specimens as follows: (1) Neighbor-Joining (NJ) and (2) ML (maximum likelihood) model parameters were estimated; distances were calculated applying Tamura and Nei's (1993) method using a parameter of the gamma distribution. Bootstrap analysis was carried out 1000 and 100 in the NJ and ML analysis respectively. Non-parametric bootstrap analyses with 1,000 replicates were performed to obtain estimates of support for each node of the NJ trees. For ML analyses, the informative sites were analyzed using equally weighted characters and were searched by heuristic option with a stepwise starting tree, and random stepwise addition of 1,000 replicates. Gaps were treated as missing data. If this heuristic search option yielded more than one of the most parsimonious trees, they were joined in one tree of the semi-strict. Finally, the statistical support for recovered nodes was assessed using a non-parametric bootstrap analysis with 1,000 replicates. This algorithm was used for the data set that has more than twenty samples of taxa.

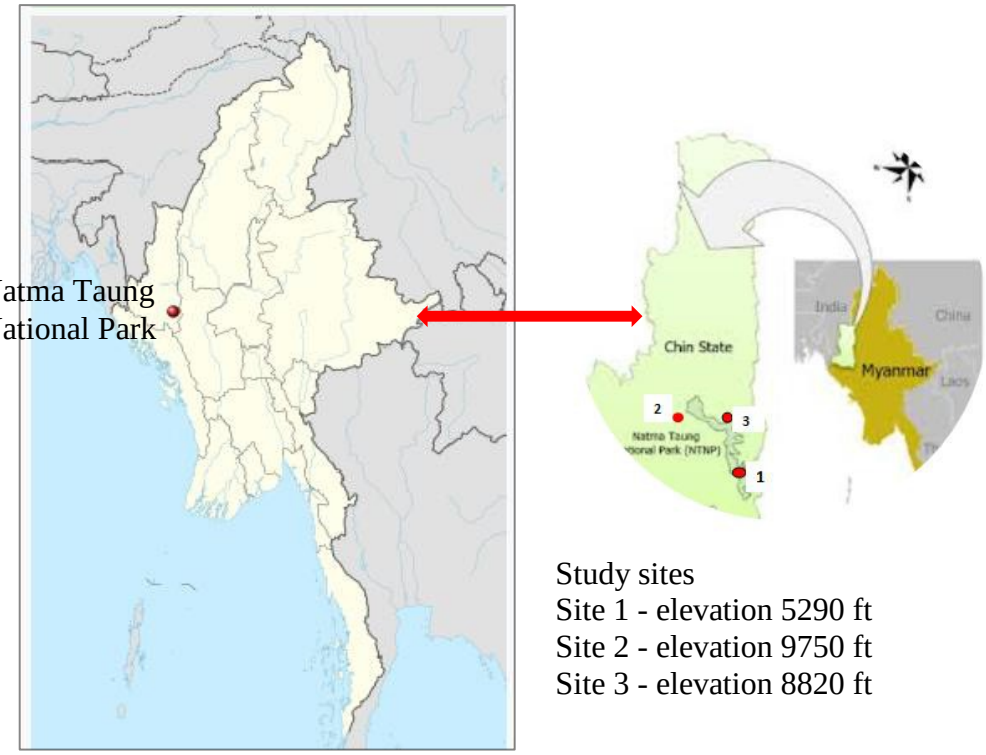


Figure 1 Map of Natma Taung National Park

Site 1 Near human habitation (Elevation 5290 ft)

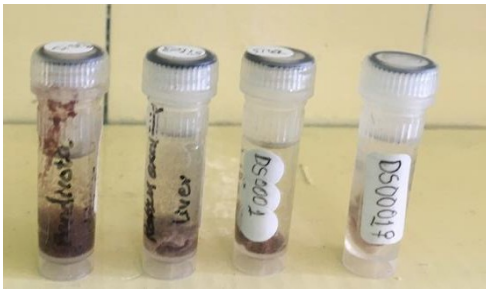
Site 2 Forest (Elevation 9750 ft) **Site 3** Near forest camp (Elevation 8820 ft)

Plate 1 Trapping sites 1, 2 and 3 in the Natma Taung National Park

A. Local snap

B. Sherman trap

Plate 2 Different kinds of small mammal's traps



Liver tissue sample kept in 70 % ethanol

Plate 3 Biological samples for DNA extraction

Results and Discussion

Specimen collection

A total of seven live specimens were collected from three different study sites at Natma Taung National Park. The specimens coded as NMT 1, 2, 3, 4, 5, and 6 were collected from site 2 (9750ft elevation) and NMT 7 was collected from site 3 (8820ft elevation). Detailed data are in Table 1. The DNA extracted from the specimen NMT1 was not enough to use in further steps of molecular biological study, and it was expelled in the list.

Morphometric measurements

The data set of morphometric measurements were shown in Table 2. Based on the morphometric measurements and the photos of live specimens, the specimens were tentatively identified as *Apodemus* sp. (NMT 6,7), *Ethenomys* sp. (NMT 2,3,4), and *Episorculus* sp. (NMT5) (Table 2, Plate 3).

Molecular Biological analysis

Molecular Biological analysis was conducted to confirm the species of the studied specimen and also to explore their molecular phylogenetic status and relationship with those found in the neighboring countries.

(a) Genetic distances

After sequence alignment, the genetic distance between the studied specimens and their GenBank references was obtained.

The genetic distance between NMT2 of *Eothenomys eleusis* compared with Gen Bank references of HM 165381 China was about 0.022 and NMT3 and KT 899700 China was 0.033. Gen Bank reference of KY 997347 China and NMT4 genetic distance was 0.016 (Table 4). Genetic distance within NMT5 *Episoriculus caudutus* and Gen Bank references of MK 962210

China, MK 962225 China, and MK 997347 China were 0.00439, 0.00183, and 0.00739 respectively (Table 5). The Genetic distances of NMT6 compared with MK JF503232 China and JF503234 China, JF 503240 China, and AY 389018 China were 0.005, 0.004, 0.007, and 0.0289 respectively (Table 6).

(b) Phylogenetic tree analysis

Maximum Likelihood (ML) trees were constructed based on the *Cytb* gene (669 bp) for the three species; *Apodemus ilex*, *Ethenomys eleusis*, and *Episoriculus caudutus* recorded in the present study separately due to the spacing because of the long tree if showed in a single tree (Fig 1,2,3).

In the ML analysis of the *Cytb* gene of *Episoriculus caudutus* (NMT 5) and MK 962210 Gen Bank from China, the bootstrap values of similarity percentage were about 0.04%. The value between the clade of *Episoriculus caudutus* and *Episoriculus leucops* GU 981281 China was about 80% (Fig. 2 A). The ML analysis of the *Cytb* gene of *Eothenomys eleusis* (NMT2,3,4) and *Eothenomys miletus* exhibited a notably high degree of similarity (0.02% and 0.05 % respectively) (Fig. 2 B). The ML analysis of the *Cytb* gene of *Apodemus ilex* (NMT6,7) haplotypes clustered compared with GenBank references from China; they within the species were 0.01%. *Eothenomys eleusis* haplotypes and HM 165381 China from Gen Bank reference within the species is 0.03 %. In this case, the internal branches were extremely supported by bootstrap analysis (99%) (Fig. 2 C).

Table 1 List of specimens recorded and collected from the study sites

Sr No.	Specimen code	GPS position	Location	Place	Elevation	Sample site
1.	NMT 2	N 21˚ 13' 25" E 93˚ 55' 17"	Nat ma Taung National Park	Forest	9750ft	2
2.	NMT 3	N 21˚ 13' 25" E 93˚ 55' 17"	Nat ma Taung National Park	Forest	9750ft	2
3.	NMT 4	N 21˚ 13' 25" E 93˚ 55' 17"	Nat ma Taung National Park	Forest	9750ft	2
4.	NMT 5	N 21˚ 13' 25" E 93˚ 55' 17"	Nat ma Taung National Park	Forest	9750ft	2
5.	NMT 6	N 21˚ 13' 25" E 93˚ 55' 17"	Nat ma Taung National Park	Forest	9750ft	2
6.	NMT 7	N 23˚13' 9" E 93˚ 56' 26 "	Nat ma Taung National Park	Near Forest camp	8820ft	3

Table 2 Morphometric measurements of collected specimens

Sr No.	Specimen Code	TL(mm)	T(mm)	W(gram)	Hfcu(mm)	E(mm)	HB(gram)	Sex
1.	NMT 2	155	45	25.6	20	13	66	Male
2.	NMT 3	145	46	2.1	16	12	63	Male
3.	NMT 4	145	46	2.3	16	12	63	Female
4.	NMT 5	110	48	7.4	15	12	52	Female
5.	NMT 6	134	42	22.4	20	12	58	Male
6.	NMT 7	169	68	29.6	23	18	79	Female

Live specimen of *Episoriculus caudutus*

Live specimen of *Eothenoys eleusis*

Dorsal view of *Eothenomys eleusis*

Ventral view of *Eothenomys eleusis*

Dorsal view of *Apodemus ilex*

Ventral view of *Apodemus ilex*

Plate 4 Collected species

Plate 5 Skin taxidermy preparation of the collected species

Table 4 Genetic distances between *Eothenomys eleusis* and Gen Bank references

NMT 2	Natma Taung National Park	vs	HM	165381	China	0.0223
NMT 3	Natma Taung National Park	vs	KT	899700	China	0.0331
NMT 4	Natma Taung National Park	vs	KY	997347	China	0.0162

Table 5 Genetic distances between *Episoriculus caudutus* and Gen Bank references

NMT 5 Natma Taung National Park	vs	MK	962210	China	0.00439
NMT 5 Natma Taung National Park	vs	MK	962225	China	0.00183
NMT 5 Natma Taung National Park	vs	MK	962220	China	0.00739

Table 6 Genetic distances between *Apodemus ilex* and Gen Bank references

NMT 6 Natma Taung National Park	vs	JF	503232	China	0.005
NMT 6 Natma Taung National Park	vs	JF	503234	China	0.004
NMT 7 Natma Taung National Park	vs	JF	503240	China	0.007
NMT 7 Natma Taung National Park	vs	AY	389018	China	0.028

Cytb gene Maximum Likelihood tree (ML)

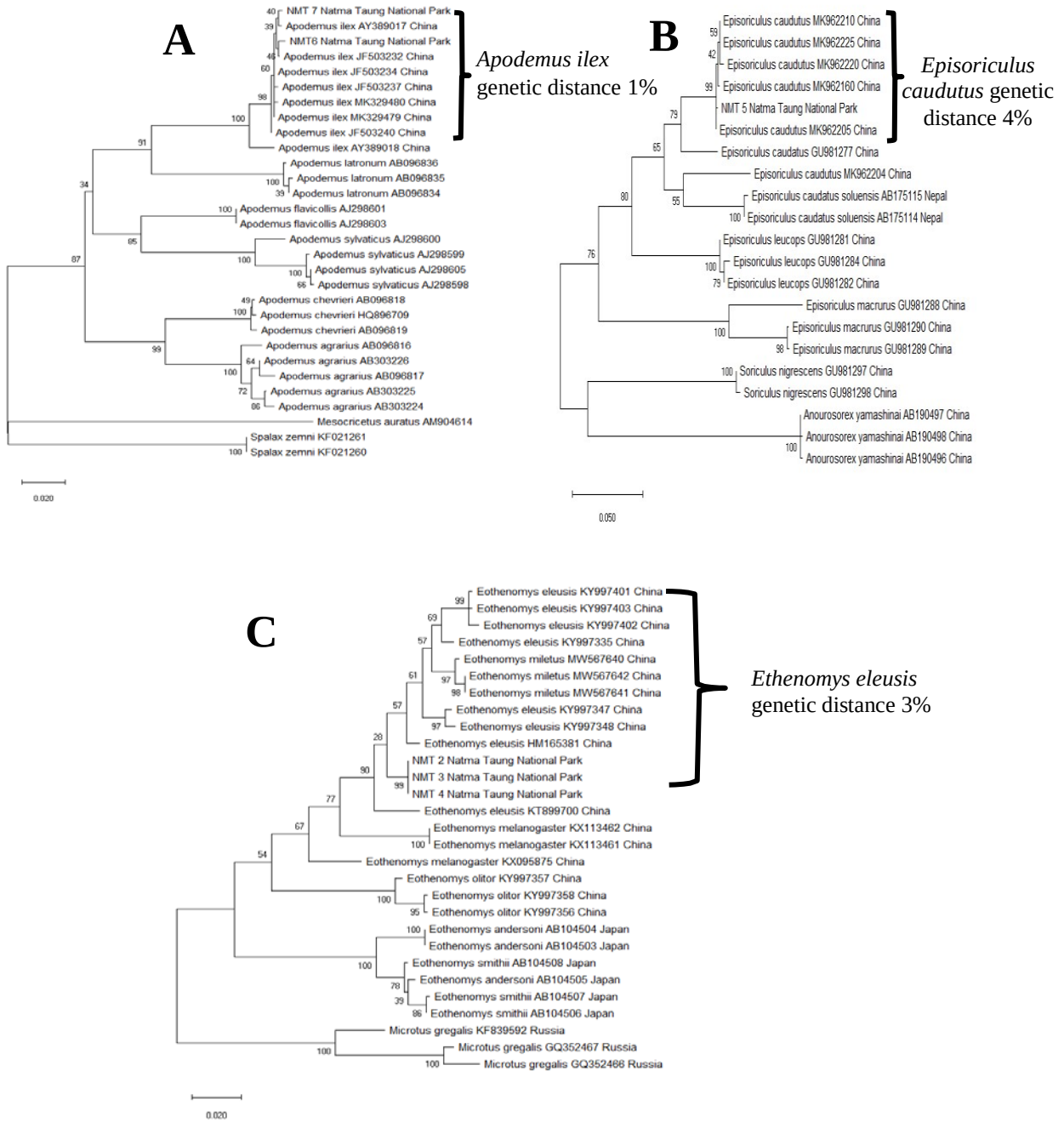


Figure 2 Maximum Likelihood (ML) tree constructed using mtDNA *Cytb* data of the (A) *Episoriculus caudutus* (B) *Ethenomys eleusis*, and (C) *Apodemus ilex* from Natma Taung National Park of Myanmar. Bootstrap values, expressed as a percentage of 1000 replications, are given at each node. The scale indicates, the number of nucleotide substitutions per site.

Discussion

Molecular phylogenetic analysis confirmed the discovery of 2 species of family Muridae; *Apodemus ilex* and *Ethenomys eleusis* and one species of the family Soricidae; *Episoriculus caudatus* as the new records of the biodiversity-rich Natma Taung National Park.

Abramov *et al*., (2017) reported the first record of *Episoriculus caudatus* from Vietnam. They reported the *cytb* (1140 bp) and nuclear *ApoB* (518 bp) and *RAG2* (730) genes which were used to estimate the phylogenetic relationships in Asiatic red-toothed shrews (Soricidae,Episoriculus). Based on molecular data, the genus *Episoriculus* seems to consist of at least seven valid species: *E. baileyi*, *E. caudatus*, *E.leucops*, *E.macrurus*, *E. sacratus*, *E. soluensis*, and *E. umbrinus*. Genetic distances among all of them are found to be of 8–16%, with the only low distance (3.4%) being that between E. baileyi and *E. leucops*. Taiwanese shrew *E. fumidus* shows high genetic divergence (16–17% for *Cytb*) from other species of *Episoriculus*. The record of finding *Episoriculus caudatus* (family Soricidae) is to agree with the report of Hoffmann (1986) mentioning that shrews of this group are widely distributed in Asia from northern China, southward to northern Vietnam and Myanmar, and from Kashmir to Taiwan. Abramov *et al*., (2017) mentioned the Kachin State of Northern Myanmar as one of the distribution sites of *Episorticulus caudatus* in their report.

Based on the review by Hoffmann (1986), four species are recognized in *Episoriculus*: *E.caudatus* (Horsfield, 1851) distributed from Kashmir to northern Myanmar and south-western China; *E.leucops* (Horsfield, 1855) distributed from central Nepal, Sikkim, and Assam to southern China and to northern Myanmar and northern Vietnam. *Episoriculus* caudate includes three subspecies (*cadatus*, *sacratus*, and *umbrinus*) (Hoffmann 1986, Hutterer, 2005).

In this study, the genetic distance between the collected specimen NMT5 and the Gen Bank reference (MK 962210 China) showed 0.04%. The record of the finding *Episoriculus caudatus* (family Soricidae) is in agreement with the report of Abramov *et. al.*, (2017) and Hoffmann (1986), mentioning that shrews of this group are widely distributed in Asia from northern China, Southward to Northern Vietnam and Myanmar, and from Kashmir to Taiwan.

Oriental voles are traditionally included in the genus *Ethenomys* (Muridae: Clethrionomyini), and inhabit in the Trans-Himalayan Ranges of Southwest China, small parts of Northeast Burma, and the Assam province in India (Suzuki *et al*.,2004). Miller (1896) first proposed the genus *Eothenomys* (which included Oriental and Japanese red-blacked voles) and Hilton (1923, 1926) subsequently designated it as a valid genus. The results of *Cytb* gene sequences from these two species were nearly identical. The result of pair-wise distances between these two species was 0.02 to 0.05%. In this result, the phylogenetic relationship of *Eothenomys eleusis* is more closely related to each other than to *E. miletus* (Suzuki *et al*., 2004).

Although *E.eleusis* and *E. miletus* were proposed as separate subspecies or species (Allen, 1940; Hinton, 1923; Musser and Carelon, 1993; Thomas, 1912a, b; Wang and Li, 2000), the *Cytb* sequences from these two taxa were nearly identical. This evidence suggested that *E.eleusis* and *E.miletus* should not be considered separate species at the genetic level. It should be cautioned, however, phylogenetic relationships inferred from single gene studies might be biased due to gene-tree effects, and evidence from additional molecular markers (i,e, nuclear genes) is required to independently assess this finding (Chen *et al*., 2003), and address the potential problem of hybridization events between these " species" (Sang and Zhong, 2000). However, more detailed information about the distribution of these endemic species and the geography of this area, together with additional taxon sampling is still required to develop the evolutionary history of Oriental voles in Southeast Asia (Suzuki *et al*., 2004). As the murine rodent (family Muridae), Genus *Mus* species were not found at Natma Taung National Park.

Khin Myat Myat Zaw *et al*., (2019) reported the Murine rodents, *Mus musculus, Mus. fragilicauda, Mus nitidulus, Mus musculus,* and *Mus lepidoides.* They are distributed in the Central dry zone and Southern Part of Myanmar. The finding of *Mus. fragilicauda* is a new record in the study of Khin Myat Myat Zaw *et al*., (2019). The comparison with *Cytb* sequences to assess the phylogenetic relationship of *Apodemus ilex* at Natma Taung National Park and within GenBank references (AY389018, JF503232, JF503234, JF503240) found that sequence variability was relatively low (0.01%). The species *Ethenomys eleusis, Episoriculus caudatus,* and *Apodemus ilex* were first recorded at different elevations at Natma Taung National Park where many species diversities depend on geographic conditions. In this study, *Apodemus ilex* and *Ethenomys eleusis* species were found in Natma Taung National Park in the Northern part of Myanmar. However, genus *Mus* species were not found at Natma Taung National Park. It may be species distribution and diversity depending on geographic barriers and weather conditions.

Based on a recent Molecular phylogenetic analysis of small mammals, the last 10-20 million years were very important in establishing the current distribution of extant species (eg. Michaux et al. 2002., 2003, 2004., Serizawa *et al*.2000.,2004). Mitochondrial cytochrome b (mt*Cytb*) gene sequences were performed to elucidate the genetic structure of the studied species and provide insight into the factors shaping their genetic structure (Khin Myat Myat Zaw *et al*., 2018). The new record of *Apodemus ilex*, *Ethenomys eleusis*, and *Episorculus caudatus* was analyzed by a molecular phylogenetic approach that consists of one rapidly evolving mitochondrial *Cytb* gene. The results of *Cytb* sequences data found two new genus-species from Natma Taung National Park.

According to the results of the molecular phylogenetic analysis of this present study, the recorded species in this research are closely related to those recorded in China. The distribution and biodiversity depend on the geographic barriers, weather conditions, and flora as the niche of the regarded fauna. Natma Taung National Park is cold weather conditions until summer with the temperature ranged 22˚C to 25C. The temperature varies with the elevation and in this study, the recorded species seem to inhibit at different levels of elevation. Study more specimens with ecological conditions is needed to understand the distribution of the small mammal population in Natma Taung National Park and also their genetic relationship with each other and with those recorded in neighboring countries of Myanmar.

Conclusion

The study of genetic variations and molecular phylogeny of the species will support a better understanding of the ecology, species diversity, and geographic distribution of the studied species. The evolutionary relationships of the studied specimens should be investigated in the future. More specimens should also be studied to get more information about Myanmar's small mammal diversity in Natma Taung National Park. This information on the small mammal species in the study area would be useful for future researches.

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REPRODUCTIVE BIOLOGY OF INDIAN MACKEREL, *RASTRELLIGER KANAGURTA* (CUVIER, 1816) FROM COASTAL REGION, MYANMAR

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Abstract

The present study related to the reproductive biology of Indian mackerel, *Rastrelliger kanagurta* (Cuvier, 1816) was conducted from January 2021 to December 2021. A total of 360 specimens of *Rastrelliger kanagurta* were collected randomly to study their length-weight relationship, sex ratio, gonadosomatic index (GSI), hepatosomatic index(HSI), fecundity and stages of gonad maturity. During the study period, the sex ratio of male to female was 1:1.02. Significant correlation existed between fish length and weight in males ($W = 0.0277L^{3.2159}$, $R^2 = 0.7455$) and females ($W = 0.0293\ L^{3.7046}$, $R^2 = 0.7652$). The highest GSI values of male and female were found in May, August, October and December, 2021. The lowest GSI values of male and female were observed in January and June, 2021. The highest value of HSI was found in March, 2021 whereas the lowest HSI value was in October, 2021 in both sexes. The GSI and HSI values were inversely correlated. Two reproductive cycles in male and female were observed with similar reproductive development. The fecundity of females varied between 2300 eggs and 78000 eggs. Total length, body weight and ovary weight were in linear relationship with fecundity. Macroscopic observation based on the appearance of the sample gonads could be classified into six maturity stages; immature, maturing1, maturing2, mature, spawning and spent. Six developmental stages of oogenesis were classified based on the chromatin nucleolus, perinucleolar, cortical alveolar, vitellogenesis, maturation and ovulation conditions. Six developmental stages of spermatogenesis were found as primary and secondary spermatogonia, primary and secondary spermatocyte, spermatid and spermatozoa. Understanding the breeding season of *Rastrelliger kanagurta* is a crucial necessity in obtaining scientific knowledge on artificial propagation process.

Keyword Reproductive biology, Maturity stages, Rastrelliger kanagurta

Introduction

Fishes play an important role in healthy nutrition for humans' consumption. In Myanmar, the total production of fish and shell fish in 2016 was more than 100,000 tonnes (FAO, 2018). Among them, 47% are freshwater varieties and 53% from the sea. Marine aquaculture has been developed in Myanmar in Rakhine Coastal and Tanintharyi Coastal water. Aquaculture sector is interested in the species and culture in the coastal area of Myanmar.

Among the commercially important species, *Rastrelliger kanagurta* (Indian mackerel) is one of the valuable economic species in Myanmar. It is commonly known as Pa-Lar-Tue in local and it belongs to Scombridae family. This fish is valued for its highly nourishing quality and fish oil was extracted for use in food or pharmaceutical industry (Ferdosh, *et al.*, 2012). The flesh of *Rastrelliger kanagurta* was used for marketed fresh, frozen, canned, dried salted, and smoked products. The study of reproductive biology of fishes is essential for conservation and selecting fish candidates of aquaculture from the wild. Fish reproductive biology plays an important role for fishery management and sustainable aquaculture. Histological observation describes the progression of the gonadal development cycle for both males and females during reproductive season.

There are few reports on the reproductive aspects of *Rastrelliger kanagurta* in Myanmar. Research of reproductive biology of *Rastrelliger kanagurta* will enhance the artificial breeding of *Rastrelliger kanagurta* which has high market demand for export. Nowadays, fisheries sectors

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have been changed to sustainable aquaculture production. Information provided in the present research will be important knowledge for the aquaculture farmers who want to start *Rastrelliger kanagurta* culture in coastal area of Myanmar.

The present study was carried out for the reproductive biology based on the various aspects such as length-weight relationship, sex ratio, GSI, HSI, fecundity and analysed the different stages of ovary and testis through morphological and histological examination of *Rastrelliger kanagurta*.

Materials and Methods

Study period and collection sites

The study was conducted from January 2021 to December 2021. Fish samples were monthly obtained from Nyaungdan jetty, Pazundaung Township, Yangon Region, where the fishes were transported from Rakhine Coastal Region.

Sample Collection

A total of 360 Indian mackerels were collected during the study period. A total of 30 specimens in different sizes were sampled monthly. Fish were randomly chosen and samples were put in ice box and transported to the Laboratory of Fisheries and Aquaculture, University of Yangon. Total lengths and body weight were recorded individually. Gonad weight and liver weight were taken by an electronic balance of 0.001 g accuracy. Size, color and appearance of the gonads were noted.

Identification and classification

Identification and classification of *Rastrelliger kanagurta* (Cuvier, 1816) were based on Day (1878), Talwar and Jhingram (1992) and Fishbase (2013).

Length-weight relationship

Length-weight relationship for males and females were calculated using a formula,

$W = aL^b$ (Le Cren, 1951),

W = body weight of fish

L = total length of fish,

a = constant (intercept)

b = the length exponent (slope)

Sex ratio

All fishes were examined for monthly sex ratio using the following formula;

$$\text{Sex ratio} = \frac{\text{Total number of male}}{\text{Total number of female}}$$

Analysis of Gonadosomatic Index (GSI) (Agarwal, 1996)

Monthly conditions of ovaries were checked and recorded. Calculation of GSI was conducted in order to estimate the peak and decline of the breeding conditions.

Gonadosomatic Index (GSI) was calculated using the formula;

$$\text{GSI} = \frac{\text{Gonad weight}}{\text{Whole body weight}} \times 100$$

Analysis of Hepatosomatic Index (HSI) (Wingfield and Grimm, 1977)

$$\text{HSI} = \frac{\text{Weight of liver}}{\text{Whole body weight}} \times 100$$

Fecundity (Bagenal, 1978)

In the present study the fecundity of *R. kanagurta* was determined from the investigation of 30 fishes with a total length range of 18 - 30 cm. The gravimetric method was used and checked the ripe ovary for estimating fecundity. The ovary (1 g) was weighed and number of eggs was counted. The relationships of total length and fecundity, body weight and fecundity and ovary weight and fecundity were calculated.

$$\text{N} = \frac{\text{Wt} \times \text{Ns}}{\text{Ws}} \quad \text{(Bagenal, 1978)}$$

Where, Wt = Total weight of ovary; Ws = Weight of subsample;

Ns = Number of oocytes in the subsample

Maturity stages of gonads

Maturity stages were recorded based on gross morphology of gonads development. The characters used for the classification of the gonads were the appearance, color, size of the ovary (bulging, half shrink and the presence of blood vessels on the ovary). The macroscopic examination of the gonads could be classified as five stages of maturity which were categorized as immature, maturing, mature, spawning, spent in both males and females. Percentage of different stages was recorded from slides sections of ovaries.

Histological study of gonads

Gonad samples were taken for further histological examination by using double staining method according to Harris's Haematoxylin and Eosin methods. Gonad tissues were dehydrated with a series of Ethanol and cleared in xylene. They were embedded in paraffin and the fixed tissues were serially sectioned at 5µm thickness and stained with Haematoxylin and Eosin. They were then mounted in DPX.

The sectioned ovaries and testes were observed under the compound microscope (Olympus – CX131). Spermatogenesis could be classified into five stages involved the spermatogonia, primary and secondary spermatocyte, spermatid and spermatozoa. Five oocyte developmental stages of oogenesis were classified as chromatin nucleolus, perinucleolar, cortical alveolar, vitellogenesis, and maturation (Pradhan and Palekar, 1956; Ravaglia and Maggese, 2002; Agrawal, 2004).

Data analysis

Linear regression method was used to analyze the length-weight relationship of male and female, relationship between fecundity and body weight, total length and ovary weight of the female. The sex ratio was tested by using chi-square test.

Male

Female

Figure 1 Morphology of *Rastrelliger kanagurta*

Results

Monthly Length-weight variations of males and females of *Rastrelliger kanagurta*

The regression equations for the length-weight relationship of males and females were calculated using the data described in Table 1 and 2, however, undifferentiated individuals were excluded.

Table 1 Monthly variation in body parameters of male *Rastrelliger kanagurta*

Month	Male		
	Sample number	Total length(cm)	Body weight(g)
January	12	24.13± 2.13	158.78 ±49.29
February	14	22.5 ±2.29	132.34 ±44.44
March	13	25.07± 2.58	188.7±63.98
April	15	24.3 ±2.21	160.52 ±52.15
May	14	22.35 ±2.93	137.57± 58.48
June	16	23.59± 0.88	144 ±20.51
July	16	22.56 ±2.01	130.68 ±33.04
August	13	23.61 ±3.60	159.31 ±67.32
September	13	24.77 ±2.77	185.36 ±60.33
October	15	22.65 ±6.38	150.86 ±59.66
November	14	23.89± 0.73	158.65 ±13.27
December	12	24.91± 1.93	187.44 ±46.29

The total length (TL) of male ranged from 18.0cm to 30.0cm with a mean value of 23.81±2.48 cm. The body weight (BW) of male ranged from 68.3g to 296.9g with a mean value of 158.08±52.08g. The length-weight relation for male was $W = 0.0277L^{3.2159}$ (b=3.2159, $R^2 = 0.7455$, n=167) (Fig. 2).

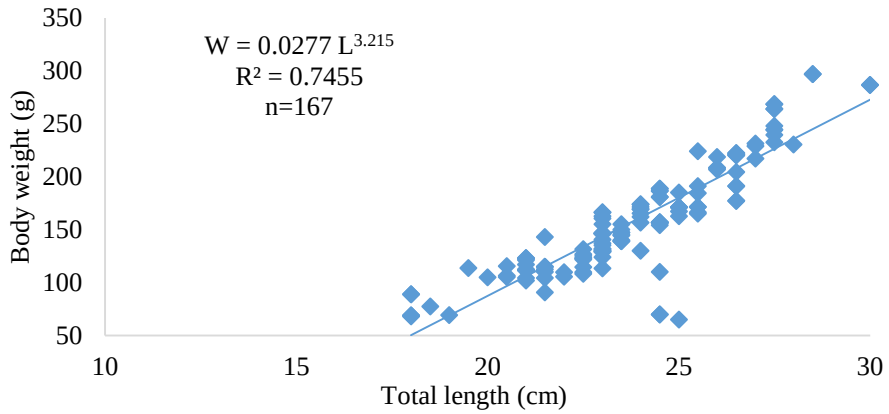


Figure 2 Length-weight relationship of male *Rastrelliger kanagurta*

Table 2 Monthly variations in body parameters of female *Rastrelliger kanagurta*

Month	Female		
	Sample number	Total length(cm)	Body weight(g)
January	15	23.43 ± 1.28	139.85 ± 32.67
February	13	22.42 ± 3.06	143.03 ± 58.12
March	17	23.94 ± 2.42	166.70 ± 59.23
April	13	23.07 ± 1.20	129.9 ± 28.67
May	16	24.71 ± 2.55	185.42 ± 47.40
June	11	23.72 ± 1.40	148.18 ± 24.50
July	13	21.96 ± 1.54	120.38 ± 24.34
August	17	22.06 ± 3.38	144.69 ± 69.68
September	17	23.76 ± 3.02	169.28 ± 55.10
October	15	23.8 ± 2.21	160.36 ± 45.78
November	16	24.18 ± 0.89	162.85 ± 10.53
December	16	23.97 ± 1.34	158.30 ± 43.04

The total length (TL) of female ranged from 18.0cm to 30.0cm with a mean value of 23.85± 2.45 cm during the studied period. The body weight (BW) of female ranged from 68.5g to 332.6g with a mean value of 160.88±52.43 g. The length-weight relation for female was $W = 0.0293\ L^{3.7046}$ (b=3.7046, $R^2 = 0.7652$, n=179) (Fig. 3).

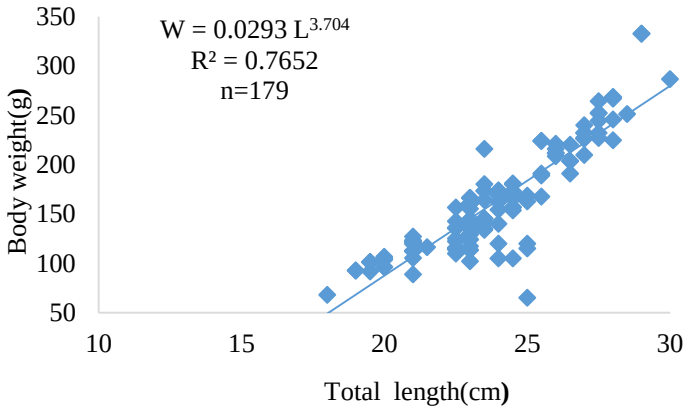


Figure 3 Length-weight relationship of female *Rastrelliger kanagurta*

Sex ratio of *Rastrelliger kanagurta*

The observed sex ratio was not different during the studied period. The percentages of the male and female populations were 49% and 51% respectively during the studied period. The overall sex ratio of male to female was approximately 1:1.The monthly distributions of the sex ratio were presented in Table 3. Monthly variation of sex ratio of fish was described in (Fig.4).

Table 3 Monthly variations of sex ratio of *Rastrelliger kanagurta*

Months	Total number	Number of male	Number of female	Undifferentiated number	Sex ratio (M:F)
January	30	12	15	3	0.8:1
February	30	14	13	3	1.08:1
March	30	15	11	4	1.36:1
April	30	15	13	2	1.15:1
May	30	15	15	0	1:1
June	30	16	11	3	1.45:1
July	30	15	14	1	1.07:1
August	30	13	17	0	0.76:1
September	30	13	15	2	0.86:1
October	30	14	16	0	0.87:1
November	30	14	16	0	0.87:1
December	30	12	16	2	0.75:1
Total	360	168	172	20	0.99:1

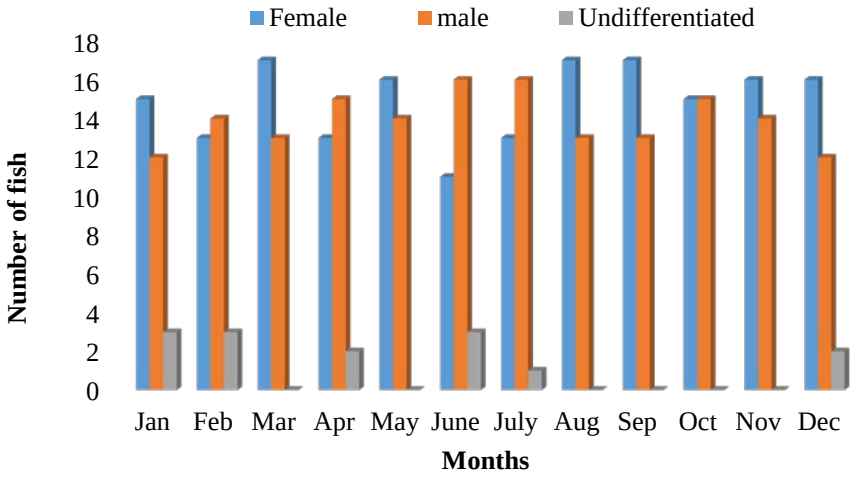


Figure 4 Monthly variation of sex ratio in *Rastrelliger kanagurta*

Fecundity of *Rastrelliger kanagurta*

In the present study, the 30 ripe females were evaluated for fecundity. The fecundity was correlated with the total length, body weight and ovary weight. The fecundity of *Rastrelliger kanagurta* was determined ranging from 18.0 to 30.0cm in total length and 68.5g to 332.6g in body weight. The fecundity of females varied between 2300 eggs and 78000 eggs (Table 4).

Table 4 Fecundity in ripe individuals of *Rastrelliger kanagurta*

Sample number	Total length(cm)	Body weight(g)	Ovary weight(g)	Number of mature ova (Fecundity)
1	18	68	2.6	2860
2	22.5	115.7	9.8	29400
3	24.5	172.6	7.3	25500
4	27	239.7	9.6	28650
5	28	224.7	8.3	27190
6	28	245.6	10.5	42000
7	21	119.7	5.9	13200
8	29	332.6	21.9	77600
9	26	221.2	6.7	15260
10	27	231.9	5.2	7800
11	27	226.3	3.6	2800
12	23.5	173.5	3.7	6510
13	26	215.8	4.7	5570
14	24.5	167.3	3.9	4960
15	28	266.6	29.2	52000
16	27.5	227.2	6.2	3360
17	23.5	134	4.8	2310
18	27.5	231.9	6.5	7680
19	24	169.6	4.9	5980
20	23	137.8	3	2700
21	25	65	3.2	3400
22	24.5	172.8	8.5	8680
23	26.5	203.6	9.3	5950
24	28	268.4	13.1	13500
25	27.5	252.2	11.3	9000
26	30	286.5	13.2	19300
27	23	160.5	4.5	6100
28	24.5	180.2	3.7	3500
29	24.5	167	5.1	3600
30	25.5	224	12.8	49700

The relationship between the fecundity (F) and total length (TL)

The relationship between fecundity (F) and total length of fish (TL) was calculated and the result was $F = 2891.6\ TL - 57486$ ($R^2 = 0.1652$). The relationship between fecundity and total length was found in linear form (Fig.5).

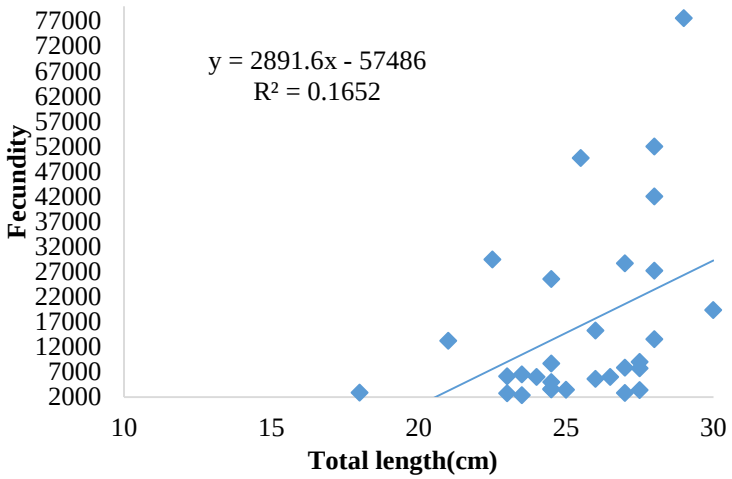


Figure 5 Total length and fecundity relationship of *Rastrelliger kanagurta*

The relationship between the fecundity (F) and body weight (BW)

The relationship between fecundity (F) and body weight of fish (BW) was calculated and it was F = 161.78 BW – 15626 (R^2 = 0.2981). The linear relationship between fecundity and fish weight showed that the fecundity increased in direct proportional to fish weight (Fig. 6).

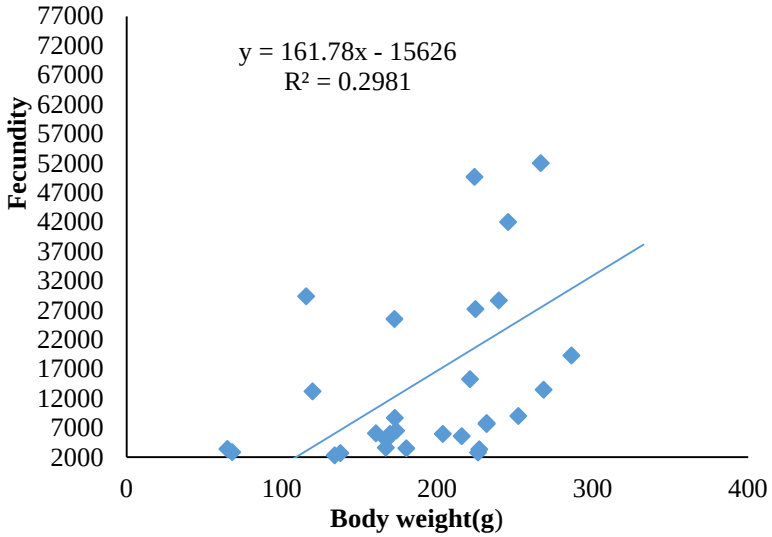


Figure 6 Body weight and fecundity relationship of *Rastrelliger kanagurta*

The relationship between the fecundity (F) and ovary weight (OW)

The relationship between fecundity (F) and ovary weight (OW) was calculated and it was F = 2582.2 OW + 4713.7 (R^2 = 0.6647). The relationship between fecundity and ovary weight was found in linear and fecundity generally increased with the increase in ovary weight. (Fig. 7)

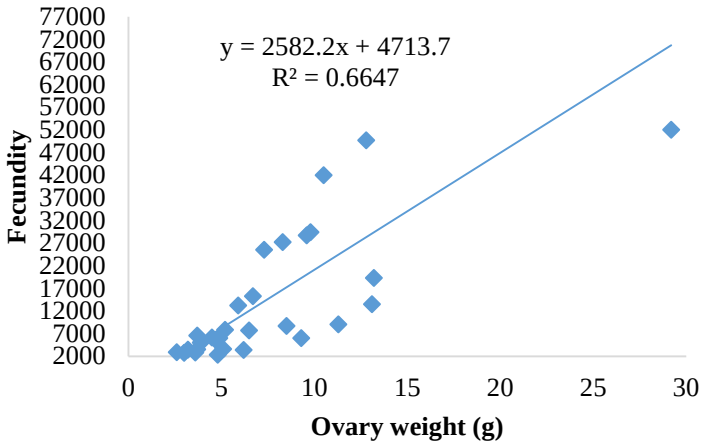


Figure 7 Ovary weight and fecundity relationship of *Rastrelliger kanagurta*

Body length and body weight increased with its fecundity. The number of ova generally increased with increase in length and weight. The calculated R^2values showed better correlation between the fecundity and ovary weight. In this study period, the correlation between fecundity and ovary weight showed more correlation than that of total length and fish weight. The results indicated that the number of eggs per female increased with the increase of length, body weight and ovary weight.

Reproductive condition of *Rastrelliger kanagurta*

Two reproductive cycles in males and females were observed with similar reproductive development. The values of GSI and HSI in males and females were described in Table (5).

Table 5 Monthly GSI and HSI values of males and females of *Rastrelliger kanagurta*

Months	Male		Female	
	GSI (%)	HSI (%)	GSI (%)	HSI (%)
January	1.2	0.9	1.5	1.4
February	2.1	0.8	2.3	0.9
March	1.7	1.5	1.5	1.7
April	2.0	0.9	1.9	1.1
May	2.9	1.3	3.8	1.2
June	1.2	1.4	1.4	1.5
July	1.7	1.1	2.5	0.9
August	2.8	1.2	3.9	1.1
September	1.9	1.1	2.6	0.9
October	2.7	0.5	3.4	0.7
November	1.2	0.7	1.9	0.8
December	2.8	0.6	4.2	1.0

The highest GSI value of female was found in May 3.8%, August 3.9%and October3.4% and Dec 4.2%. The lowest GSI value of female was in June 1.4%. The highest GSI value of male was found in May 2.9%, August 2.8% and October 2.7% and the lowest in January and June (1.2%). The spawning period was determined by monthly evaluation of the gonadosomatic index and maturity stages of oocytes.

The highest values of HSI in males and females (1.7%) were found in March. The lowest HSI value 0.7% was in October. GSI value was negatively correlated with HSI (Fig.8, 9).

GSI was highest for both males and females during the month of May, August, October and December showing occurrence of more ripe individuals.

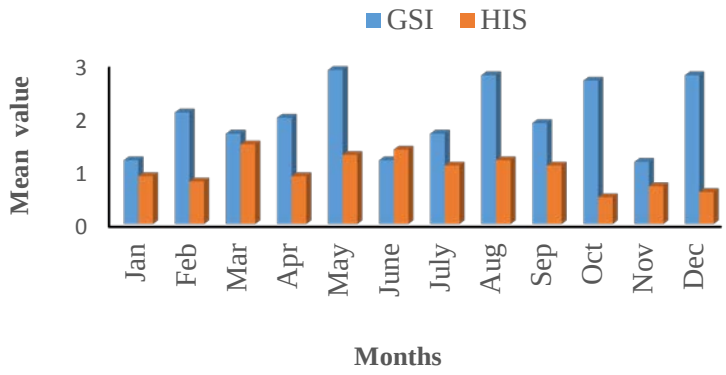


Figure 8 Monthly mean GSI and HSI of male *Rastrelliger kanagurta*

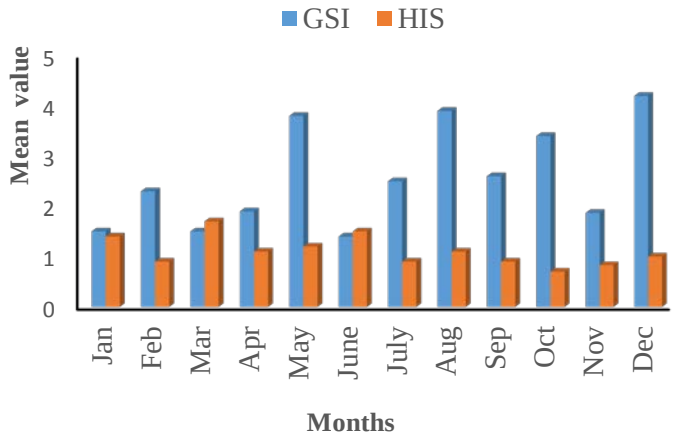


Figure 9 Monthly mean GSI and HSI of female *Rastrelliger kanagurta*

Maturity stages of *Rastrelliger kanagurta*

The maturity stages of males were classified into six stages. Stage I was dominant in January (30%). Stage V was dominant in February (40%), May (55%) and August (50%) and December (45%). Stage VI was not found in May, August, October and December (Fig.10).

The gonadal maturity of females during the studied period was classified as stages I, II, III, IV, V and VI. The stage V was the most dominant in May 53.46%, August 54.25%, October 50.81% and December 50.2%. Stage I (immature stage) was observed dominant in January 29 %. Stage VI (spent stage) was observed in February 20% and June 31%. The stage I, II, III and IV were observed throughout the year. All the six stages occurred in the month of January, February, September and November (Fig. 11).

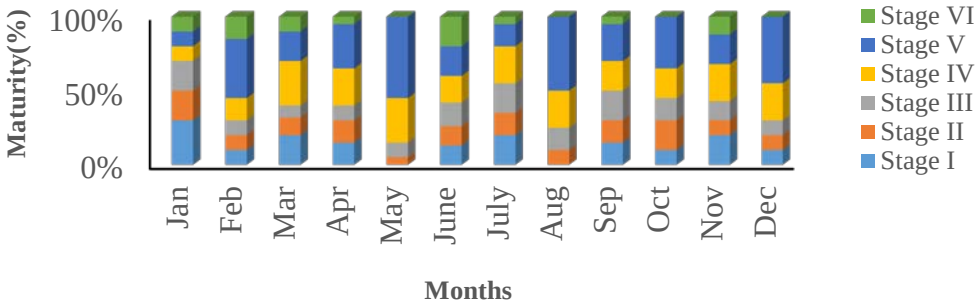


Figure 10 Monthly maturity stages of male *Rastrelliger kanagurta*

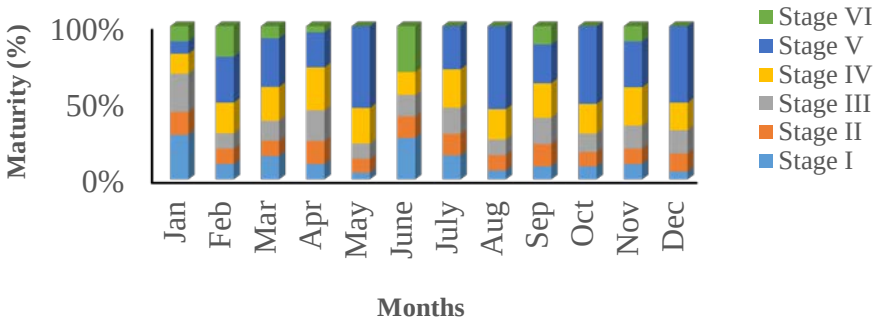


Figure 11 Monthly maturity stages of female *Rastrelliger kanagurta*

Gross morphology of gonad development of male *Rastrelliger kanagurta*

Testes morphology of male *Rastrelliger kanagurta* was classified into six stages based on its external appearance.

Immature- -Testes were elongated, pinkish-white, and transparent membrane. The testis occupied one-third of the body cavity.

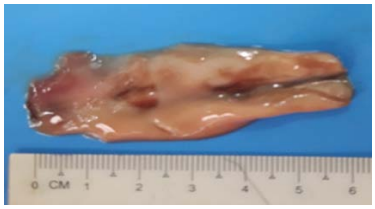
Maturing 1-Testes were in less lobular shape, pinkish white, semi-transparent and easily visible to the naked eye and occupied one-half of the body length.

Maturing 2-Testes were creamy-white, occupying about third-fourth of the body cavity.

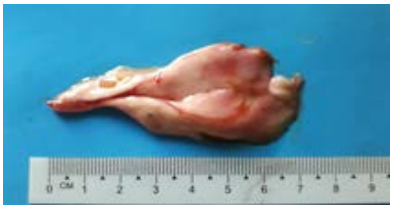
Mature -Testes were large, white to creamy, drops of milt produced when pressed, two stages.

Spawning -Testes were creamy white, soft, lobulation and transparent. Weight and volume increased than mature stage, two third of the body cavity and the pressure to the abdomen, milt flowed out. The left was very slightly larger than the right.

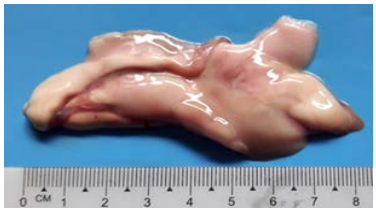
Spent -Testes were shrunken, flaccid, dirty white in color and one-third of the body cavity.



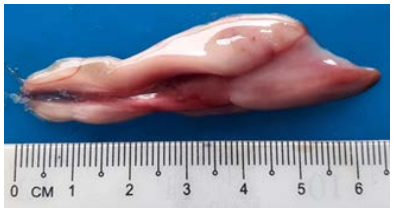
(A) Immature



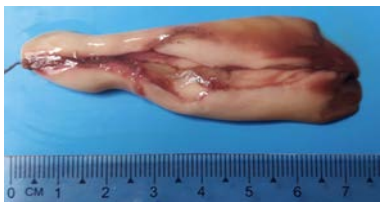
(B) Maturing 1



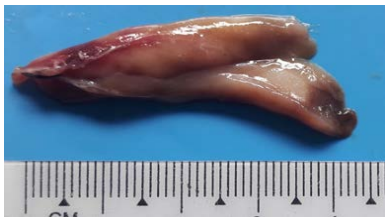
(C) Maturing 2



(D) Mature



(E) Spawning



(F) Spent

Plate 1 Macroscopic developmental stages of male *Rastrelliger kanagurta*

Histological observation of gonad development of male *Rastrelliger kanagurta*

Primary spermatogonia (Stage 1) -This was primary stage of spermatogenesis, which had the largest germ cell, irregularly shaped in the germinal epithelium tissue. These cells were divided and formed primary and secondary spermatocytes.

Secondary spermatogonia (Stage II) - They were structurally similar to spermatogonia except in their sizes. These cells divided and formed primary and secondary spermatocytes.

Primary spermatocytes (Stage III)-They were spherical or oval shape and presented either singly or in small groups. Spermatocytes were more in number with the spermatogonia. Distinctive changes from spermatogonia to spermatocytes was found and decreased in size. They produced secondary spermatocytes.

Secondary spermatocytes (Stage IV) -They were morphologically similar to primary spermatocytes though somewhat smaller and darker. The major difference was the slight reduction in size of the secondary spermatocytes.

Spermatids (Stage V)-Secondary spermatocytes produced spermatids. Spermatids were strongly basophilic spherical cells. They became uniformly condensed in this stage.

Spermatozoa (Stage VI) - Few spermatozoa in sperm duct and more spermatocytes were found. Accumulation of mature spermatozoa was found in the lumen of seminiferous lobules. This stage was the final maturation of the spermatogenesis.

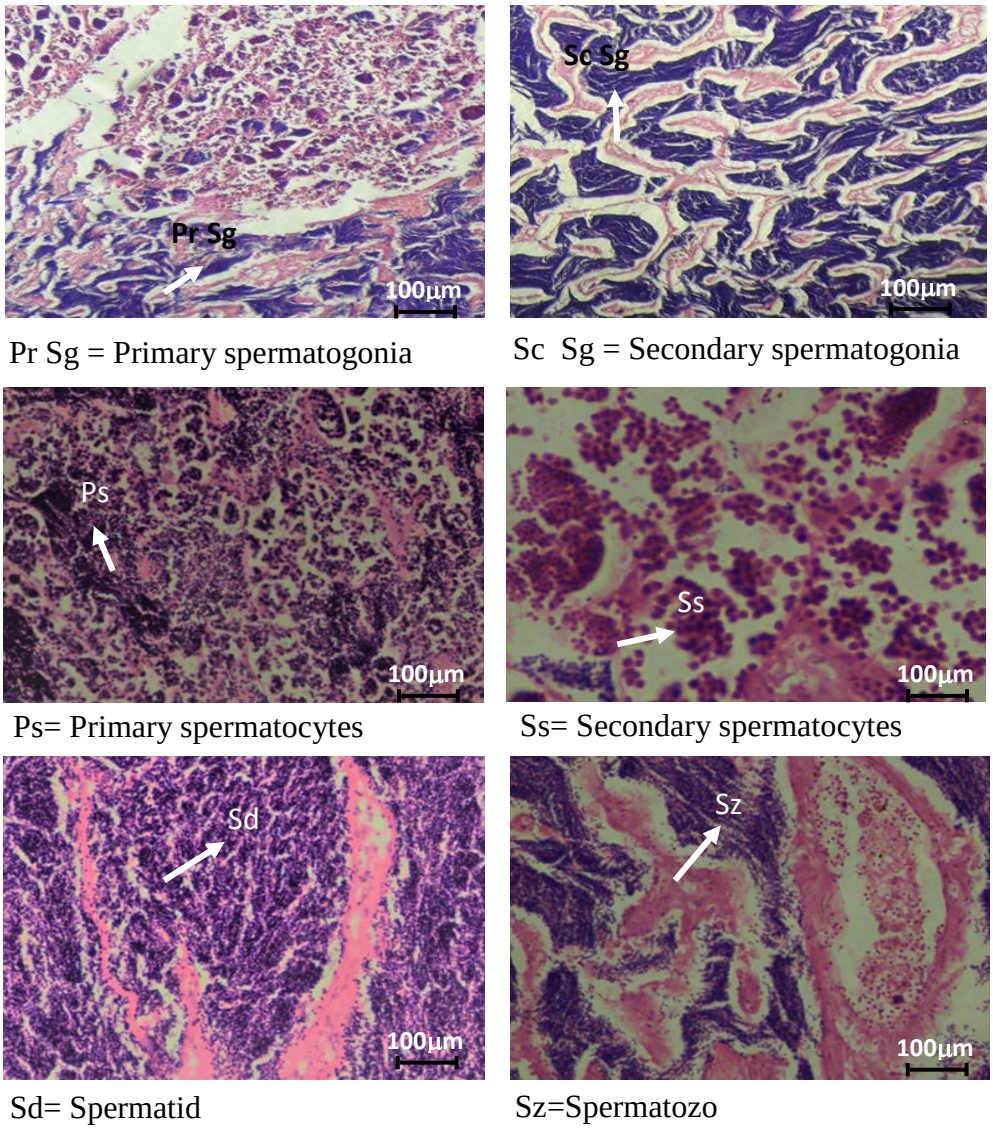


Plate 2 Microscopic developmental stages of male *Rastrelliger kanagurta*

Gross morphology of gonad development of female *Rastrelliger kanagurta*

Ovaries morphology of female *Rastrelliger kanagurta* was classified into six stages based on its external appearance.

Immature- Ovary was relatively small, translucent; Pinkish in color not visible to the naked eye; Occupied about one fourth of the body cavity.

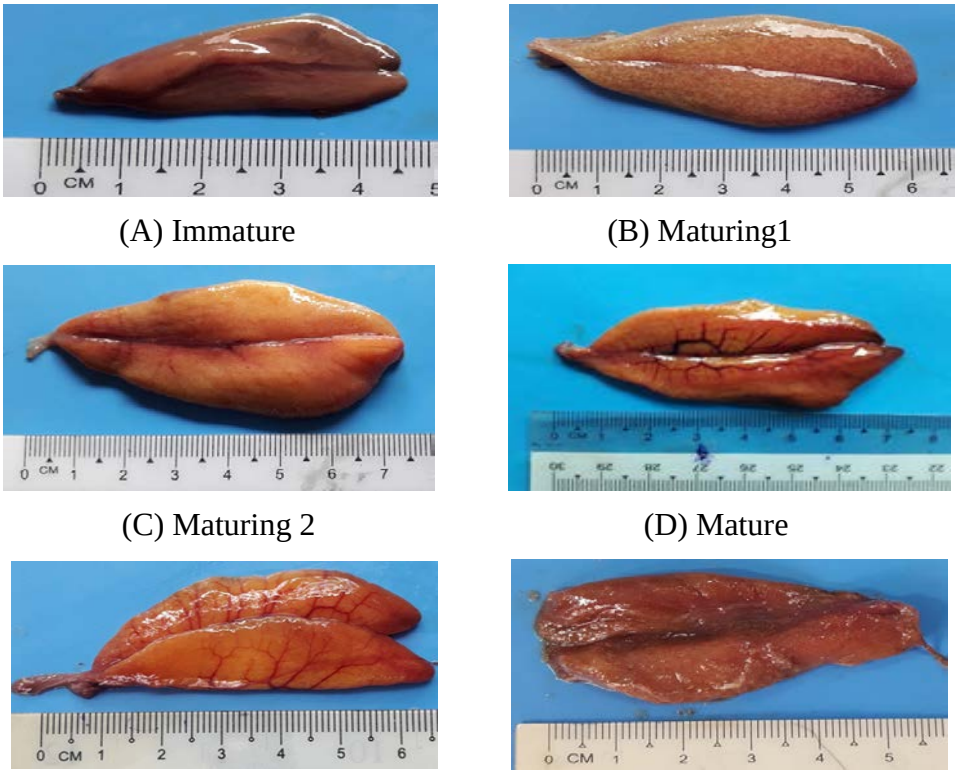
Maturing 1 - The ovary became pale yellow in color and increased in size. Ova were not clearly visible to the naked eye. Blood capillaries were conspicuous and occupied one third of the body cavity of the fish.

Maturing 2 -The ovary became pale yellowish in color and increased in size; Blood vessels visible in dorsal side and occupied one half of the body cavity of the fish.

Mature - Yellowish enlarged ovary occupied nearly two third of the body cavity round section and transparent prominent vascularization. Ovaries appeared granular due to the eggs that were visible to the naked eye. Eggs were not extruded with pressure on the abdomen.

Spawning -The ovary was golden yellow in color filled up with ripe eggs. Blood vessels were still prominent. Transparent ripe ova were clearly visible through the thin ovary wall.

Spent: Ovary was shrunken and flaccid, contained a few whitish residual eggs. Blood vessels were not prominent. Ovaries were smaller and lighter in color than in previous stages, but still reddish brown in color.



(A) Immature (B) Maturing1

(C) Maturing 2 (D) Mature

(E) Spawning (F) Spent

Plate 3 Macroscopic developmental stages of female *Rastrelliger kanagurta*

Histological observation of gonad development of female *Rastrelliger kanagurta*

Chromatin nucleolus (Stage I)- The cytoplasm of these cells were thin and the nucleus was large and rounded, the chromatin nucleolus oocytes, which were observed only in immature females. Oocytes were more abundant than perinucleolar stage oocytes. The oocytes nucleus contained the nucleolus.

Perinucleolus (Stage II) - The cytoplasm was more basophilic than in the previous stage, with a gradual decrease according to the increase in cell size. This stage was characterized by more regular in shape and increased in size of the oocytes, caused by enlargement of the nucleus as well as the cytoplasm. The increase in number of nucleoli indicated increasing nuclear activity.

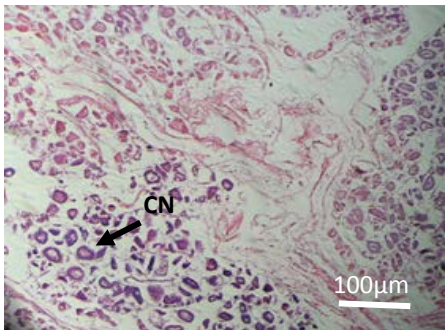
Cortical alveoli (Stage III) – It appeared in the periphery of the cytoplasm. The nucleus and cytoplasm increased compared to the previous stage. Nucleoli and the number of yolk vesicle also increased. A few oil droplets were observed in the cytoplasm around the nucleus. The wall of the ovary was thicker and some oocytes beginning to undergo vitellogenesis with yolk granules on the cytoplasm.

Vitellogenesis (Stage IV) - The deposition of yolk granules was seen at the periphery of the cytoplasm. The vitelline membrane became thicker and follicular cells grew. In this stage, yolk granules rapidly increased in size and number. Yolk granules were densely packed and occupied

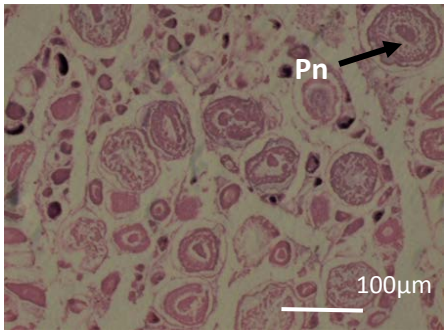
the total volume of the cytoplasm. Large number of nucleoli was observed around the peripheral of nucleus.

Maturation(Stage V) -The nucleus retained with very small nucleoli, lost its spherical shape and shrunk. The vitelline membrane was radially arranged around the oocyte. In this stage, yolk granules and oil droplets continued to increase in size and number, primary and secondary yolk stage also numerous, vacuoles gradually increased in the periphery and central zones. The size of the nucleus was small .The tertiary yolk stage oocytes underwent maturation.

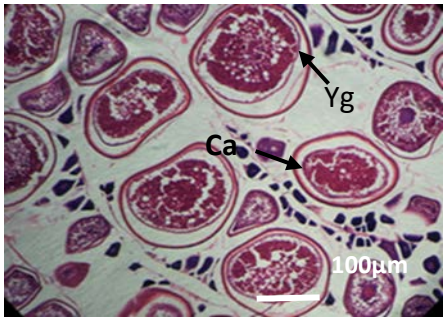
Ovulation(Stage VI)- The ovary showed atretic and discharged follicles, along with stage I and II of oocytes. Post-ovulatory follicles were clearly seen.



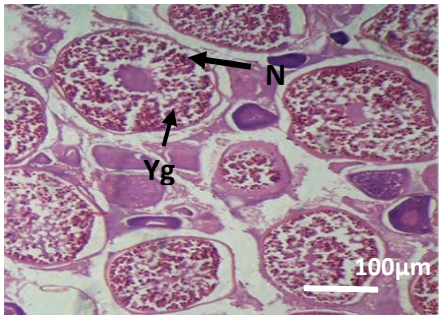
STAGE I. Chromatin nucleolus



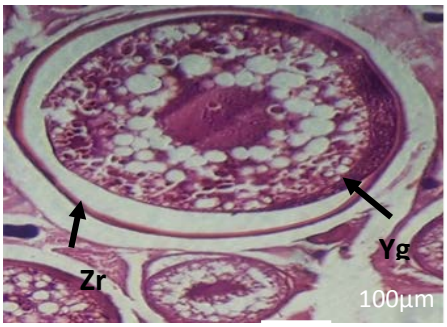
STAGE II. Perinucleolus



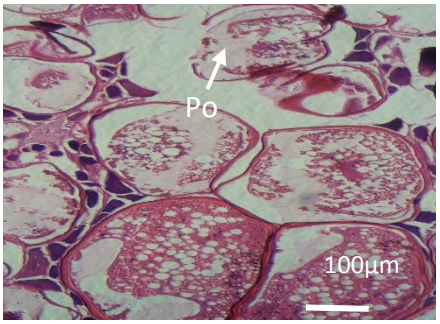
STAGE III. Cortical alveoli
Yg=yolk granule , Ca=cortical alveoli



STAGE IV.Vitellogenesis
Yg=yolk granule, N=nucleus



STAGE V. Maturation
Yg=yolk granule,Z =zona radiata



STAGE VI. Ovulation
Po=postovulatory follicle

Plate 4 Microscopic developmental stages of female *Rastrelliger kanagurta*

Discussion

In the present study, the fish length and weight relationships of male ($W = 0.0277\ L^{3.2159}$, $R^2 = 0.7455$) and female ($W = 0.0293\ L^{3.7046}$, $R^2 = 0.7652$) were investigated. The value of b>3 was found in this study. Length-weight relationship of Indian mackerel was $W = 0.005L^{3\cdot21}$ and having linear growth (Abdurrahman *et al*., 2004 and Gupta, 2004). Hulkoti (2005) reported that length-weight relationship did not show significant difference in both sexes of *R. kanagurta* in the Western Coast of India. Pradhan (1956) stated that length-weight relationship of Indian mackerels was allometric growth. The result supported to the prior researches.

During the studied period, the sex-ratio of *R. kanagurta* showed that the most of the months had equal proportion of males and females. The overall male and female ratio was 1:1.2. Hulkoti (2005) observed the sex ratio of *R. kanagurta* of male: female as 1:0.9 which is in agreement with the present finding. Observation of the fish sex ratio is important for population structure studies. The sex ratio of male and female (1: 1) indicates that the population is in a balanced state in nature.

The spawning period was May (3.8%), August (3.9%), October (3.4%) and December (4.2%) at that time as indicated by GSI peak values. Hulkoti (2005) assessed the values of GSI peaks in July and August indicating the spawning period of this fish. In the present investigation, the gonado somatic index (GSI) increased more obviously in females than males. The monthly GSI values of males and females were in a similar mode, higher values of GSI coincided with spawning period. Male always showed lower gonad somatic index (GSI) values than female due to higher ovary weight compared to testes. Basically, the spawning season determined the occurrence of maturing, mature, and spent stages of individuals. Higher values of GSI are regarded as indicative of spawning season. The present study showed that *R. kanagurta* spawned during all the year, especially with high values in May, August, October and December.

Maturity estimations were based on monthly changes in gonado somatic index in both sexes. Yohannan and Abdurahiman (1998) observed that the indian mackerel spawned in succession and recruitment during the monsoon period in India. In this study the peak reproductive action is coinciding with the monsoon season since it has abundant food supply and the favorable period for larval surviving. Rainboth (1991) pointed out that the rainy season and spawning peaks of fishes were highly correlated.

In the present study, the relation between the spawning period of *Rastrelliger kanagurta* and annual cycle of rainfall was found. The highest GSI was found in July and August which is typically associated with the raining season in Myanmar.

The estimation of fecundity was generally determined by the number of vitellogenic oocytes. During the present study, the relationships between fecundity and total length, body weight, ovary weight of fish were found to be linear. It showed that the fecundity increased with increasing body length, body weight and gonad weight. These observations are in agreement with the observations of Yohannan and Sivadas (2003). During the present study, the absolute fecundity of ripe females was found with 2300 eggs to 78000 eggs. Rao (1967) stated that absolute fecundity of mackerel ranged from 20,911 to111, 000 eggs and Antony Raja and Bande (1972) presented the average fecundity of 38,000 eggs.

Five different stages of maturity were found in most of the months however spent stage was observed in June. Abdussamad, *et al*., (2010) indicated that the spawning stage of Indian mackerel was highly abundant during March to May. Moreover, Hulkoti (2005) observed that spawning season of mackerel extended from June to September.

Longhurst and Pauly (1987) stated that the maturation is a continuous process resulting in the occurrence of mature fishes throughout the year. Abdussamad, *et al.,* (2006) reported the

progress of gonad that depended on the environmental condition occurring throughout the year. In the same fish species of the different areas, the peak of ovarian maturation may be variable due to divergence ecological conditions.

It can be concluded that the females of *R.kanagurta* have three peaks of spawning time in May, August and October. It is recommended to define batch spawners. Size of both sex organs increased as it attained the stage of maturity to produce fully matured gametes. *Rastrelliger kanagurta* was capable of spawning for multiple times.

Lowe-Mc Connell (1987) observed that fish species in tropical river systems were generally noted for very rapid maturation and multiple spawning behavior which was an adaptive response to fluctuations in water level. Wootton (1990) also stated that the liver weight (HSI) decreased as the ovary weight increased during vitellogenesis. The release of energy from the liver into the ovary supports the condition that GSI and HSI values were inversely related with each other as recorded in the present study. Hepatosomatic index and gonadosomatic index were inversely proportional to each other and showed a high hepatic activity. This showed that HSI has reverse action on GSI.

Seifaddini *et.al* (2014) stated the reproductive cycle of the female Indian mackerel, *Rastrelliger kanagurta*, where it demonstrated six stages of ovary maturity and six oocyte developmental stages in the northern Persian Gulf and Oman Sea. In the present study, six different stages of maturity were found in most of the months however spent stage was observed in January and June. Histological examinations indicated the similar gonadal development having six stages of spermatogenesis and oogenesis in the present study.

Conclusion

Understanding the reproductive biology of the species is an important requirement for scientific advice on artificial breeding of Indian Mackerel. The present investigation will contribute to the long-term productivity of *Rastrelliger kanagurta*. Our findings revealed that the studied fish had four distinct spawning peaks in May, August, October and December. Gonad development suggested a pattern of continuous breeding. These results have important implications for the breeding of *Rastrelliger kanagurta* as they demonstrate the potential for induced breeding without being constrained by seasonal variation in reproductive activity. Further research in this area could lead to significant advances in production and management of *Rastrelliger kanagurta*.

Acknowledgements

We would like to thank Dr. Tin Maung Tun, Rector, University of Yangon for his permission to conduct this research.

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EFFECT OF DIFFERENT CULTURE MEDIA FOR THE GROWTH OF SOME MARINE MICROALGAE

May Thu Rein Oo[1], Cho Cho Thin[2], July Maung Maung[3], Aye Aye Khaing[4], Kyaw Naing Than[5], Saw Soe Moe Thu[6], Kay Lwin Tun[7]

Abstract

Microalgae play an important role in supporting the development of aquaculture because they can be used as natural feed for larvae due to their high nutrient value. The present study was to develop a cost effective and optimal growth of some marine microalgae such as *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. by using two different culture media in laboratory condition. conway media for experiment I A_1, B_1 and C_1, and agricultural fertilizer media for experiment II A_2, B_2 and C_2 were used to culture microalgae. Three replications were prepared for each media. The cell densities of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. in different culture media were counted daily through the culture period. The results showed that the maximum population densities of *Nannochloropsis* sp. (8.80 x 10^6 cells/ml), *Chlorella* sp. (8.37 x 10^6 cells/ml), and *Tetraselmis* sp. (8.29 x 10^6 cells/ml) occurred on the fourth day with agricultural fertilizer media, while it was observed on the fifth day with conway media. The population densities of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. started to decrease in agricultural fertilizer media on fifth day while on sixth day in conway media. However, the agricultural fertilizer media had higher density than the conway media. It was concluded that the agricultural fertilizer media was the best for growth rate of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp.

Introduction

Algae are primary producers of the oceans, rivers, streams and lakes (Stottrup & McEvoy, 2003). Microalgae are microorganism such as diatoms, blue green algae and flagellates and primary producers of the oceans, rivers, streams and lakes that change sunlight, water and carbon dioxide in to algal biomass (Stottrup & McEvoy, 2003). The algae are manufactured for complex nutritive molecules including proteins, starches, fatty acids and oils and used as an excellent diet for early stages of mollusks, farmed shrimp, crustacean and fish (Stahl, 2009).

Micro-algae are cultured intensively for direct or indirect feeding through production of zooplanktons and *Artemia nauplii*. Sea water was supplemented with commercial nitrate and phosphate fertilizers, and a few other essential micro nutrients, are commonly used for growing marine microalgae. The elements required for the growth of green algae are N, P, K, Mg, Ca, S, Fe, Cu, MN+, and Zn and these elements are added in the form of salts (Sen *et al*., 2005).

Certain nutrients in appropriate quantities are needed in culture media for the algae to multiply. All media possess nitrogen, phosphorous and carbonate as major nutrients and lack trace metals, vitamins and other mineral nutrients. Some algae require trace metals and minor nutrients for better growth.

The most common microalgae species are *Nannochloropsis* sp., *Scenedesmus* sp., *Isochrysis* sp., *Pavlova* sp., *Dunaliella* sp., *Spirulina phaeodactylum, Chlorella* sp., *Rhodomonas* sp., *Tetraselmi*s sp., *Skeletonema* sp., and *Thalassiosira* sp. (Parrish et al., 2012).

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Among them, *Nannochloropsis* sp. is a single-celled sea microalga that can be used as the live feed for larvae cultivation of shrimp, fish, and shellfish. *Nannochloropsis* sp. is used in aquaculture as a valuable feed, providing polyunsaturated fatty acids, essential vitamins, and amino acids, along with energy. *Nannochloropsis* sp. has high nutrition value, and it is used widely as aquaculture hatchery industry for food of larvae and juvenile of bivalve, rotifer, as well as fish larvae (Tawfiq et al., 1999).

The *Chlorella* sp. is perfect food for shrimps, marine fish and all other ornamental fish, crustacean and also serves as a food for zoo-planktons such as daphnia, moina and rotifer. It is also used in food industry, cosmetics and pharmaceutical industry (Sergejevová and Masojidek, 2011).

Tetraselmis has been one of the microalgae most frequently recommended as a feed for early life stages of shrimp. Providing natural feed usually arises when organisms live in a cultivation environment.

In Myanmar fish farmers have limited experience to cultivate the microalgae such as *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. which is necessary for marine fish and shrimp hatchery. However, fish farmers are facing the high cost of culture medium used in optimal growth of the algae .It is one of the main problems related to the large scale culture of microalgae.

The present study has been conducted to produce the three marine microalgae such as *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. using Conway and agriculture Fertilizers media.

Materials and Methods

Study site and Study period

The present study was conducted in the Live Food Laboratory of Fisheries and Aquaculture in the Research and Innovation Center, University of Yangon .The microalgae was collected from Department of Fisheries (DoF) and cultured in the laboratory from January 2022 to September 2023.

Microalgae collection

The pure strains of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were purchased from Department of Fisheries (DoF). The cultivation of *Nannochloropsis* sp. *Chlorella* sp. and *Tetraselmis* sp. were conducted in Live Food Culture Laboratory of Fisheries and Aquaculture, Center for Research and Innovation, University of Yangon.

Preparation of apparatus

All apparatus (beakers, bottles, measuring spoons and sea water) were covered by aluminum foil and autoclaved at 120 °C for 25 mins to avoid contaminations. The seawater was measured and diluted with distilled water to obtain 25‰ salinity. They were then filtered with Millipore (0.45 μm) filter paper. The solution was then kept in dark and cold place.

Preparation of Conway media

For conway media, three type of solution: solution A, B and C were prepared separately. They were then mixed prior to cultivation of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. For solution A, all chemical substances (Disodium Hydrogen Phosphate, Boric Acid, Ferric Chloride, Manganese Chloride, Na2EDTA and Sodium nitrate) were weighed by using digital balance and added in the beaker that contained 900ml of distilled water.

After adding the substances in the beaker, the solution was stirred by using a magnetic stirrer. (Table1).

Table 1 Composition of conway media (Solution A)

Chemical	Amount
Disodium Hydrogen Phosphate (NaH_2PO_4)	20 g
Boric Acid	33.6 g
Ferric Chloride	1.3 g
Manganese chloride	0.36 g
Na2 EDTA	40 g
Sodium nitrate	100 g
Distilled water	900 ml

For solution B, all chemical Substances (Zinc Chloride, Calcium chloride Ammonium paramolybdate tetrahydrate and Copper II sulfate) were weighed by using digital balance and added in the beaker that contained 100ml of distilled water. The solution was stirred by using a magnetic stirrer (Table 2).

Table 2 Composition of conway media (Solution B)

Chemical	Amount
Zinc Chloride	2.1 g
Calcium chloride	2.1 g
Ammonium paramolybdate tetrahydrate	2.1 g
Copper II sulfate	2 g
Distilled water	100 ml

For solution C, 3mg of Vitamin B1 and 10mg of B12 were added in the beaker that contained 200ml of distilled water.

All the solutions (A, B and C) in Conway media were prepared separately. 900 mL of Solution I was mixed 1 mL of Solution II (Trace metal) into a 1000 mL beaker. Then, the solutions were autoclaved in an autoclave machine for 121°C at 25 mins, the solutions were taken out when the temperature dropped until 80°C. The solutions were added Solution III (Vitamin) and stirred. Then, the solution was stored in a sterilized bottle and kept in refrigerator.

Preparation of agricultural Fertilizer Media

Agricultural fertilizers such as 100 g of Urea, and 20 g of Triple Super Phosphate (TSP) were weighed by using digital balance and added in the beaker that contained 1000ml of distilled water. After adding the substances in the beaker, the solution was stirred by using a magnetic stirrer. The solutions were sterilized in an autoclave machine at 121°C for 25 mins. The solutions were then taken out when the temperature dropped until 80°C. Then, the solutions were stored in a sterilized bottle and kept in refrigerator to avoid contaminations for further use.

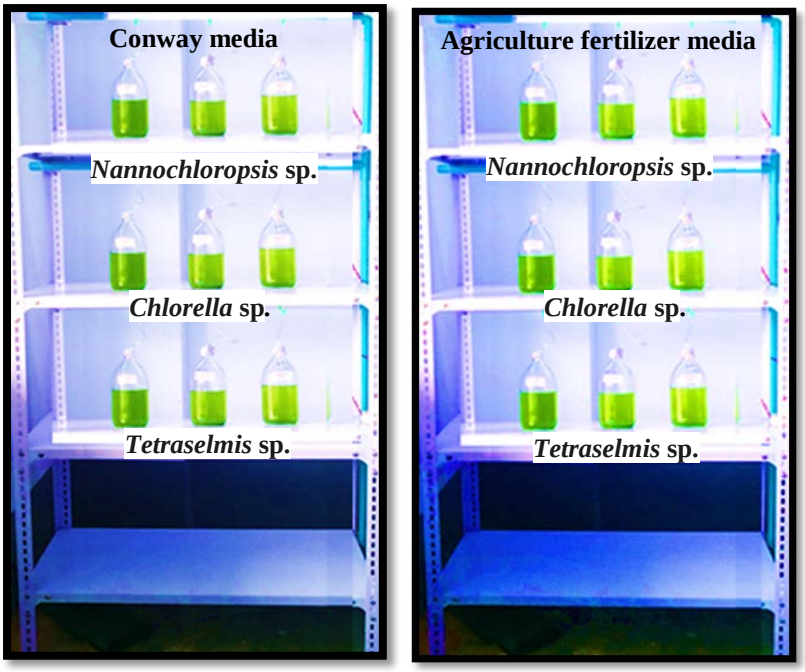
Microalgae Inoculant Preparation

Inoculant preparation of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were started by using the sterilized plankton culture glass bottle (2000 mL). The bottle was filled with 1500 mL of sterilized 25% seawater and add 300 mL of pure strain *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. A total of 3ml of the nutrient agriculture fertilizers media was added for each experiment. Each culture bottle was sealed with aluminum foil and labeled with

date and time. All culture bottles were kept at air-conditions room at 25°C with light by fluorescent tubes and cultured for 4 days so that the microalgae were ready to be used.

Cultivation of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. using conway and agricultural fertilizer media

Nannochloropsis sp., *Chlorella* sp. and *Tetraselmis* sp. were treated with conway and agricultural fertilizer media. The sterilized plankton culture glass bottle (1000 mL**)** was filled with 500 mL of sterilized 25% seawater and 100 mL of pure strain *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were added into each glass bottle. Then 1ml of conway media was added to each glass bottle for experiment I A_1, B_1 and C_1, while 1ml of the agricultural fertilizer media was added to experiment II A_2, B_2 and C_2. Three replications were prepared for each experiment (Plate 2). Each culture bottle was sealed with aluminum foil and labeled date and time. The Plankton culture bottles were arranged on cultivation shelf and aerated with blower. All culture bottles were kept at air-conditions room at 25°C with light by fluorescent tubes. The experiments were extended for 10 days. The population density of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were calculated every day by collecting 1ml of subsample from each bottle.



(A) Conway media (B) Agriculture fertilizer media

Plate 2 Cultivation of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. using conway and agricultural fertilizer media

Determination of growth conditions and cell density of *Nannochloropsis sp., Chlorella sp., and Tetraselmis sp.*

The growth of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp.were estimated by counting cell density using hemocytometer. The subsamples of 1-mL from each bottle were collected without replacement. One drop cell suspension was placed in the central counting chamber of hemocytometer (Thoma, Grmany) and covered with cover glass (22 mm) carefully to avoid the formation of bubbles between the cover glass and hemocytometer. The chamber was placed under light microscope (CX 31, Olympus) at 100× magnification. The counting of cell

density was started from the first day of culture period until the 10^{th} days and calculated using the formula (Taw, 1990).

$$\text{Cell count (cells/mL) for 25 squares} = \frac{\text{total number of cells counted}}{\text{Number of blocks}} \times 4 \times 10^{6}$$

The growth of culture of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. was characterized by five phases (Creswell, 1993). The detail descriptions of phases are;

1. Lag phase or induction phase*:* After the addition of stock culture inoculum for the subculture of micro algae, there was no cell division phase for few hours which was known as lag or induction phase.

2. Exponential phase*:* after the lag phase, the cells were acclimatized and started dividing, grow fast by utilizing nutrients, aeration and light. This growth phase was called exponential phase and reaches maximum cell concentration during this period.

3. Declining phase*:* After reaching the growth phase, the cells showed less growth or slow growth. This stunted growth stage was known as declining phase.

4. Stationary phase*:* The declining phase continued for few days without any cell division and this period was known as stationary phase. Sometimes, the culture might divide the cells with suitable conditions.

5. Death phase*:* Prolonged stationary phase would lead to the death phase, where algal cells would lose their viability and the cells died. This phase was called death phase (Fig. 3).

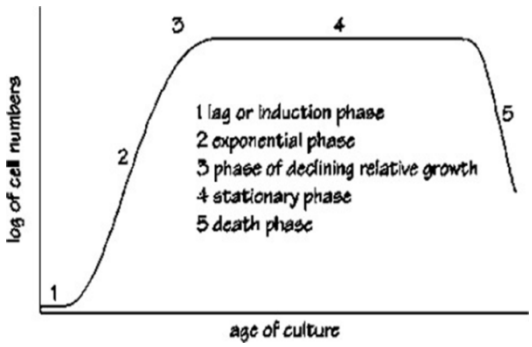


Figure 3 Growth phase of Algae (Creswell, 1993)

Determination of water quality

Water quality analysis was conducted for temperature and salinity at the early stage and at the end of culture.

Data Analysis

Cell densities were expressed as the average number of cell.ml^{-1} ± standard deviation. Growth curves for each experiment were prepared by plotting the average cell density *vs* corresponding cultivation time. Curves were prepared by using EXCEL computer program.

Results

The systematic position of marine microalge *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. studied in the present research were as follow.

Nannochloropsis sp.

Phylum: Ochrophyta
Class: Eustigmatophyceae
Order: Eustigmatales
Family: Monodopsidaceae
Genus: *Nannochloropsis* D.J.Hibberd, 1981

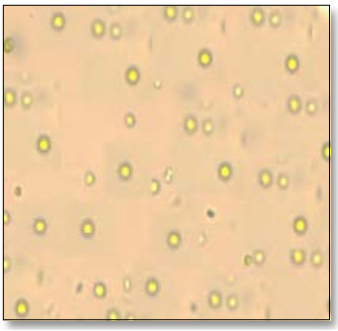


Plate 3 *Nannochloropsis* sp. (400 x magnifications)

Chlorella sp.

Phylum: Chlorophyta
Class: Trebouxiophyceae
Order: Chlorellales
Family: Chlorellaceae
Genus: *Chlorella* M.Beijerinck, 1890

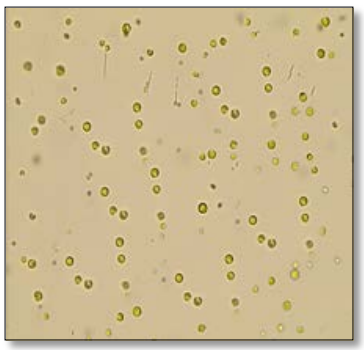


Plate 4 *Chlorella* sp. (400 x magnifications)

Tetraselmis sp.

Phylum: Chlorophyta
Class: Chlorodendrophyceae
Order: Chlorodendrales
Family: Chlorodendraceae
Genus: *Tetraselmis* F.Stein, 1878

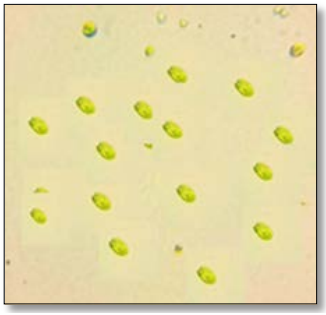


Plate 5 *Tetraselmis* sp. (400 x magnifications)

Population density of *Nannochloropsis* sp. *Chlorella* sp. and *Tetraselmis* sp. with conway and agricultural fertilizer media

The present experiments were performed to produce the *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. in laboratory condition using agricultural fertilizers media and conway media. The initial stocking density of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were (1.5 x 10^6 cell/ml), (1.38 x 10^6 cell/ml) and (1.42x 10^6 cell/ml) in first day, respectively. The cell density of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. were described in table 6.

The population density of *Nannochloropsis* sp. cultured with conway media started to increase from the second day to fifth day. However, it decreased in sixth day. In agricultural fertilizeral media, the population density of *Nannochloropsis* sp. started to increase from the second day to the fourth day. And it decreased on fifth day.

The maximum population density of cultured *Nannochloropsis* sp. occurred in conway media (8.47 x 10^6 cell/ml) while (8.8 x 10^6 cell/ml) in agricultural fertilizer media (Table 3 and Fig.1).

Table 3 Population densities of *Nannochloropsis* sp. with conway and agricultural fertilizer media

Time (Day)	Conway media	Agricultural fertilizer media
Day 1	1.50±0.01	1.50±0.01
Day 2	3.02 ± 0.25	3.20 ± 0.18
Day 3	5.39 ± 0.15	6.53 ± 0.13
Day 4	7.83 ± 0.30	**8.80 ± 0.25**
Day 5	**8.47 ± 0.07**	7.93 ± 0.11
Day 6	7.56 ± 0.2	6.56 ± 0.2
Day 7	5.54 ± 0.25	5.18 ± 0.10
Day 8	3.46 ± 0.10	2.46 ± 0.10
Day 9	1.78 ± 0.10	1.41 ± 0.06
Day 10	0.99 ± 0.26	0.69 ± 0.01

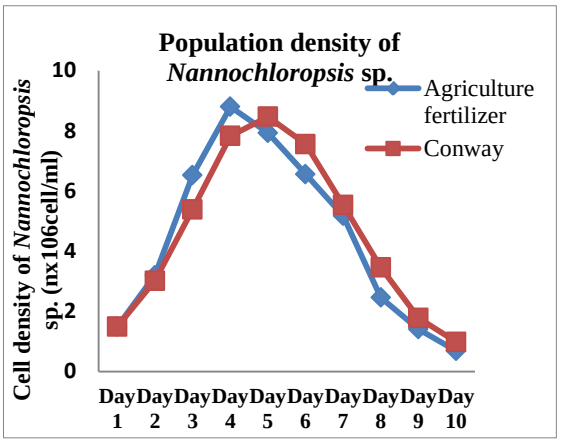


Figure 1 Population density of *Nannochloropsis* sp.

The population density of *Chlorella* sp. cultured with conway media started to increase from the second day to fifth day and it started to decrease in the sixth day. In agricultural fertilizer media, the population density of *Chlorella* sp. started to increase from the second day to the fourth day and then it started to decrease in the fifth day.

The maximum population density of cultured *Chlorella* sp. occurred in conway media (8.12 x 10^6 cell/ml) while ((8.37 x 10^6 cell/ml) in agricultural fertilizer media (Table 4 and Fig.2)

Table 4 Population densities of *Chlorella* sp. with conway and agricultural fertilizer media

Time (Day)	Conway media	Agriculture fertilizer media
Day 1	1.38±0.10	1.38±0.10
Day 2	2.55 ± 0.10	2.88 ± 0.05
Day 3	4.72 ± 0.30	5.43 ± 0.06
Day 4	6.74 ± 0.21	**8.37 ± 0.12**
Day 5	**8.12 ± 0.18**	7.54 ± 0.10
Day 6	7.00 ± 0.42	6.65 ± 0.3
Day 7	5.31 ± 0.15	4.68 ± 0.15
Day 8	3.74± 0.21	2.61± 0.36
Day 9	2.34± 0.06	1.48± 0.22
Day 10	1.32 ± 0.17	0.42 ± 0.36

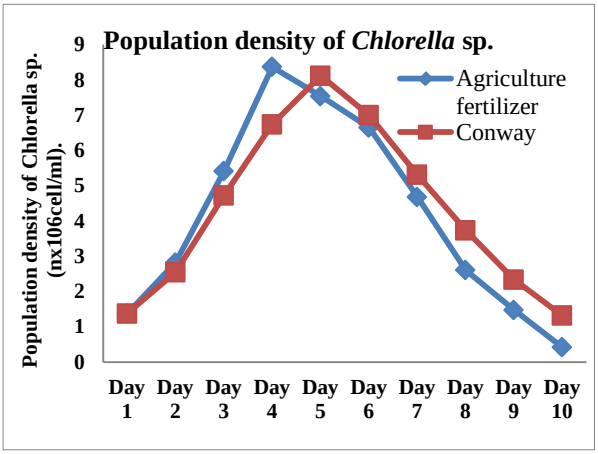


Figure 2 Population density of *Chlorella* sp.

The population density of *Tetraselmis* sp cultured with conway media started to increase from the second day to fifth day while it decreased in sixth day. In agricultural fertilizer media, the population density of *Tetraselmis* sp. started to increase from the second day to the fourth day then it decreased on fifth day.

The maximum population density of cultured *Tetraselmis* sp. was found in conway media (8.06 x 10^6 cell/ml) while it was (8.29 x 10^6 cell/ml) in agricultural fertilizer media. (Table 5 and Fig.3).

Table 5 Population densities of *Tetraselmis* sp. with conway and agricultural fertilizer media

Time (Day)	Conway media	Agriculture fertilizer media
Day 1	1.42±0.02	1.42±0.02
Day 2	3.38 ± 0.07	3.37 ± 0.07
Day 3	5.35 ± 0.11	6.02 ± 0.08
Day 4	6.95 ± 0.06	**8.29 ± 0.10**
Day 5	**8.06 ± 0.08**	7.65 ± 0.3
Day 6	7.02 ± 0.01	6.80 ± 0.2
Day 7	5.27 ± 0.16	4.61 ± 0.4
Day 8	4.01 ± 0.43	2.87 ± 0.06
Day 9	2.07± 0.38	1.74± 0.21
Day 10	1.08 ± 0.10	0.84 ± 0.06

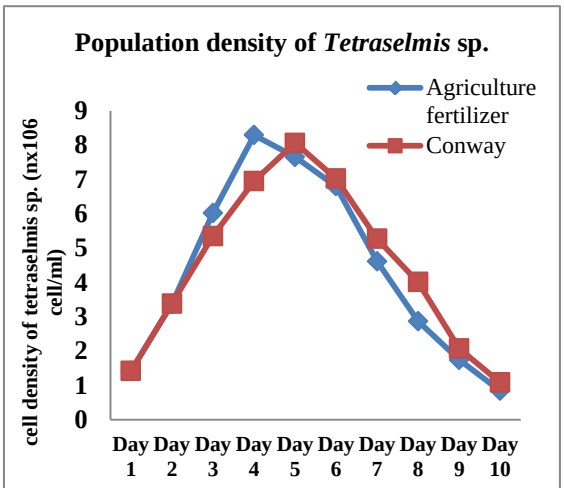


Figure 3 Population density of *Tetraselmis* sp.

The population density of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. cultured with conway media were shown in Table 6. The peaks of *Nannochloropsis* sp. (8.47 x 10^6 cell/ml), *Chlorella* sp. (8.12 x 10^6 cell/ml) and *Tetraselmis* sp. (8.06 x 10^6 cell/ml) populations with conway media occurred on fifth day. The cell densities of *Nannochloropsis* sp. (7.56 x 10^6 cell/ml), *Chlorella* sp, (7.00 x 10^6 cell/ml), and *Tetraselmis* sp. (7.02 x 10^6 cell/ml) decreased on sixth day (Table 6 and Fig.4).

Table 6 Population densities of *Nannochloropsis* sp., *Chlorella* sp.and *Tetraselmis* sp.in conway media during the period of 10 days cultivation

Time (Day)	*Nannochloropsis* sp.	*Chlorella* sp.	*Tetraselmis* sp.
Day 1	1.50±0.01	1.38±0.10	1.42±0.02
Day 2	3.02 ± 0.25	2.55 ± 0.10	3.38 ± 0.07
Day 3	5.39 ± 0.15	4.72 ± 0.30	5.35 ± 0.11
Day 4	7.83 ± 0.30	6.74 ± 0.21	6.95 ± 0.06
Day 5	**8.47 ± 0.07**	**8.12 ± 0.18**	**8.06 ± 0.08**
Day 6	7.56 ± 0.2	7.00 ± 0.42	7.02 ± 0.01
Day 7	5.54 ± 0.25	5.31 ± 0.15	5.27 ± 0.16
Day 8	3.46 ± 0.10	3.74± 0.21	4.01 ± 0.43
Day 9	1.78 ± 0.10	2.34± 0.06	2.07± 0.38
Day 10	0.99 ± 0.26	1.32 ± 0.17	1.08 ± 0.10

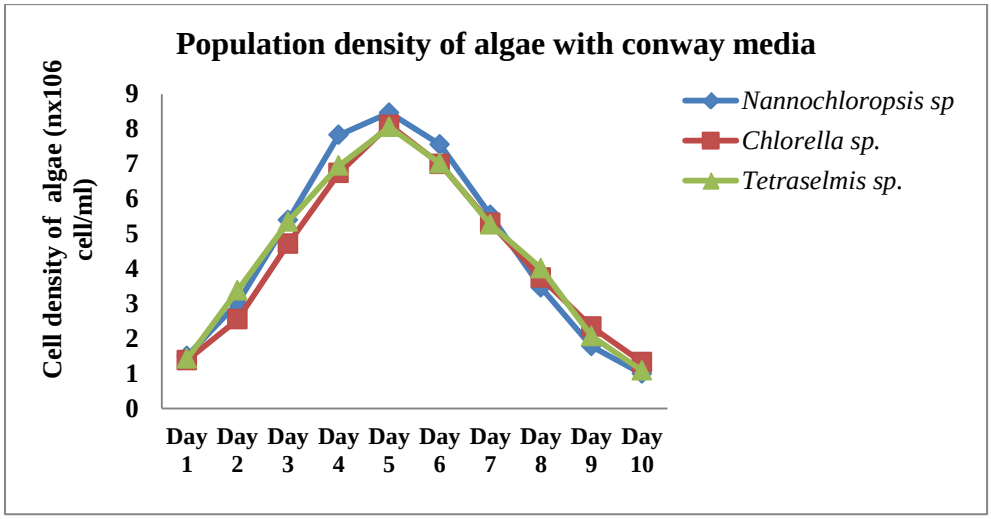


Figure 4 Population densities of *Nannochloropsis* sp., *Chlorella* sp.and *Tetraselmis* sp.in conway media

The population densities of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. cultured with agricultural fertilizer media was shown in Table 7. The maximum population densities of *Nannochloropsis* sp.(8.80 x 10^6 cell/ml), *Chlorella* sp. (8.37 x 10^6 cell/ml) and *Tetraselmis* sp. (8.29 x 10^6 cell/ml) with agricultural fertilizer media occurred on fourth day. The cell densities of *Nannochloropsis* sp. (7.93 x 10^6 cell/ml), *Chlorella* sp, (7.54 x 10^6 cell/ml), and *Tetraselmis* sp. (7.65 x 10^6 cell/ml) decreased on fifth day (Table 7 and Fig.5).

Table 7 Population densities of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. in agricultural fertilizer media during the period of 10 days cultivation

Time (Day)	*Nannochloropsis* sp.	*Chlorella sp.*	*Tetraselmis sp.,*
Day 1	1.50±0.01	1.38±0.10	1.42±0.02
Day 2	3.20 ± 0.18	2.88 ± 0.05	3.37 ± 0.07
Day 3	6.53 ± 0.13	5.43 ± 0.06	6.02 ± 0.08
Day 4	**8.80 ± 0.25**	**8.37 ± 0.12**	**8.29 ± 0.10**
Day 5	7.93 ± 0.11	7.54 ± 0.10	7.65 ± 0.3
Day 6	6.56 ± 0.2	6.65 ± 0.3	6.80 ± 0.2
Day 7	5.18 ± 0.10	4.68 ± 0.15	4.61 ± 0.4
Day 8	2.46 ± 0.10	2.61± 0.36	2.87 ± 0.06
Day 9	1.41 ± 0.06	1.48± 0.22	1.74± 0.21
Day 10	0.69 ± 0.01	0.42 ± 0.36	0.84 ± 0.06

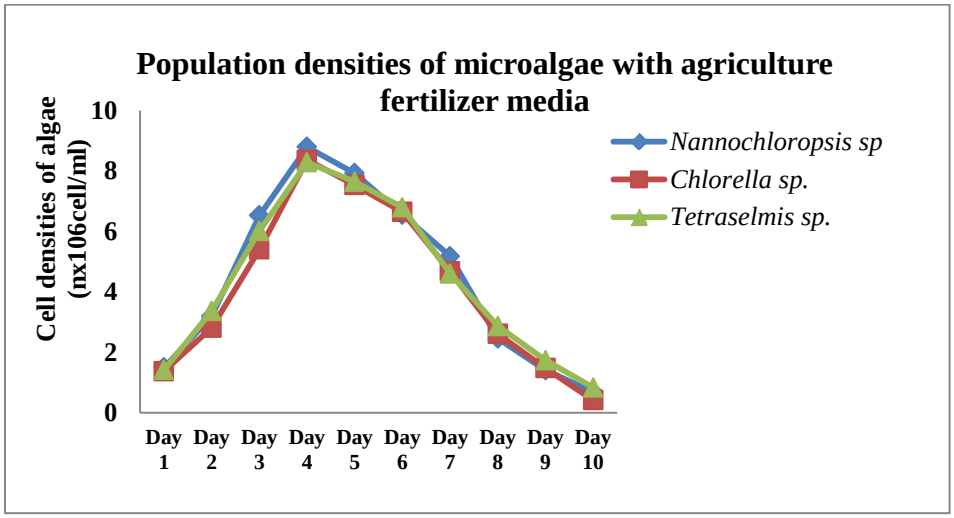


Figure 5 Population densities of *Nannochloropsis* sp., *Chlorella* sp.and *Tetraselmis* sp.in agricultural fertilizer media

Determination of water quality

In conway media, the water temperature in culture bottles ranged from 26.4 -27.6 ^{o}C while salinity ranged from 25-27.3 ppt. In agricultural fertilizer media, it ranged from 26.4 -27. 86 ^{o}C while salinity ranged from 25-27.1 ppt. Similar water quality was maintained for the culture (Table 8).

Table 8 Water salinity and temperature during the culture period

		Conway media		Agriculture fertilizermedia	
No.	**Algae**	**Temparature (^{o}C)**	**Salinity (ppt)**	**Temparature (^{o}C)**	**Salinity (ppt)**
1.	***Nannochloropsis* sp.**	26.4 - 27.6	25-27	26.4 - 27.9	25-27
2.	***Chlorella* sp.**	26.6 - 27.23	25-27.3	26.6 - 27.86	25-27.1
3.	***Tetraselmis* sp.**	26.6 -27.3	25-27	26.6 -27.76	25-27

Discussion

The variation of cell densities of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. was observed using the conway media and agricultural fertilizer media. The growth rates of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. were estimated by population density.

In the present study, the maximum population densities of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. cultured with agricultural fertilizer media were found on the fourth day of cultivation while it reached their maximum densities on the fifth day during the cultivation period using Conway media.

Creswell, (1993) stated that the Growth phases of plankton consist of 4 phases namely; lag phase, exponential phase, stationary phase, and death phase. Tugiyono, (2018) stated that the growth phase of *Nannochloropsis* sp. with agricultural fertilizer media occurred from second day to the fourth day as called the exponential growth phase. The population densities of microalgae *Nannochloropsis* sp. and *Tetraselmis* sp. showed exponential phase on fourth day.

In the present study, the population densities of *Nannochloropsis* sp.,*Chlorella* sp., and *Tetraselmis* sp. cultured with agricultural fertilizer media showed the exponential phase on fourth day. Therefore, the present findings agreed with the previous findings of Tugiyono, (2018).

The population densities of *Nannochloropsis* sp., *Chlorella* sp. and *Tetraselmis* sp. with conway media started to decrease on sixth day while cell density of *Nannochloropsis* sp., *Chlorella* sp, and *Tetraselmis* sp. cultured with agricultural fertilizer media decreased on fifth day. Rahardini *et al.*, (2018) revealed that the growing population of *Chlorella* sp. occurred when nutrients concentration was enough, and the cell division occurred rapidly (exponential phase). The cell population decreased when the nutrients run out.

In this study, the decline was caused by the decreasing of nutrients in the culture medium and it was eventually lost so that the cells deprived nutrients for growth. The present finding was agreed with the previous finding by Rahardini *et al.*, (2018).

Sivakumar and Rajendran, (2013) stated that microalgae require nutrients for their growth because nitrogen is a major nutrient for microalgal cultivation. The principal nutrients for algae are nitrogen (N), potassium (K), carbon (C), and phosphorus (P). In addition, microalgae growth also requires appropriate temperature, adequate sun rays, and an optimal combination of NPK. In the present study, the agricultural fertilizer media composed of nitrogen (N) and phosphorus (P) for microalgal cultivation.

Rahardini *et al.*, (2018) described that temperature is an important limiting factor for organism life, because every organism has limiting ability to tolerate temperature change in environment. The temperature and salinity in the present study were 26.4 -27.6 ^{o}C and 25-27.3 ppt respectively .Therefore, water quality in the present study was appropriate for marine algae culture.

Agriculture fertilizers mainly composed of macro-elements as nitrogenous and phosphorus and some of other elements such as iron, sodium, and potassium. However, they do not contain, in general, all micro-nutrients and vitamins necessary for the growth of microalgae.

Acién *et al.*, (2018) stated that the *Tetraselmis* sp. consumed nitrogen and phosphate in the fertilizers based-media during the culture period.

Elnabris (2012) also described that agricultural fertilizer such as urea, calcium superphosphate, ammonium sulfate, micronutrient, and vitamin solutions greatly supported the

growth of *Nannochloropsis* sp. and confirmed that using agricultural grade fertilizers could substitute the F/2 media which was commonly used for culture in commercial aquaculture.

In the present study, the population densities of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. with agricultural fertilizer media were higher than those of conway media. Nitrogen contained urea (fertilizer Media) which is a more dominant factor in stimulating *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. growth than in conway media.

According to the Canter *et al* ., (2015) several studies have investigated the performance of low-cost culture media based on commercial fertilizers for the cultivation of marine microalgae. The preparation of pure chemical media for mass algae culture was very expensive. Therefore, the cheaper commercial fertilizers were used for microalgae culture, this could contribute to the further development of large scale microalgae culture for different fields.

The results of the present study indicated that agricultural fertilizer media was cost effective than the conway media.

Conclusion

The best growth and cell densities of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. were found in agricultural fertilizer media when compared to conway media. The combination of agricultural fertilizers such as urea and triple superphosphate strongly supported the growth of of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp.

The success of fingerlings production of fish in the hatchery for stocking in the grow-out production system is largely dependent on the availability of suitable microalgae. The mass production of *Nannochloropsis* sp., *Chlorella* sp., and *Tetraselmis* sp. using agricultural fertilizer will benefit to get maximum growth and survival of marine fish larvae, fry, and fingerlings.

Acknowledgement

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OVARIAN DEVELOPMENT IN THE FRESHWATER PRAWN *MACROBRACHIUM JOHNSONI* RAVINDRANATH, 1979 (DECAPODA, PALAEMONIDAE)

Nway Yu Aung[1], Win Mar[2]

Abstract

A study on the characterization of the ovarian development in the freshwater prawn *Macrobrchium johnsoni* was conducted. The specimens were from Ayeyarwady segment of Myinmu Township. A total of 1621 specimens (550 ovigerous females and 1071 non-ovigerous females) of *M. johnsoni* were collected. A total of 20 female prawns were randomly chosen to study the reproductive biology. Four stages of ovarian development were recorded; Stage I ovary (Predeveloping), Stage II ovary (Developing), Stage III ovary (Maturing) and Stage IV ovary (Ripening) according to both morphological and histological observations. The results indicated that freshwater prawn *M. johnsoni* can spawn the whole year round according to the record of occurrence of ovigerous females, the fluctuation of GSI values and occurrence of oogonia and maturing oocytes throughout the study period. It was assumed that the reproduction of the study species may be continuous type. The present study provides important information on the reproductive biology of crustaceans in general and also can serve as a significant foundation for aquaculture of a freshwater prawn species.

Keywords ovarian development, ovigerous female, GSI, HSI, reproductive

Introduction

The prawns, *Macrobrachium* species belonging to the family Palaemonidae are decapod crustacean of high economic importance world-wide and have been subjected to intense aquacultural practices especially in Asia and America. Moreover this genus is particularly important among palaemonid prawns including species of major scientific and economic interest. Therefore, understanding the reproductive periodicity of this group is imperative to develop management and culture programs (Arimoro & Meye, 2007).

The reproductive process is an essential part of the biology of the species. In addition an understanding of the gonadal development and reproductive cycles is of fundamental importance for the conservation of natural stocks and for culture purposes (Chellappa, *et al.,* 2005).

White, *et al.,* (2003) also stated that a more precise estimate of spawning season may be determined from microscopic gonad stage. Therefore, histological analysis of gonad should be investigated in order to confirm the sexual maturity. Determination of oocyte diameter with histological tools provides basic information on classification of ovarian development (Revathi, *et al.,* 2012).

Present study is to investigate ovarian developments of the fresh water prawn *M. johnsoni* such as spawning period, morphological and histological observation of ovary development, GSI and HSI from Ayeyarwady river segment of Myinmu Township.

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Materials and Methods

Study Area

The study area was Ayeyarwady River Segment of Myinmu Township, situated in Sagaing Region on the northern bank of Ayeyarwady River.

Study Period

The study was carried out from April, 2019 to March, 2020.

Developmental stages of the Ovary

After sampling and dissection, ovaries of female *M. johnsoni* were observed. The gonadosomatic index and hepatosomatic index were determined according to (King, 1995).

$$\text{GSI} = \frac{\text{Gonad weight} \times 100}{\text{Body weight}}$$

$$\text{HSI} = \frac{\text{Liver weight} \times 100}{\text{Body weight}}$$

Determination of the developmental stages of ovary

For histological examination, 20 prawns at different ovarian developmental stages and five individual of each ovarian stage were used. Respective stages of each ovarian tissues were fixed in 10 volumes of 5 % formalin for 12-24 hours. Afterwards the tissues were dehydrated in alcohol and cleared in xylene, embedded in paraffin and sectioned at 7 μm. The sections were stained with haematoxylin - eosin and mounted permanently in Canada balsam for microscopic analysis. The developmental stages of germ cell were determined according to (Huang, *et al.*, 2010).

Analysis of Ovigerous Female

Ovigerous females were separated from other females. A total of 550 ovigerous females (Berried female) were observed and gathered data for analysis. The data referring to the total length and weight of the females were noted.

Results

After sampling and dissection, ovaries of female *M. johnsoni* were classified by their external morphology and were further examined histologically. The ovary of *M. johnsoni* was a two lobed organ situated above the hepatopancreas and below the pericardial sinus and the heart. The anterior and posterior ends of the ovaries are touching each other and leaving a gap at the middle in immature ovary. The colour, shape and size of the ovaries vary according to developing stages. In this study, four ovarian developmental stages were initially classified based on the external morphology, including the size, shape and colour.

Morphological appearance of developmental stages of ovary

Stage I (Pre- developing): creamy white, thin, small, and its anterior end remained close to the stomach and extending to the posterior border of hepatopancreas (Plate 1, A).

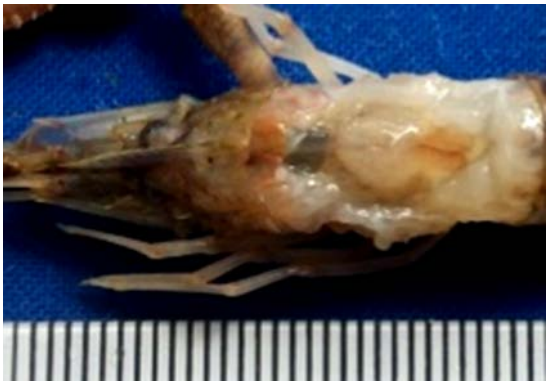
Stage II (Developing): pale yellow, oval shaped and its anterior end of ovary reached just behind the stomach and the posterior end extended to the anterior end of the first abdominal segment of the prawn (Plate 1, B).

Stage III (Maturing): orange in colour, distinctly expanded laterally and its anterior end of the ovary covered stomach and the posterior end distinctly bi-lobe and extended to the anterior end of the first abdominal segment of the prawn (Plate1, C).

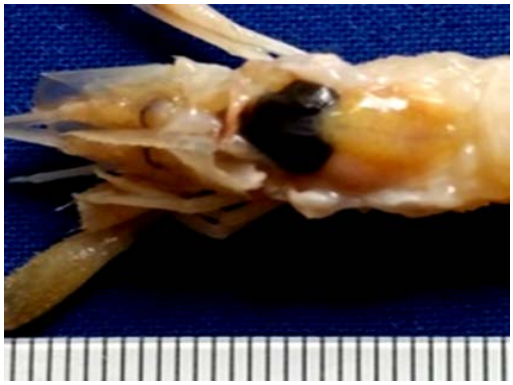
Stage IV (Ripening): deep green in colour, and characterized by the great increased size and the presence of visible oocytes. Anterior end of the ovary became more elongated, extending beyond the penultimate spine of dorsal rostral teeth (Plate1, D).

Table 1 Total ovary length, weight, gonadosomatic index (GSI), hepatosomatic index (HSI) and oocytes size of each ovarian stage of *M. johnsoni* from Myinmu Township

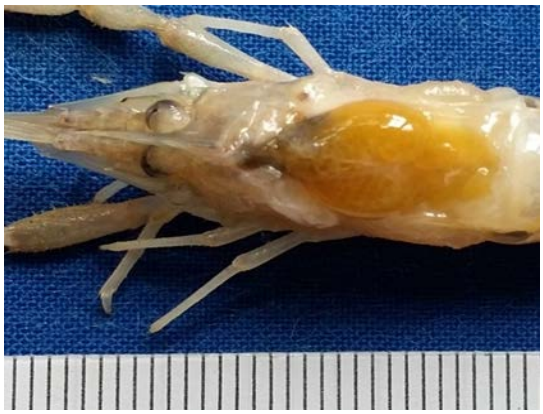
Ovarian	NS	Total length (mm)	Body weight (g)	Ovary Weight (g)	GSI	HSI	Oocyte Length (mm)	Oocyte width (mm)
Stage I	114	39 - 64 (51.34 ± 4.66)	0.86 - 5.15 (2.55 ± 0.67)	0.01 - 0.2 (0.04 ± 0.05)	1.61 - 12.95 (1.54 ± 2.35)	2.02 - 10.49 (5.66 ± 1.72)	0.05 - 0.65 (0.14 ± 0.19)	0.0025 - 0.45 (0.09 ± 0.12)
Stage II	31	44 - 61 (53.55 ± 4.33)	1.65 - 4.48 (2.80 ± 0.76)	0.02 - 0.2 (0.05 ± 0.05)	0.51 - 8.87 (1.90 ± 1.77)	1.84 - 8.33 (4.96 ± 1.47)	0.005 - 0.64 (0.16 ± 0.18)	0.0025 - 0.44 (0.09 ± 0.1)
Stage III	60	39 - 64 (52.73 ± 4.98)	1.46 - 5.13 (2.68 ± 0.81)	0.01 - 0.25 (0.08 ± 0.67)	0.30 - 14.93 (3.55 ± 3.53)	1.75 - 10.68 (5.10 ± 1.93)	0.005 - 1.05 (0.25 ± 0.31)	0.0025 - 0.55 (0.14 ± 0.17)
Stage IV	35	37 - 58 (50.57 ± 4.87)	1.64 - 4.3 (2.48 ± 0.67)	0.02 - 0.3 (0.1 ± 0.06)	1.01 - 8.66 (4.42 ± 1.68)	1.77 - 8.47 (4.63 ± 1.60)	0.25 - 0.8 (0.56 ± 0.16)	0.15 - 0.61 (0.38 ± 0.14)



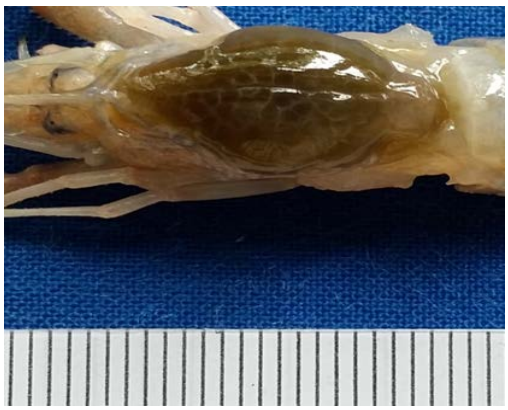
A. Stage I



B. Stage II



C. Stage III



D. Stage IV

Plate 1 Morphological appearance of developmental stages of ovary

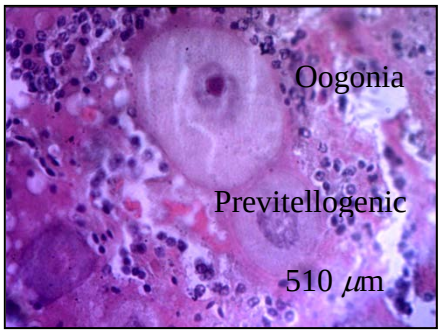
Histological appearance of developmental stages of ovary

Stage I: Histologically the ovaries consisted of oogonia (99.84%), previtellogenic oocytes (0.11%) and vitellogenic oocytes (0.06%). The oogonia had a well- defined nucleus with clear nucleoplasm and surrounded by ooplasm. In previtellogenic oocytes, the nucleus was large and surrounded by large and apparent cytoplasm. In vitellogenic oocytes, nucleus was smaller than that of oogonia and previtellogenic oocytes. The oocytes had a few lipid droplets and yolk granules were present in cytoplasm of vitellogenic oocytes. The oocytes with thick follicle cells were also noted (Plate 2, A).

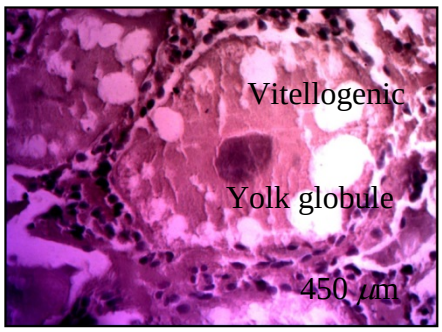
Stage II: The ovaries composed of oogonia (99.31%), previtellogenic oocytes (0.41%), vitellogenic oocytes (0.25%) and mature oocytes (0.03%). In mature oocytes, nucleus was small and cytoplasm with lipid droplets and yolk globules. The mature oocytes were surrounded by thick follicle cells (Plate 2, B).

Stage III: The ovaries composed of oogonia (96.86%), previtellogenic oocytes (0.64%), vitellogenic oocytes (1.41%) and mature oocytes (1.10%). In this stage, the nucleus of mature oocytes became vestigial or disappeared and surrounded by thick follicle cells (Plate 2, C).

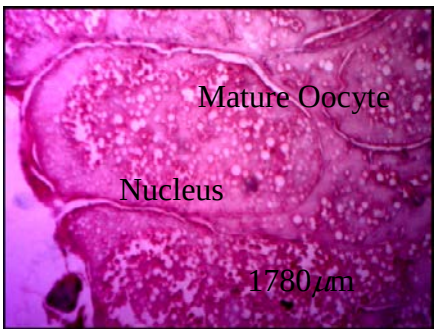
Stage IV: The ovaries composed of previtellogenic oocytes (2.78%), vitellogenic oocytes (1.89%) and mature oocytes (78.33%). The major feature of mature oocytes was the enlarged size, which was the maximum compared to other type of germ cells. Large lipid droplets and yolk globules were distributed in the cytoplasm of mature oocytes. Follicular cells became very thin or disappeared. If present, it was fully elongated and adhered tightly to the surface of the oocytes (Plate 2, D).



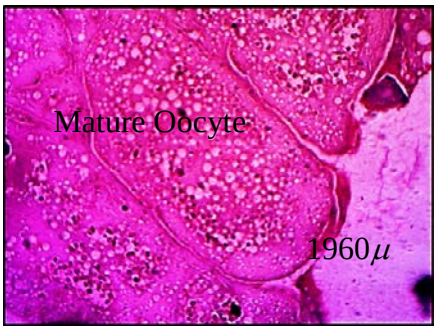
A. Cross section of Pre-developing stage



B. Cross section of Developing stage



C. Cross section of maturing stage



D. Cross section of ripening stage

Plate 2 Histological appearance of developmental stages of ovary

Diameter of oocyte size varied according to the different stages of development, from 0.005 mm to 1.05 mm in length and 0.0025 mm to 0.61 mm in the width. In female, the range of total length was (37-64 mm), body weight (0.86-5.15 g), gonadosomatic index (0.30-14.93), and hepatosomatic index (1.75-10.68) (Table 1).

The results showed that GSI increased to significant ($p< 0.01$) level during the development of the ovary from stage I-IV. The stage IV ovary showed higher GSI values than other stages. The results exhibited significant increase in oocytes diameters from stage I to IV. Conversely, HSI values decreased significantly ($p<0.01$) during the development of the ovary through the stage I-IV (Figure 1).

Occurrence of Ovigerous Female

Ovigerous females (berried female) were observed in all month of study periods. The lowest and highest percentage of berried females were 0.56% in November and 74.58% in other months.

Sexual Maturity

A total of 240 females were examined to determine the length at first maturity stage. The smallest female carrying the eggs was found to be 37 mm in length while nonovigerous females with mature gonads (Stage IV ovary) were at a maximum size of 58 mm in total length (Table 1).

Spawning Season

Ovigerous females were observed throughout the year during the study period, the highest percentage of ovigerous females prawns occurred in May (74.58%). Moreover females with stage III ovary were observed throughout the year.

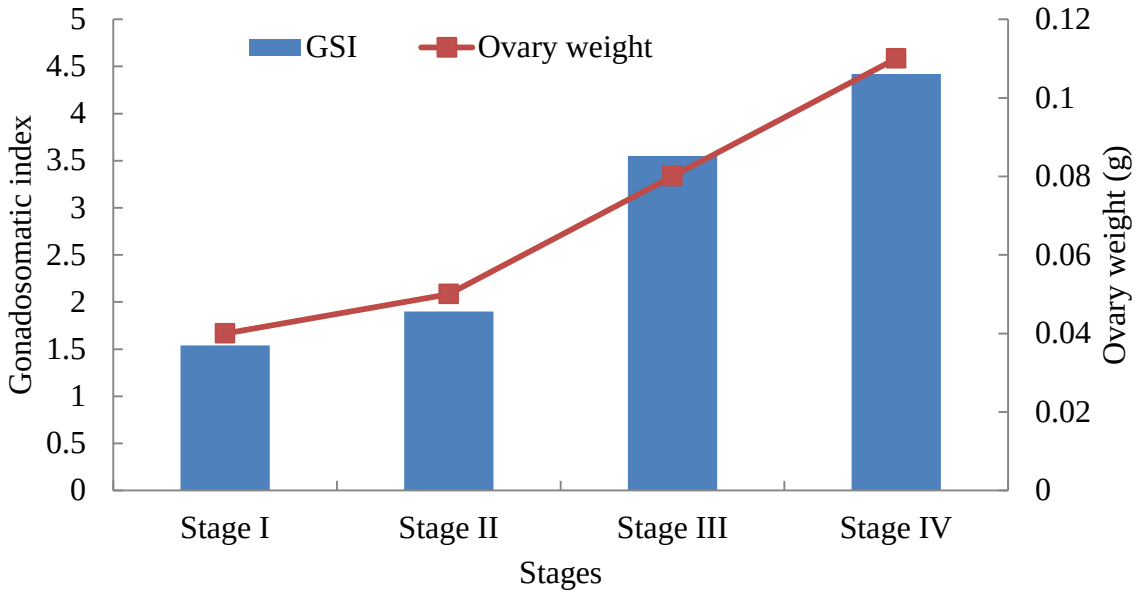


Figure 1 Ovary weight and gonadosomatic index (mean ± SD) of prawns (N = 20) with each ovarian developmental stages of *M. johnsoni*

Discussion

The present study was conducted on the ovarian development of the freshwater prawn *Macrobrachium johnsoni* from Ayeyarwaddy river segment of Myinmu Township. In the present study, ovarian development was categorized into four stages, based on external morphological differences (colour, position and size of the ovary). In the ovarian development of the other species of *Macrobrachium*, such as *M. amazonicum* and *M. rosenbergii*, was divided into four to six stages (Chang & Shih, 1995).

Similarly, the ovarian developments could be histologically classified into four stages according to the presence and amount of nucleus, yolk globules, yolk vesicles, follicle cells and oocyte sizes. Oogonia and yolk vesicle stages of oocytes were observed throughout the study period. In Stage I, ovaries and oogonia were present mostly in the germ layer.

Gonadosomatic Index (GSI) and Hepatosomatic index (HSI) are common tools used as quantitative methods to verify the gonadal development and represent the percentage of these organs to the total weight of the animal. Gonadosomatic index (GSI) is the most common index used to define reproductive cycling in animal population (Magalhaes, *et al.*, 2012).

Khin Khin Lay, (2007) recorded that the mean GSI values of both males and females of *M. malcolmsonii* were high from March to August. According to Win Mar, (2007), the highest GSI value of females *M. palaemonoides* was found in March and continued to increase between January to April.

According to above results, it was determined that higher GSI values occurred during the hot season but fluctuated in other months. Nevertheless, it can be assumed based on the data collected during the study that *M. johnsoni* is a continuous breeder.

Four developmental stages of ovaries could be discerned in *M. johnsoni* based on both morphological and histological considerations. Ovigerous females were observed throughout the study period. As reproducing females (female with mature ovary and carrying eggs) were found throughout the year, so that *M. johnsoni* is regarded as a continuous breeder, and thus recommended as a prospective candidate for aquaculture practice.

Conclusion

The present study elucidated the ovarian development of freshwater prawn *M. johnsoni* from Ayeyarwady River of Myinmu Township. A total of 240 females were examined for ovary condition, and mean ovary size increased in accordance with stages I, II, III and IV. From the histological observation, the mature oocytes had been found in the stages of II, III and IV. Moreover, the gradual increase of GSI values was observed from stage I to IV in this study. Females with mature ovaries (Stage III) occurred in all months in this study. So, it was assumed that females spawned the whole year round.

This study contributes to the reproductive biology of freshwater prawn *M. johnsoni* and the findings will be useful for the culture of the prawn. It is further recommended that reproductive biology of other freshwater prawn species from the same study area should be investigated.

Acknowledgements

We are greatly indebted to Dr. Khin Maug Oo, Rector, University of Magway for his encouragement to conduct this research. We are very grateful to Dr. Than Aung Htwe, Pro-Rector, University of Magway for his advices and constant encouragement. Special gratitude goes to Dr. Daw Oo Than Kyi, Professor and Head, Department of Zoology, University of Magway for her permission to conduct this research.

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USING OF MACRO-AQUATIC INSECTS IN WATER QUALITY ASSESSMENT OF TAUNGTHAMAN LAKE, AMARAPURA TOWNSHIP, MANDALAY REGION

Nyunt Lwin[1], Thandar Saw[2]

Abstract

An investigation using macro-aquatic insects in water quality assessment was carried out at Taungthaman Lake, during July 2019 to June 2020. The specimen collection was conducted using D-framed net by drifting method. A total of 3196 individuals of 30 species, belonging to 29 genera, 21 families under nine orders were recorded. The water quality was assessed as fairly poor in the studied lake. The highest degradation of water quality (BI) was (7.34) examined at Site I in cold season, and the lowest degradation (BI) was (6.67) at Site II in rainy season. Nowadays, Taungthaman Lake is important to maintain the healthy water body because human invaded and utilized the land for settlement around the lake. The lake water was disturbed by the human activities. Thus, the water quality was degraded to unusable condition.

Keywords Water quality, aquatic insects, Taungthaman Lake.

Introduction

Aquatic insects involved on land and then evolved to water (Ward, 1992). About 45,000 insect species are known to diverse freshwater ecosystem (Balaram, 2005). Aquatic insects are one of the important components for food web in freshwater ecosystem (Resh and Rosengerg, 1984). In the food web of Lake ecosystem, aquatic insects are the main prey of nekton and have a role as decomposer of organic matter (Bouchard, 2004; Choudhury and Gupta, 2015).

Aquatic insects serve as reliable indicators of ecological characteristics of water. Some aquatic insect families such as Ephemeroptera, Plecoptera, Tricoptera, Diptera, etc., may serve as good indicators of water pollution; and have been used in fresh water bio-monitoring and assessment of environmental impacts (Arimoro and Lkomi, 2008). They also played an important role in removing nutrients from the polluted waters (Resh and Rosenberg, 1984). When insects emerge from the water as adults, they remove some of the nutrients that were in the water, they remove 1-14% of Phosphorus and 1-10% of Nitrogen from the water (Resh and Rosenberg, 1984). They have population fluctuation and this character is mainly related to seasonal inundation (Hamilton, 2002; Mayora *et al.,* 2013).

Taungthaman Lake is located in Amarapura Township, Mandalay Region. Tropical floodplain plays a significant role in providing highly productive ecosystem services (Pettit *et al.,* 2011), vital to a range of ecosystem processes (Hamilton, 2002). However, industrial and domestic waste discharge (Azrina *et al.,* 2006) often contributes to river water quality deterioration in the tropical regions (Harun *et al.,* 2015). By knowing some of the important factors in aquatic insect fatalities, people will be more able to solve pollution problems of lake to keep the insects from dying out forever. Thus, Taungthaman Lake is selected to conduct a research work; with the objectives to record the occurrence of aquatic insects and to make assessment of water quality at Taungthaman Lake.

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Materials and Methods

Study area and study sites

The study was conducted in Taungthaman Lake. Taungthaman Lake is situated in Amarapura Township of Mandalay Region. It lies between 21° 53′ N to 21° 54′ N latitude and 96° 03′ E to 96° 05′ E longitude (Fig.1) and it covers an area of about 495 km^2. The climate of the study area is characterized by three seasons: hot season (March - June), rainy season (July - October) and cold season (November - February). Two study sites were allocated as Southern part (Site I), it is deep area of lake and Northern part (Site II), which is related with inlet channel from downtown.

Figure 1 A map of study area and study sites (Source: Google earth, 25-11-2020)

Data collection

Study period was from July 2019 to June 2020. The specimens were collected in the morning from 8:00 am to 10:00 am at the littoral zone of each study site. The study sites were visited every month in the study period. The collection was undertaken using D-framed net by drifting method. The net opening is 35 cm wide and 25 cm deep. Once drifting, it takes two minutes and three times in each study site. After drifting, examine, wash and discharged large pieces of plant, woody debris, stones, making sure to retain any aquatic insects observed.

Identification of species

Collected specimens were identified and classified followed after Garrison *et al.* (2006), Easton *et al*. (2012). Koh (1989), Subramanian and Sivaramakrishnan (2007), Verma (1999) and Harrlod and Guarlnick (2010).

Analysis of Data

Biotic index (BI)

BI was used to determine pollution level in the lake. BI is calculated fallowed after Hilsenhoff (1982).

$$BI = \frac{\sum ni.ai}{N}$$ Where, BI = Biotic index

Ni = number of specimen in each taxon
Ai = tolerance score for each group of taxon
N = number of individuals of insects in a samples

Each family has a pollution tolerance on a scale of 1-10 in which 1 is not tolerance and 10 is extreme tolerance to pollution.

Table 1 Evaluation of water quality using the family-level biotic index (Hilsenhoff, 1988)

Biotic index	Water quality	Degree of organic pollution
0.00-3.50	Excellent	No. apparent organic pollution
3.51-4.50	Very good	Possible slight organic pollution
4.51-5.50	Good	Some organic pollution
5.51-6.50	Fair	Fairly significant organic pollution
6.51-7.50	Fairly poor	Significant organic pollution
7.51-8.50	Poor	Very significant organic pollution
8.51-10.00	Very poor	Severe organic pollution

Results

Occurrence of aquatic insects

A total of 3196 individuals of aquatic insects were collected at two study sites, in Taungthaman Lake, during July 2019 to June 2020. Of these individuals, a total of 30 species, belonging to 29 genera, 21 families under nine orders were recorded. Among these, 1175 individuals of 25 species, 24 genera from 18 families under eight orders at Site I and 2021 individuals of 28 species, 26 genera from 18 families under seven orders were recorded at Site II. Regarding the seasonal investigation, the highest number of 21 species (246 individuals) in the rainy season, followed by 13 species (274 individuals) in the cold season and the lowest 10 species (655 individual) were recorded at Site I, while in the hot season, 20 species (714 individuals) in the rainy season was observed as the highest occurrence and followed by 19 species (360 individuals) in the cold season and the lowest 15 species (947 individuals) were recorded in the hot season (Fig. 2).

Water quality as biotic index (BI)

There were 11 families of aquatic insects to support the assessment of water quality as biotic index (tolerance level, 1-10). The families were Libellulidae (9), Coenagrionidae (9), Capniidae (1), Gerridae (8), Corisxidae (9), Syrphidae (10), Hydrophilidae (5), Chrysomelidae (5), Dytiscidae (4), Ephydridae (6) and Chironomidae (Red) (8). In this study, the water quality was assessed as fairly poor in both study sites. The highest degradation of water quality (BI) 7.34 in cold season at Site I, followed by 7.31 in hot season at Site II, 7.19 in cold season at Site II, 6.67 in hot season at Site I and 6.47 in rainy season at Site II were recorded (Table 1-3).

Table 1 Recorded biotic index (BI) levels at two study sites

Sites	Season	BI	Water quality	Degree of organic pollution
Site I	Rainy season	6.57	Fairly poor	Significant organic pollution
	Cold season	7.34	Fairly poor	Significant organic pollution
	Hot season	6.67	Fairly poor	Significant organic pollution
Site II	Rainy season	6.47	Fair	Fairly significant organic pollution
	Cold season	7.19	Fairly poor	Significant organic pollution
	Hot season	7.31	Fairly poor	Significant organic pollution

Table 2 The computation of water quality biotic index (BI) at Site I during the study period

No.	Order	Family	Tolerance level (1-10)	Rainy season			Cold season			Hot season		
				No. of species	No. of individuals	Tolerance total value	No. of species	No. of individuals	Tolerance total value	No. of species	No. of individuals	Tolerance total value
1.	Odonata	Libellulidae	9	7	3	27	2	70	630	0	0	0
2.		Coenagrionidae	9	1	2	18	0	0	0	0	0	0
3.	Plecoptera	Capniidae	1	1	3	3	0	0	0	0	0	0
4.	Hemiptera	Gerridae	8	1	15	120	0	0	0	1	80	640
5.		Corixidae	9	1	20	180	1	44	396	1	71	639
6.	Hymenoptera	Syrphidae	10	1	1	10	1	1	10	0	0	0
7.	Coleoptera	Hydrophilidae	5	1	1	5	2	26	130	1	4	20
8.		Chrysomelidae	5	0	0	0	0	0	0	0	0	0
9.		Dytiscidae	4	0	0	0	0	0	0	0	0	0
10.	Diptera	Ephydridae	6	2	119	714	2	98	588	1	393	2358
11.		Chironomidae (Red)	8	0	0	0	1	0	0	0	0	0
						1077			1754			3657
			biotic index (BI) level			6.57		BI	7.34			6.67

Table 3 The computation of water quality biotic index (BI) at Site II during the study period

No.	Order	Family	Tolerance level (1-10)	Rainy season			Cold season			Hot season		
				No. of species	No. of individuals	Tolerance total value	No. of species	No. of individuals	Tolerance total value	No. of species	No. of individuals	Tolerance total value
1.	Odonata	Libellulidae	9	7	16	144	3	68	612	3	4	36
2.		Coenagrionidae	9	1	3	27	0	2	18	0	0	0
3.	Plecoptera	Capniidae	1	1	1	1	0	0	0	0	0	0
4.	Hemiptera	Gerridae	8	0	0	0	1	22	176	1	336	2688
5.		Corixidae	9	0	0	0	1	33	297	0	69	621
6.	Hymenoptera	Syrphidae	10	1	1	10	1	1	10	1	16	256
7.	Coleoptera	Hydrophilidae	5	2	0	0	2	7	35	2	11	55
8.		Chrysomelidae	5	1	1	5	1	2	10	0	0	0
9.		Dytiscidae	4	0	0	0	1	13	52	0	0	0
10.	Diptera	Ephydridae	6	2	102	612	1	122	732	1	360	2160
11.		Chironomidae (Red)	8	1	2	16	1	0	0	0	6	48
						815			1942			5864
			biotic index (BI) level			6.47			7.19			7.31

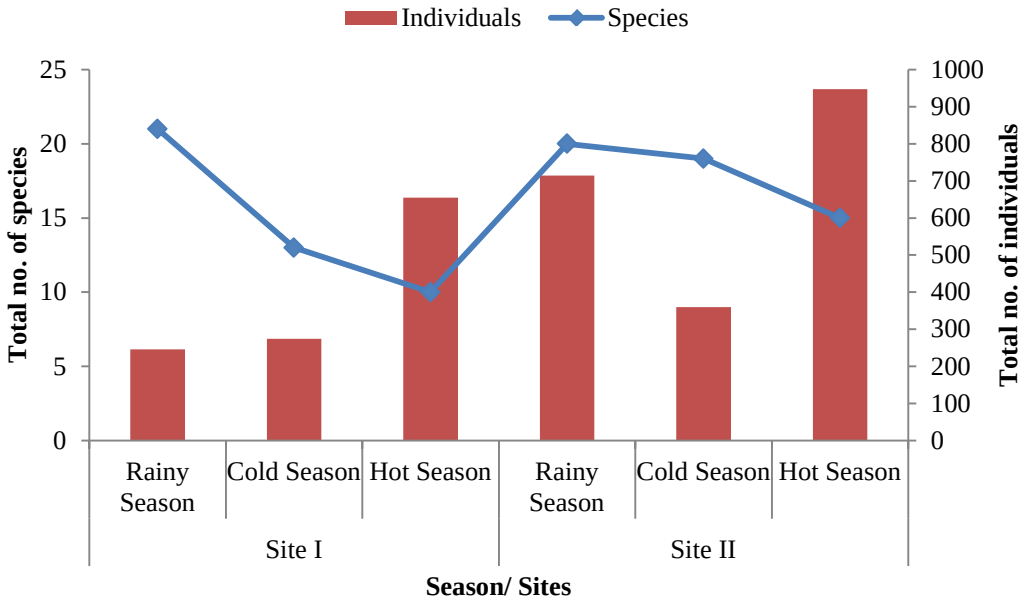

Figure 2 seasonal occurrences of insect species at two study sites

Discussion

In the littoral zone, in which light reaches well, the benthos and macrophytes can grow, and insect diversity is at its maximum. Many differentiated microhabitats are available and physico-chemical factors are less restricting than in the dark, cold, and perhaps anoxic conditions of the deeper waters (Gullan and Cranston, 2010). In the study area, the selected two study sites have various abiotic conditions in each season. The highest precipitation was obviously recorded in rainy season, especially in July to August (4.57 meters) compared to other seasons. The depth in littoral area of study Site II is always lower than that of Site I, which showed the slow water and more varied types of habitat growth. The range of water temperatures, pH and DO were 23.4-34.5°C, 7.1-8, 0.22-7.88 mg/L respectively. The range of turbidity was found from 10.8 to 217 and the highest was found in the hot season and the lowest was found in cold season.

There are 3276 aquatic insects of 30 species, 29 genera, 21 families, under 9 orders were collected from Taungthaman Lake in the study period. The largest occurrence was found in November and the smallest occurrence in March and April at the study Site I. At study Site II, the largest occurrence was found in November and the lowest in July. It is found that November had favorable conditions to survive for aquatic insects while it is in March and April, the water level was at least condition, the water drained flooded water from Ayeyawady River in July.

High abundance of insects was recorded with respect to the quality and size of the water body (Arimoro and Keke, 2016). In the present seasonally study, the highest water volume was found in rainy season and the lowest was in hot season. It clearly indicated the agreement with that stated by Arimoro and Keke (2016). Corixidae and Notonectidae prefer lentic aquatic system as their habitat (Ambrose, 2015, cited by Priawandiputra, *et al.*, 2016). Both Corixidae and Notonectide showed different patterns of abundance (Priawandiputra, *et al.*, 2016). Corixidae is widely distributed in many types of lake such as oligotrophic, mesotrophic and eutrophic lakes (Jastrey, 1981; cited by Priawandiputra *et al.*, 2016). Notonectidae is a predator, which prefer the shallow water (Merrit and Cummins, 1996, Hilsenhoff, 1975). These results were provided by Ambrose (2015) and stated by Priawandiputra *et al.*, (2016). *Notonecta* sp. was found mostly in shallow water. The species, *Orchelimum gladiator* and *Harmonia axyridis*

at Site I and species, *Eocapnia nivalis*, *Spathosterninae prasiniferum* and *Lopidea confluent* at Site II were commonly recorded.

The insect composition and the physcio-chemical parameter values of the water are correlated, where fairly high abundance of insects with respect to the water quality and size of the stream was stated (Adjarho *et al.,* 2013 and Udebuana *et al.,* 2015). Regarding the relationship of aquatic invertebrate and water quality, Tachet *et al.* (2003) also stated that aquatic insects improved the health of a stream, pond, river or a lake and they were good indicators of water quality because they were affected by the physical, chemical, and biological conditions of the water body. The vast occurrence and abundance of Odonata in two sampled stations may be attributed to their diverse feeding pattern and the abundance of aquatic vegetation which have been known to provide good oviposition sites for the member of this order (Braccia *et al.,* 2007). In the present investigation, a total of six orders of aquatic insects were used as assessment tool of lake water quality. The orders include Odonata, Plecoptera, Hemiptera, Hymenoptera, Coleoptera and Diptra. In water quality assessment, organic pollution level ranged from 6.36 of fair to 7.79 of poor quality.

Odonata has relationship with water quality, such as temperature, pH, TDS, DO, total alkalinity and total hardness etc (Azrina *et al.,* 2006) and used as indicators for wetland conservation (Bried *et al.,* 2007). The order Odonata has good indicator species, which are taxonomically well known, relatively easy to identify and having distinct habitat requirements (Krebs, 2001). They are constituted the third most abundant group of insect fauna. Odonata prefer fresh water habitat with rich oxygen so their abundance is seen in winter because there is high dissolved oxygen in freshwater ecosystem in this season (Choudhary and Ahi, 2015). In the present research, their occurrence was recorded from June to November and the most abundance (0.1205) species was *Brachythemis contaminate.* The biotic organic level was 6.36-6.88 (fairly - fairly poor) indicating significant organic pollution. The investigation indicates that Odonata can live in polluted as well as fairly clean water. On the other hand, Odonata shows least diversity and were very sparse in distribution, indicating their preference for freshwater, non- contaminated and well oxygenated habitats (Choudhary and Ahi, 2015). In the present study, the value of dissolved oxygen reached up to 0.22-7.88 mg/L and temperature decreased from 23.4-31.5°C.

Members of Plecoptera are used as biological indicators of water quality, especially for dissolved oxygen levels, thus deteriorating populations of stoneflies mean that poor water quality and it threatens the aquatic insects. The absence of Plecoptera indicates the water quality degradation and physical alteration (Choudhary and Ahi, 2015). In the present result, the plecoptera species, *Eocapnia nivalis* was found in September and October in Site I and August in Site II. It clearly indicated that the water quality level is fair because the flooded water from the Ayeyawady River drained into lake and the level of water is high in two study sites and then reduced while the water level was saved in Site I and reduced in Site II in other months. Thus, the water quality of Taungthaman Lake was fairly clean in August in Site II, and September to October in Site I.

Aquatic Hemipteras stand out as an important group of aquatic insects, which are considered important in environmental reclamation of aquatic habitats and are often used to gauge toxins in an environment (Jansson, 1987; Papacek, 2001; Wollman, 2001). The Hemipterans are associated with macrophytes, their diversity is high during winter as the increasing growth of macrophytes (Choudhary and Ahi, 2015). In the present study, the result was agreed with Choudhary and Ahi (2015); they were more abundance in cold season (winter).

The members of order Hymenoptera inhabit an extremely wide range of habitats and biological environments. Some are parasites, while others are predators, herbivores, gall-formers,

fungus feeder, leaf miners or nectar and/ or pollen gatherers (Britton, 2018). In the present study, the Hymenopterans species were found from October to November in cool dry period, and from March to June in dry period. They prefer the flowering time of aquatic vegetation along the edges of lake in the two study sites.

Among coleopteran, the Hydrophilids are predominant in rivers and streams. The members of family Dytiscidae have adapted perfectly well to aquatic life. All adults and larvae are aquatic organisms. The members of family Gyrinidae are found in fresh water ponds, lakes, open flowing streams etc. The members of Haliplidae live among aquatic vegetation along the edges of ponds, lakes, streams and creeks. The abundance occurs in summer because of high rate of decomposition of organic matter due to high temperature which reaches up to 28-30°C. Coleoptera forms the second most abundant group of insect fauna. This group was represented by *Cybister* sp., *Dystiscus* sp., and *Hydrophilus* sp (Choudhary and Ahi, 2015). In the present investigation, dominance of Coleopteran species was found in cold and hot seasons. During this time, most of aquatic plants were found along the edges of habitat and the water temperature ranged from 23.4°C to 34.5°C. The present findings are in conformity with the finding of Choudhary and Ahi (2015).

Presence of Saprophilic species of Diptera indicates that water bodies are grossly polluted with poor water quality characterized by low oxygen and high nutrient concentration, large numbers of pollution tolerant Chronomids which are often indicative of poor water quality. Excellent water quality conditions are often characterized by relatively low densities and high species diversity. The high abundance of *Chironomus* sp. in aquatic body indicates eutrophic nature of water body (Choudhary and Ahi, 2015). In the present study, during hot season, less dissolved oxygen levels of 0.22 mg/L in Site II and 0.35 mg/L in Site I were recorded. The occurrence of Chronomids larvae indicated that the water quality is fairly poor (BI = 7.19- 7.31) when the temperature reached from 23.4°C to 34.4°C.

Conclusion

There were four significant organic pollution levels in this water quality assessment; the water quality of this study lake represented fairly poor at Site I in all seasons and at Site II in the cold season while the good water quality was recorded at Site II in the rainy season and the bad water quality at Site I in the hot season. The present findings indicated that the water quality was dependent on water depth and size of water body, where Site II was inlet water and possessed the larger size of water body in the rainy season and Site I was deeper than that of Site II. Taungthaman Lake is famous for U-Bain Bridge in Mandalay, the second largest city of Myanmar. It is important to maintain the lake in healthy conditions because the lake provides many ecosystem products for the community. The findings of the present research will be helpful for the regular monitoring of the water quality in future in aspect of freshwater ecosystem conservation.

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INCIDENCE OF SOME INSECT SPECIES ON *SOLANUM MELONGENA* L., 1753 IN MAGWAY TOWNSHIP

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Abstract

Eggplant (*Solanum melongena* L.) also called aubergine or brinjal, is one of the top ten vegetables in the world. Eggplant is also one of the most important vegetables in Asia. The present study was conducted at eggplant plantations of Magway Township during December 2019 to August 2020. A total number of (3743) insects were recorded, and they were listed under 24 species of 24 genera belonging to 16 families of six orders during the study period. Among the recorded species, 20 species were identified as pests and the other four species as beneficial or predatory insects. In the present study, eight species of Lepidoptera (33.33%), seven species of Hemiptera (29.17%), and six species of Coleoptera (25%), while Orthoptera, Neuroptera and Diptera were recorded only one species (4.17%) each during the study period. In the present study, the maximum number of individuals was recorded in order Hemiptera by (1032) individuals (73.71%), while the minimum number of individuals in Orthoptera as one individual (0.07%) was recorded in the study site I. The maximum number of 1032 individuals (73.71%) was recorded in order Hemiptera while the minimum number of one individual (0.07%) in Orthoptera was recorded in the study site I. The maximum number of 917 individuals (74.92%) was collected in order Hemiptera, and the minimum number of eight individuals (0.65%) was collected in Neuroptera in the study site II. In the study site III, the maximum number of 851 individuals (76.05%) was recorded in order Hemiptera, while the minimum number of ten individuals (0.89%) in Neuroptera was recorded during the study period. Hemipterans species were predominant on eggplant growing in the study area.

Keywords Brinjal, insects, beneficial, predatory, Hemipterans

Introduction

Insects are the largest group of animals on earth by far: about 926,400 different species have been described. They are more than half of all known living species. They may be over 90% of animal species on Earth. Estimate of the total number of species ranged from 2 million to 30 million. The most species live in tropical area (Rasnitsyn and Quicke, 2002).

Several insect species are predators or parasitoids on other harmful pests and others are pollinators, decomposers of organic matter or producers of valuable products such as honey or silk. The majority of insects are directly important to humans and the environments. Less than 0.5 percentage of the total number of the known insect species are considered pests, and only a few of these can be a serious menace to people **(**Kyerematen *et al*., 2014). It is susceptible to several pests, especially the eggplant fruit and shoot borer. In the tropics, eggplant production is severely constrained by several insects and mite pests. Shoot and fruit borer, jassid, aphids, leaf roller and stem borer were the most common pests of eggplant in Madhay Pradesh, India (Bhadauria *et al*., 1999).

The cultivation of eggplant is more than 1600000 ha producing around 50 million Mt (Metric ton) throughout world, among which ninety percent of production from five countries, of which China shares 58 percent of output, India, 25 percent, followed by Iran, Egypt and Turkey. Insect pests inflict damage to humans, farm animals and crops. Herbivorous insects are said to be responsible for destroying one fifth of the world's total crop production annually (FAO, 2012).

Eggplant is by far the major vegetable representing some 41% by weight of all vegetables produced, occupying 19% of the land used to cultivate them. More than 4 million acres are

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devoted to the cultivation of eggplant in the world with total production of 32,072,972 tones. Eggplant is said to be native of India with secondary center as China and extensively grown in South East Asia (NHB, 2010).

However, eggplant production is in threat in recent years due to increased cost of production on management of insect pest complex. It also reduces the content of vitamin C in fruit up to 80 percent. Hence, many farmers were leaving growing eggplant because of this pest (Chakraborti *et al*., 2011).

Eggplant contains nutrients such as dietary fiber, folate, ascorbic acid, vitamin K, B_6, pantothenic acid, potassium, iron, magnesium, phosphorus and copper (USDA, 2009).

Eggplant occupies an important position in every day diet due to its high nutritive, dietary and medical values as 100 g of edible fruits contains 92.7 g water, 1.3 g carbohydrates, 1.4 g protein, 0.3 g mineral matter and 4 g calcium. It is low in calories and fats (Varmudy, 2011).

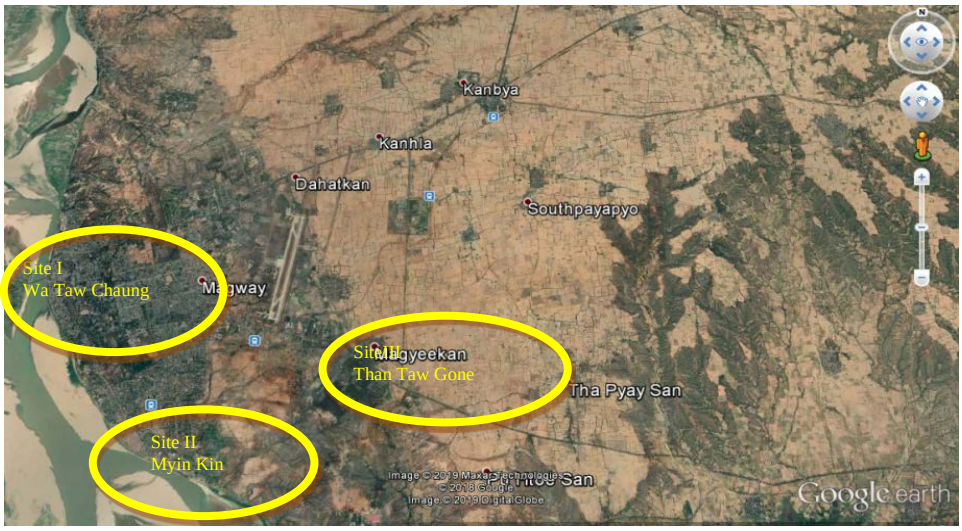
There is no previous research that focused on insect species of eggplant in Magway area. Therefore, the objectives of the present study were: to record and identify the insect species on eggplant in Magway Township, to examine the species composition of different orders of recorded insects and to determine the insects which are pests or beneficial insects of the eggplant.

Materials and Methods

Study Area

The study was conducted in Magway Region, which is situated on the East of the Ayeyarwady River and it has an area of 17, 305 square miles.

In Magway Region, the three villages were chosen as the present study sites. Study site I is Wa Taw Chaung Village (20° 10'41.33" N and 94° 54'52.34 " E), study site II is Myin Kin Village (20° 07' 0.71" N and 94° 56' 37.02" E) and study site III is Than Taw Gone Village (20° 08'03.42" N and 94° 58'22.81" E) (Fig.1).



(Source: Google earth, 2020)

Figure 1 Map of the study area and the locations of the study sites

Study period

The study was carried out from December 2019 to August 2020.

Collection of specimens

Specimens were collected during the day time. The study sites were visited once in every week for nine months. Some insects were easily collected by hand-picked and some were caught by insect net. Photographic records were taken from fresh specimens.

Preparation of specimens

The collected specimens were transferred into the killing bottle which contained cotton wool soaked with chloroform. Some were preserved in small plastic bottles, containing 70 percent alcohol with glycerin. Dissecting microscope was used for identification of insect species. Under each specimen, a label was added bearing the name of the species, locality and date of capture and then it was transferred into the insect box (Plate.1).

Data Analysis

Species composition was calculated using the method of Bisht *et al*. (2004).

$$\text{Species composition} = \frac{\text{Number of a recorded species}}{\text{Total number of all recorded species}} \times 100$$

Identification of specimens

The identification was carried out according to Borror and Delong, (2005), Gullan and Cranston, (2010), Awasthi, (2016) and Debbie, (2018).

A. Insect net

B. Collecting bottles

C. Dissecting microscope

Plate 1 Equipment used for collection and identification

Results

A total of 24 insect species belonging to 24 genera, 16 families and six orders were recorded during the period from December 2019 to August 2020 (Table.1).

Occurrence of insect species on eggplants in the three study sites

Total numbers of insect species (3743) individuals were recorded from three study sites throughout the nine months survey. During the study period, a total number of (1400) individuals were collected. The collected insects belonged to 23 species of insects, distributed among 23 genera, 15 families and six orders which occurred at the eggplant cultivation in study site I (Wa Taw Chaung Village) (Table.4).

In study site II (Myin Kin Village), a total number of (1224) individuals were collected and they belonged to 18 species of insects, distributed among 18 genera, 13 families and five orders occurring at the eggplant cultivation (Table.5).

A total number of (1119) individuals belonging to 15 species of insects, distributed among 15 genera, 10 families and five orders were collected from eggplant cultivation in the study site III (Than Taw Gone Village) (Table.6).

Order-wise species composition of insect species recorded in eggplant cultivation of three study sites

Among the 24 recorded insect species, the order Lepidoptera was represented by eight species, followed by Hemiptera with seven species, six species in Coleoptera, while Orthoptera, Neuroptera and Diptera were recorded with only one species each.

Out of six orders, the highest species composition (33.33%) was observed in order Lepidoptera, followed by order Hemiptera (29.17%) and then Coleoptera (25%), and the lowest Orthoptera, Neuroptera and Diptera (4.17%) (Table.2) (Fig.2)

Beneficial insects and insect pest species from the three study sites

Among the total of 24 insect species recorded, four species of insects were observed as the beneficial insects namely, *Antilochus coquebertii, Chrysoperla carnea, Menochilus sexmaculatus,* and *Micraspis discolor,* while the rest 20 species were represented as pest species.

Fifteen species, (*Bemisia tabaci, Amrasca biguttula biguttula, Antilochus coquebertii, Dysdercus koenigii, Creontiades pallidus, Chrysoperla carnea, Aulacophora foveicollis, Monolepta signata, Menochilus sexmaculatus, Micraspis discolor, Henosepilachna sumbana, Bactrocera cucurbitae, Lineodes integra, Luecinodes orbanalis,* and *Plutella xylostella*), were collected in all three study sites during the study period.

Six species (*Schistocera nitens, Cletus bipunctatus, Scirpophaga nivella, Earias vittella, Utetheisa pulchella, and Anomis flava*) were recorded only in study site I and *Protaetia fusca* was collected in site II during the study period (Table.3 and Fig.3).

Table 1 Recorded insect species from the three study sites

No.	Order	Family	Species	Common name
1.	Orthoptera	Acrididae	*Schistocera nitens*	Large gray bird grasshopper
2.	Hemiptera	Pentatomidae	*Nezara viridula*	Southern Green stink bug
3.		Aleyrodidae	*Bemisia tabaci*	Sweet potato Whitefly
4.		Cicadellidae	*Amrasca biguttula biguttula*	Okra leafhopper
5.		Pyrrhocoridae	*Antilochus coquebertii*	True bug
6.		Pyrrhocoridae	*Dysdercus koenigii*	Red cotton bug
7.		Coreidae	*Creontiades pallidus*	Sheddeer bug
8.		Coreidae	*Cletus bipunctatus*	Spined legume bug
9.	Neuroptera	Chrysopidae	*Chrysoperla carnea*	Green lacewing
10.	Coleoptera	Scarabaeidae	*Protaetia fusca*	Mango flower beetle
11.		Chrysomelidae	*Aulacophora foveicollis*	Red pumpkin beetle
12.		Chrysomelidae	*Monolepta signata*	White spotted leaf beetle
13.		Coccinellidae	*Menochilus sexmaculatus*	Six spotted zigzag Ladybird
14.		Coccinellidae	*Micraspis discolar*	Ladybird beetle
15.		Coccinellidae	*Henosepilachna sumbana*	Cucurbit ladybird
16.	Diptera	Tephritidae	*Bactrocera cucurbitae*	Melon fly (fruit fly)
17.	Lepidoptera	Crambidae	*Cydalima perspectalis*	Box tree moth
18.		Crambidae	*Lineodes integra*	Leaf-roller moth
19.		Crambidae	*Scirpophaga nivella*	Sugarcane top borer
20.		Pyralidae	*Leucinodes orbanalis*	Eggplant fruit and shoot borer
21.		Nolidae	*Earias vittella*	Spiny bollworm

22.	Erebidae	*Utetheisa pulchella*	Crimson-speckled moth
23.	Erebidae	*Anomis flava*	Orange cotton moth
24.	Plutellidae	*Plutella xylostella*	Cabbage moth

Table 2 Species composition of insects from the eggplants of the three study sites

Order	Number of Family	Number of Genus	Number of Species	Composition of species in order (%)
Orthoptera	1	1	1	4.17
Hemiptera	5	7	7	29.16
Neuroptera	1	1	1	4.17
Coleoptera	3	6	6	25
Diptera	1	1	1	4.17
Lepidoptera	5	8	8	33.33
Total	16	24	24	100

Table 3 Status of insect species from the three study sites

No.	Scientific name	SiteI	SiteII	SiteIII	Status
1.	*Schistocera nitens*	+	-	-	Pest
2.	*Nezara virudula*	+	+	-	Pest
3.	*Bemisia tabaci*	+	+	+	Pest
4.	*Amrasca biguttula biguttula*	+	+	+	Pest
5.	*Antilochus coquebertii*	+	+	+	Beneficial
6.	*Dysdercus koenigii*	+	+	+	Pest
7.	*Creontiades pallidus*	+	+	+	Pest
8.	*Cletus bipunctatus*	+	-	-	Pest
9.	*Chrysoperla carnea*	+	+	+	Beneficial
10.	*Protaetia fusca*	-	+	-	Pest
11.	*Aulacophora foveicollis*	+	+	+	Pest
12.	*Monolepta signata*	+	+	+	Pest
13.	*Menochilus sexmaculatus*	+	+	+	Beneficial
14.	*Micraspis discolar*	+	+	+	Beneficial
15.	*Henosepilachna sumbana*	+	+	+	Pest
16.	*Bactrocera cucurbitae*	+	+	+	Pest
17.	*Cydalima perspectalis*	+	+	-	Pest
18.	*Lineodes integra*	+	+	+	Pest
19.	*Scirpophaga nivella*	+	-	-	Pest
20.	*Leucinodes orbanalis*	+	+	+	Pest
21.	*Earias vittella*	+	-	-	Pest
22.	*Utetheisa pulchella*	+	-	-	Pest
23.	*Anomis flava*	+	-	-	Pest
24.	*Plutella xylostella*	+	+	+	Pest

(+) found, (-) not found

Table 4 Monthly occurrence of insect species and number of individuals from the study site I (Wa Taw Chaung Village)

No.	Order	Scientific name	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Total
1.	Orthoptera	*Schistocera nitens*	0	0	0	1	0	0	0	0	0	1
2.	Hemiptera	*Nezara viridula*	0	1	0	0	0	0	0	0	0	1
3.		*Bemisia tabaci*	0	25	15	9	5	0	7	10	14	85
4.		*Amrasca biguttula biguttula*	33	53	195	150	170	80	85	80	45	891
5.		*Antilochus coquebertii*	0	1	0	0	0	2	1	0	2	6
6.		*Dysdercus koenigii*	5	3	0	3	1	6	0	2	4	24
7.		*Creontiades pallidus*	4	2	2	2	2	0	3	0	3	18
8.		*Cletus bipunctatus*	0	1	2	1	0	1	0	0	2	7
9.	Neuroptera	*Chrysoperla carnea*	1	0	3	0	0	2	1	1	2	10
10.	Coleoptera	*Aulacophora foveicollis*	3	1	2	9	0	5	2	5	8	35
11.		*Monolepta signata*	0	0	3	0	0	2	0	0	0	5
12.		*Menochilus sexmaculatus*	0	0	4	0	0	0	3	6	7	20
13.		*Micraspis discolar*	2	4	0	3	0	2	0	2	3	16
14.		*Henosepilachna sumbana*	0	0	0	0	0	0	2	4	6	12
15.	Diptera	*Bactrocera cucurbitae*	0	5	6	7	10	4	0	0	1	33
16.	Lepidoptera	*Cydalima perspectalis*	0	1	0	0	0	0	1	0	0	2
17.		*Lineodes integra*	0	0	2	1	0	0	0	0	0	3
18.		*Scirpophaga nivella*	0	1	0	0	0	0	0	0	1	2
19.		*Luecinodes orbanalis*	23	36	45	30	25	5	10	12	9	195
20.		*Earias vittella*	0	0	0	1	0	2	0	0	2	5
21.		*Utetheisa pulchella*	0	0	0	3	1	2	2	0	4	12
22.		*Anomis flava*	0	1	0	0	0	0	0	0	3	4
23.		*Plutella xylostella*	0	0	2	4	1	1	0	0	5	13
Number of individuals			71	135	281	224	215	114	117	122	121	1400
Number of species			7	14	12	14	8	13	11	9	18	

Table 5 Monthly occurrence of insect species and number of individuals from the study site II (Myin Kin Village)

No.	Order	Scientific name	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Total
1.	Hemiptera	*Nezara viridula*	0	2	0	0	0	0	0	0	0	2
2.		*Bemisia tabaci*	0	15	10	8	5	0	5	7	10	60
3.		*Amrasca biguttula biguttula*	33	50	180	150	170	95	70	45	30	823
4.		*Antilochus coquebertii*	0	0	0	2	0	0	0	0	1	3
5.		*Dysdercus koenigii*	3	2	0	2	0	5	0	0	3	15
6.		*Creontiades pallidus*	3	2	0	3	1	0	2	0	3	14
7.	Neuroptera	*Chrysoperla carnea*	2	0	2	1	0	0	1	0	2	8
8.	Coleoptera	*Protaetia fusca*	0	0	0	0	0	0	2	4	6	12
9.		*Aulacophora foveicollis*	0	0	3	6	0	0	1	3	5	18
10.		*Monolepta signata*	0	0	2	0	0	0	0	0	0	2
11.		*Menochilus sexmaculatus*	0	0	2	0	2	0	0	3	5	12
12.		*Micraspis discolar*	1	3	2	3	0	1	0	2	3	15
13.		*Henosepilachna sumbana*	0	0	0	0	0	0	0	3	5	8
14.	Diptera	*Bactrocera cucurbitae*	0	3	4	7	8	5	0	0	0	27
15.	Lepidoptera	*Cydalima perspectalis*	0	0	1	0	0	0	0	0	0	1
16.		*Lineodes integra*	1	0	1	0	0	0	1	0	0	3
17.		*Luecinodes orbanalis*	20	32	30	35	28	18	16	10	5	194
18.		*Plutella xylostella*	1	0	0	3	0	1	0	0	2	7

Number of individuals	64	109	237	220	214	125	98	77	80	1224
Number of species	8	8	11	11	6	6	8	8	13	

Table 6 Monthly occurrence of insect species and number of individuals from the study site III (Than Taw Gone Village)

No.	Order	Scientific name	Dec.	Jan.	Feb.	Mar.	Apr.	May.	Jun.	Jul.	Aug.	Total
1.	Hemiptera	*Bemisia tabaci*	0	10	8	10	4	0	3	6	5	46
2.		*Amrasca biguttula biguttula*	30	35	170	145	150	100	65	50	30	775
3.		*Antilochus coquebertii*	0	0	1	0	0	0	0	1	1	3
4.		*Dysdercus koenigii*	3	2	1	1	0	0	2	0	3	12
5.		*Creontiades pallidus*	4	0	2	3	0	1	0	2	3	15
6.	Neuroptera	*Chrysoperla carnea*	0	3	2	3	0	0	0	1	1	10
7.	Coleoptera	*Aulacophora foveicollis*	0	2	0	4	0	0	2	0	3	11
8.		*Monolepta signata*	1	0	0	1	0	0	0	0	0	2
9.		*Menochilus sexmaculatus*	0	0	0	1	2	1	1	0	3	8
10.		*Micraspis discolar*	2	3	0	4	0	1	1	0	1	12
11.		*Henosepilachna sumbana*	0	0	0	0	0	0	0	3	4	7
12.	Diptera	*Bactrocera cucurbitae*	0	2	4	6	7	4	0	0	0	23
13.	Lepidoptera	*Lineodes integra*	0	0	0	1	0	1	0	1	0	3
14.		*Luecinodes orbanalis*	20	28	25	30	32	22	15	10	5	187
15.		*Plutella xylostella*	0	0	0	2	0	0	1	0	2	5
Number of individuals			60	85	213	211	195	130	90	74	61	1119
Number of species			6	8	8	13	5	7	8	8	12	

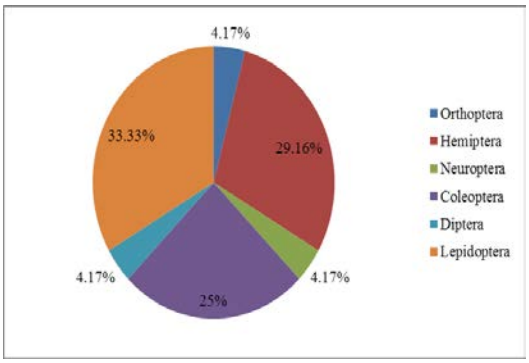


Figure 2 Composition of insect species in different orders from the three study sites

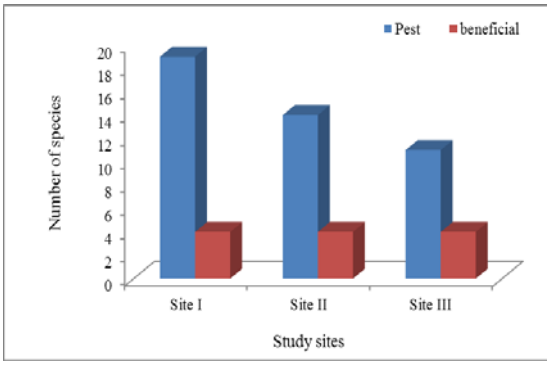


Figure 3 Pest and beneficial species from the three study sites during the study period

Discussion

A total of 24 insect species belonging to 24 genera, 16 families and six orders were recorded during December 2019 to August 2020. In the present study, eight species of Lepidoptera, seven species of Hemiptera, and six species of Coleoptera were recorded while only one species of each of Orthoptera, Neuroptera and Diptera was recorded from the three study sites.

Among the recorded species, the highest number was observed in order Lepidoptera (33.33%) follow by Hemiptera (29.17%), Coleoptera (25%), and the lowest number of Orthoptera, Neuroptera and Diptera (4.17%) was recorded from the three study sites during the study period. Lepidopterans and Hemipterans therefore appeared to predominate in the three study sites, while Orthopterans, Neuropterans and Dipterans appeared as rare species.

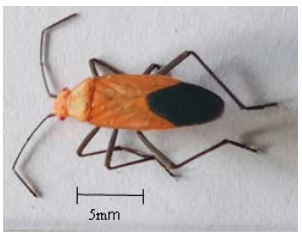
Among the recorded species, 20 species were identified as pests and the other four species as beneficial or predatory insects. The recorded species in orders Coleoptera, namely *Menochilus sexmaculatus*, *Micraspis discolor*, and members of order Neuroptera, and *Chrysoperla carnea*, were regarded as beneficial insects while those of orders Orthoptera, Hemiptera, except *Antilochus coquebertii*, Diptera and Lepidoptera appeared as pests.

A. *Schistocera nitens*

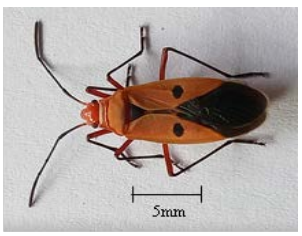
B. *Nezara viridula* (3rd instar)

C. *Bemisia tabaci*

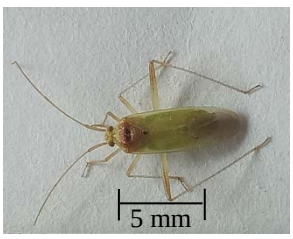
D. *Amrasca biguttula biguttula*



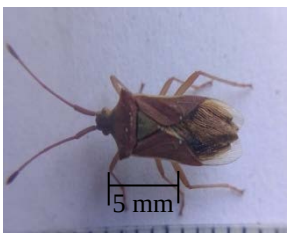
E. *Antilochus coquebertii*



F. *Dysdercus koenigii*

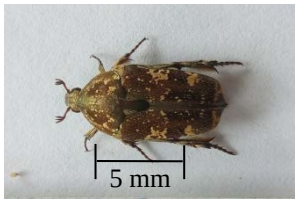


G. *Creontiades pallidus*



H. *Cletus bipunctatus*

I. *Chrysoperla carnea*



J. *Protaetia fusca*

K. *Aulacophora foveicollis*

L. *Monolepta signata*

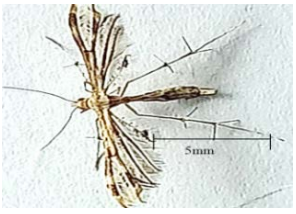
M. *Menochilus sexmaculatus*

N. *Micraspis discolar*

O. *Henosepilachna sumbana*

P. *Bactrocera cucurbitae*

Q. *Cydalima perspectalis*



R. *Lineodes integra*

S. *Scirpophaga nivella*

T. *Leucinodes orbanalis*

U. *Earias vittella* V. *Utetheisa pulchella* W. *Anomis flava* X. *Plutella xylostella*

Plate 2 Recorded insect species of Order Orthroptera, Hemiptera, Neuroptera, Coleoptera, Diptera and Lepidoptera

Mote (2003) reported that *Amrasca biguttula biguttula*, commonly known as jassid and *Leucinodes orbonalis*, commonly called as brinjal shoot and fruit borer were the serious pests of eggplant. In the present study, these species were destroying the eggplants.

Amrasca biguttula biguttula is an important sucking pest of brinjal crop recorded from July to November. Both nymphs and adults of Jassid have a piercing and sucking type of mouth parts and suck the sap from the lower surface of the leaf. When the several insects suck the sap from same leaf, yellow spot appear on the leaf followed by crinkling, curling and "hopper burn" as reported by Borah *et al*. (2017).

Bharadiya *et al*. (2005) have also reported that *Leucinodes orbonalis* was most destructive and major insect pest of brinjal in North Gujarat. The incidence of this insect started soon after transplanting of the seedlings and continued till the harvest of the crop. The larvae of this insect initially bored the tender shoots and fed internally resulting in weathering and drying of the shoot. In the present study, Larvae of *Leucinodes orbonalis* destroyed the eggplants in the three study sites. This resulted that the fruit became unfit for human consumption and fetched fewer prices in the market. During the present study, two out of the 20 pest species, *Amrasca biguttula biguttula* (2489 individuals) and *Leucinodes orbonalis* (576 individuals) appeared as predominant pests from the three study sites.

Schaefer and Ahmad (2000) described that Heteropteran predators were important natural enemies of phytophagous insects and have thus potential in biological control of crop pests. The red cotton bug, *Dysdercus koenigii* and its natural predator *Antilochus coquebertii* were recorded as the most abundant insects in Asian cotton agro-ecosystem, suggesting that the importance of *Antilochus coquebertii* in controlling *Dysdercus koenigii*. During the present study, *Antilochus coquebertii* (predator) and *Dysdercus koenigii* (prey) were recorded in eggplant cultivation from the three study sites in Magway Township. The result showed predator-prey relationship.

Chakraborti *et al*. (2011) stated that eggplant fruit and shoot borer, *Leucinodes orbonalis* was the key pest of eggplant, inflicting sizeable damage in the eggplant growing area and it's most destructive, especially in South East Asia. In the present study, *Leucinodes orbonalis* was the most important pest from the three study sites.

Aye Aye Myint (2014) stated that three predators species of *C. transversalis*, *M. sexmaculata* and *H. octomaculata* fed on aphids, eggs of Lepidopterans and other soft bodied insect pests. In the present study, two predators species of *Menochilus sexmaculata* and *Micraspis discolor* were collected on eggplants from the three study sites.

Concerned with study sites, 23 species were recorded at site I (Wa Taw Chaung Village) followed by 18 species of site II (Myin Kin Village) and 15 species of site III (Than Taw Gone Village). Moreover, total numbers of insects (3743 individuals) were recorded from all sites

throughout the nine months survey in which the highest species number was found in site I. This place situated on river alluvial plain near Wa Taw Chaung Village, it might be more suitable place for insect species.

Maximum numbers of individuals were collected under order Hemiptera from the three study sites during the study period. Hemipterans were the largest order of insect species and the insects in this order were extremely diverse. Minimum number of individuals on the other hand was collected under order Orthoptera from site I, Neuroptera from site II and site III. Highest numbers of insect species were collected in the month of August; it might be assumed that some of the pests were active during the rainy season. Lowest number was collected in the month of December during the study period. Cold season seems to be a suitable season of the pests.

Conclusion

A total of 24 insect species belonging to 24 genera, 16 families and six orders were recorded on eggplant growing area of Magway Township. Twenty species of the insects were recorded as the insect pests and four species of the insects were found as the beneficial insects. A total of 1400 individuals in site I, 1224 individuals in site II and 1119 individuals in site III were recorded in the study period. Lepidopteran species were predominant on eggplant cultivation in the study area. Since Lepidopteran species inhabit the agricultural lands, they are of economic importance. This research contributes some information concerning with insect pest and beneficial species of eggplants and suggests the potential of biological control of insect pests in agricultural field.

Acknowledgements

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MYXOZOSPOREAN INFECTION IN THE MUSCLES OF *CIRRHINUS MRIGALA* (HAMILTON, 1822)

Yu Yu Aye[1], Thant Zin[2], Kay Lwin Tun[3]

Abstract

The two types of *Myxobolus* species were recorded in the muscles of *Cirrhinus mrigala* collected from Yezin Fisheries Station, Nay Pyi Taw. The plasmodia of *Myxobolus* sp. containing with mature spores in the central part of the plasmodia were bounded by single-unit membrane in contact with the muscle tissues. Spores of *Myxobolus rohitae*, measured 11.6µm ± 1.8µm in length and 9.1µm ± 0.9µm in width and appeared elongated ellipsoidal in valvular view. Polar capsules were pyriform, equal and sporoplasm granular present. Spores of *Myxobolus* sp., measured 11.6µm ± 1.1µm in length and 7.6µm ± 0.8µm in width in valvular view. Two polar capsules were slightly pyriform and unequal in shape, larger 5.0µm ± 1.1µmx3.3µm ± 0.5µm and smaller 3.5µm ± 0.8µmx3.3µm ± 0.5µm in size. The highest prevalence of *Myxobolus rohitae* infection (48%) was recorded in July, 2019 with the highest mean intensity 2.5. The prevalence of *Myxobolus* sp. infection was highest 62% in August with the highest mean intensity 3. Histopathological changes such as loss of epidermis, dermis split and separated from muscle and hemorrhage were found in muscle layer. The present study represents the first report of *Myxobolus* infection in the muscle tissues of *Cirrhinus mrigala* in Myanmar.

Introduction

Fishery and aquaculture has an important role in the local economy in Myanmar. Fish diseases may cause severe losses of fish farms through reduced fish growth and production. The parasites may involve in the serious outbreak of disease in fish farms (Kayis *et al*., 2009). It is a major problem that carrying heavy infestation of parasites of freshwater fishes in aquaculture. They have been receiving considerable scientific attention due to serious damage to fisheries resources by them (Ravichandram *et al*., 2009). Infectious diseases of cultured freshwater carps are one of the major problems to successful aquaculture industry. The outbreak of various types of disease is one of the important reasons of reduction in the fish production. Therefore, proper health management procedures should be followed with appropriate control measures to boost up aquaculture production. In the high stocking condition, particularly if the fishes are stressed, the parasites multiply rapidly.

Parasites of the phylum Cnidarian have been described in lower vertebrate hosts, mainly in fish and in some amphibians. They are the most common fish parasites, infecting both marine and freshwater fish (Eiras *et al*., 2005). The genus *Myxobolus* is the richest group among Myxosporidia, containing about 744 nominal species (Eiras *et al*., 2005). The ability of some Myxosporea species to transmit as a barrier between fish and humans (Boreham *et al*., 1998; Moncada *et al*., 2001) does not prevent the possibility that the *Myxobolus* sp. may also be transmitted to humans. The zoonotic potential of *Myxobolus* sp. cannot be ignored. Infection in humans is associated with the consumption of raw and undercook fish containing live parasites. The *Cirrhunus mrigala* has been an important commercial fish species in Myanmar for a long time. However, parasitic diseases are a significant restricts for the development of the carps culture industry, among which especially myxobolosis, which has become one of the most notably growing parasitic infections. The genus *Myxobolus* was first established by having spores with or without an iodinophilous vacuole and with one or two polar capsules (Butschli, 1882). This genus has the global distribution and highest number of Myxosporidia.

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The infection by *Myxobolus* sp. is characterized by the formation of cysts in the tissues of fish with mature spores. The presence of these cysts has been associated with tissue lesions, gross deformities, and organ malfunction (Feist 2008). Myxospores have been incidentally found in human fecal samples, which were collected to investigate the intestinal diseases caused by protozoa of medical importance (Bradbury *et al*. 2015). The showing of myxospores in human feces associated with ingestion of contaminated food or undercooked fish (Kawai *et al*., 2012). However, Japanese food types, like sushi and sashimi are now consumed worldwide. This has resulted in an increase of food-borne diseases caused by the ingestion of raw fish (Barralet *et al*., 2004).

Muscle is the main component of the fish body. It is a common site of establishment for various myxosporeans in infecting fishes. In a synopsis of *Myxobolus* species of the world, 54 of the 751 characterized species have been described from the muscles (Eiras *et al*., 2005). In another synopsis on *Henneguya* species, 6 of the recorded 146 species were found to infect muscles (Eiras 2002). In addition, muscle cells contain blood vessels, nerves and connective tissue as well as cartilaginous and bony elements (Ferguson 1989). In several fish, myosepta are supported by bony portions formed by a dense connective tissue which has been calcified in some fish. Therefore, due to their different tissue affinities, myxosporeans can occur in different locations and might affect muscle cells, connective tissue, bones and blood vessels. *Myxobolus sandrae* infected to the intermuscular connective tissue in the pike-perch, *Sander lucioperca* was observed (Molnar and Szekely, 2014). Examination of parasitic infections in muscles of *Cirrhinus mrigala* in Myanmar is still required to improve production. The present study was therefore undertaken to detect the myxozoan parasites infected to the muscles of *Cirrhinus mrigala* in Yezin fishery station, one of the biggest *C. mrigala* hatchery in Myanmar and to evaluate the histopathological alterations caused by Myxosporean parasitic infestation in muscles of *Cirrhinus mrigala*.

Materials and Methods

Study Area

Yezin Fishery Station is a government owned fish seed multiplication center. It is situated at 19° 50' 14.9" N and 96° 16' 36.8" E about 19 km away from Pyinmana city, and it is located near the Yezin Dam. It is one of the biggest *Cirrhinus mrigala* hatcheries in Myanmar, and also distributes fry/fingerling *C. mrigala* through the country.

Study Period

The research work was carried out from August 2018 to September 2019.

Sample Collection and Examination of Parasites

Cirrhinus mrigala fingerlings were cultured in experimental pond (8.3mx33.3m) at Yezin Fishery Station as extensive culture system. Thirty fish were collected monthly to examine the occurrence of parasites. A total of 30 fish samples were carried to the laboratory of Department of Aquaculture and Aquatic Diseases, University of Veterinary Science or laboratory of Aquatic Bioscience, University of Yangon with oxygen filled plastic bags. The total length, standard length and body weight of each specimen were immediately measured and recorded. Fish were dissected and muscle tissues were collected to examine the parasites. The muscle tissues were used for smear slide preparation and histological slides preparation. For smear slide preparation, the muscle tissues of fish were checked under stereomicroscope for the cyst formation of myxosporean. Muscles were squeezed with cover slip with 1 drop of normal saline (0.9% NaCl). Occurrence of parasites was examined under light microscope, Olympus – CX 31.

Identification of parasites

Identification of myxosporean parasites was conducted on the various morphological structures of spore including shape, size, number of polar capsules, length and number of coils of polar filaments, intercapsular process presence or not, number of nuclei and iodinophilous vacuole in the sporoplasm, etc. according to the guidelines of Lom and Dykova (1992) and Kalavati and Nandi (2007). They were measured and photographed using the light microscope (Olympus CX 31) under x100 magnification.

Data analysis for parasites

Prevalence of parasitic infection was calculated in accordance with the following methods (Bush *et al*., 1997).

$$\text{Prevalence (\%)} = \frac{\text{Number of infected host}}{\text{Total number of host examined}} \times 100$$

Mean intensity of infection was classified four stages according to Culloty *et al*. (1999).

Stage (I): 1-20 parasites observed within five minutes of screening under x40 magnification

Stage (II): 21-40 parasites observed within five minutes of screening under x40 magnification

Stage (III): 41-60 parasites observed within five minutes of screening under x40 magnification

Stage (IV): 1-10 parasites in all field of region observed immediately in screening under x40 magnification

$$\text{Mean Intensity} = \frac{\text{Total Number of parasites recovered}}{\text{Total number of infected fishes}}$$

Preparation of Histopathological Slides

To understand the histological changes of infested tissues of muscles, and infected tissue with cyst formation were fixed with 10% neutral buffered formalin. After fixation for 48 hours, the tissues were cut in order to obtain a size of 1 cm^3. The prepared tissues were dehydrated through a graded series of ethanol, cleared in xylene, and infiltrated in the paraffin. Sections were cut at 5μm in thickness on a microtome (TBS SHUR/Cut 2500) fitted with a sharpened microtome knife. These sections were then stained with Hematoxylin-Eosin. The permanent mounting of the slides was made by DPX (distyrene, plasticizer and xylene). Histopathological lesions were examined and photographed at different magnifications with the help of binocular microscope with digital camera and attached monitor (Olympus – CX 31).

Results

Myxobolus spp. infection in the muscles of *Cirrhinus mrigala*

Myxobolus rohitae and *Myxobolus* species were recorded in the muscles of *Cirrhinus mrigala* collected from Yezin Fisheries Station. *Myxobolus* sp. was identified according to Lom and Arthur (1989), Lom and Dykova (1992) and Kalavati and Nandi (2007).

Plasmodia

The plasmodia of *Myxobolus* sp. were bounded by single-unit membrane in contact with the muscle tissues of the host (Plate 1, A and Plate 2, A). The central part of the plasmodia contained the mature spores.

Morphometry of *Myxobolus rohitae*

Spores are roughly oval to elongate ellipsoid in front view, sometimes with a semicircular ledge or mucus envelope at the posterior end. Anterior end of the spore is extremity pointed and posterior end is rounded. Spores of *Myxobolus rohitae*, measured 11.6µm±1.8µm in length and 9.1µm±0.9µm in width and appeared elongated ellipsoidal in valvular view. Polar capsules were pyriform, equal and polar filament well marked inside the capsule in fresh spores, sporoplasm granular present. Length and width of polar capsules were 3.2µm±0.4µmx2.3µm±0.4µm in size (Plate 1).

Morphometry of *Myxobolus* species

Spores of *Myxobolus* sp., measured 11.6µm±1.1µm in length and 7.6µm±0.8µm in width and appeared elongated ellipsoidal in valvular view. Two polar capsules were slightly pyriform and unequal in shape with 4 to 6 filaments, larger 5.0µm±1.1µmx3.3µm±0.5µm and smaller 3.5µm±0.8µmx3.3µm±0.5µm in size (Plate 2). Sporoplasm was finely granular and occupied most of the extracapsular cavity of spore. Spores were elongated and ellipsoid in valvular view with mucus envelope around the posterior end. Two polar capsules were slightly pyriform and unequal in shape with 4 to 6 filaments. Sporoplasm was finely granular and occupied most of the extracapsular cavity of spore.

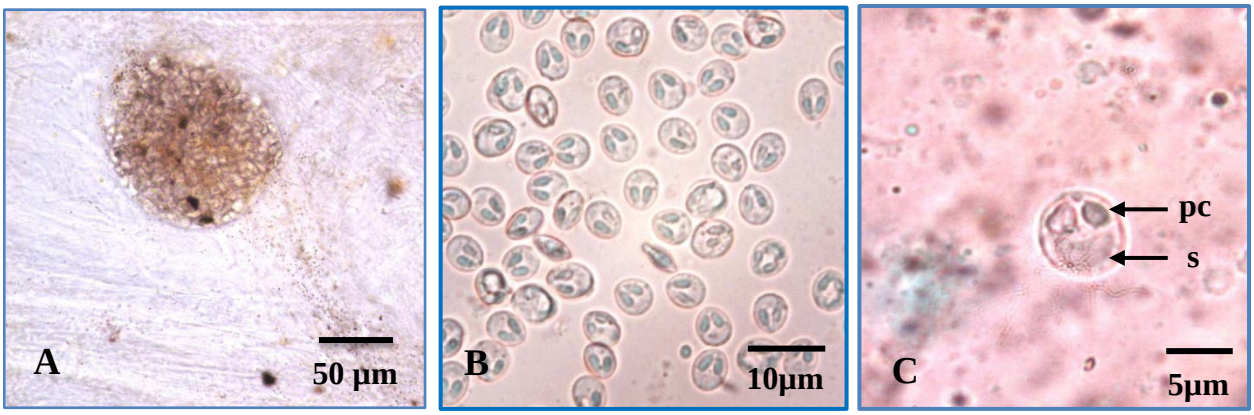

Plate 1 *Myxobolus rohitae* recorded from the muscle of *Cirrhinus mrigala* (Wet mount)
(A) Cyst of *M. rohitae* infested to the muscles of *C. mrigala*, (B) Spores of *M. rohitae* recorded in the muscles of *C. mrigala*, (C) Spore of *M. rohitae* (pc = polar capsule, s = sporoplasm)

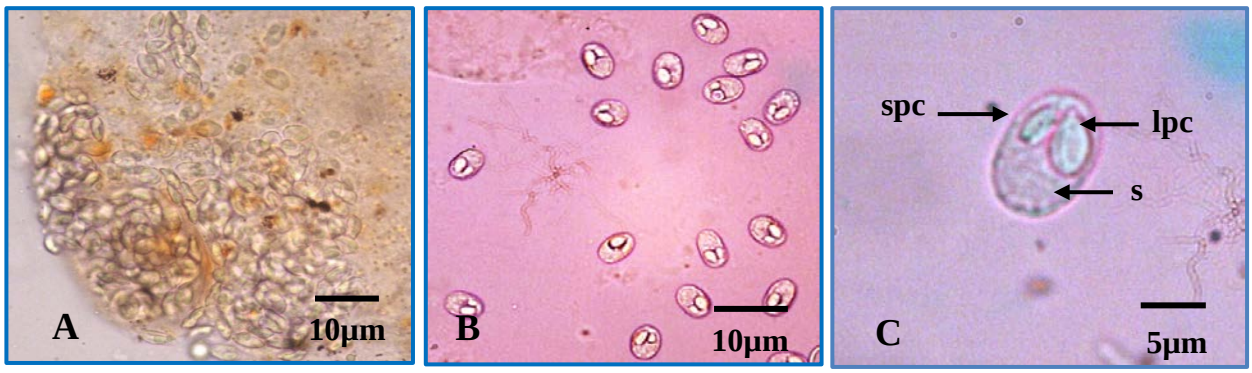

Plate 2 *Myxobolus* sp. recorded from the intestine of *Cirrhinus mrigala* (Wet mount)

(A) Plasmodia of *Myxobolus* sp. infested to intestine of *C. mrigala* (B) Spores of *Myxobolus* sp. (C) Spore of *Myxobolus* sp. (lpc = large polar capsule, spc = small polar capsule, s = sporoplasm)

Prevalence and mean intensity of *Myxobolus* spp.

The prevalence and mean intensity of *Myxobolus* species infested in *C. mrigala* were recorded from September 2018 to August 2019. The prevalence of *Myxobolus rohitae* in muscles was found 3% in December 2018 and gradually increased to the highest prevalence 48% in July 2019 and slightly decreased to 28% in August 2019. The prevalence of *Myxobolus* sp. was initially recorded only 8% in November 2018. The prevalence of *Myxobolus* sp. infection was 14% in March 2019 and it was markedly increased to 54% in May 2019. Then, it was slightly decreased to 48% in June and minimally increased to 54% in July and 62% in August 2019.

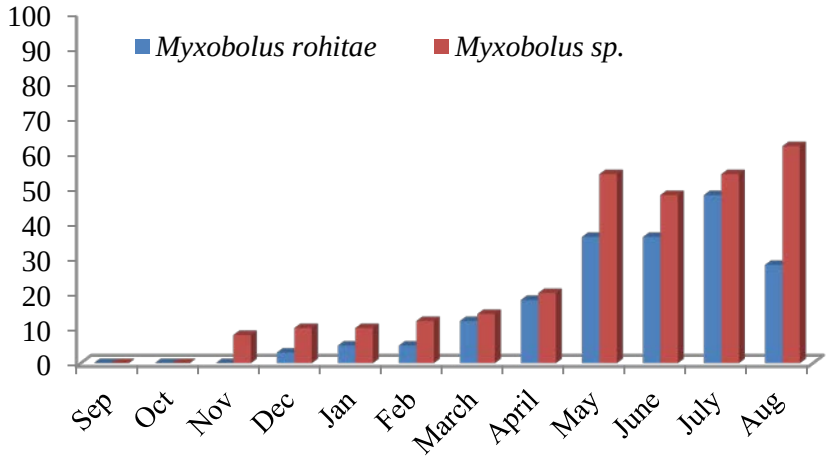

Figure 1 Prevalence of myxosporean infections in the muscles of *Cirrhinus mrigala*

The mean intensity of *Myxobolus rohitae* was recorded at stage 1 in December 2018 to March 2019 and gradually increased to highest intensity (2.5) in June 2019. *Myxobolus* sp. infested in muscle with mean intensity 1 in November 2018 to February 2019 and gradually increased to 1.5, 2.1, 2.9, 2.5, 2.6 and 2.6 in March, April, May, June, July and August 2019 respectively.

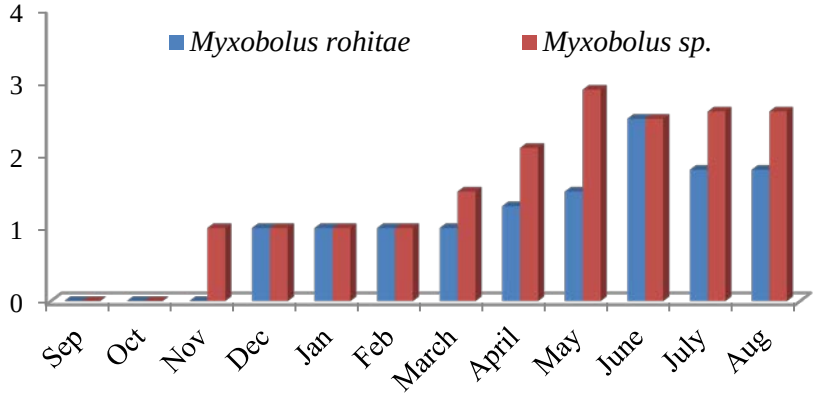

Figure 2 The mean intensity of myxosporean infections in the muscles of *Cirrhinus mrigala*

Histopathological Changes of the Muscles infested with *Myxobolus* species

In the study, plasmadia were found intracellularly in muscle cells of infested fish. In infested muscle, loss of epidermis, dermis split and separated from muscle, parasitic infestations and hemorrhage were found in muscle layer (Plate 3). Epidermis sloughed off from dermis, dermis split from muscle, myotomes were necrotic, *Myxobolus* spesies cyst and vacuums were found in many places (Plate 3, A).

In the same way, histopathological lesions and attached of *Myxobolus* spesies cyst were observed in fibrous connective tissue of dermis layer (Plate 3, B). Muscle tissue showed histoarchitectural loss in fishes that were characterized by the increased changes in the muscle fiber along with intercellular edema, necrosis, atrophic myocytes and mass of *Myxobolus* sp. mature spores (Plate 3, C). Whereas muscle structure was almost normal except the *Myxobolus* spesies cyst and any disintegrated muscle could not be seen obviously (Plate-3, D). Although, the muscle seems to be lost the myoseptum that separate within the myotomes in the muscle tissue (Plate 3, E). Each muscle bundle was irregular in shape and possesses peripheral nuclei and encysted parasites were enveloped by a loosened fibrous tissue (Plate 3, F). The damaged muscle caused by myositis which was characterized with a defect in the muscle. Necrosis is the death of cells caused by acute cell damage.

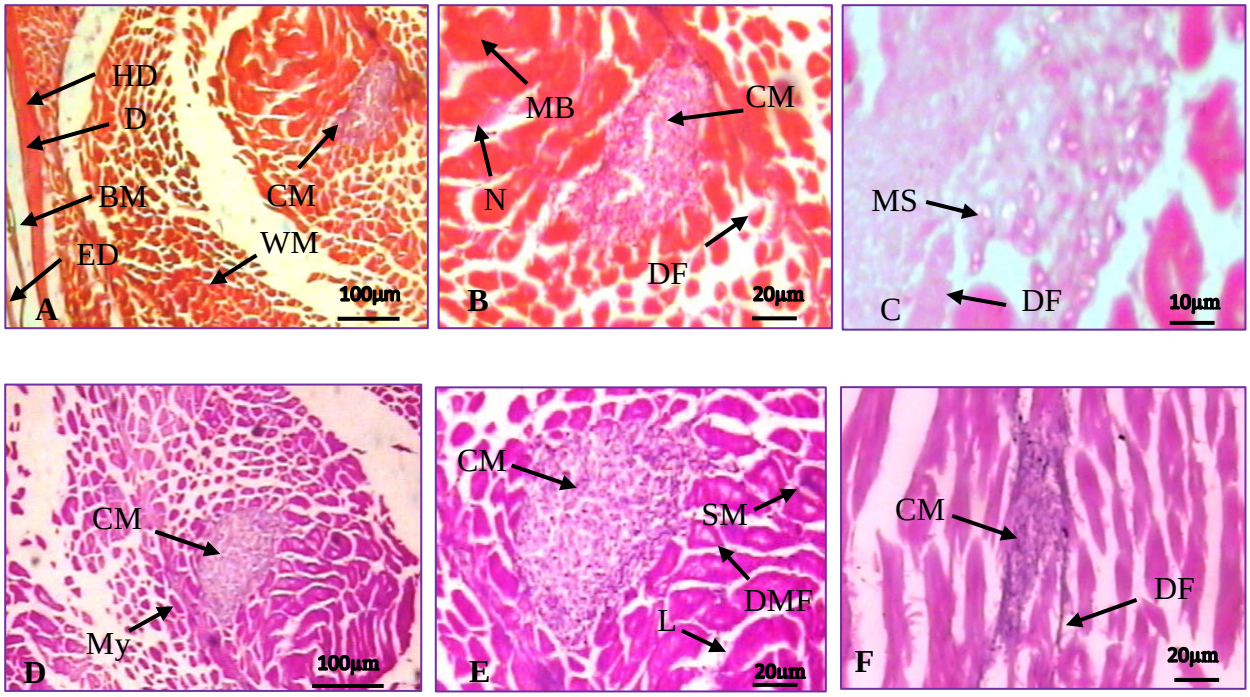

Plate 3 Histopathology in muscle tissues of *Cirrhinus mrigala* caused by *Myxobolus* species infestations (A) Developing intracellular plasmodium filled by sporogonic stages and young spores of *Myxobolus* species in a cross-sectioned muscle cell of *Cirrhinus mrigala.* (ED=Epidermis, D=Dermis, BM=Basement membrane, HD=Hypodermis, WM=White muscle, CM= cyst of *Myxobolus* sp.) (B) A mass of *Myxobolus* sp. spores in the intermuscular space, released from a disrupted plasmodium of the damaged host cell. (N=Necrosis, MB=Muscle bundle, CM= cyst of *Myxobolus* sp., DF=Disintegrated fibers) (C) Mass of *Myxobolus* sp. mature spores and disintegrated muscle (MS=Mature spore of *Myxobolus* sp, DF=Disintegrated fibers) (D) A *Myxobolus* species plasmodium located in the intermuscular septa of the muscle of *Cirrhinus mrigala*. (My=Myofbrils, CM= cyst of *Myxobolus* sp.) (E) *Myxobolus* sp. spores among cross-sectioned muscle cells of roach *Cirrhinus mrigala* encapsulated by a thick connective tissue layer (MC= *Myxobolus* sp. cyst, L=Lesion, DMF=Disintegrated myofibrils, SM=Split muscle) (F) Longitudinal section of the muscle cells of *Cirrhinus mrigala* infected with elongated mature plasmodia of *Myxobolus* sp. (MC= *Myxobolus* sp. cyst, DF=Disintegrated fibers)

Discussion

To establish the identity of the present specimens, they were compared with all the species of *Myxobolus* described so far, and therefore including all the species described from hosts in Myanmar. Lom and Dykova, 2006 estimated that, the world of myxosporidia fauna was composed of about 2180 species gathered within 62 genera among which the genus *Myxoblus* represented about 36.33% of species (792 species). *Myxobolus* is the predominant species group within the phylum Cnidarian. Most of the species infect primarily fish, both freshwater and marine species and a few numbers of species were found in amphibians (Eiras *et al*., 2005). The importance of site selection as a diagnostic characteristic for myxosporean species infecting gills, fins and kidneys have demonstrated (Molnar, 2007). The relatively low number of reported muscle infections might be due to the technical difficulties of examining muscles. Examining the myxosporeans infection in the muscle is rather difficult. While even small plasmodia can readily be studied in the gills, plasmodia infecting the muscles must be detected in muscle samples. In Asia, *Myxobolus lentisuturalis*, a pathogenic species infecting the gibel carp in China, is the best-studied species developing intracellularly in muscle (Dykova *et al*., 2002). In Indian, 7 out of the 97 *Myxobolus* species have been located in muscle (Kalavati and Nandi, 2007). The present study represents the first report of *Myxobolus* infection in the muscle tissues of *Cirrhinus mrigala* in Myanmar.

Passage of myxozoan parasites into human feces has been described (Reis *et al*., 2019). They reported that 13% of the 97 samples, the parasitological examination of fecal samples from adults (22 to 71 years old) resulted in the detection of myxozoan spores in the Amazonas State, Brazil. In this state, consumption of fish is very high, and is the main source of protein, particularly for people living in rural areas and in riverine communities (Lopes *et al*., 2016). Another Myxozospoean, *Kudoa septempunctata*, was responsible for outbreaks of food poisoning caused by the consumption of raw fish in Japan (Kawai *et al*. 2012). Consuming raw, undercooked, or smoked fish causes dipyllobothriasis in humans due to *Diphyllobothrium latum*, a cestode (Emmel *et al*. 2006). The habit of eating raw fish such as sushi and sashimi has spread throughout the world. Fish consumption has increased worldwide including Myanmar because it is a healthy source of nutrients that is rich in proteins, minerals, and essential fatty acids. Therefore, it is possible that food-borne illness caused by myxosporean parasites will occur in Myanmar.

In the present study, the plasmodia of *Myxobolus* sp. containing with mature spores in the central part of the plasmodia were observed in the muscle tissues of *Cirrhinus mrigala*. Myxosporean spores have been shown to be very resistant to a range of environmental conditions and can survive entrance through the alimentary tracts of piscivorous vertebrates (El-Matbouli *et al*., 1991). They observed that myxosporean cysts and spores have been damaged by freezing or cooking due to the release of proteolytic enzymes by postmortem myoliquefaction of fish fillets.

In the present study, parasitic prevalence of *Myxobolus rohitae* in muscles was highest (48%) stated as “stage 2” in July 2019. *Myxobolus* sp. was found in the muscles with the highest prevalence 62% stated as “stage 3” in August 2019, which was nearly similar to the finding of Deva (2016). The author stated that *Myxobolus* sp. infection in the gills of *Labeo rohita* reached peak stage (52%) during the raining season.

The shape and dimension of *Myxobolus* spp., in the present study were compared to those of other *Myxobolus* spp. reported by Eiras *et al*. (2014). *Myxobolus rohitae* recorded in the present study is superficially similar to *Myxobolus eirasi* infected in caudal fin and *Myxobolus guangzhouensis* infested in scales of *Cirrhinus mrigala*. The shape and size of *Myxobolus* sp. detected in this study is also similar to *Myxobolus* sp. A total of 7 fish infected in gills and kidneys of *Cirrhinus mrigala* from Kantawgyi Lake were recorded by Pa Pa Win (2007) and

Myxbobolus sp. infected to the gills of *Labeo rohita* from Lay Daung Kan Fish Farm was recorded by Su Su Mon (2014). Although length and width of the spores of *Myxobolus* species were not differed from those species; the size of polar capsules was slightly different.

The histological changes associated with the present infection caused slight distention of the muscularis and led to replacement of the muscle tissues with developing plasmodia. In the infected portions of the muscles, histological observation showed that some alterations of muscle tissues like necrosis and abnormalities in the muscle fiber. Hence, the severe infection may indicate a health risk to the infected fish. The invading ectoparasites cause significant necrotic changes in skin and muscle tissue, produce lesion and ultimately result in the formation of dermal ulcers (Ahmed, *et al*., 2007). Furthermore, Das and Chandra, (2018) observed that partial or total loss of epidermis and dermis, dermal splitting, necrosis, pyknosis, vacuolation, hemorrhage and presence of parasites and fungal granuloma occurred mainly in skin and muscle pathology. In addition, almost similar pathological symptoms in skin and muscle of various freshwater species in Bangladesh were also found by Hossain, *et al*. (2009) and Moniruzzaman (2000). The result agreed with the finding of Chandra *et al*. (2012) who observed that pathologically, skin and muscle were almost normal structure in carps.

According to Golder, *et al*. (1987) presence of *Chilodonella* sp., *Trichodina* sp., *Dactylogyrus* sp., *Ichthyophthirius multifiliis*, myxosporean spores and fungal granuloma with necrosis, pyknosis and hemorrhage in muscle of *Nandus nandus* were significant pathology as recorded from ponds. *Myxoblus lintoni* caused marked changes in the epidermis and hypodermis of the skin of the host *Cyprinodon vreiegates*, characterized by invasion of fibroblasts (Dykova and Lom, 1978). According to Molnar and Kovacs-Gayer, (1985), *Myxobolus* sp. is a typical intracellular parasite of muscle cells, which their spores are found in other organs. The spores will spread to the organs throughout blood circulation.

In the present study, histopathological lesions and attached *Myxobolus* sp. cyst were observed in fibrous connective tissue of dermis layer. Each muscle bundle is irregular in shape and possesses peripheral nuclei and encysted parasites are enveloped by a loosened fibrous tissue. Furthermore, disintegrated tissue like intercellular edema, necrosis, atrophic myocytes and mass of *Myxobolus* sp. spores in the muscle tissue of host fish were found out. Maftuch, *et al*. (2018) said that necrosis is the death of cells caused by acute cell damage. It was characterized by the death of muscle tissue that still attached to the fish body surface. Similarly, Plumb, (1994) said that necrosis was characterized by the death of cells or tissue that accompanied in cell degeneration in animal life and it was the final stage of irreversible degeneration. Therefore, clinical signs and pathogenesis in muscle and other important organs of host fish might be due to the *Myxobolus* infestations.

The histopathological findings of the present study were consistent with the infection of the muscles of *Cirrhinus mrigala* by *Myxobolus* species, with clear evidence of clinical signs in the fish specimens. The necropsy revealed extensive damage to the host organism, with fibrocystic infections established in the muscle fibers, resulting in histopathological finding.

Conclusion

The muscles of *Cirrhinus mrigala* collected from Yezin Fishery Station were infected with *Myxobolus* species. High prevalence of infection was recorded during the raining season. Histopathological lesions and attached *Myxobolus* species cyst were observed in fibrous connective tissue of dermis layer. The loss of epidermis, dermis split and separated from muscle, and hemorrhage were found in muscle layer of *Cirrhinus mrigala*. These parasites were infecting economically important fish species although not seriously dangerous for humans. Thus, an

important action to prevent the spread of these parasites is removing dead and infected fish, and using water from parasite-free sources.

Acknowledgements

We would like to thank Dr. Win Ohnmar Kyaw, Professor and Head of Department of Aquaculture and Aquatic Diseases, University of Veterinary Science for her encouragements to carry out this study. Profound indebtedness are due to U Khin Maung Maw, Director General (Retired), Department of Fisheries, for allowing us to conduct the study at Yezin Fishery Station; U Soe Paing, Assistant Director, Yezin Fishery Station, Department of Fisheries, for supporting in handling of fish specimens and fish pond during the research work at Yezin Fishery Station, Nay Pyi Taw.

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EMBRYONIC DEVELOPMENTAL STAGES OF *NOTOPTERUS NOTOPTERUS* IN INDUCED BREEDING

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Abstract

The present study was conducted to investigate the embryonic developmental stages of *Notopterus notopterus* in the induced breeding. The breeders of *Notopterus notopterus* were collected from the Hlawgar fish farm, Yangon Region in September 2020. The injected hormone was buserelin acetate (Suprefact) 10µg per 1kg for the first injection and the second injection was 30 µg per 1kg. The hormone was introduced two times of injection to the female but it was introduced single dose to the male. After 52 hours of injection, female laid eggs on the surfaces of the bricks. The present work showed that the fertilized eggs had yellowish color. The eggs were enveloped with many ridges around the micropyle. The cleavage of fertilized eggs was at 1 hour after laying of the eggs. The early morula (1cell, 2 cells, and 4 cells) stage lasted 2:54 hours and morula (16 cells and 32 cells) stage was found in 4:50 hours. Blastula stages of the dome formation, late blastula and multicellular layer occurred at 6:54, 8:10, and 10:01 hours respectively. The latest cell division of embryonic stage was found at 40:25 hours. The embryonic phase was completed at 50:21 hours. The results of the present work indicated the different phases of embryonic stages of *Notopterus notopterus* and the time periods of cell division of the eggs.

Keyword Induced breeding, cleavage, fertilized eggs, embryonic developmental stages

Introduction

In recent years, aquaculture had been recognized as an important strategy to meet the growing demands of fish protein all over the world. Artificial propagation of fish is the most promising and reliable way of ensuring availability of good quality fish seed all year round and sustainability of the aquaculture industry. It involved the use of natural (hypophysation) or synthetic hormones to induce ovulation and spawning in farmed fishes (Salami, *et. al*., 2003).

Aquaculture activities are suitable solutions to reduce fish exploitation from nature for human consumption. The oviparous bronze featherback (knife-fish), *Notopterus notopterus*, is a popular food fish with ornamental value, thrives well in freshwater rivers, ponds and lakes. Bronze featherback (*Notopterus notopterus*, Pallas 1769) is one of the native species which had high economic value.

Bronze feather knife fish have high economic value and favored by society to be consumed and made ornamental fish. It had a very hardy fish and can be reared in aquarium, stagnant water and aquaculture system on a variety of feeds. The fish *N. notopterus* in the wild had been categorically kept in the list of the threatened species.

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High-fat content makes bronze featherback had a delicious and distinctive taste, in addition this fish also had high protein and vitamin A content. In Myanmar, people consumed as traditional food by cooking, frying and making fish ball of bronze featherback knife fish.

Induced breeding is a producing of fish larvae through artificial stimulation using synthetic hormone. The artificial fecundation and ranching are the envisaged strategies for conservation and rehabilitation of endangered species. The stimulation promotes timely release of sperms and eggs.

In Myanmar, artificial spawning has been performed in fishes by giving hormone stimulation to the parent with buserelin acetate (suprefact). The use of buserelin acetate had been successfully performed on many fish species especially *Labeo rohita*, *Cyprinus mrigala*, *Cyprinus carpio, Clarias. spp* and *Anabas testudineus.* The use of buserelin acetate was also performed successfully in induced breeding of *Prochilodus lineatus* and *Osteobrama belangeri* recently (Kalayar Win Maung, *et. al.*, 2019, Than Than Myint, *et. al.*, 2019).

The aim of the present study was using synthetic hormone to perform induced breeding of *Notopterus notopterus* including embryonic developmental stages of *Notopterus notopterus*. The understanding on hormone stimulation on breeders of *Notopterus notopterus* is valuable for the commercially mass production of important fish species.

Materials and Methods

Study site

Breeders were collected from Hlawgar fish farm located at 16˚ 58' 15.58" N and 96˚ 06' 36.40" E. It is also situated in Minglagdon Township, Yangon Region (Fig. 1).

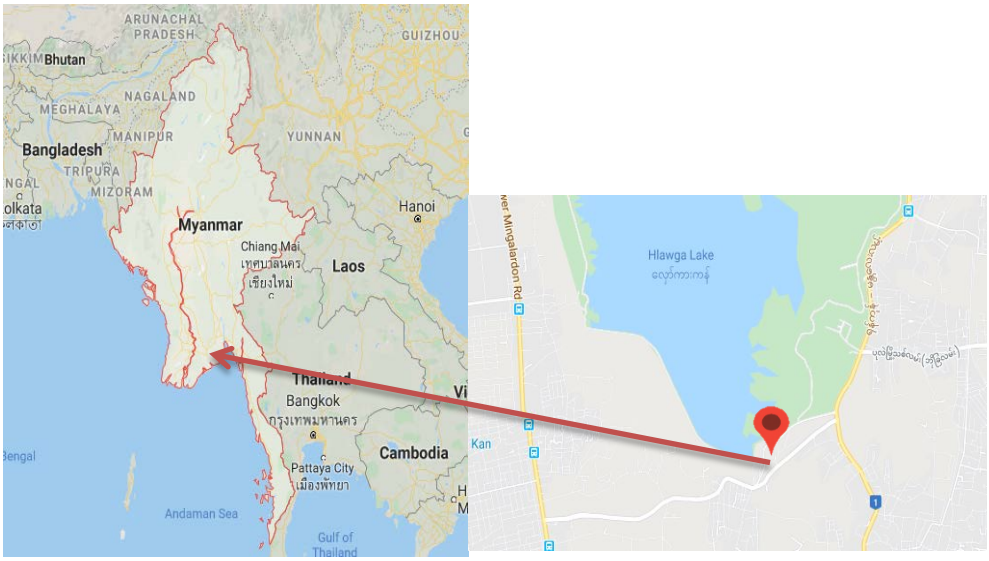


Figure 1 Map showing Hlwagar Fishery Station. (Source: Google)

Study period

The study period was from 2019 May to 2020 January.

Collection of Breeders

Notopterus notopterus was collected from the earthen ponds of Hlawga fishery station. The collected breeders included 23 females and 45 males. They were then transferred to concrete tank before preparation for the induced breeding.

Maintenance of Breeders

The breeders were maintained in the concrete tank for 14 days before induced breeding. The measurement of concrete tanks was (240 x 240 x 90) cm^3. The water level was maintained at 45 cm with running system. All the breeders were fed with formulated feed produced by Green Feed Co. Ltd with 10% of body weight. Nutrient contents in Green Feed were crude protein 40%, fiber 11 %, calcium 2.5% phosphate 1.5% and lysine 2.0%. The ingredients contained soybean meal, fish meal, squid meal, wheat, broken rice, amino acid, minerals and vitamins. They were fed twice a day, 9 am in the morning and 7 pm at night.

Preparation of Spawning Sites

A tank (240 x240 x 90) cm^3 was prepared for spawning after injection. Before using the tank, the concrete tank was cleaned with (30%) of salt water for disinfection. In the tank, four different spawning sites were created at four corners for the nesting and hiding places of fish. Bricks, stone and wooden blocks were used for hiding.

Selection of Breeders

Among the 68 individuals of collected breeders, four healthy males and four females were selected for induced breeding. The sexes of *Notopterus notopterus* were differentiated by the shape of the genital papillae (Weitkamp, 2005). The male had narrow genital papilla of reddish color and longer than the rudimentary pelvic fin. Female had broader and whitish color papilla and shorter than its pelvic fin (Plate 1).

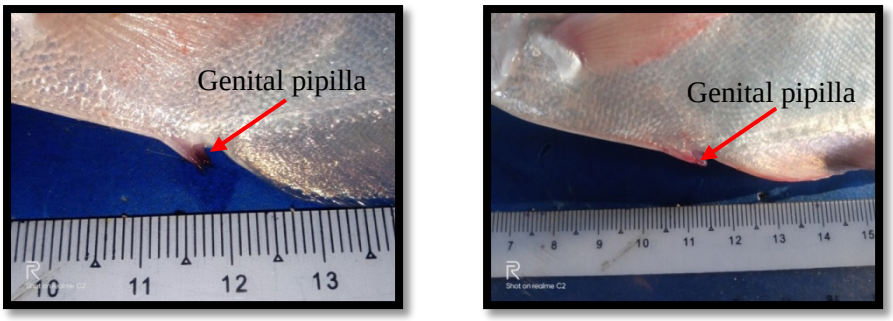
Hormone preparation and Injection of *Notopterus notopterus*

All the breeders were injected with Buserelin acetate (suprefact). The sex ratio of breeders was (1:1). Two doses were prepared for female while one dosage was prepared for male. Females were injected 10µl/kg in first injection. In second injection, dose of 10 µl/kg was injected for male while 30µl/kg for female. Weight of breeders and concentration of hormone were described in Table 1.

Hormone injection of *Notopterus notoprerus*

Hormone injection of *Notopterus notopterus* was carried out in 17 July, 2019. The hormone stimulant was injected at the base of pectoral fin. First injection was carried out in the evening (6 pm) at water temperature of 27˚C. The second injection was conducted in 12 am at the water temperature of 29˚C. Courtship behavior was observed after the injection.

A. Breeder of *Notopterus notopterus*



B. Male C. Female

Plate 1 Breeder *Notopterus notopterus* and genital Papilla of Male and Female

Table 1 Preparation of hormone dosage for female and male of *Notopterus notopterus*

No.	Sex (M=Male F=Female) (g)	Body weight (g)	1st doses (Suprefact)		Interval (hr)	2nd doses (Suprefact)	
			(Suprefact) µg/kg	DOM (mg/kg)		(Suprefact) µg/kg	DOM (mg/kg)
1.	F	250	2.5	1.25	6	7.5	1.25
2.	F	323	3.2	1.61	6	9.7	1.61
3.	F	198	1.9	1.05	6	5.9	1.05
4.	F	230	2.3	1.15	6	6.9	1.15
5.	F	130	1.3	0.65	6	3.9	0.65
6.	M	280	-	-	-	2.8	1.4
7.	M	220	-	-	-	2.2	1.1
8.	M	314	-	-	-	3.14	1.57
9.	M	270	-	-	-	2.7	1.35
10.	M	302	-	-	-	3.02	1.51

Results

Mating Behavior of *Notopterus notopterus*

Among the five pairs of fish, only one pair showed mating. The female displayed a swollen belly, indicating female genital papilla was ready to mate, while the male appeared visibly drawn to the female. Around fourteen hours after the second injection, the male approached the female, swimming alongside. Male and a female stayed in a corner of the tank where bricks were set up. The successful spawning event of the breeding was observed 52 hours after hormone injection and fish spawning was found only in bricks set. The female laid eggs on the surface of brick (Plate 2). The female deposited approximately 355 eggs. The eggs laid by female were attached to the surface and bottom side of the bricks .The diameter of fertilized egg had 4 mm.

Plate 2 Eggs attached to the surface of brick

Embryonic Developmental Stages of Eggs

The fertilized eggs displayed a spherical shape, measuring 4 mm in diameter, and were characterized by a yellowish coloration. The egg envelope presented multiple external ridges, predominantly clustered around the micropyle (Plate 3-a, b).

The embryonic period

Zygote-one cell - After fertilization, cell division in the zygote began at 1:00 hour, resulting in the emergence of a distinct brownish pattern on the top of the eggs (Plate 3-c). The germinal disc exhibited characteristic bipolar differentiation, with the cytoplasm tightly connected to the yolk through the formation of the prospective internal yolk layer.

Two-blastomere - The germinal disc underwent its initial cleavage, was found at 2:00 hours, resulting in the division of the germinal disc into two identical blastomeres, marking the formation of the two-cell stage (Plate 3-d).

Four-blastomere and Eight-blastomere At 2:32 hours, the subsequent cleavage furrow formed at a right angle to the first cleavage furrow, resulting in the emergence of four equivalent blastomeres. The third cleavage took place at 2:54 hours, giving rise to eight blastomeres arranged in two parallel rows of four cells. (Plate 3-e, f)

Early morula and Late morula - The fourth cleavage displayed an orientation that was approximately horizontal to oblique. By the 4-hour, the germ comprised 16 blastomeres. The fifth cleavage occurred at 4:58hr. Subsequent cleavages involved the nonsynchronous development of furrows, resulting in the production of blastomeres of unequal sizes. At this stage, the germ consisted of approximately 32 blastomeres (Plate 3-g, h).

Blastula - At 6:54 hours, the germinal disc exhibited a distinct pebbled appearance, with the upper exterior side forming a dome shape. The yolk cell displayed a ring-like projection, indicating the formation of the internal yolk layer (Plate 3-i).

Flat blastula and Late blastula - The germinal division underwent into two distinct types of cells, at 8:10 hours. These cells primarily originated from the superficial cells of the blastoderm, forming an epithelial sheet consisting of a single layer. The deep layer of cells resided beneath the internal surface of the enveloping layer. The margin of the blastoderm expanded outward. The deep cells had undergone multiplication, resulting in the formation of a multicellular layer at 10:01 hours. As cleavage progressed, surface of the yolk cell underlying the blastoderm became flattened, and most of the yolk vacuoles were located internally (Plate 3-j, k).

The embryonic phase

50% Epiboly and 75% epiboly notochord-formation - The blastoderm expanded and enveloped approximately 50% of the yolk surface. As the blastoderm elongated vegetally, the nuclei of the external yolk syncytial layer migrated beneath the blastoderm, dispersing throughout the yolk mass. The boundary between the blastoderm and the germ ring became distinguishable at 26:14 hours. At 28:08 hours, the blastoderm had covered approximately 75% of the yolk surface, marking the completion of this stage. The neural ridges in the prospective head region rose above the epidermal yolk sac covering. The notochord was already visible along the midline of the neural plate, establishing the morphological definition of the future embryo's rostral-caudal axis. The initial three somites appeared (Plate 3-l, m).

Embryonic shield – At 29:15 hours, the periphery of the blastoderm continued to expand across the yolk boundary, leading to the emergence of a noticeable embryonic shield as a slender protrusion. The longitudinal orientation of the embryonic shield was subsequently identified as the potential embryo (Plate 3-n).

Wedge-shaped neural plate - The blastoderm underwent further expansion, spreading and encompassing nearly 90% of the yolk surface, while the yolk plug noticeably diminished in size compared to the previous stage. The neural plate continued its lateral extension and transformed into a wedge shape at 40:25 hours (Plate 3-o).

Latest epiboly - The blastoderm enveloped nearly the entirety of the yolk surface, leaving a small exposed yolk plug. The embryonic ring was enlarged all around and the main yolk sac cavity was elongated anteriorly and posteriorly beneath the complete neural plate to form the segamentation cavity at 42:35 hours (Plate 3-p).

Spoon-shaped embryo - Epiboly was accomplished, and the yolk mass was completely enveloped by both the blastoderm and the yolk syncytial layer. Progression of a neural groove became apparent along the central axis of the plate, forming a concave shape at the front end. Shortly thereafter, the neural folds had converged at the midline, resulting in the neural plate acquiring a shape resembling a dumbbell (Plate 3-q).

Early trunk-tailed bud - The lateral boundaries of the neural plate were conspicuous and bent inward towards the central line of the developing organism during a specific time period. Most of the posterior segment of the neural precursor and the underlying mesoderm extended from the surface covering of the skin-like yolk sac, giving rise to an early stage of the trunk-tail region. At this stage, the embryo possessed approximately 10-13 somites. The initial muscular movements were observed in the posterior part of the trunk-tail area at 64:05 (Plate 4-a).

Tail bud-bent - During this stage, the distinguishing feature was the independent trunk-tail formation. The posterior tail section of the developing organism detached itself from the yolk sac and arched downward, following the contour of the egg membrane, at the time of 90:00 hours (Plate 4-b).

Optic placode and heart-beat - The auditory placode formed its initial appearance. Heart contraction was observed. The straightening of the developing organism and the existence of blood cells facilitated the circulation of blood. The blood cells circulated through a series of structures in a specific arrangement: a heart with two chambers, a dorsal aorta extending from the heart to the rear boundary of the yolk at 94:20 hours (Plate 4-c).

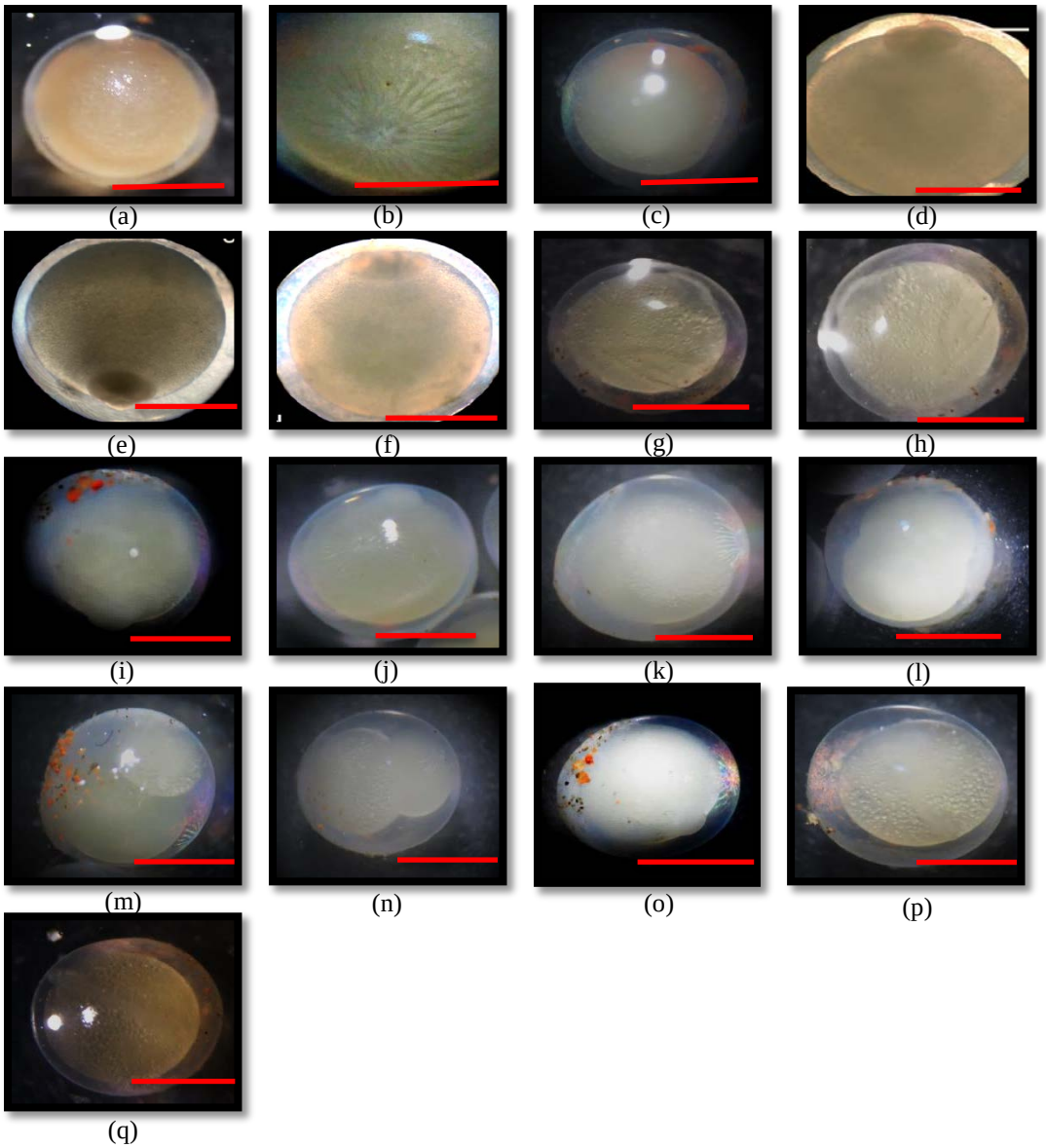


Plate 3 The cleavage phase and embryonic phases of *Notopterus notopterus* (a. fertilized egg, b. micropylar ridges, c. one blastomere, d. two two blastomere, e. four two blastomeres, f. eight blastomeres, g. early morula, h. late morula, i. blastula, j. flat blastula, k. late blastula, l. 50% epiboly, m. 75% epiboly, n. embroyoic shield, o. Wedge-shaped neural plate, p. latest epiboly and q. spoon-shaped embryo.) Scales bar= 2mm

Eye pigment - The eye exhibited widespread pigmentation. Numerous tiny unbranched melanophores, which harbored dispersed melanin pigment, were evenly dispersed across the entirety of the head. The muscular contractions of the developing organism transformed into vigorous motions encompassing the entire body. The vascular fin fold network expanded throughout the entire fin fold area at 122:05 hours (Plate 4-d)

Pre-hatching - The yolk cavity had expansion, while the entire front portion of the developing organism remained attached to the yolk sac covering. Under these circumstances, the tip of the curved trunk-tail area extended as far as the uppermost part of the head. During movements, it was possible for this tip to come into contact with or even surpass the head. The embryo executed robust and energetic rotations and movements. These actions proved

adequate to alter the positioning of the embryo within the egg envelop. The embryo assumed a twisted posture at 146:50 hours (Plate 4-e).

Hatchling – Hatching occurred approximately at 192 hours after spawning. Despite a consistent temperature, the hatching process exhibited variability across different reproductive occurrences, and even within the same group of eggs, spanning a duration of several minutes to half an hour. Typically, the tail emerged initially, and following a partial breakage of the egg membrane, the unhindered embryos, often with the head portion still enclosed, remained within the egg envelope (Plate 4-f).

Jaws and branchial arches - The concentrated clusters of mesenchyme forming the jaws and branchial arches were seen in the region between the head and yolk mass. Upon stimulation, hatched embryos performed swift and conspicuous motions, spinning in circles with the yolk sac movement. The newly hatched embryos were deliberated directional movement within the first 10 days (Plate 4-g).

The Protopterygiolarval phase

Mouth opening - The head process had already experienced a substantial elevation. Numerous structures are now more distinctly delineated. The dorsal and caudal fins emerged concurrently as more concentrated masses of mesenchyme within the fin fold. Melanophores dispersed considerably, not only on the cranial section but also made their initial appearance on the trunk and tail region. The cranial region was elevated and experienced additional growth and alignment. The mandible had moved forward in a straightened manner. The mouth aperture exhibited “&” configuration, with evident demarcation between the upper and lower jaws in day 10 (Plate 4-h).

Progressive median fin-fold regression and formation of distinct fins - The dorsal and caudal fins were extended along the embryonic fin fold. The continuous decline of the dorsal median fin fold assisted in defining the form of the body, including the dorsal, anal, and caudal fins. The embryos progressively gained mobility and appeared to be drawn towards clusters of nourishing organisms. The embryo attained the ability to completely elevate its head and actively initiate the opening or closing of its oral cavity. The yolk-sac's capability had markedly reduced in days 12 (Plate 4-i).

Late embryo - The height of the dorsal embryonic fin fold was a substantial reduction. The pectoral fins were generated with a proximal bud, and a fan was sustained by the segmented lepidotrichial rays. They were operational and employed, as in numerous other teleostean larvae, for directional impulsion. At this phase, the initial movement of active respiration of the gill cover occurred in day 20 (Plate 4-j).

The larval period

Exogenous feeding – In the initial stage of the larval stage, external exogenous feeding took place simultaneously with internal nutrient utilization. The residual yolk sac was still evident but nearly absorbed. The dorsal fin had undergone growth, displaying formed fin-rays. The definitive form of the tail fin was achieved through the prominent narrowing and regression of the remaining dorsal fin fold at the tail's end. The pectoral fins were adequately developed and exhibited high mobility by day 25 (Plate 4-k).

Eye differentiation - The larvae exhibited high mobility and actively sought out nourishment. The pigmented outer layer of the eye had been formed, appearing as deep-black, with visible ocular motion as the larvae swim in close proximity to the seabed. On day 25, a vivid yellow-orange cluster occupied the digestive tract (Plate 4-l).

Anal and caudal lobe formation - The dorsal fin was detached from the fin fold near the tail and the elevated count of bony rays became evident in the fold of the fin on the posterior side, merging with the caudal fin. The concentration of melanins had significantly increased around the entire body, particularly beneath the pectoral fins and the region above the digestive tract in day 40 (Plate 4-m).

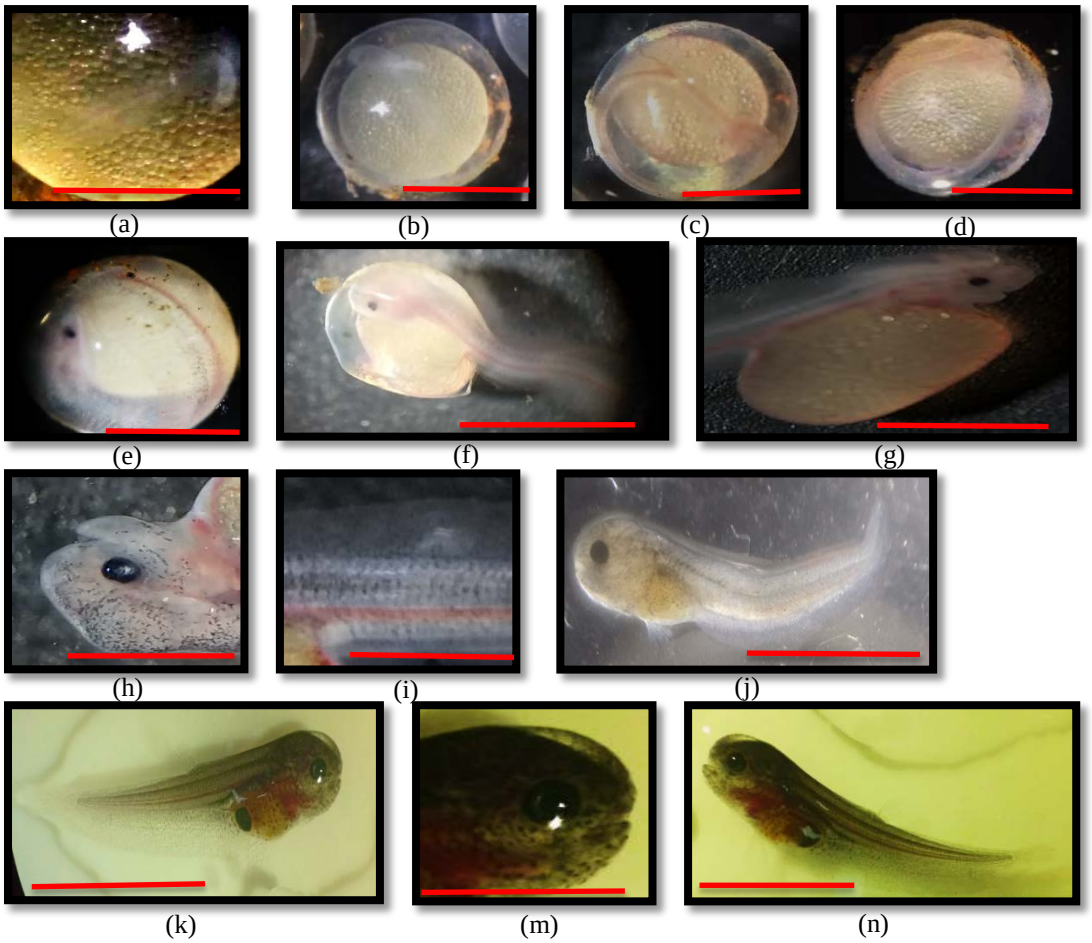


Plate 4 Embryonic phases, protopterygiolarval phase, and larval period of *Notopterus notopterus* (a. early trunk bud, b. tail-bud bent, c. otic placode, d. eye pigmentation, e. prehatchling, f. hatchling g. branchial arches formation, h. mount opening, i. median fin-fold regression and formation, j. late embryo, m. eye differentiation, n. fin lobe formation.) Scale bar = 2mm

Discussion

In the present study, Induced breeding performed in one native indigenous fish species *Notopterus notopterus.* Female fish laid eggs after 52 hours of the second injection.

The eggs of *Notopterus Notopterus* possessed an adhesive characteristic, and the present study confirmed the species as a button spawner, producing relatively sizable eggs. The eggs were partially transparent in nature, encased within a yellowish yolk mass. It was documented that *Chitala chitala* exhibited an adhesive egg envelope (Axelrod and Burgess, 1981).

The egg of *Notopterus notopterus* was found with external ridges which were centered round the single micropyle located at the animal pole. Based on the four classifications of micropyle of *N*otopterus *notopterus*, it exhibited a spiral arrangement of raised lines that partially

terminated in the micropylar regoin (Kunz, 2004). The configuration grooves on the surface of the species followed a radial pattern, extending from the animal pole to the vegetal pole. The eggs of the Loricariidae family's *Sturisoma aureum* and the cyprinid *Barbus conchonius* also possessed a micropyle with furrows and ridges directed towards the micropylar canal (Amanze and Iyengar 1990).

Spawning in *N. notopterus* occurred mostly at night during study. Friese (1980) also reported that spawning in *Notopterus notopterus* tended to occur in the early morning. Similarly, Pinxteren (1974) reported the spawning of *Chitala* and *Notopterus* species occurring mainly at night. The number of the eggs laid by the female *Notopterus notopterus* was 355 eggs.

Southwell and Prashad (1919) reported that the higher number of eggs laid by *Chitala chitala* was 300–500 eggs. In the present study, it was found that *Notopterus notopterus* possessed the longer body than the other fish species. The fertilized eggs of *Notopterus notopterus* hatched after 192 hrs (8 days) at 23˚C. Yanwirsal, *et. al.,* (2017) had shown that *N. notopterus* hatched at 168 hrs (7 days) after spawning at temperature of 27 °C. Srivastava, *et. al*., (2012) reported that the hatching period of *Notopterus notopterus* was 5–6 days after spawning.

According to present results, eye differentiation in *Notopterus notopterus* occurred within five day. Eye pigmentation of *Notopterus notopterus* lasted five or six days. Diedhiou *et. al*., (2007b) showed that eye pigmentation of *Notopterus notopterus* was found on the fourth day after hatching.

The melanophore or pigment melanin was diffused on the integument of *Notopterus notopterus* when the embryo was in 5 days old. In fishes of the *Paramormyrops magnostipes*-complex, black melanophores developed one day after hatching (Nguyen, 2011). Similarly, Yanwirsal *et al*. (2017) had shown the onset of melanophore development in the integument of *N. notopterus*. It became apparent on the frontal region before hatching, which occurred at five to six days.

Induced breeding of *Notopterus notopterus* was conducted using busereline acetate (suprefact). Yulindra *et. al*., (2017) examined the effect of different dosage of ovaprim in induced breeding of knife fish (*Notopterus notopterus*) and they reported that ovaprim 1.5 ml/kg is the best dosage for *N. notopterus*. According to the present study, busereline acetate (suprefact) can be used for induced breeding of *Notopterus notopterus.*

Conclusion

Induced breeding of *Notopterus notopterus* was succeeded using Buscerelin acetate (suprefact). This experiment was conducted to study on embryonic and larval development of Bronze featherback knife fishes by artificial propagation. The embryonic developmental stages from the morula to the hatchling lasted (146-192) hours which was longer than approximately 120 hours of carp species.

Acknowledgements

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SEASONAL OCCURRENCE OF *AEDES AEGYPTI* (LINNAEUS, 1762) LARVAE IN DIFFERENT WATER STORAGE CONTAINERS IN SIX AREAS OF HINTHADA DISTRICT, AYEYARWADY REGION

Min Zaw Latt[1], Maung Maung Mya[2], Tha Zin Hlaing[3]

Abstract

The seasonal study was carried out in six areas of Hinthada District, Ayeyarwady Region, from 2018 to 2020 using descriptive field investigation method. All potential breeding sites were examined seasonally by different larval positive container types (major, minor and miscellaneous) from 50 households in each village of all study areas and the occurrence of *Aedes aegypti* larvae was investigated in accordance with different seasons. Seasonal variations of *Ae. aegypti* in key containers and key premises in all areas were compared. In different positive containers such as concrete jars, earthen pots and bamboo bowls were found to be the most positive and predominated ones for breeding sources throughout the survey period. The larval occurrences in positive containers were significantly different among six areas in accordance with different seasons. Seasonal prevalence of *Ae. aegypti* larvae in various containers was investigated and found to be higher in wet season than other seasons. Hence, this study revealed that wide-spread breeding of *Ae. aegypti* occurred in cleaned and uncovered containers, and untreated water. Information on reduction of breeding sites of *Ae. Aegypti* such as daily practices with covering, emptying out, changing and filtering of water containers, and awareness of vector-borne diseases namely Dengue and Dengue Haemorrhagic Fever (DF and DHF endemicity) could be contributed to local community.

Keywords seasonal occurrence, *Aedes aegypti* larvae, water storage containers, DF, DHF

Introduction

Aedes aegypti and *Aedes albopictus* are belonging to the subgenus *Stegomyia* and they are closely associated with peri-domestic environments (Balasubramanian *et al.,* 2015). The species *Aedes aegypti* is one of the world's most widely distributed mosquitoes and is of considerable medical importance as a major vector of dengue, dengue haemorrhagic fever and dengue shock syndrome (DF, DHF and DSS) in many tropical and subtropical countries throughout the world (Akram *et al.,* 2010).

The abundance of dengue is closely associated with the abundance of vectors and environmental factors (rainfall, temperature and relative humidity). Infestation of vectors to new geographical areas, and related to warm and humid climate, increased population density, water storage pattern in houses and storage of trash, for instance, recyclable materials can serve as risk factors for dengue virus infections (Simmons *et al.,* 2012).

Aedes density, as well as the number of dengue cases, increased in the wet season in Malaysia, India, Sri Lanka, Myanmar, Indonesia, Philippines and Thailand. A severe outbreak of DHF occurred for the first time in Yangon in 1970. Ayeyarwady Region is the second position wise DF/DHF cases and deaths in 2015-2019 (Vector Borne Disease Control Program (Myanmar), 2016). The number of DF/DHF cases and deaths usually were higher in raining season than other seasons (Maung Maung Mya *et al.,* 2016).

There are several factors that influencing on *Aedes* mosquitoes, including water container types, seasons and socio-culture practices, topographic, climatic and vectoral factors (Chumsri *et al.,* 2018). Container type is probably the most important factor determining breeding sites of

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mosquito species (Rajesh *et al.,* 2013). The positive number of various water containers was higher in rainy season than other seasons (Maung Maung Mya *et al.,* 2016). *Aedes aegypti* breeds in a wide assortment of domestic containers whereas *Aedes albopictus* more likely to be found in actual containers (Scott *et al.,* 1993).

Key containers are the primary source of adult *Aedes* mosquitoes. The use of oviposition traps or ovitraps was to estimate the vector population and this technique was recognized by WHO as it can attract female *Aedes* to oviposit.

Prevention of DHF outbreaks is based on long-term anti-mosquito control measures mainly in household and environmental sanitation with emphasis on larval source reduction (Xu *et al.,* 2016). In drawing up strategy for *Aedes* control, it is essential that distribution and density of the mosquitoes should be studied and clearly understood. Thus, prevention of mosquito bite by personal protection and control of vectors are the only methods available to prevent dengue fever (DF) and dengue haemorrhagic fever (DHF) (Maung Maung Mya *et al.,* 2016).

The human population of Ayeyarwady Region is over six million and the Region comprises six districts and 26 townships, and it has relatively high temperature, population and humidity which serve as favorable conditions for the existence of *Aedes aegypti* mosquito during the rainy season especially in July and August. Among six districts, Hinthada District is involved in high risk areas of dengue endemic disease in Ayeyarwady Region because it has yearly high rainfall, temperature, relative humidity and human population that serve as favorable conditions for the distribution, breeding and existence of *Aedes* mosquitoes. The present survey areas have not been studied yet by previous researchers.

Therefore, the aim of the present study was to provide the basic information on the larval densities in the six study areas where children predominated in the population. This study was conducted to determine the seasonal occurrence of *Aedes aegypti* larvae in different water storage containers in selected six areas.

Materials and Methods

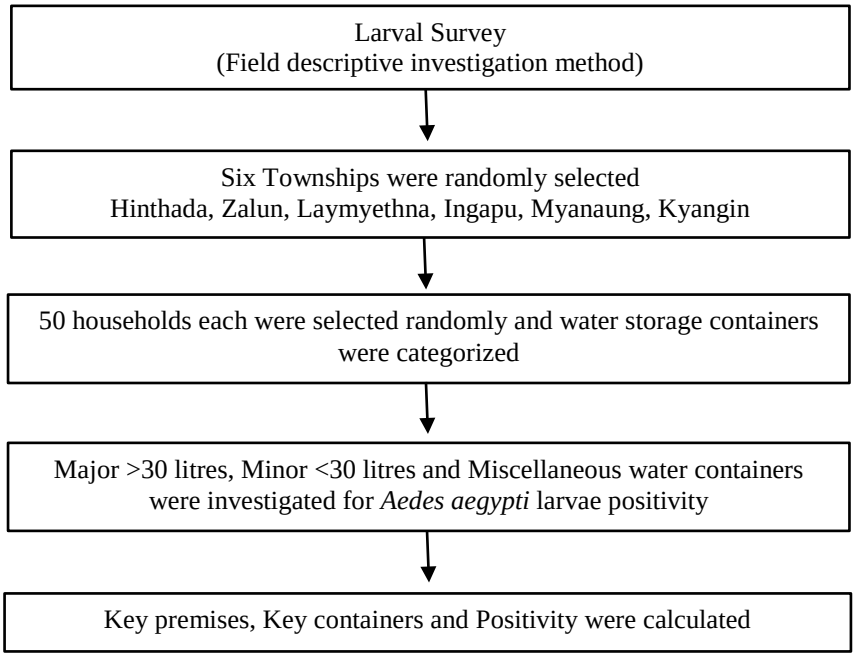
Study sites

The present study was conducted in six field areas such as Ywa Thit village in Hinthada Township (17° 38′ N and 95° 22′ E), Set Kyi Tan village in Zalun Township (17° 28′ N and 95° 33′ E), Lal Tan Ngal village in Laymyethna Township (17° 41′ N and 95° 41′ E), Tha Phan Pin village in Ingapu Township (17° 48′ N and 95° 15′ E), Set Kwin village in Myanaung Township (18° 17′ N and 95° 17′ E) and Shwe Taung Su village in Kyangin Township (18° 20′ N and 95° 15′ E). All study areas were included in Hinthada District, Ayeyarwady Region (Fig.1).

Study period

The present study was carried out from 2018 to 2020.

Study design



Utilization of equipment

Torch light, hand lens (magnification of ×4 & ×6), plastic cups, stereomicroscope, dissecting microscope, sweeper, plastic pipette, measuring slender tube, thermometer, litmus paper and thermo-hydrometer were utilized.

Larvae collection

Larvae collection was conducted by using sweeping method (Tun Lin *et al.*, 1995).

Species identification

Identification of collected *Aedes aegypti* larvae followed after Peyton and Harrison (1980), and Reid (1967).

Weather parameters

Weather parameters were obtained from Department of Meteorology and Hydrology in Hinthada Township.

DHF cases and death

DHF cases and death in six areas were obtained from Rural Health Center (RHC) and Public Health Center in Hinthada District.

Larval indices

Larval examination method of Sheppard *et al.* (1969) was used to confirm the presence of larvae in the different containers.

(a) Key container = 500 and above larvae positive per container

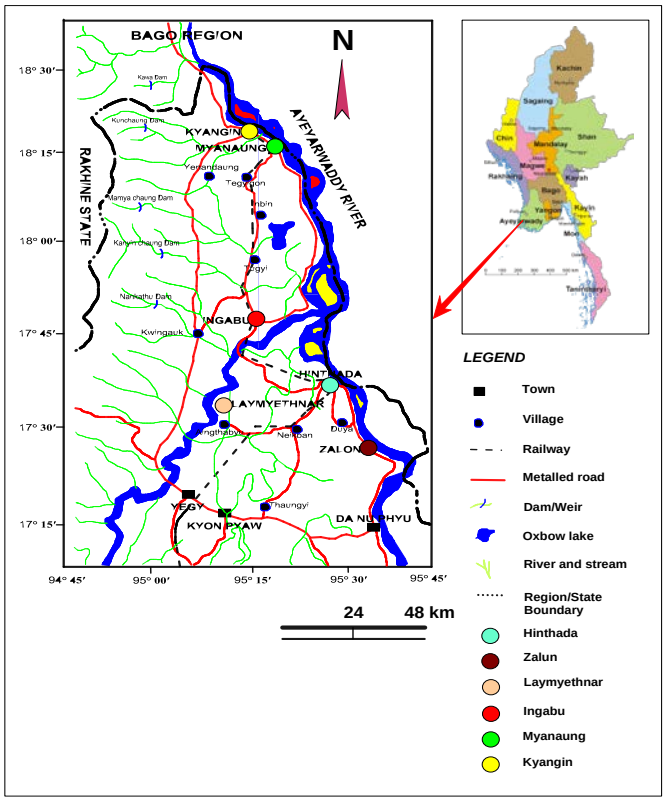
(b) Key premises = 3 and above positive containers with *Aedes* larvae per house

Data collection form

Standard sheet for data collection was developed adjusting that of Department of Medical Research, Yangon).

Data analysis

Key containers, key premises and percentage (%) were calculated.



(Source - Geology Department of Hinthada University, 2007)

Figure 1 Location map of the study area

Results

Seasonal occurrence of different containers harboring *Aedes aegypti* larvae in six areas of Hinthada District

In seasonal distribution of *Ae. aegypti* larvae, the highest number of positive containers was found in three container types as 28, 18 and eight in wet season and the lowest was 16, six and one in cool season (2018). The positivity rates of *Ae. aegypti* larvae in houses were recorded as 21 (42%), 40 (80%) and 21 (42%) in dry, wet and cool seasons (2019) respectively. In seasonal distribution of *Ae. aegypti* larvae, the highest number of positive containers was recorded in three types as 46, 25 and 27 in wet season though the lowest was 19, seven and two in cool season in Hinthada Township (Table 1). In Zalun Township, the highest number of positive containers was found in all three container types as 30, 19 and one in wet season and the lowest was nine, seven and three in dry season (2018). The highest number of positive containers was recorded in three types as 37, 13 and four in wet season though the lowest was nine, six and only one in dry season (2019) (Table 2). In Laymyethna Township, the highest number of positive containers was found in all three container types as 51, seven and five in wet season and the lowest was 24, six and five in dry season (2018). The highest number of positive containers was recorded in three types as 33, 30 and 13 in wet season though the lowest was 30, nine and three in dry season (2019) (Table 3). In Ingapu Township, the highest number of positive containers was found in all three container types as 38, 21 and eight in wet season and the lowest was 30, nine and one in dry season (2018). The highest number of positive containers was recorded in three types as 42, 33 and 20 in wet season though the lowest was 25, 13 and one in

dry season (2019) (Table 4). In Myanaung Township, the highest number of positive containers was found in all three container types as 26, 16 and three in wet season and the lowest was seven, four and two in dry season (2018). In seasonal distribution of *Ae. aegypti* larvae, the highest number of positive containers was recorded in three types as 23, 16 and three in wet season though the lowest was eight, six and one in dry season (2019) (Table 5). In Kyangin Township, the highest number of positive containers was found in all three container types as 44, 41 and four in wet season and the lowest was four, two and one in dry season (2018). The highest number of positive containers was recorded in three types as 27, 36 and one in wet season though the lowest was two, seven and one in dry season (2019) (Table 6).

Seasonal variation of recorded key containers harboring *Aedes aegypti* larvae in six areas of Hinthada District

In seasonal variation of key containers, one container each in dry, wet and cool season (2018) was recorded as the breeding habitats of *Aedes aegypti* larvae. Among three seasons in 2019, only one container was studied in cool season (2019) in Hinthada Township. The number of key containers in cool season (2018) was investigated to be higher than other seasons. The number of key containers in wet season was investigated to be higher than other seasons in Zalun Township. The highest number occurred in cool season while the lowest was in dry season (2018). The number of key containers in cool season was higher than other seasons (2019) in Laymyethna Township. The highest number occurred in dry season while the lowest was in wet season (2018). The number of key containers in cool season was studied to be higher than other seasons (2019) in Ingapu Township. The highest number was determined in wet season while the lowest was in dry season (2018). Among three seasons in 2019, two containers were studied only in cool season (2019) in Myanaung Township. The highest number was found in wet season (2018). Among three seasons in 2019, one container was recorded only in cool season (2019) in Kyangin Township

In seasonal variation of key containers in six areas from dry season (2018) to cool season (2019), the number of key containers was higher in Ingapu Township than other areas in dry season (2018). In wet season (2018), the highest number was recorded in Kyangin Township. The highest number of key containers was found in Laymyethna Township while the lowest in Hinthada Township in cool season (2018). Among all study areas, Laymyethna Township was found to have the highest number of key containers in dry season (2019). In wet season (2019), the highest number was observed in Ingapu Township. In cool season (2019), the number of key containers was determined to be higher in Ingapu Township than other areas. (Table 7 and 8)

Seasonal variation of recorded key premises harboring *Aedes aegypti* larvae in six areas of Hinthada District

The highest number of key premises was found in both dry and wet seasons while the lowest was in cool season (2018). The number of key premises in wet season was determined to be higher than other seasons (2019) in Hinthada Township. The number of key premises in wet season was found to be higher than other seasons (2018). The highest number was recorded in wet season while the lowest was in dry season (2019) in Zalun Township. The highest number was recorded in both wet and cool seasons while the lowest was in dry season (2018). The highest number was observed in wet season while the lowest was in cool season (2019) in Laymyethna Township. The highest number was found in wet season while the lowest was in dry season (2018). The highest number was determined in wet season while the lowest was in dry season (2019) in Ingapu Township. The highest number was found in wet season, followed by cool season and the lowest was in dry season (2018). The highest number occurred in wet season, followed by cool season and the lowest was in dry season (2019) in Myanaung Township. The number of key premises in wet season (2018) was observed to be higher than

other seasons. The number of key premises in wet season was found to be higher than other seasons (2019) in Kyangin Township.

In seasonal variation of key premises in six areas from dry season (2018) to cool season (2019), the number of key premises in Hinthada Township occurred to be higher than other areas in dry season (2018). In wet season (2018), the highest number was recorded in Kyangin Township and the lowest was in Hinthada Township and Myanaung Township. The highest number of key premises was found in Kyangin Township while the lowest was in Zalun Township in cool season (2018). The number of key premises in Laymyethna Township was found to be higher than other areas in dry season (2019). In wet season (2019), the highest number was determined in Ingapu Township while the lowest was in Zalun Township. In cool season (2019), the highest number was observed in Ingapu Township although the lowest was in Hinthada Township (Table 9 and 10).

Seasonal variation of positive different containers in Hinthada District

The highest number of major and minor positive containers occurred in wet season (217, 208, 122 and 153), 2018 and 2019 while the lowest was found in dry season (110, 94, 35 and 49), 2018 and 2019. In miscellaneous type, the highest number of positive containers was found in wet season (29 and 68), 2018 and 2019 although the lowest was recorded as 13 in cool season (2018) and nine in dry season (2019). Relationship between various container types and weather parameters was shown in Figure 2.

Seasonal occurrence of DHF patients in six areas of Hinthada District

The highest numbers of DHF patients occurred in wet season (840 and 741) while the lowest were found in dry season (52 and 47) in all areas (2018 and 2019). Among six study areas, the highest number of DHF patients (1165) was found in Hinthada Township and the lowest was in Kyangin Township (68). Relationship between DHF patients and weather parameters was shown seasonally in Figure 3.

Table 1 Number of different water storage containers harboring *Aedes aegypti* larvae in Hinthada Township

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	29(58%)	81	36(44.44%)	115	11(9.57%)	7	6(85.71%)
Wet season (2018)	50	32(64%)	83	28(33.73%)	123	18(14.63%)	13	8(61.54%)
Cool season (2018)	50	16(32%)	77	16(20.78%)	94	6(6.38%)	3	1(33.33%)
Dry season (2019)	50	21(42%)	66	20(30.30%)	95	8(8.42%)	4	2(50%)
Wet season (2019)	50	40(80%)	81	46(56.79%)	126	25(19.84%)	30	27(90%)
Cool season (2019)	50	21(42%)	65	19(29.23%)	99	7(7.07%)	3	2(66.67%)

Table 2 Number of different water storage containers harboring *Aedes aegypti* larvae in Zalun Township

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	15(30%)	56	9(16.07%)	91	7(7.69%)	3	3(100%)
Wet season (2018)	50	26(52%)	56	30(53.57%)	86	19(22.09%)	1	1(100%)
Cool season (2018)	50	29(58%)	52	23(44.23%)	71	11(15.49%)	1	1(100%)
Dry season (2019)	50	11(22%)	55	9(16.36%)	98	6(6.12%)	2	1(50%)
Wet season (2019)	50	32(64%)	63	37(58.73%)	98	13(13.27%)	4	4(100%)
Cool season (2019)	50	19(38%)	55	20(36.36%)	86	9(10.47%)	1	1(100%)

Table 3 Number of different water storage containers harboring *Aedes aegypti* larvae in Laymyethna Township

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	22(44%)	102	24(23.53%)	78	6(7.69%)	8	5(62.5%)
Wet season (2018)	50	30(60%)	113	51(45.13%)	60	7(11.67%)	9	5(55.56%)
Cool season (2018)	50	31(62%)	96	36(37.5%)	88	16(18.18%)	4	3(75%)
Dry season (2019)	50	24(48%)	90	30(33.33%)	62	9(14.52%)	7	3(42.86%)
Wet season (2019)	50	35(70%)	114	33(28.95%)	112	30(26.79%)	21	13(61.90%)
Cool season (2019)	50	26(52%)	94	30(31.91%)	73	10(13.70%)	6	3(50%)

Table 4 Number of different water storage containers harboring *Aedes aegypti* larvae in IngapuTownship

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	30(60%)	144	30(20.83%)	80	9(11.25%)	2	1(50%)
Wet season (2018)	50	33(66%)	133	38(28.57%)	77	21(27.27%)	11	8(72.73%)
Cool season (2018)	50	28(56%)	135	25(18.52%)	71	14(19.72%)	9	5(55.56%)
Dry season (2019)	50	28(56%)	136	25(18.38%)	72	13(18.06%)	1	1(100%)
Wet season (2019)	50	38(76%)	145	42(28.97%)	114	33(28.95%)	23	20(86.96%)
Cool season (2019)	50	24(48%)	147	41(27.89%)	71	8(11.27%)	4	2(50%)

Table 5 Number of different water storage containers harboring *Aedes aegypti* larvae in Myanaung Township

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	10(20%)	137	7(5.11%)	60	4(6.67%)	5	2(40%)
Wet season (2018)	50	22(44%)	141	26(18.44%)	54	16(29.63%)	3	3(100%)
Cool season (2018)	50	12(24%)	145	10(6.90%)	100	10(10%)	14	2(14.29%)
Dry season (2019)	50	12(24%)	132	8(6.06%)	50	6(12%)	1	1(1000%)
Wet season (2019)	50	21(42%)	142	23(16.20%)	63	16(25.40%)	8	3(37.5%)
Cool season (2019)	50	14(28%)	131	12(9.16%)	53	13(24.53%)	2	1(50%)

Table 6 Number of different water storage containers harboring *Aedes aegypti* larvae in Kyangin Township

Survey	Total no. of houses	Positive houses	Containers					
			Major		Minor		Miscellaneous	
			Inspected	Positivity	Inspected	Positivity	Inspected	Positivity
Dry season (2018)	50	6(12%)	93	4(4.30%)	73	2(2.74%)	1	1(100%)
Wet season (2018)	50	41(82%)	88	44(50%)	77	41(53.25%)	4	4(100%)
Cool season (2018)	50	27(54%)	91	3(3.30%)	127	55(43.31%)	1	1(100%)
Dry season (2019)	50	9(18%)	88	2(2.27%)	38	7(18.42%)	1	1(100%)
Wet season (2019)	50	25(50%)	93	27(29.03%)	54	36(66.67%)	1	1(100%)
Cool season (2019)	50	20(40%)	86	13(15.12%)	110	31(28.18%)	1	1(100%)

Table 7 Seasonal variation of recorded key containers in six areas of Hinthada District (2018)

Areas	Key containers		
	Dry (2018)	Wet (2018)	Cool (2018)
Hinthada Township	1	1	1
Zalun Township	0	6	9
Laymyethna Township	3	6	13
Ingapu Township	8	3	5
Myanaung Township	3	14	5
Kyangin Township	2	19	2

Table 8 Seasonal variation of recorded key containers in six areas of Hinthada District (2019)

Areas	Key containers		
	Dry (2019)	Wet (2019)	Cool (2019)
Hinthada Township	0	0	1
Zalun Township	0	2	1
Laymyethna Township	1	0	5
Ingapu Township	0	6	8
Myanaung Township	0	0	2
Kyangin Township	0	0	1

Table 9 Seasonal variation of recorded key premises in six areas of Hinthada District (2018)

Areas	Key premises		
	Dry (2018)	Wet (2018)	Cool (2018)
Hinthada Township	5	5	2
Zalun Township	0	6	1
Laymyethna Township	1	8	8
Ingapu Township	1	10	2
Myanaung Township	1	5	2
Kyangin Township	0	15	11

Table 10 Seasonal variation of recorded key premises in six areas of Hinthada District (2019)

Areas	Key premises		
	Dry (2019)	Wet (2019)	Cool (2019)
Hinthada Township	1	14	1
Zalun Township	1	7	2
Laymyethna Township	6	9	5
Ingapu Township	1	15	10
Myanaung Township	1	8	3
Kyangin Township	0	11	9

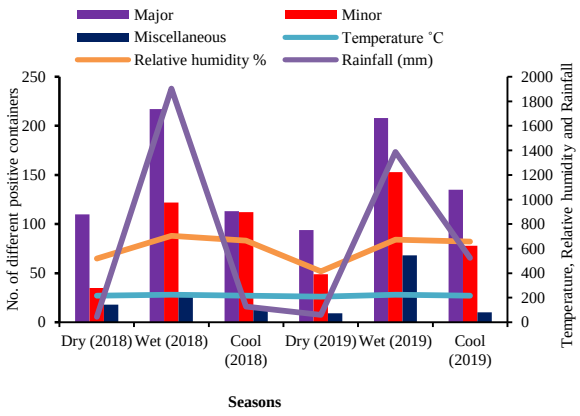


Figure 2 Relationship between positive different containers and weather parameters

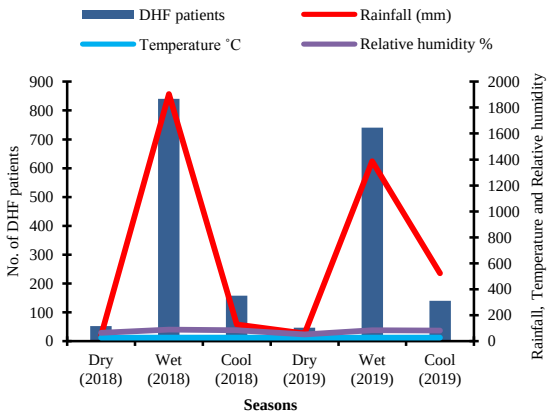


Figure 3 Relationship between DHF patients and weather parameters

Discussion

Aedes aegypti is a vector of dengue in urban areas but now the species are distributed in rural areas in Myanmar. Mosquito-borne diseases are a major public health problem which threats in Myanmar. The highest number of DHF patients also occurred in wet season while the

lowest was found in dry season in all six study areas of Hinthada District according to public health center report in Hinthada Township, in 2020.

In the present study, the highest number of positive containers and key containers, and highest positivity rate were recorded in concrete jars of major type followed by bago jars, plastic drums, concrete barrels, metal drums and concrete tanks which were found to be more widely used for storing water, and not completely covered and treated of water, and concrete jars were predominated ones for larval breeding in all areas of Hinthada District. Among major containers, the concrete jars were found to be more widely used to store water for multipurpose in Hinthada District. Other researcher revealed that same positivity of water storage containers and metal drums were recorded as key containers in Hpa-an Township, Kayin State. Moreover, these containers were mostly placed under the roof gutters to keep rainwater in the wet season (Maung Maung Mya *et al.,* 2015).

In minor type, the highest number of positive containers and key containers, and highest positivity rate were observed in small bago jars, followed by earthen pots in Laymyethna Township; earthen pots, followed by flower bidets in Zalun Township; and earthen pots, followed by plastic bowls and plastic buckets in the remaining areas in three seasons. The earthen pots were widely used for multipurpose and found to be higher positive number amongst the different minor containers in Hinthada District. These containers were also examined to be not carefully cleaned or changed of water and became good breeding places of larvae. Similarly, it revealed that the highest number of larvae positivity was in small bago jars in Thakayta Township, Yangon Region (Ni War Lwin, 2013).

In positivity of miscellaneous containers, the highest number and positivity were observed in bamboo bowls, followed by broken earthen pots, broken plastic bowls, broken bago jars, discarded tins, coconut shells, discarded tires and milk tins; and key containers were bamboo bowls, broken bago jars and discarded tires in Hinthada District. These positive containers were examined to be not carefully discarded and filled with clean water and they became attractive breeding sources of *Aedes aegypti* larvae. The highest number of positive containers was found in wet season and the lowest in dry season. Cutting bamboo stumps or bowls were mainly full of rain water in wet season which provided high positive containers or good breeding habitats for *Aedes* mosquitoes in Hinthada District.

Maung Maung Mya *et al.* (2015) also found that the bamboo stumps were key containers in Hpa-an area. Gould *et al.* (1970) mentioned that the natural breeding sites are more difficult to control than artificial containers, but for disease and pest control, it will be necessary to reduce or possibly eliminate these sources of vector species. The present survey found that the bamboo bowls in miscellaneous type were widely distributed and highly positive as the breeding places under natural condition which were more difficult to reduce and eliminate ones for disease and vector control than artificial containers.

In seasonal occurrence of *Aedes aegypti* larvae in different types of container, the present finding pointed out that the positive rate of households in wet season was higher than other seasons in all study areas. In number of positive containers in three container types, the highest number of positive containers was found in major type, followed by minor type whereas the lowest was in miscellaneous type in all seasons. In positivity rate of different container types, the positive rate was recorded to be higher in miscellaneous than other container types in all seasons. The highest seasonal occurrence of *Ae. aegypti* larvae was found in wet season because it was well collected rainwater in various containers which serve as the harboring places for appropriate breeding activities of *Aedes* mosquitoes. Maung Maung Mya *et al.* (2015) also stated that the distribution of *Aedes* larvae in wet season was higher than in cool and dry seasons.

The present observation showed that the seasonal variation of recorded key containers in positive containers from 50 houses was significantly found to be higher in cool season than other seasons. Because, the breeding of *Aedes aegypti* larvae was examined to be the highest in various water storage containers that stored rain water without changing or cleaning of water or covering completely with lids and with retaining of water for long periods for a requirement of domestic usage. Similarly, Ahmed *et al.* (2019) reported that the containers that retained water for long periods of time make good suitable breeding habitats for mosquitoes such as the different water holding artificial containers. In the present finding, the seasonal variation of recorded key premises in positive households from 50 houses was higher in wet season than other seasons in all six study areas. In addition, the high number of households was observed that had three and above positive containers without completely covering of lids and placed under roof gutter to store rainwater in this season. Tin Mar Yi Tun (2007) described that the high number of key premises in all areas was high in rainy season than other seasons.

Among six study areas, Hinthada Township was a high-risk area of DHF. Among six study areas, the highest number of DHF patients was found in Hinthada Township and the lowest was in Kyangin Township. Hinthada District had highest rainfall (1387 to 1904 mm), maximum relatively humidity (84-88%) and moderate temperature (28ºC) which were favorable for the breeding of *Aedes* mosquitoes; increased the maximum number of positive containers and caused to the high number of DHF patients. The suitable breeding, distribution and oviposition of *Aedes* mosquitoes are mainly depending on the availability of rainfall. Wongkoon *et al.* (2013) similarly mentioned that rainfall, daily maximum rainfall and minimum/ maximum/ mean temperatures were associated with the dengue incidence.

Large containers may contain a more permanent aquatic habitat than smaller containers (Harrington *et al.,* 2008). It was agreed that the present study revealed the large water containers (such as concrete jars, concrete barrels, concrete tanks, bago jars, plastic and metal drums, etc.) were highly positive with optimal harboring larvae for favorable long period of breeding, resting and existing; and found to be more permanent aquatic habitats for *Aedes* mosquitoes than smaller water containers.

Conclusion

The results of the present study indicated that the seasonal variations of positive houses, positive containers, larval positivity rates and larval indices were significantly different among study areas according to three seasons and observed to be higher in wet season than other seasons. The abundance of dengue and dengue haemorrhagic fever (DF and DHF) was closely associated with the seasonal prevalence and breeding of *Aedes* larvae in different water storage containers which were widely distributed and permanently served for harboring and breeding places. The monthly or weekly container assessment survey will be able to promote the daily practices and contribute an awareness of vector-borne diseases for the local people seasonally, and may be useful for elimination of breeding habitats and oviposition sites, and reduction of eggs, larvae and pupae of *Aedes* mosquitoes in various water storage containers.

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Cytogenetic Analysis of Silver Carp, *Hypophathalmichthys molitrix* (Valenciennes in Cuvier and Valenciennes, 1844) and Thai Silver Barb, *Barbonymus gonionotus* (Bleeker, 1850)

Thidar Aung[1], Win Win Mar[2]

Abstract

Cytogenetic investigation on silver carp *Hypophthalmichthys molitrix* and silver barb *Barbonymus gonionotus* belonging to family Cyprinidae from Thatyetkone Fisheries Station, Mandalay Region, was carried out. The optimal effect of colchicine concentration was observed at 0.50 % for duration 5 hrs with 0.56 % hypotonic solution treatment of 1 hr 30 mins period in silver carp and colchicine concentration 0.50 % for 6 hrs with 1 hr duration of hypotonic treatment in silver barb. The diploid chromosome number of silver carp *H. molitrix* was 2n = 48 with karyotype formula 2 m (metacentric) + 4 sm (submetacentric) + 6 a (acrocentric) + 12 t (telocentric) and fundamental arm number 60. The chromosomal number of silver barb *B. gonionotus* was 2n = 50 with karyotype formula 1 m (metacentric) + 9 sm (submetacentric) + 2 a (acrocentric) + 12 t (telocentric) and fundamental arm number 68.

Keywords *Hypophthalmichthys molitrix, Barbonymus gonionotus,* karyotype, frequency

Introduction

Myanmar is rich in natural resources such as flora as well as fauna. Not only the indigenous freshwater fishes distributed throughout the rivers, ponds and lakes, but also marine fishes are populated in costal, beaches and bays of Myanmar.

Among the fish species, silver carp *Hypophthalmichthys molitrix* (Valenciennes in Cuvier and Valenciennes, 1844) is a freshwater fish native to East Asia and introduced around the world for aquaculture and control of algal blooms. Silver barb *Barbonymus gonionotus* (Bleeker, 1850) is recorded from Vietnam Mekong delta and the Dong Nai River, the Mekkong basin in Lao PDR, Cambodia and Thailand. These fishes are introduced from Malaysia, Peninsular Malaysia and distributed in Southeast Asia.

A karyotype is the arrangement of pairing a set of metaphase chromosomes according to their sizes and shapes. The precise number of complete sets of metaphase chromosomes would be observed within the nucleus of a cell of an individual species, genus or other grouping (Levan *et al.,* 1964). Cytogenetic studies are very useful for taxonomic, genetic, cyto-toxicology, race improvement and biotechnological investigations (Nandini *et al.,* 2014).

The detail information on karyological studies of these fishes from Thatyetkone Fisheries Station is necessary to fill the gap of research areas. In addition, the different methodological approaches on cytogenetic studies have been limited in various research areas. Therefore, these fishes are selected to investigate the effects of colchicine on the inhibition of metaphase checkpoint of cells in *H. molitrix* and *B. gonionotus,* and to evaluate the metaphase checkpoint and karyotypes of these fishes.

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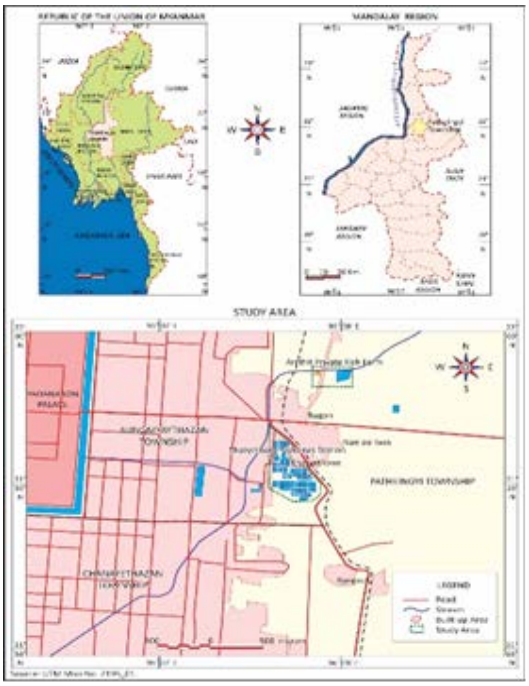


Figure 1 Study map of Thatyetkone Fisheries Station, Mandalay Region (Source: UTM)

Materials and Methods

Study site

Thatyetkone Fisheries Station is situated around 21° 59' 28.53" N, and 96° 7' 44.60" E, Patheingyi Township, Mandalay Region (Fig. 1).

Study period

The study period was from January to August 2022.

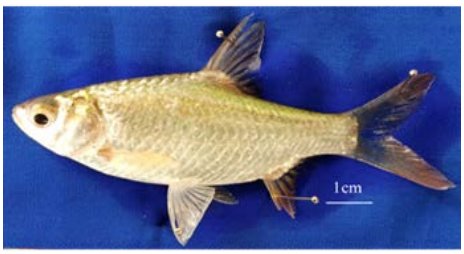
Specimen collection

Twenty fishes for each species were collected from Thatyetkone Fisheries Station and cultured in the Laboratory, Department of Zoology, University of Mandalay (Plate 1 A and B; 2 A). The fishes were fed twice a day with formulated commercial feeds. The water was changed twice a week and kept in well-aerated aquarium.

Identification of species

The species identification followed after Talwar and Jhingran (1991) and Integrated Taxonomic Information System (ITIS, 2022).

A. Lateral view of *Hypophthalmichthys molitrix*



B. Lateral view of *Barbonymus gonionotus*

Plate 1 Collected fishes

Injection technique

The total length and standard length of fish were measured by plastic ruler to the nearest 0.1 cm. The body weight of fish was recorded by digital balance to the nearest 0.01 g. Depending on the weight of fish (1ml / 100 g), the different concentrations of colchicine (Avi Chem, India) solutions such as 0.10 % for duration 1 hr 30 mins, 2 hrs, 2 hrs 30 mins; 0.30 % for 2 hrs and 0.50 % for 3 hrs, 4 hrs, 4 hrs 45 mins, 5 hrs and 6 hrs were applied to block the metaphase stage of cells. Fishes were injected at the base of pelvic fin (Plate 2 B and C).

Collection of blood and tissues

The blood was collected directly from colchicine treated fish by using syringe (0.5 cm) and diluted into the culture tube with 0.56 % hypotonic solution (24 Well Cell Culture Cluster, USA)**.** Then, each fish was sacrificed with one or two drops of 10 % formaldehyde solution (Laba Chemie Put. Ltd, India). The sample tissues such as liver, heart, kidney, oral cells and gill filaments were taken out for further study (Plate 2 D).

Extraction of cells

Each sample was kept in block-cup filled with 0.56 % hypotonic solution and incubated for various duration 10 mins, 20 mins, 30 mins, 45 mins, 1 hr, 1 hr 30 mins and 2 hrs 45 mins. After that, these samples were mixed homogeneously by Advanced Vortex mixer (2 × 3, Nanova**)** and centrifuged (Firlabo.sa, CBA 1015**)** at 1000 rpm for 10 mins (or) 2000 rpm for 5 mins. The supernatant was discarded and the pellets were treated with Carnoyl's fixative (3 methanol: 1 acetic acid) and kept for 15 mins. Then, the samples were centrifuged again at 1000 rpm for 10 mins (or) 2000 rpm for 5 mins. The supernatant was excluded from the test tube by pipette. After that, the pellets were treated with fixative solution again for stabilization of the cells (Plate 2 E, F, G and H).

Preparation of slide and Giemsa stain

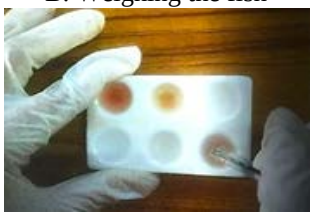
One or two drops of samples were placed onto the pre-warmed slides with a far distance and dehydrated at room temperature. Then, these slides were stained with undiluted Giemsa stain (Avi Chem, India) for 10 mins, washed under running tap water and dried at room temperature. The permanent slides were prepared by dropping one or two drops of immersion oil, covered with cover slip and coated with Canada balsam (Kanto Chemical Co. Inc, Tokyo, Japan) (Plate 2 I, J, K and L).

A. Fish rearing in aquarium

B. Weighing the fish

C. Injection of fish

D. Extraction of blood



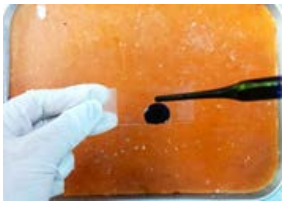
E. Mechanical dissociation of tissue

F. Homogenization of tissues

Plate 2 Preparation of cytological process from fish tissues

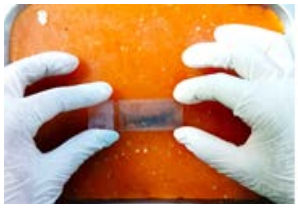
G. Centrifugation of pellets

H. Discarding the supernatant from the test tube



I. Staining on the slide

J. Wash under tap water



K. Covering with coverslip

L. Examination under microscope

Plate 2 Preparation of cytological process of fish tissues

Chromosomal analysis and preparation of karyotype

The microphotographs were recorded by biological microscope with attached camera (G303p, Taiwan), (x1000). The metaphase stages were counted and the chromosomal numbers were recorded by ImageJ. (1.52a, USA). Acrocentric and telocentric chromosomes were scored as uniarmed, metacentric and submetacentric chromosomes which were suggested as biarmed. Chromosome pairs were arranged in decreasing order of largest to smallest ones according to their sizes from each cell point.

Statistical analysis

The frequency and the range of metaphase chromosomes spreads from silver carp and silver barb were analyzed using Microsoft Excel (2010).

Results

Different tissues of fish such as liver, oral cells, kidney, heart, gill filaments and blood were taken out from the fish. Among these tissues, the kidney tissue was the best for karyological studies in these fishes. The optimal concentration of colchicine was 0.50 % for 5 hrs and hypotonic treatment was 0.56 % hypotonic solution for 1hr 30 mins in silver carp. But the best duration of colchicine solution was 6 hrs and duration of 0.56 % hypotonic solution treatment was 1 hr in silver barb.

Systematic position of *Hypophthalmichthys molitrix*

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Actinopterygii
Order	-	Cypriniformes
Family	-	Cyprinidae
Subfamily	-	Xenocyprididae
Genus	-	*Hypophthalmichthys* (Bleeker, 1860)
Species	-	*H.molitrix*
Common name	-	Silver Carp
Vernacular name	-	Ngwe-Yaung-Nga-Gyin

Hypophthalmichthys molitrix with mean total length 14.3 ± 0.80 cm, standard length 11.45 ± 0.70 cm and the body weight 23.3 ± 6.06 g (n = 4) was used. The mean meristic

characters of silver carp were dorsal fin (D) – I 7, pectoral fin (P) – i 14, ventral fin (V) – i 7, anal fin (A) – ii 10 and caudal fin (C) – 26.

Systematic position of *Barbonymus gonionotus*

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Actinopterygii
Order	-	Cypriniformes
Family	-	Cyprinidae
Genus	-	*Barbonymus*
Species	-	*B.goinonotus* (Bleeker, 1850)
Common name	-	Silver barb or Java barb
Vernacular name	-	Nga-khone-ma-kyee

Barbonymus gonionotus with mean total length 10.00 ± 1.20 cm, standard length 7.98 ± 1.10 cm and the body weight 9.25 ± 3.40 g (n = 4) was used. The mean meristic characters of silver carp were dorsal fin (D) – II 9, pectoral fin (P) – i 13, ventral fin (V) – i 7, anal fin (A) – I 7 and caudal fin (C) – 20.

Frequency distribution of metaphase chromosomes

In silver carp *Hypophthalmichthys molitrix*, the highest percentage of chromosome counts was found in the range of 46 – 50 (32.61 %, n = 15) with 39.13 % cumulative frequency (CF), followed by 51 – 55 (23.91 %, n = 11) and 56 – 60 (21.74 %, n = 10). The ranges of chromosomes with 40 – 45 and 61 – 65 (6.52 %, n = 3 each), 66 – 70 and 71 – 75 (4.35 %, n = 2 each) were observed (Table 1 and Fig 2).

In silver barb *Barbonymus gonionotus*, the highest range of chromosome count n = 17 was found in 46 – 50 (38.64 %) with 52.28 % cumulative frequency (CF), followed by 51 – 55 (22.73 %, n = 10), 40 – 45 (13.64 %, n = 26), 61 – 65 (11.36 %, n = 5) and the lowest percentage was found in 66 – 70 and 71 – 75 (2.27 %, n = 1 each) (Table 2 and Fig 3).

Table 1 Percent and frequency distribution of diploid number of chromosomes counts in silver carp *Hypophthalmichthys molitrix*

Treatment	Range	Frequency	Percentage (%)	Cumulated frequency (CF)
0.50 %	40-45	3	6.52	6.52
Colchicine	46-50	15	32.61	39.13
(5 hr)	51-55	11	23.91	63.04
0.57 %	56-60	10	21.74	84.78
KCL	61-65	3	6.52	91.30
(1hr 30	66-70	2	4.35	95.65
min)	71-75	2	4.35	100.00

Table 2 Percent and frequency distribution of diploid number of chromosomes counts in silver barb *Barbonymus gonionotus*

Treatment	Range	Frequency	Percentage (%)	Cumulated frequency (CF)
0.50 %	40-45	6	13.64	13.64
Colchicine	46-50	17	38.64	52.28
(5 hr)	51-55	10	22.73	75.01
0.57 %	56-60	4	9.09	84.10
KCL	61-65	5	11.36	95.46
(1hr 30	66-70	1	2.27	97.73
min)	71-75	1	2.27	100.00

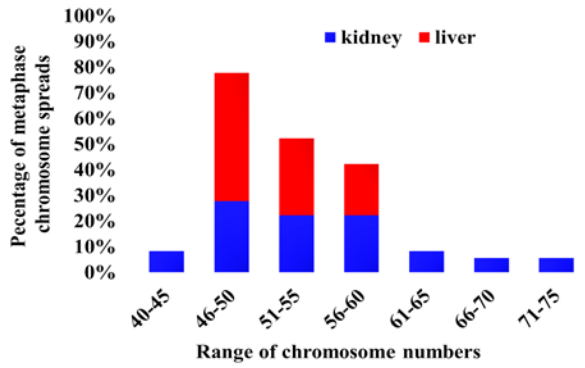


Figure 2 Frequency distribution of metaphase chromosomes spreads from liver and kidney cells of silver carp *Hypophthalmichthys molitrix*

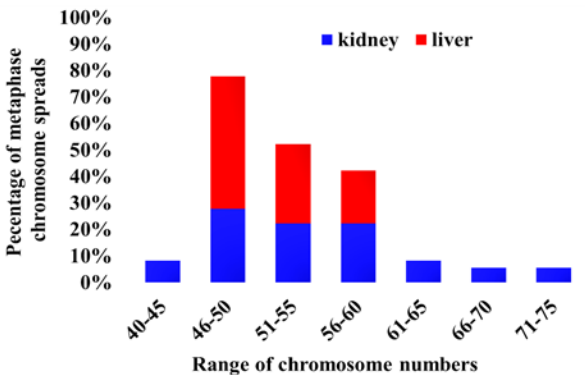
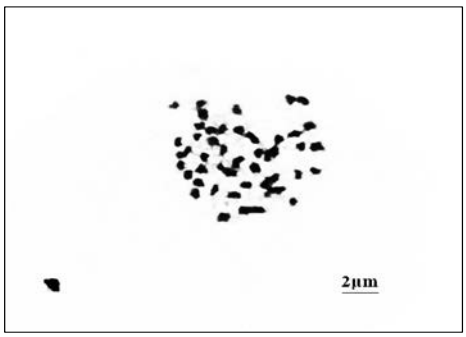


Figure 3 Frequency distribution of metaphase chromosomes spreads from liver and kidney cells of silver carp *Hypophthalmichthys molitrix*

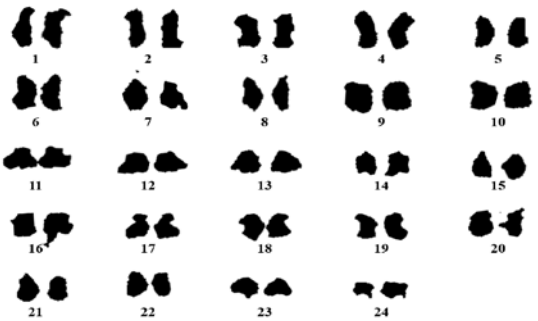
Karyotype

Forty-six well spread metaphase complements of chromosomes in silver carp *Hypophthalmichthys molitrix* and forty-four in silver barb *Barbonymus gonionotus* were selected and counted to construct the karyotypes of these fishes. The number of diploid chromosomes ranged from 40 - 74 in silver carp and 40 - 70 in silver barb (Plate 3A and 4A).

In silver carp, the modal diploid chromosome numbers was 2n = 48 consisting with 2 metacentric, 4 submetacentric, 6 acrocentric and 12 telocentric with 60 fundamental arm numbers (Plate 3 B). In silver barb, the chromosomal formula was 2n = 50 with 1 metacentric, 9 submetacentric, 2 acrocentric and 12 telocentric with 68 fundamental arm numbers (Plate 4 B).

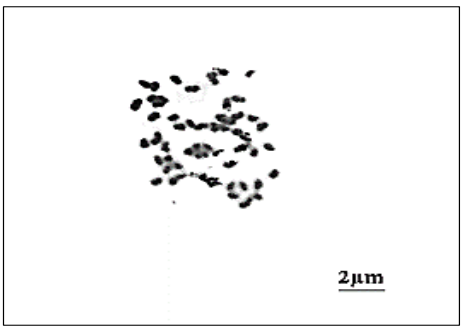


(A) Mitotic metaphase chromosome spread (x1000)

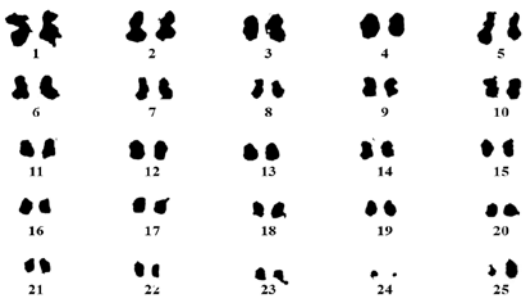


(B) Karyotype

Plate 3 Silver carp *Hypophthalmichthys molitrix*



(A)Mitotic metaphase chroosome spread (x1000)



(B) Karyotype

Plate 4 Silver barb *Barbonymus gonionotus*

Discussion

In silver carp *Hypophthalmichthys molitrix*, late prophase stages of cells were more observed in colchicine (0.50 %) for 3 hrs treatment with 0.56 % hypotonic solution for 45 mins. However, the injection of colchicine treatment 4 hrs with 0.56 % hypotonic solution for 1 hr generated the early metaphase stages of chromosomes through the breakdown of nuclear envelope.

When fish were treated with colchicine solution 5 hrs injection with 1 hr fixation of hypotonic solution, the early and middle metaphase stages were observed. The complete set of metaphase chromosomal configurations was observed in 5 hrs colchicine treatment with hypotonic treatment for 1 hr 30 mins. This is the optimum treatment for desired condensation degree of metaphase chromosomes in silver carp. To sum up these consecutive tests on blocking the metaphase stage of cells in these two fishes (silver carp and silver barb), the effects of optimal concentration on colchicine concentration and hypotonic solution did not synchronize on the mitotic checkpoints of cells.

The condensed chromosomal configuration was observed in 0.50 % colchicine concentration for 4 hrs 45 mins duration with 0.56 % hypotonic solution for 1 hr indicating the optimal stage of metaphase check point of cells in silver barb. The middle stage of metaphase stage was observed in injection duration for 5 hrs and hypotonic treatment duration for 1 hr. The complete metaphase stages were observed in colchicine injection duration for 6 hrs and hypotonic solution treatment for 1 hr. However, the desired metaphase chromosomes were not observed in 6 hrs colchicine solution with hypotonic treatment duration for 1 hr 30 mins.

In this study, the duration of Carnoyl's fixative for 15 mins was the optimal condition for the preservation and suspension of the cells in silver carp and silver barb. The good shapes of miotic chromosome spreads were observed by using pre-warmed slides and stained with undiluted Giemsa stain for 10 mins. Undiluted Giemsa stain was very effective for getting the good shape of chromosomes in prepared slides.

The model chromosome number of silver carp was optimized as 2n = 48. The chromosomal formula of silver carp *Hypophthalmichthys molitrix* has 2 metacentric (m), 4 submetacentric(sm), 6 acrocentric (a) and 12 telocentric (t) with fundamental arm number (60) in this study. The similar result was not found in other researchers. Nandini, *et al.* (2014) stated that the chromosomal formula of *H. molitrix* has 11 pairs (metacentric), 11 pairs (submetacentric), and 2 pairs (acrocentric), 11 m, 7 sm and 6 t (Márián, and Krasznai, 1979); and 4 m , 12 sm and 8 st-a (Sember *et al.*, 2020). The diploid chromosomal number of silver barb *Barbonimus gonionotus* was 2n = 50 with a chromosomal formula 1 metacentric (m), 9 submetacentric (s.m), 2 acrocentric (a) and 12 telocentric (t) with fundamental arm number (68). This study does not support to Magtoon and Arai (1989) who described that the karyotype composition of silver barb was 2 (m), 20 (s.m), 4 (st) and 24 (a). The fundamental arm number of this fish was 72. The differential chromosomal configurations on these fishes may be due to the researcher's assumption on karyological study even though the same methodological approach.

The present result on the range of diploid chromosome number on each cell varied from 40 - 74 (n = 46) in silver carp and 40 – 70 (n = 44) in silver barb with various chromosomal configurations in each well spread cell. This was due to the result of losses or additions of chromosomes during karyotype preparation including splashing due to their downfall from various heights from nearby cells (Ganai and Yousuf, 2011). Therefore, Biswal, *et al.* (2008) reported the broad spectrum of karyotype of some fishes is needed to assay with an aid of DNA maker.

Conclusion

Silver carp, *Hypophathalmichthys molitrix* had the optimal chromosomal number 2n = 48 while that of silver barb, *Barbonymus gonionotus* had the modal diploid number 2n = 50. And the basic information on karyotypes of these fishes was indication of further genetic researches in diverse areas. Concerning with the chromosomal configurations in selected fishes, the chromosomal preparation is vital process to designate the karyomorphological studies on haploid or diploid species.

Acknowledgements

We are grateful to Dr Nwe Nwe Khaing, Professor and Head of Zoology Department of Mandalay University, Dr Thant Zin, Professor, Curriculum Central Unit, Department of Education Research, Planning and Training, Yangon (Former Professor and Head, Department of Zoology, University of Mandalay), Dr Kay Thi Mya and Dr Ni Ni Win, Professors, Department of Zoology, Mandalay University for their suggestions and comments on this paper.

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KARYOTYPE ANALYSIS OF DECCAN CARP, *LABEO POTAIL* (SYKES, 1839) AND STRIPED CATFISH, *PANGASIANODON HYPOPHTHALMUS* (SAUVAGE, 1878)

Thawdar Aung[1], Win Win Mar[2], Htin Zaw Latt[3]

Abstract

The deccan carp *Labeo potail* and striped catfish *Pangasianodon hypophthalmus* were obtained from Arrthit Private Fish Farm, Patheingyi Township to investigate their chromosomal characteristics by blocking the metaphase stage of mitotic division during January to August 2022. The spreads of metaphase stages of chromosomes were observed in the colchicine concentration 0.50 % for duration 4 hrs 30 mins in *L. potail* and 5 hrs 30 mins in *P. hypophthalmus* with 0.56 % hypotonic solution for duration 1 hr. The various organs of fishes were used for chromosome preparation. Karyological analysis revealed that 2n = 56 having 1 m (metacentric) + 3 sm (submetacentric) + 4 a (acrocentric) + 20 t (telocentric) with fundamental arm number (64) in *L. potail*, and 2n = 60 having 1 m (metacentric) + 8 sm (submetacentric) + 4 a (acrocentric) + 17 t (telocentric) with fundamental arm number (78) in *P. hypophthalmus*. The best metaphase spreads of chromosomes were observed in kidney and gill filaments compared with the liver, blood cells and oral cells in both of the fish species.

Keywords *Labeo potail, Pangasianodon hypophthalmus,* karyotype, colchicine

Introduction

Labeo is a large genus having several species which are of considerable importance as an article of food. Some of the species of the *Labeo* genus are reared for ornamental purpose, some as food species, some for extracting oil and some are considered to be of medicinal value. Among them, *Labeo potail* has good market value and high consumer preference, importance to fisheries and in aquaculture activities (Sarma *et al*., 2017). *Pangasianodon hypophthalmus* is one of the major fish species in the Mekong River Fishery, one of the largest and most important inland fisheries in the world. Striped catfish is also riverine freshwater species that can be found in Ayeyarwady Basin of Myanmar. Myanmar and other Asian countries followed after Vietnam in export sector (Griffiths *et al*., 2020).

A karyotype is the complete diploid set of chromosomes grouped together in pairs, arranged in order of decreasing in size within a nucleus of every cell in eukaryotes and haploid set of chromosomes observed in prokaryotes. The global fish fauna consists of about 2, 8900 species of fishes which 2,200 species are cytogenetically studied (Biswal *et al*., 2010).

The chromosomal characteristics of an individual species vary under the same genus or species. These characters may be different depending on the researchers' assumptions to identify chromosomal patterns (Levan *et al*., 1964).

Cytogenetic research on *L. potail* and *P. hypophthalmus* has not been carried out yet in Myanmar. That is why, these fishes from Arrthit Private Fish Farm were selected to investigate their chromosomal characteristics of these fishes.

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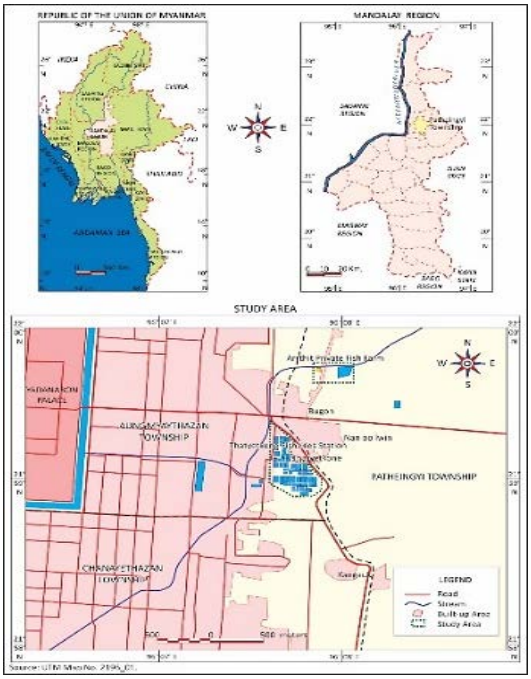


Figure 1 Map showing Arrthit Private Fish Farm, Patheingyi Township, Mandalay Region (Source from Universal Thermatic Map)

Materials and Methods

Study site

Arrthit Private Fish Farm is located around 21°59´50.90"N and 96° 07´50.23"E, Patheingyi Township, Mandalay Region (Fig. 1).

Study period

The study period was from January to August 2022.

Collection of the fishes

Forty fishes were collected from Arrthit Private Fish Farm and reared in the laboratory, Department of Zoology (Plate 1 and 2). The fishes were fed twice a day with formulated commercial feeds. The water was changed twice a week and kept in well-aerated aquarium (Plate 3 A).

Identification of species

The identification of fish species was conducted following after Talwar and Jhingran (1991) and Khamees *et al*. (2013).

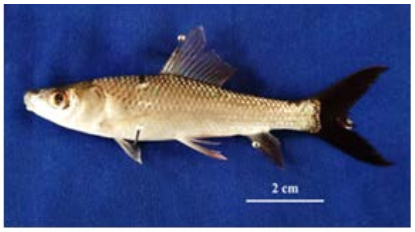


Plate 1 Lateral view of *Labeo potail*

Plate 2 Lateral view of *Pangasianodon hybpphthalmus*

Injection technique

Each fish was weighed with balance (kitchen scale) to the nearest 0.01 g and measured their standard length and total length to the nearest 0.1cm by using scaled ruler. The two different concentrations of colchicine (AVI CHEM, AC01318, India) solutions, 0.30 % and 0.50 %, were prepared and treated intramuscularly to fishes depending upon their weight (1ml /100 g) (Plate 3 B and C).

Collection of tissues

After injection with colchicine, fishes were placed into the aerated bowl to attain the designated time points. Blood was extracted from caudal peduncle by a syringe. Then, fishes were anesthetized with one or two drops of 10 % formaldehyde solution onto the head. The tissues samples such as oral cells, gill, heart, liver and kidney were harvested immediately for chromosomal preparation (Plate 3 D and E).

Extraction of cells

The sample tissues were incubated in hypotonic solution (0.56 % KCL) (MERCK Limited, Worll, Munbal) for 15 mins, 20 mins, 1hr and 1hr 30 mins and then minced these tissues with glass rod to get very fine particles. The incubated samples were mixed thoroughly with vortex mixer (NANOVA) by adding 3 methanol (Pure Chemical Industries., Ltd): 1 acetic acid (BDH Chemicals Ltd Poole England) and centrifuged (Firlabo) for 5 mins at 2000 rpm to get the pellets. The supernatant was discarded by using pipette. This procedure was repeated again (Plate 3 F, G and H).

Preparation of slides

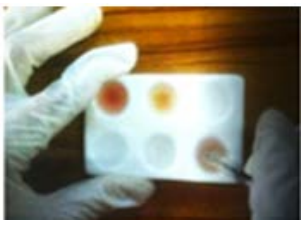
The slides were heated in the oven (Gallenhamp). One or two drops of pellets were placed onto the pre-warmed slides in a far distance. These slides were stained with undiluted Giemsa stain (AVI CHEM, India) for 10 mins, 15 mins and with diluted Giemsa stain for 20 mins. The stained slides were washed under running tap water. To prepare the permanent slides, the stained slides were covered with pre-cleaned coverslips and finally coated with Canada balsam (Kanto Chemical Co., Inc, Tokyo, Japan) (Plate 3 I, J and K).

A. Fish reared in glass tank

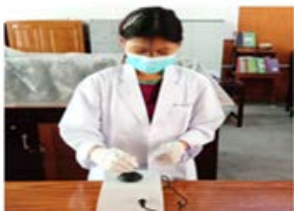
B. Weighing the fish

C. Injection of fish

D. Extraction of blood



E. Mechanical dissociation of tissues

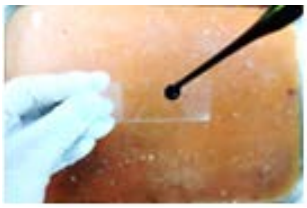


F. Homogenization of tissues

Plate 3 Preparation of cytological process from fish tissues

G. Centrifugation of pellets

H. Sucking the pellet from test tube to drop on slide



I. Dropping the Giemsa stain onto the slide

J. Washing the slide under tap water

K. Covering with cover slip

L. Examining the tissue

Plate 3 Preparation of cytological process from fish tissues

Chromosomal analysis and preparation of karyotype

The microphotographs were recorded with biological microscope with attach camera (G-303P, Taiwan) (x1000). Good quality metaphase spreads were recorded to analyse various stages of metaphase spreads, counting chromosomal numbers and microphotographed for karyotyping in mitotic division. The chromosomal numbers were counted by using ImageJ (1.52a, USA). All chromosome pairs were arranged linearly from biggest to smallest ones according to their size. The chromosome patterns were classified into metacentric (m), submetacentric (sm), acrocentric (c), and telocentric (t) with visual observation according to their designated chromosomal patterns (Levan *et al*., 1964) (Plate 3 L).

Statistical analysis

All recorded data was performed by Microsoft Excel 2010 to check the effects of mitotic inhibitors on mitotic division of cells from different tissues of designated fishes.

Results

The same concentration of 0.50 % colchicine solution was exposed to different organs of deccan carp *Labeo potail* and striped catfish *Pangasianodon hypophthalmus* such as gill filaments, oral cells, kidney, blood and liver. The effect of colchicine solution varied depending upon the type of somatic tissues. The optimum colchicine concentration and duration of *Labeo potail* was 0.50 % for 4 hrs 30 mins in gill filaments and 0.50 % for duration 5 hrs 30 mins in *Pangasianodon hypophthalmus* especially in the kidney tissues rather than other organs.

The best treatment of hypotonic solution was 1 hr on all extracted organs in *L. potail* and 1 hr 30 mins especially in kidney tissues of *P. hypophthalmus*. When staining with undiluted Giemsa stain on prepared slides, chromosomal patterns were clearly distinguished to designate the stage of mitotic division.

Systematic position of fishes

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Actinopterygii
Order	-	Cypriniformes
Family	-	Cyprinidae
Genus	-	Labeo
Species	-	*L.potail* (Sykes, 1839)
Common Name	-	Deccan Carp

In *Labeo potail*, the mean total length of deccan carp (n = 10) used was 16.80 ± 1.09 cm, standard length 13.90 ± 3.70 cm and the body weight 10.32 ± 3.40 g. The meristic characters of *Labeo potail* were dorsal fin (D) ii 13, pectoral fin (P) i 13-14, ventral fin (V) - i 8, anal fin (A) ii 5-6, caudal fin (C) 22-26, lateral line scale (Lts) 37-38 and branchiostegal rays 3 (Plate 1).

Kingdom	-	Animalia
Phylum	-	Chordata
Class	-	Actinopterygii
Order	-	Siluriformes
Family	-	Pangasiidae
Genus	-	Pangasianodon
Species	-	*P.hypophthalmus* (Sauvage, 1878)
Common name	-	Striped catfish
Vernacular name	-	Nga Khu Zin Nga Dan

In *Pangasianodon hypophthalmus*, the mean total length of striped catfish (n =4) used was 22.80 ± 1.37 cm, standard length 21.00 ± 1.19 cm and the body weight 37.90 ± 0.97 g**.** The meristic characters of *P. hypothalamus* were dorsal fin (D) I 7, pectoral fin (P) I 10, ventral fin (V) i 7, anal fin (A) 29, caudal fin (C) 31 and lateral line scale (Lts) 50-51 (Plate 2).

Effects of solutions on blocking stages of cells

The metaphase stages of cells were observed at 4 hrs 30 mins with 0.56 % hypotonic solution in kidney cells and gill filaments, 5hrs in liver, oral cells and blood cells in *L. potail.* The range of chromosome count 56 – 60 (41.66 %) in gill filaments, 20 % in kidney cells and 36.36 % in liver cells. Among three tissues, gill filaments were the best for karyotype analysis, followed by liver cells (36.36 %) and kidney cells (20 %) (Fig. 2)

In *P. hypophthalmus*, the effect of colchicine solution on blood cells and kidney tissues with duration 5 hrs 30 mins and other tissues with 5 hrs were operated at the metaphase stages of chromosomes. Especially, the kidney cells generated the metaphase stage of cells with the optimal range of 56 – 60 (32.25 %), followed by the range 45 – 50 (25.80 %), 40 – 45 (19.35 %), 71 – 75 (12.90 %) and 61– 65 (3.22 %) (Fig. 3)

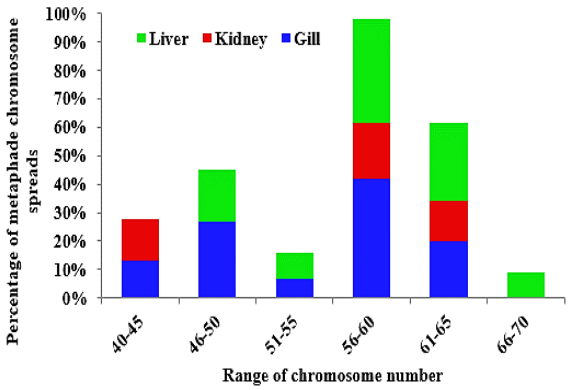


Figure 2 Frequency distribution of metaphase chromosome spread from different tissues of deccan carp *Labeo potail*

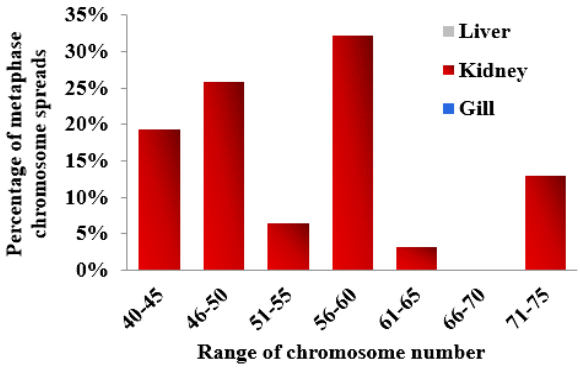


Figure 3 Frequency distribution of metaphase chromosome spread from different tissues of striped catfish *Pangasianodon hypophthalmus*

Percent and frequency distribution

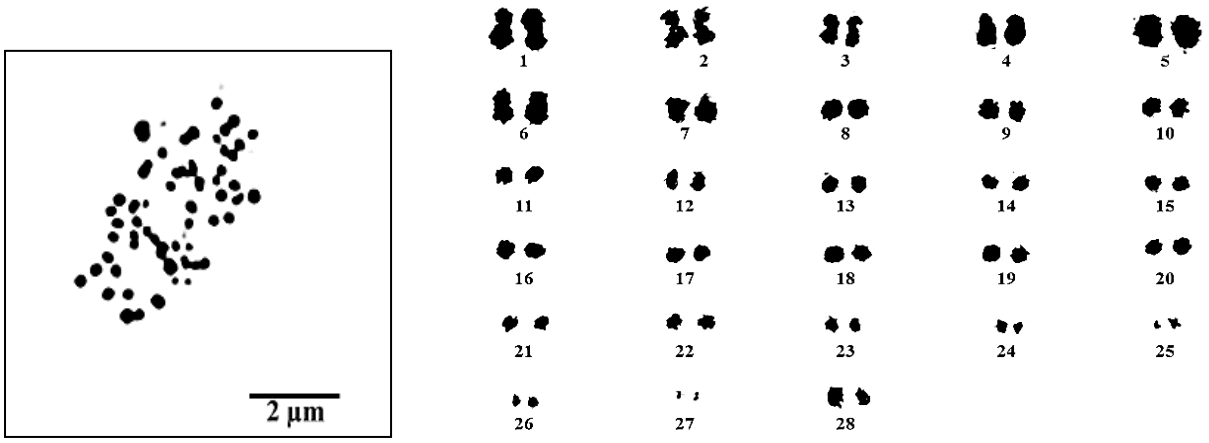
Sixty-three from *Labeo potail* and sixty-one from *P. hypophthalmus* well-spread metaphase complements were counted. In *L. potail*, the highest range of metaphase chromosome count was 56 – 60 with 43 % (n =14) having the cumulative frequency 76, followed by 61 – 65 with 21 % (n = 7), 46 – 50 with 18 % (n = 6), 40 – 45 with 9 % (n = 3) and 66 – 70 with 3 % (n = 1) except the range of 71 – 75. In *P. hypophthalmus*, the range of metaphase count 56 – 60 was the highest percentage 32 % (n = 10) with cumulative frequency 84 and the lowest range of 61 – 65 had 3 % (n = 1) with cumulative frequency 87. The second largest range of chromosome count was 46 - 50 with 26 % (n = 8). Unfortunately, the metaphase chromosome was not observed in the range of 66 – 70. However, the range 71 – 75 had 13 % (n = 4) of metaphase stage of cells (Table 1).

Table 1 Percent and frequency distribution of diploid number of chromosomes counts in deccan carp *Labeo potail* and striped catfish *Pangasianodon hypophthalmus*

Deccan carp *Labeo potail*					Striped catfish *Pangasianodon hypophthalmus*				
Treatment	Range	Frequency	Percentage (%)	Cumulative Frequency	Treatment	Range	Frequency	Percentage (%)	Cumulative Frequency
0.50 % Colchicine (4 hrs 30 mins) 0.56 % KCL (1 hr)	40-45	3	9	9	0.50 % Colchicine (5hrs 30 min) 0.56 % KCL (1 hr 30 mins)	40-45	6	19	19
	46-50	6	18	27		46-50	8	26	45
	51-55	2	6	33		51-55	2	7	52
	56-60	14	43	76		56-60	10	32	84
	61-65	7	21	97		61-65	1	3	87
	66-70	1	3	100		66-70	0	0	87
	71-75	0	0	0		71-75	4	13	100

Karyotype

The numbers of diploid chromosomes ranging from 40 - 65 in *Labeo potail* and 40 - 75 in *Pangasianodon hypophthalmus* were observed. The modal diploid chromosome number was 2n = 56 as it constituted 38 % and 2n = 64, constituted 14 % of counted plates in *Labeo potail* consisting of 1 metacentric, 3 submetacentric, 4 acrocentric and 20 telocentric with fundamental arm number (64) (Table 1, Plate 4 and 5).

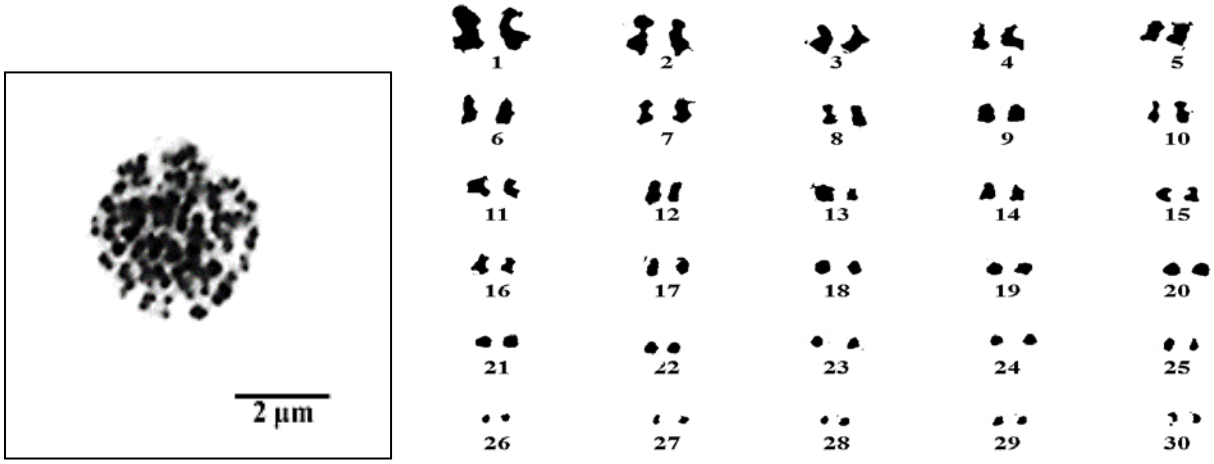


A. Mitotic metaphase chromosome Spread (x1000)

B. Karyotype

Plate 4 Chromosomes of deccan carp *Labeo potail*

The optimal diploid chromosome number was considered as 2n = 60, constituted 49 % and 2n = 74 with 13 % of metaphase plates in striped catfish *Pangasianodon hypophthalmus*. The modal diploid chromosome number of this fish was 1 metacentric (m), 8 submetacentric (sm), 4 acrocentric (a) and 17 telocentric (t) with fundamental arm number (NF = 78).



A. Mitotic metaphase chromosome Spread (x1000)

B. Karyotype

Plate 5 Chromosomes of striped catfish *Pangasianodon hypophthalmus*

Discussion

Fishes were widely cultivated in Arrthit Private Fish Farm, Patheingyi Township, Mandalay and distributed to local fishermen through Upper Myanmar. *Labeo potail* and *Pangasianodon hypophthalmus* were collected from Arrthit Private Fish Farm and investigated their genetics configuration on each species in this study.

Mahfuj *et al.* (2014) described that the variations of chromosomal characteristics are largely dependent on methods of chromosome preparation, staining procedure, tissue source of the body where the dividing cells. In the present study, the methods of chromosome preparation were based on testing with different concentrations of colchicine solutions using on various organs of fishes. The optimum colchicine concentration for *L. potail* and *P. hypophthalmus* was 0.50 % to block the metaphase checkpoint.

The successful outcome of check point was observed in liver tissues of *L. potail*. This result is an agreement with Mahfuj *et al.* (2014) who described that the liver tissues from two- days old larvae of *L. rohita* generated rather than using other somatic tissues.

The kidney tissues generated the best degree of chromosomal condensation rather than other tissues by using 0.50 % colchicine concentration for treatment 5 hrs 30 mins than 4 hrs 30 mins and 5 hrs. Therefore, this research is approved to John *et al.* (2017) who reported that the diploid number of chromosomes for *Labeo coubie* occurred more in the kidney plates than the liver plates.

In *Pangasianodon hypophthalmus*, the optimum concentration of colchicine solution 0.50 % for duration of 5 hrs 30 mins was good for kidney tissues and 5 hrs for other tissues. The present study is inconsistent with John *et al*. (2017) who stated that the best metaphase stages in *L. coubie* was 0.02 % colchicine solution for 4 hrs. The kidney tissues were the best tissues for metaphase spreads of mitotic division.

The hypotonic treatment of 0.56 % KCL for 1 hr was good for explosion of nuclear membrane (Win Win Mar and Thant Zin, 2020). According to the results of this study, longer treatment of 0.56 % KCL solution for 1 hr generates enough explosion of nuclear envelope and cytoplasmic membrane observed in all tissues and blood cells in *L. potail*. However, the same technique did not support the explosion of cells from all tissues in *P. hypophthalmus* expect the blood and oral cells. When the exposure time was raised up to 1 hr 30 mins, the check point of cells was observed.

Another important factor for cytogenetic analysis is Carnoyl's fixative (3 acetic acid: 1 methanol). In this study, Carnoyl's fixative was used to treat the extracted cells for 10 mins duration. The good shape of chromosomal configuration of *Labeo potail* and *Pangasianodon hypophthalmus* were obtained clearly leading to count and differentiated the chromosomal structure in detail.

Mahfuj *et al*. (2014) who described that their proposed standard karyotype for *Labeo rohita* was 18 m , 6 sm and 1 st chromosome; *L. coubie* was 2n= 10 m + 11 sm + 5 st + 24 t (John *et al.*, 2017) and *Labeo rohita* 8 m+ 6 sm + 4 st + 30 a (Bhatnagar *et al.*, 2014). The modal diploid chromosome number of *Labeo potail* was 2n = 56 with the range of 40 - 65. Standard karyotype of this fish consisted of 1 m+ 3 sm + 4 a + 20 t with fundamental arm number NF 64. And the karyological structure of *Pangasianodon hypophthalmus* was the diploid number 2n = 60 having 1 m + 8 sm + 4 a + 17 t with fundamental arm number (78).

Therefore, the karyological data of studied fishes are standardized for their population. Hence, these data highly recommended for further phylogenetic studies and should research on the strains with various karyotype formulae within the same species.

Conclusion

The effect of colchicine concentrations 0.50 % was better than 0.30 % with various durations of 4 hrs 30 mins, 5 hrs and 5 hrs 30 mins in *Labeo potail* and *Pangasianodon hypophthalmus.* The cells extracted from kidney tissues and gills filament induced the metaphase spreads in *L. potail*, however, it was by blood cells in *P. hypophthalmus.* The modal diploid number of *L. potail* was 2n = 56 with fundamental arm number (64), and 2n = 60 with fundamental arm number (78) in *P. hypophthalmus* was observed.

Acknowledgements

We would like to extend our grateful thanks to Dr Nwe Nwe Khaing, Professor and Head of Zoology Department of Mandalay University, Dr Thant Zin, Professor, Curriculum Central Unit, Department of Education Research, Planning and Training, Yangon (Former Professor and Head, Department of Zoology, University of Mandalay), Dr Kay Thi Mya and Dr Ni Ni Win, Professors, Department of Zoology, Mandalay University, for their various supports, valuable suggestions and comments.

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RELATIONSHIP BETWEEN TOXIC METAL CONCENTRATION AND SIZE OF FISH AND TRANSFER FACTOR OF AQUATIC ENVIRONS IN THANLWIN RIVER SEGMENT, MON STATE

Yee Yee Win[1], Kay Lwin Tun[2], Cho Cho Thin[3]

Abstract

Seasonal variations of As, Pb and Cd concentrations in muscle tissues of *Lates calcarifer*, *Otolithoides pama* and *Polynemus paradiseus* captured from Thanlwin River segment of Mon state were investigated during November 2019 to October 2020. Metal concentrations in water and sediment samples of the study sites were also assessed. Metal concentrations in extracts were determined by using Flame Atomic Absorption Spectrophotometry (FAAS) (Perkin Elmer AA Analyst 800 and Winlab – 32 software) at University Research Center (URC) in University of Yangon. Heavy metal concentrations in muscle tissues of all study fish species were below WHO standard limits. Concentration of As in water of the study site I in all seasons as well as those of the study site II also in all seasons were found higher than the WHO standard limits. As concentration in sediment of the study site I in all seasons as well as those of the study site II also in all seasons were found to be higher than the WHO standard limits. Concentration of Pb in water of the study site I in all seasons as well as those of the study site II also in all seasons were found higher than the WHO standard limits. No relationship between sizes of fish and heavy metal concentrations in *L. calcarifer* while negative relationship in *O. pama* and positive relation in *P. paradiseus* were found at the study site I. In the study site II, negative relationships between size of fish and As concentration in *L. calcarifer*, no relationships in *O. pama* and positive relationships between size of fish and As, Pb and Cd in *P. paradiseus* were observed. In the present study, transfer factor from water to muscle tissue of *L. calcarifer* was observed in As and Pb at the study site I and those transfer factor from sediment to muscle tissue of *L. calcarifer* was observed in Pb and Cd at the study site II. Transfer factor from water to muscle tissue of *O. pama* and *P. paradiseus* was observed in Cd at the study site II. However, heavy metal concentrations in muscle tissues of the study fish species were not higher than maximum permissible limits of WHO. Thus, mentioned fish species captured from the study area are generally safe for human consumption.

Keyword fish muscle, arsenic, lead, cadmium, muscle tissue, water, sediment

Introduction

Heavy metal pollution of water has become a major environmental problem almost since the advent of agricultural and industrial revolution and today most water resources are still being contaminated with heavy metals released from domestic, industrial and other man-made activities (Khare and Singh, 2002; Hayat and Javed, 2008). Heavy metal contamination may have devastation impact on the ecological balance of natural water bodies including the loss of aquatic diversity (Vosyliene and Jankaite, 2006; Farombi *et al*., 2007; Hayat and Javed, 2008).

Heavy metals are environmentally ubiquitous, readily dissolved and transported by water and readily taken up by aquatic organisms (Alam *et al*., 2002). Fishes are often at the top of aquatic food chain in water ecosystems and fish living in the polluted water may accumulate toxic trace metals (Mansour and Sidky, 2002).

It is well known that fish, as a regular constituent of the human diet, can represent a dangerous source of certain heavy metals. The discharge of wastewater and industrial effluents whether treated or not can be regarded as constant pollution source that dominate water quality. Water quality parameters can produce an improved understanding of the environmental situation

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and assist policy makers to design priorities for sustainable water management (Hung *et al*., 2010).

Thanlwin River which is one of the distinguished rivers in Myanmar is the study site for the present study. However, due to the natural disasters such as soil erosion and the existence of cement factories, machine cleaning workshop and industrial factories which are located near the study site, there is always a risk for aquatic organisms including fish, water and sediments due to contaminants such as heavy metals. Moreover, as farmers use agricultural fertilizer and pesticides, contamination occurs in water and sediments.

Due to above mentioned reasons, the present study has been carried out with the following objectives; to assess the contents of toxic heavy metal residues (As, Pb and Cd) which bio-accumulated in the muscle tissues of three selected fish species, to identify the toxic metal residue (As, Pb and Cd) concentrations in River water and sediment of River in the study sites, to study the relationship between fish size and toxic level in muscle tissues of three fish species, and to evaluate the transferred factor (TF) of heavy metals from River water and sediment in the muscle tissues of the three fish species.

Materials and Methods

The present study was conducted in two different study sites (Ngan Tae village and Kyauk Tan village) situated on the Thanlwin River segment and its tributaries in Mon states. Ngan Tae village (Lat 16º 28' N and Long, 97º 39' E) and Kyauk Tan village (Lat 16º 24' N and Long, 97º 36' E) were designated as the study site I and II, respectively. Study period lasted from November 2019 to October 2020. *Lates calcarifer*, *Otolithoides pama* and *Polynemus paradiseus*, water and sediment samples were collected monthly in fish landing depots of the study sites. At least seven samples from each fish species were collected. Total length and body weight of specimens were measured. They were dissected using stainless steel scalpels and forceps. A part of the muscle (dorsal muscle) was removed and weighed. Samples were put into an oven (90 ºC) and dried to reach constant weight. After that they were stored at low temperature until digestion. Digestion of the samples was carried out according to dry method by using furnace (Model-L-3383).

Element concentrations of As, Pb and Cd in extracts were determined by using Flame Atomic Absorption Spectrophotometry (FAAS) (Perkin Elmer AA Analyst 800 and Winlab-32 software) at Universities Research Center (URC) in University of Yangon. Test results were compared with maximum permissible limit (MPL) designated by WHO.

Functional relationship between sizes of fish and heavy metal concentrations were analyzed by using regression method with the following formula (Le ren, 1951) Where,

Y	=	a + bX
a	=	Y intercept
b	=	slope of the line
r	=	regression coefficient

The transfer factor is an approach based on the water-fish transfer factor that provides a straight forward, constructive method for assessing heavy metal accumulation for the purposes of health risk assessment for humans consuming the fish. The water-fish transfer factor (TF) of the biological accumulation coefficient (BAC), which expresses the ratio of contaminant concentration in fish to the concentration in water, was used to characterize quantitatively the transfer of an element from the water to fish (Rodriguez *et al*., 2002 and Tome, V, *et al*., 2003).

To evaluate the bio-accumulation of heavy metals in fish muscle tissue from water or sediment, the transfer factor (TF) was calculated (Kalfakakour and Akrida-Demertzi, 2000; Rased, 2001). The TF formula was given as:

$$TF = \frac{\text{concentration of metal in fish muscle tissue}}{\text{concentration of metal in environ (water or sediment)}}$$

According to Kalfakakour and Akrida-Demertzi (2000), if the value of TF was is greater than 1, it indicated bioaccumulation of metals in fish muscle tissue.

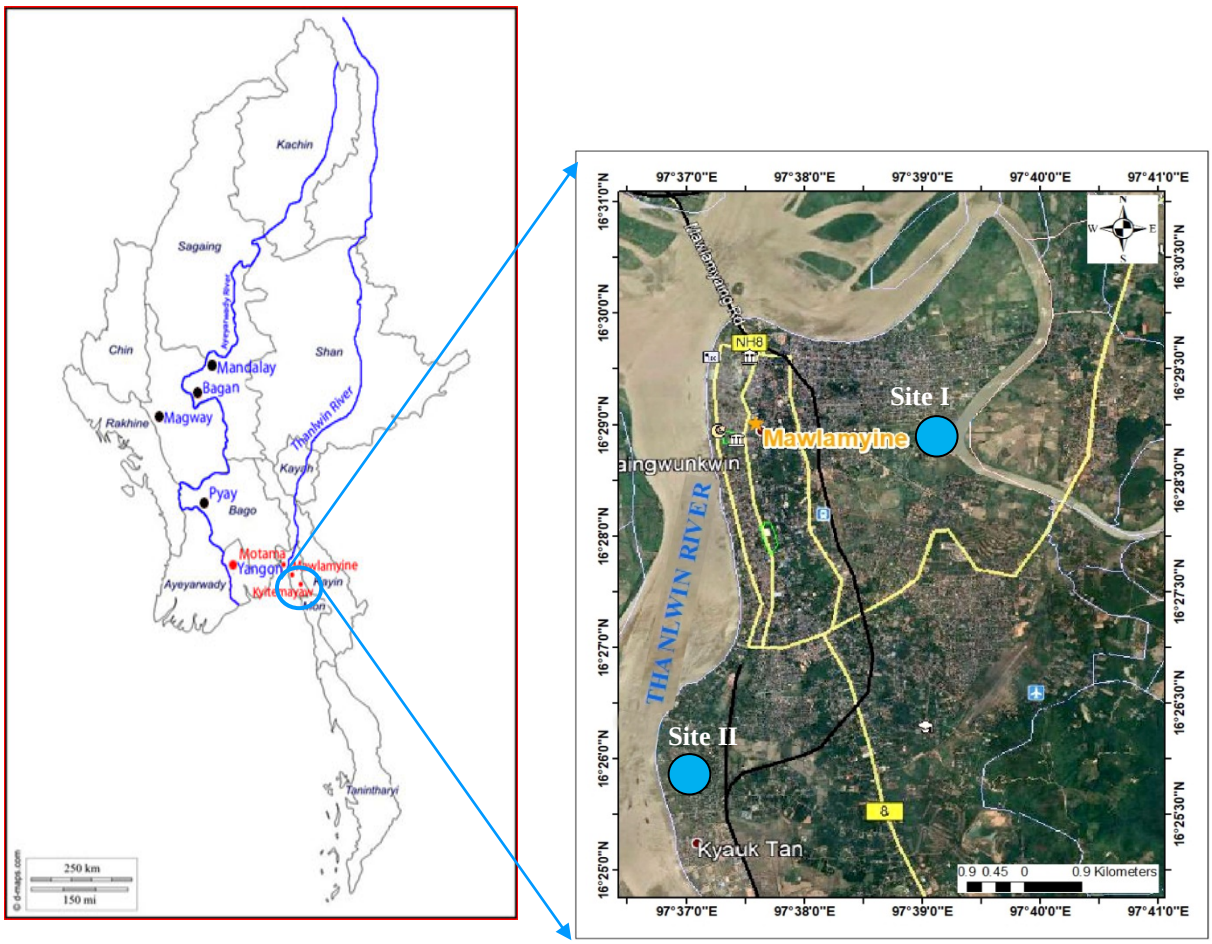

Figure 1 Map of the study sites

A. Cement Factory (Back View) Site I

B. Machine cleaning workshop (Back View) Site II

Plate 1 Environs of the study sites

A. *Lates calcarifer*

B. *Otolithoides pama*

C. *Polynemus paradiseus*

Plate 2 Studied fish species

Results

Mean sizes (total length and body weight) of three fish species, *Lates calcarifer*, *Otholithoides pama* and *Polynemus paradiseus,* which were selected to test metal concentrations in muscle tissues were different. Seasonal variations among mean total lengths of all study species in 2020 were observed (Table.1 and Table. 2).

In metal concentrations in muscle tissue of three selected fish species at the study area, Arsenic concentration in muscle tissue of *Lates calcarifer* collected from study site I was -11.99 mg/L in winter season and it was > -26.45 mg/L in summer season and > -31.43 mg/L in rainy season. Similar results were observed in study site II. The As concentration in muscle tissue of *Otolithoides pama* and *Polynemus paradiseus* was highest in winter season and followed by summer season and rainy season. The As concentrations in muscle tissues of three study fish species collected from study site I were higher than those of study site II in winter season (Table 3).

Pb concentration in muscle tissue of *Lates calcarifer* collected from study I was 0.131 mg/L in summer season, >0.110mg/L in rainy season and >-0.051mg/L in winter season. Similar results were observed in the study site II. The same conditions of Pb concentration were found in muscle tissue of *Otolithoides pama* and *Polynemus paradiseus.* However, Pb concentration in muscle tissues of three study fish species collected from the study site II were higher than those of the study site I in summer seasom (Table 4).

Cadmium concentration in muscle tissue of *Lates calcarifer* at summer season (-0.265 mg/L) was higher than those of rainy season (0.171mg/L) and winter season (-0.274mg/L) in the study site I. Similar results were observed in the study site II. The same conditions of Cd concentration were found in muscle tissue of *Otolithoides pama* and

Polynemus paradiseus. However, Cd concentrations in muscle tissues of *Lates calcarifer*, *Otolithoides pama* and *Polynemus paradiseus* collected from the study site I were higher than those of the study site II (Table 5).

Arsenic concentrations in water and sediment at rainy season (2.965mg/L in water and 109.10mg/L in sediment) were higher than those of summer season (2.114mg/L in water and 87.84mg/L in sediment) and winter season (69.55mg/L in water and 88.01mg/L in sediment) in the study site I. Similar results were observed in the study site II. Arsenic concentrations in water collected from study site I was higher than that of study site II. As concentrations in sediment collected from the study site II was higher than that of the study site I (Table 6 and Table.7).

Lead concentration in water at winter season (0.137mg/L) was higher than those of rainy season (0.114mg/L) and summer season (0.088 mg/L), but in sediment at rainy season was the most higher (0.231 mg/L) than those of summer (0.152mg/L) and winter (0.200mg/L) in the study site I. Although Pb concentration at rainy season (0.468mg/L in sediment) in site II was higher than those of rainy season (0.231mg/L) and winter season (0.200mg/L) in study site I. However, Pb concentrations in water and sediment collected from study site II were higher than those of the study site I in summer season (Table 6 and Table.7).

Cadmium concentration in sediment of summer season (-0.268mg/L) was higher than those of rainy season (-0.291mg/L) and winter season (0.291mg/L) in the study site I. Similar results were observed in the study site II. Cd concentration of water in winter season (0.292mg/L) was higher than those of summer season (0.294mg/L) and rainy season (-0.293mg/L) in site I. However, Cd concentrations in water and sediment collected from the study site I were higher than those of the study site II in all seasons (Table 6 and Table.7).

WHO/FAO maximum permissible limits of As, Pb and Cd in muscle tissue of fish are 0.01 mg/L, 1 mg/L and 0.2 mg/L respectively. Concentrations of As, Pb and Cd in muscle tissues of the study three fish species were found under the maximum levels permitted by WHO/FAO (Table 3,4,5 and Fig. 3,4,5).

In the study for transfer factors of heavy metals from water and sediment to muscle tissues of three fish species, transfer factor (TF) of Cdamium in site I from sediment of aquatic environs to muscle tissues of *L. calcarifer* was (1.29), Cdamium in site II of *L. calcarifer* and *O. pama* were 1.38 and 1.38 respectively and Cadmium in *P. paradiseus* of site II (1.43) was also higher than index value 1 in 2020. (Table 9).

In the study for relation between size of fish and metals concentrations in muscle tissue of fish, regression coefficient of total length of *L. calcarifer* and concentrations of As (y=-0.1762x-18.685, r = 0.195), Pb (y=0.0021x+0.0086, r= 0.236) and Cd (y=1E-04x-0.2725, r= 0.236) were observed. Total length of *L. calcarifer* and As, Pb and Cd concentrations were found to have no relation in the study site I. In site II, As (y=0.0919x-17.112, r=0.321) Pb (y=-0.0002x-0.0498, r=0.024) and Cd (y=1E-05x-0.2904, r = 0.017) were observed. Total lengths of *L. calcarifer* and As concentrations were found to have negative relation in the study site II (Table 8).

Regression coefficient of total lengths of *O. pama* and concentrations of As (y=1.11x-54.619, r=0.336), Pb (y=0.0149x-0.4214, r=0.374) and Cd (y=0.0014x-0.2962, r=0.372) in muscle tissue of this species in site I were observed. In site II, As (y=-0.2614x-19.106, r= -0.176) Pb (y=-0.0007x-0.0328, r=0.024) and Cd (y=8E-05x—0.2949, r = 0.060) were observed. Total lengths of *O. pama* and As and Pb concentrations were found to be negatively related in the study site I (Table 8).

Regression coefficient of total lengths *P. paradiseus* and concentrations of As (y=-1.7043+13.34, r= -0.429) Pb (y=0.033x-0.5404, r=0.631) and Cd (y= 0.0033x-0.323,

r = 0.635) in muscle tissue of this species in site I were observed. In study site II, As (y=3.4211x-87.336, r=0.760) Pb (y=-0.0033x-0.0189, r=-0.501) and Cd (y=-0.002x-0.2611, r=-0.522) were observed. In site I and II, total lengths of fish and As, Pb and Cd concentrations were found to be positively related (Table. 8).

Regression coefficient of body weights of *L. calcarifer* and concentrations of As (y=0.0066x-21.575, r=0.225) Pb (y=9E-05x+0.0397, r=0.314) and Cd (y=5E-06x-0.2713, r = 0.372) in muscle tissue of this species in site I were observed. In study site II, As (-0.0021x-18.977), Pb (y=6E-06x-0.0531, r=0.036) and Cd (y=2E-07x-0.2901, r=0.010) were observed. Body weight of *L. calcarifer* and As concentrations were negatively related in site II (Table 8).

Regression coefficient of body weights of *O. pama* and concentrations of As (y=0.079x-35.91, r=0.404), Pb (y=0.0008x-0.1412, r=0.333) and Cd (y=7E-05x-0.2694, r=0.329) in muscle tissue of this species in site I were recorded. In the study site II, that of As (y=0.1779x-41.001, r=0.408), Pb (y=-0.0058x+0.4742, r=0.728) and Cd (y=-0.0058x-0.4742, r=-0.721) were observed. Positive relation between body weights of *O. pama* and As concentrations was observed in site I and site II. Body weights of *O. pama* and Pb concentrations were negatively related in site I and site II (Table 8).

Regression coefficient of body weights *P. paradiseus* and concentrations of As (y=-0.2811x-7.8548, r=-0.385), Pb (y=0.0058x-0.138, r=0.605) and Cd (y=0.0006x-0.284, r =0.653) in muscle tissue of this species in site I were observed. In study site II, As (y=0.6402x-46.374, r=0.746), Pb (y=-0.0006x-0.0583, r=-0.489) and Cd (y=0.0004x-0.2851, r =-0.510) were observed. Body weights of *P. paradiseus* and As concentrations were positively related in site II and those with Pb and Cd concentrations were positively related in site I (Table 8).

Table 1 Mean total length and body weight of the fish for seasonal analysis of metal concentration in the study site I

Sr No.	Species	Summer		Rainy		Winter	
		TL (cm)	Weight (g)	TL (cm)	Weight (g)	TL (cm)	Weight (g)
1.	*Lates calcarifer*	23.2±10.94	142.8±159.42	28.7±9.35	395.1±408.42	26.5±5.35	236.2±97.87
2.	*Otolithiodes pama*	23.42±1.69	85.42±15.63	25.67±1.89	123.83±23.17	24.5±2.65	114.3±54.58
3.	*Polynemus paradiseus*	14.83±0.53	15.67±1.2	17.44±0.8	30.11±5.23	16.78±1.69	25.44±9.12

Table 2 Mean total length and body weight of the fish for seasonal analysis of metal concentration in the study site II

Sr No.	Species	Summer		Rainy		Winter	
		TL (cm)	Weight (g)	TL (cm)	Weight (g)	TL (cm)	Weight (g)
1.	*Lates calcarifer*	30.10±8.14	418.6±372.09	34.9±14.6	700.6±744.98	26.8±3.31	239.5±93.47
2.	*Otolithiodes pama*	22.08±0.98	77.08±4.69	21.29±6.32	99±8.97	23.17±0.75	95.33±11.12
3.	*Polynemus paradiseus*	16.22±1.13	22.94±5.71	15.78±0.71	20.44±2.88	19.22±0.71	38.39±5.26

Table 3 Seasonal variation of Arsenic concentrations (mg/L) in muscle tissues of fish species in the study sites (2020)

Sr. No.	Species	Site I			Site II			MPL
		Summer	Rainy	Winter	Summer	Rainy	Winter	
1.	*Lates calcarifer*	-26.45	-31.43	-11.99	-19.47	-23.77	-16.53	0.26
2.	*Otolithoides pama*	-22.21	-21.81	-38.16	-28.20	-16.97	-29.54	0.26
3.	*Polynemus paradiseus*	-13.26	-22.75	-7.578	-27.86	-38.95	-18.97	0.26

Table 4 Seasonal variation of Lead concentrations (mg/L) in muscle tissues of fish species in the study sites in 2020

Sr. No.	Species	Site I			Site II			MPL
		Summer	Rainy	Winter	Summer	Rainy	Winter	
1.	*Lates calcarifer*	0.131	0.110	-0.051	0.059	-0.110	-0.116	1
2.	*Otolithoides pama*	0.071	-0.104	-0.138	0.097	-0.116	-0.125	1
3.	*Polynemus paradiseus*	0.036	0.078	-0.114	-0.059	-0.080	-0.086	1

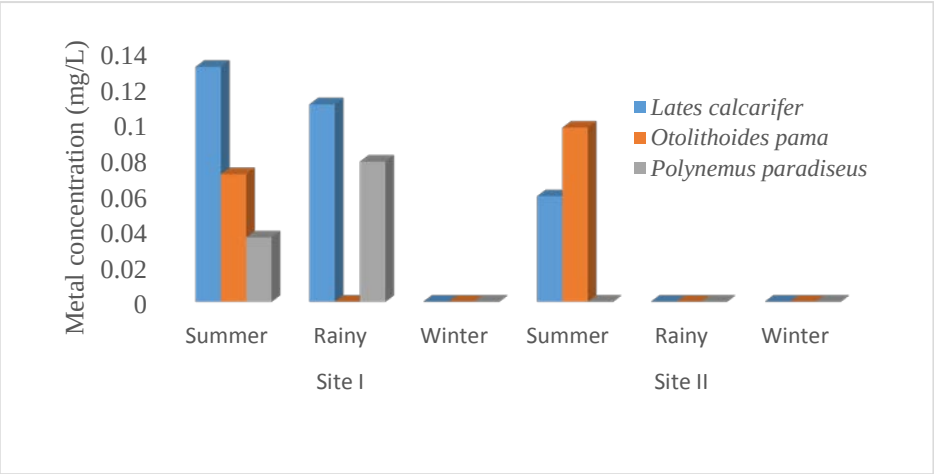

Figure 2 Lead concentrations (mg/L) in three fish species at two different study sites

Table 5 Cadmium concentrations (mg/L) in three fish species at two different study sites

Sr No.	Species	Site I			Site II			MPL
		Summer	Rainy	Winter	Summer	Rainy	Winter	
1.	*Lates calcarifer*	-0.265	-0.271	-0.274	-0.279	-0.294	-0.297	0.2
2.	*Otolithiodes pama*	-0.249	-0.266	-0.269	-0.286	-0.299	-0.294	0.2
3.	*Polynemus paradiseus*	-0.258	-0.271	-0.278	-0.286	-0.298	-0.302	0.2

MPL = Maximum permissible limit

Table 6 Heavy metal concentrations in water of two different study sites

Sr No.	Elements	Site I			Site II			MPL
		Summer	Rainy	Winter	Summer	Rainy	Winter	
1.	Arsenic	2.114	2.956	69.55	2.682	4.620	2.698	0.01
2.	Lead	0.088	0.114	0.137	0.120	0.078	0.125	0.01
3.	Cadmium	-0.294	-0.293	-0.292	-0.300	-0.317	-0.309	0.003

MPL = Maximum permissible limit

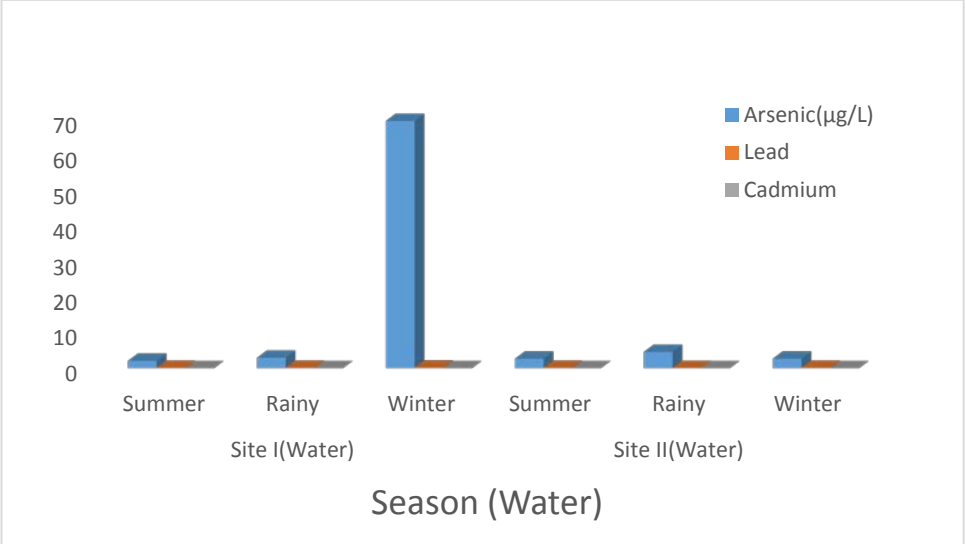

Figure 3 Heavy metal concentrations in water of two different study sites

Table 7 Heavy metal concentrations in sediment of two different study sites

Sr No.	Elements	Site I			Site II			MPL		
		Summer	Rainy	Winter	Summer	Rainy	Winter	TEC	MEC	PEC
1.	Arsenic	87.84	109.10	88.01	93.97	162.10	226.90	9.8	21.4	33
2.	Lead	0.152	0.231	0.200	0.216	0.468	0.024	36	83	130
3.	Cadmium	-0.286	-0.291	-0.291	-0.293	-0.294	-0.045	0.99	3	5

MPL = Maximum permissible limit
TEC = Threshold effect concentration
MEC = Midpoint effect concentration
PEC = Protable effect concentration

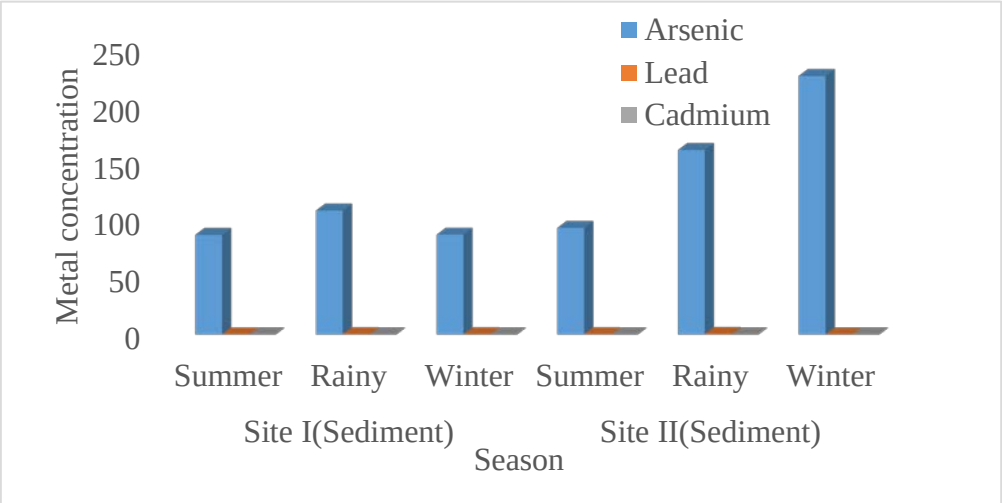

Figure 4 Heavy metal concentrations in sediment of two different study sites

Table .8 Relationship between body length (cm) and weight (g) of fish and heavy metal concentrations in muscle tissue of fish at study Site I and Site II

Species	Heavy metals	Site I			Site II		
		Length	Weigth	Remark	Length	Weigth	Remark
		Linear equation	Linear equation		Linear equation	Linear equation	
Lates calcarifer	As	y = -0.1762x - 18.685 R² = 0.0382 r=-0.195	y=-0.0066x - 21.575 R²=0.0508 r=-0.225	No relation	y=-0.0919x - 17.112 R²=0.1028 r=-0.321	y= -0.0021x - 18.977 R²=0.1333 r=-0.365	Negative relation
	Pb	y=0.0021x + 0.0086 R²=0.0556 r=0.236	y=9E-05x + 0.0397 R²=0.0988 r=0.314	No relation	y= -0.0002x - 0.0498 R²=0.0006 r=-0.024	y=-6E-06x - 0.0531 R²=0.0013 r=-0.036	No relation
	Cd	y=1E-04x - 0.2725 R²=0.0555 r=0.236	y=5E-06x - 0.2713 R²=0.1386 r=0.372	No relation	y=1E-05x - 0.2904 R²=0.017 r=0.017	y= 2E-07x - 0.2901 R²=0.0001 r=0.010	No relation
Otolithoides pama	As	y=1.11x - 54.619 R²=0.113 r=0.336	y=0.079x - 35.91 R²=0.1635 r=0.404	Negative relation	y= -0.2614x - 19.106 R²=0.031 r=-0.176	y=0.1779x - 41.001 R²=0.1666 r=0.408	Positive relation
	Pb	y=0.0149x - 0.4214 R²=0.14 r=0.374	y=0.0008x - 0.1412 R²=0.1106 r=0.333	Negative relation	y= -0.0007x - 0.0328 R²=0.0006 r=-0.024	y= -0.0058x + 0.4742 R²=0.5293 r=-0.728	Negative relation
	Cd	y= 0.0014x - 0.2962 R²=0.1387 r=0.372	y=7E-05x - 0.2694 R²=0.1085 r=0.329	No relation	y= 8E-05x - 0.2949 R²=0.0036 r=0.060	y== -0.0058x + 0.4742 R²=0.5198 r=-0.721	Negative relation
Polynemus paradiseus	As	y=-1.7043x + 13.34 R²=0.1838 r=-0.429	y=-0.2811x - 7.8548 R²=0.1484 r=-0.385	Negative relation	y=3.4211x - 87.336 R²=0.5778 r=0.760	y= 0.6402x - 46.374 R²=0.556 r=0.746	Positive relation
	Pb	y=0.033x - 0.5404 R²=0.3984 r=0.631	y=0.0058x - 0.138 R²=0.3659 r=0.605	Positive relation	y= -0.0033x - 0.0189 R²=0.2514 r=-0.501	y=-0.0006x - 0.0583 R²=0.2392 r=-0.489	Negative relation
	Cd	y=0.0033x - 0.3236 R²=0.4027 r=0.635	y= 0.0006x - 0.284 R²=0.427 r=0.653	Positive relation	y= -0.002x - 0.2611 R²=0.2724 r=-0.522	y=-0.0004x - 0.2851 R²=0.2596 r=-0.510	Negative relation

Table 9 Transfer factors of toxic metals from water and sediment to muscle tissue of different fish species in 2019-2020

Species	Metals	2019-2020			
		Site I		Site II	
		TF from water	TF from sediment	TF from water	TF from sediment
Lates calcarifer	As	0.94	-0.25	-5.98	-0.12
	Pb	0.55	0	-0.55	0.21
	Cd	0.93	**1.29**	0.94	**1.38**
Otolith0ides pama	As	-1.1	-0.29	-7.48	-0.15
	Pb	-0.55	0	-0.45	-0.21
	Cd	0.9	0.9	0.94	**1.38**
Polynemus paradiseus	As	-0.58	-0.15	-8.59	-0.18
	Pb	0	0	-0.73	-0.33
	Cd	0.93	0.93	0.97	1.43

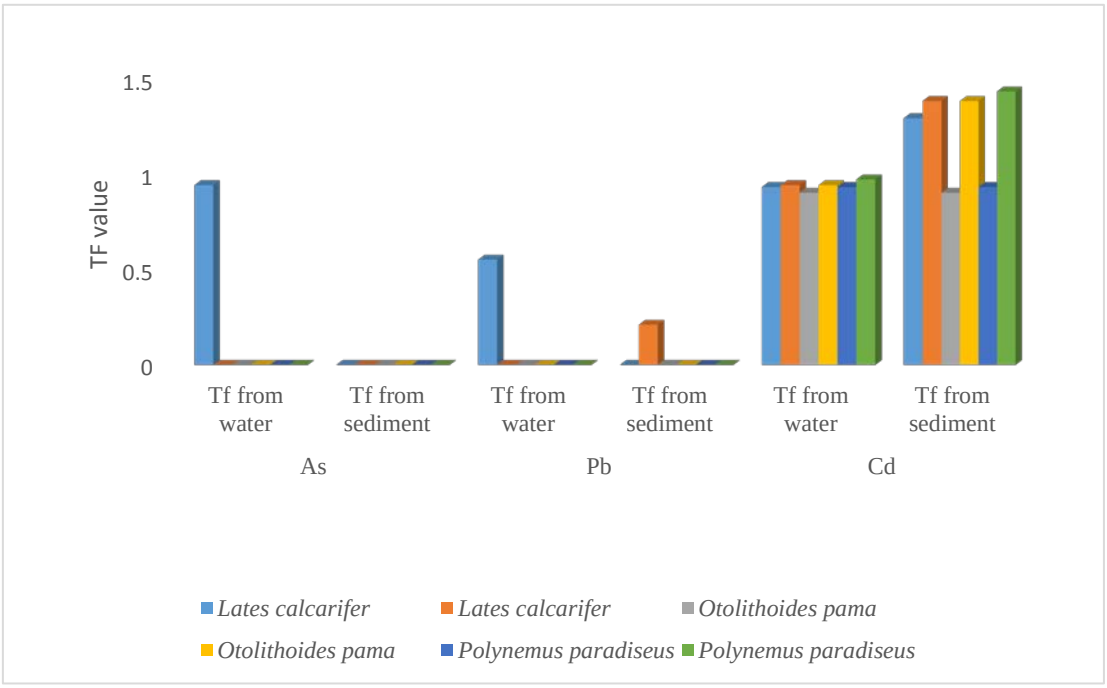

Figure 5 Transfer factors of heavy metals from water and sediment in muscle tissue of different fish species in 2019-2020

Discussion

Heavy metals (As, Pb and Cd) in muscle tissues of three fish species (*Lates calcarifer*, *Otolithoides pama* and *Polynemus paradiseus*) collected from the catch in Ngan Tae village and Kyauk Tan village situated on the Thanlwin River segment and its tributaries of Mon states were analyzed seasonally during the period from November 2019 to October 2020. Metal concentrations in water and sediment of aquatic environs of the study sites were also assessed. Arsenic is released in the environment through natural processes such as weathering, and may circulate in natural ecosystems for long time (Mol *et al*., 2010).

Toxic effects appear when arsenic is ingested in excess for long periods resulting in cancer, cutaneous malignancies, etc. Lead is toxic metal and non-essential element for human body as it causes a rise in blood pressure, kidney damage and miscarriage Cadmium injures the kidney, poor reproductive capacity, hypertension, tumors and hepatic dysfunction. (Kiran *et al*., 2011).

In the present study, concentrations of Pb in muscle tissues of *L. calcarifer, O. pama*, and *P. paradiseus* were found to be higher in summer and rainy seasons than those of winter in site I.

Similar findings of Pb concentrations in muscle tissues of *L. calcarifer* and *O. pama* were observed to be high in summer of site II. As and Cd concentrations in this species were lower than MPL limit in all season.

Thus, concentrations of As, Pb and Cd in muscle tissues of three study fish species collected from the study site I and II were not higher than the MPL recommended by WHO.

Khin Thida Kyaw (2008) stated that seasonal variation of toxic metals (As,Pb and Cd) concentrations in three fish species were higher in summer and rainy season than those of winter.

However, Cho Cho Thin (2017) stated that the level of As in fish muscle was found above the WHO standard limit but Khin Myint Mar(2011) found that the concentrations of heavy metals in studied species were lower than WHO standard limit.

Thus, present findings were not agreed with the findings of above authors and potential danger may not occur for the consumption of the study fish species from the present study area.

In relationship between fish size and toxic level, length and weight of *P. paradiseus* and As, Pb and Cd concentrations were positively related in the study site I and site II. Lengths and Pb and Cd concentrations as well as weight and Pb and Cd concentrations of three fish species in site I were positively related. Lengths and weights of *P. paradiseus* and As concentrations were positively related in the study site II. Cho Cho Thin (2017) observed that relationship between As concentrations in muscle tissues of carnivorous and omnivorous fishes and their body lengths and body weights were significant.

In the present study, transfer factor from sediment to muscle tissue of *L. calcarifer* was observed in Cd at site I and site II. Similar case of *O. pama* was observed in Cd at site II. Transfer factor from sediment to muscle tissue of *P. paradiseus* was observed in Cd at site II. Sein Moh Moh Paing (2019) stated that Cd and As concentrations in water and sediment of the study area were found above the WHO standard limits in all seasons.

Excessive releases of heavy metals into the environment due to industrialization and urbanization has posed a great problem worldwide. Water contaminants in those habitats were being discharged by industries affecting the ecosystem of the aquatic organisms including fish species. Sustainable use, public awareness, and conservation activities are important to maintain healthy aquatic environs.

The effects of pollutants may be also detected on land as a result of their bioaccumulation and bioconcentration in the food chain (Zhang *et al*., 2004; Stara *et al*., 2013). Fish cover a wide range of trophic levels and are an important link of aquatic food chains with human populations (Costa and Kehrig, 2002).

On the basis of results of present study, the levels of As, Pb and Cd concentration in three fish species were lower than those of limits recommended by WHO. However, in water of both study sites, levels of As and Pb concentration were higher than maximum permissible limits of WHO. Cd concentration level was lower than maximum permissible limit of WHO. In sediments, the level of As concentration was higher than maximum permissible limit of WHO whereas the levels of Pb and Cd concentration were lower than maximum permissible limits of WHO. Therefore the studied fish species are generally safe for human consumption. Water and sediment of the study area were not safe due to heavy metals contaminations and are therefore in alarm state for human drinking, and other domestic uses.

Conclusion

The results of this research based on the investigation of metal concentrations in *L. calcarifer, O. pama, P. paradiseus,* water and sediment of aquatic environs in Thanlwin River lower segment of Mon State revealed the heavy metal contaminations of water and sediment. In the present study As, Pb and Cd concentrations of water and sediment in all seasons during the study period were observed to be higher than the maximum permissible limits and guide line limits of WHO/FAO. Transfer factors of Pb and Cd in *Lates calcarifer, Otolithoides pama and Polynemus paradiseus* were found to be from sediment of their aquatic environs. In the present study, it might be due to soil erosion, agricultural runoff of fertilizers and pesticides, industry effect composed of organic and inorganic materials which are oxidized. People should have awareness that environment is degraded and contaminated due to human activities. Three fish species, *Lates calcarifer, Otholithoides pama* and *Polynemus paradiseus,* were selected from the catch of Ngan Tae village and Kyauk Tan village situated on the Thanlwin River segment and its tributaries of Mon State to test metal concentrations in their muscle tissues. In the present study, arsenic, lead and cadmium concentrations in muscle tissues of all studied fish species in all three seasons at two study sites were found under the WHO maximum permissible limits. However, As concentrations in water and sediment were higher than maximum permissible limit.

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INFESTATION OF VARROA MITE (*VARROA DESTRUCTOR*) IN HONEY BEE (*APIS MELLIFERA*) COLONIES AT SINTKAING TOWNSHIP, KYAUKSE DISTRICT

Nang Cherry Oo[1], Aye Aye Cho[2], Moe Nilar Tun[3]

Abstract

A total of 45 honey bee (*Apis mellifera*) colonies with adult bees and broods were investigated from November 2021 to April 2022 at Sintkaing Township, Kyaukse District. Those colonies were divided into non-treatment (15) colonies and treatment (30) colonies. Among the total bee populations of 728000 bees, a total sample of 42000 bees was observed, and it was found to be infested with some 759 varroa mites. The total number of estimated infested bees was 13422 individuals, with 2002.5 individuals from non-treatment colonies and 11419.5 individuals from treatment colonies. The infestation rates of the year 2022 were found to be higher than those of the year 2021. The highest mortality rate was 53% in January 2022 in non-treatment bee colonies. In this study, the mortality rate of bees may be correlated with the infestation of varroa mites, as well as food and the use of insecticide on the plantation.

Keywords honey bee, mite, ectoparasite, infestation, *Varroa destructor*

Introduction

Honeybees are the most efficient pollinators of 80% of the crops worldwide, and farmers prefer their services because they greatly improve crop yields. The decline of pollinators in recent decades is threatening the structure and function of natural and agricultural tree reproduction that require pollination assistance. Large-scale production of food crops in the agricultural system is, in many cases, only possible with the assistance of pollinators, primarily honeybees (USDA, 2009).

Most honey bee researchers consider the ectoparasitic mite *Varroa destructor* to be the most damaging enemy of the honey bee. It has been recently identified as one of the major factors responsible for colony losses worldwide (Broadschneider *et al*. 2010).

Honey bee health has become a primary focus of researchers in response to several episodes in which commercial colonies were lost at unusually high rates in the United State and Canada. Although not fully understood, high colony mortality stemmed from multiple factors that included the parasitic mite *Varroa destructor*, viruses vectored to bees by varroa mites, pesticide exposure, residues of agrochemicals in hives, and poor nutrition (Horris J *et al*., 2016).

Beekeepers control *Varroa* levels in colonies using synthetic acaricides, organic acids, essential oils, and a wide variety of management techniques, which has helped to improve survival rates (Rosenkranz *et al*., 2010).

Mite numbers increase slowly within a hive. It may not be until the fourth year of infestation that numbers are sufficiently high for honey bee larvae to be parasitized by several females when this occurs, newly emerged adult bees with deformed wings, legs, and abdomens may be found at the hive entrance. Patchy broad patterns may also be seen in advanced infestations. Colonies affected to this extent will usually die (Agriculture Victoria, 2022).

In this research, beekeeping sites in Sintkaing Township was chosen as a survey area to observe the occurrence of varroa mite disease in their bee colonies. Sintkaing Township

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contributes 15 percent of overall honey production, according to the Apiculture Division. Sunflower honey, palm honey, and sesame honey were highly demanded in local markets. This research will also inform the infestation of varroa mites in honeybee colonies at Sintkaing Township and their effects on local apiculture farms.

Materials and Methods

Study area

This study was conducted around latitude 21º 43' 59" North and Longitude 96º 7' 0" East with an elevation of 81 m above sea level in Sintkaing Township, Kyaukse District.

Study sites

The study sites were located at Development Apiculture Sub-department, Sintkaing Township. A total of 60 bee colonies were raised in this study sites.

Study period

The present study was carried out from November 2021 to April 2022.

Sample of bee collection

A total of 5 colonies were randomly selected from 45 colonies in each beekeeping in the study area. Samples were collected from November 2021 to April 2022. Adult bees were taken from both sides of three uncapped broad combs of 7 frames in each hive. Collected bee samples were kept individually in a bottle containing 30% ethanol and then the number of mites on the bee was counted.

The 45 colonies were divided randomly into 15 colonies of nontreatment and 30 colonies of treatment. Treatment colonies were applied with formic acid (35cc) in each colony weekly every month during the study period.

Monitoring and determining the infestation rate of bee colonies, and varroa population levels in honey bee colonies were generally estimated using two methods; (1) determining the number of mites per 100 bees (infestation rate) within a subsample of adult bees and (2) determining the colony varroa population using natural mite fall (Jack C *et al*., 2020). Infestation rates were determined by (1) collecting a subset of adult bees into a container, (2) removing the mites from the bees with a dislodging agent, and (3) counting the mites.

Methods of removing the mites from the bees

(1) Alcohol / Soap Wash method

This method involves adding isopropyl alcohol or soapy water to the container of bees, shaking the container to dislodge the mites, washing the mites from the bees, and counting the mites.

(2) Natural mite fall method

In a natural setting, *Varroa* may either be groomed off by the bee or naturally fall from the bees or combs through the action of natural hive activity. Consequently, one can sample varroa by collecting them from the below colony.

Visual signs of problematic infestation

There are several visual signs of problematic infestation, but visual inspections don't provide a reliable estimate of the potential risk to the colony; (1) decreased colony productivity, (2) abnormal or spotty brood pattern, (3) abnormal adult behavior, (4) the excessive number of

dead or discolored, sick, greasy-looking adult bees inside or outside the hive, (5) visual sightings of other pests or disease symptoms, (6) deformed adult wings and/or brood bodies, (7) failure to use supplemental food and or lack of "normal" honey bee bread reserves.

Calculation of bee population using the formula

According to Delaplane *et al*. (2013)

$$N = 3 \text{ x } \left(\frac{f}{0.0138} \right)$$

N = number of bees in the hive

F = number of bees seen leaving the nest in one minute

Note that the value of 0.0138 is based on the average amount of the spend foraging for an average honey bee colony on an average day, and that this value will actually change considerably depending on the amount of food available, weather conditions and so on.

In the present study the average amount of th spend foraging was 69 to 92 bees in one colony on an average day.

Data analysis

According to Jack. *et al*.(2020)

$$\text{Infestation} = \frac{\text{the number of mites captured}}{\text{the number of bees in sample}} \times 100\%$$

$$\text{Treatment thresholds by honey bee colony phase \%} = \frac{\text{Total mites}}{100 \text{ adult bees}}$$

Meteorologic data

Meteorological data of the study area was obtained from the Department of Meterological and Hydrology, Sintkaing Township.

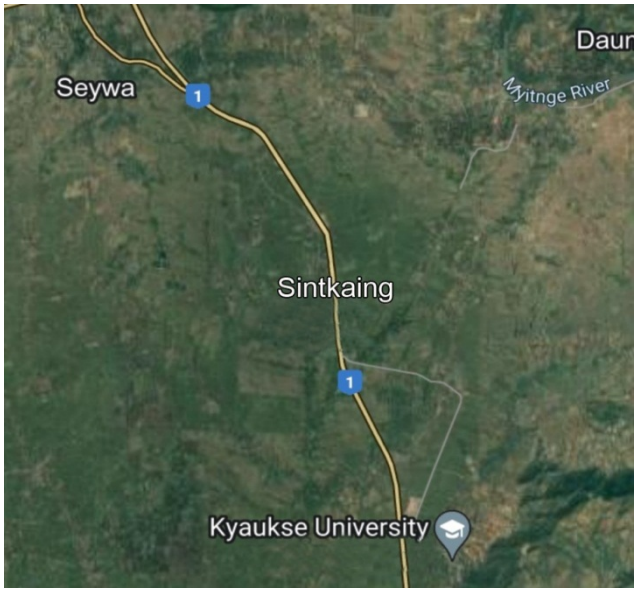

Figure 1 Location map of Sintkaing Township (Source: Google Map)

Results

The honeybee *Apis mellifera* under order Hymenoptera and the mite *Varroa destructor* belonging to order Mesostigmata were collected from the study area and identified.

The adult bees and brood with a total bee population of 162750 bees of non-treatment bee colonies and 565250 bees of treatment bee colonies from the 45 colonies were investigated. The total number of 10500 bees from non-treatment colonies and 31500 bees in treatment colonies were examined (Table 1 and 2).

Among them, a total number of 150 varroa mites in non-treatment colonies and 609 varroa mites in treatment colonies were collected. The total number of estimated infested bees of 2006.5 was collected from non-treatment colonies and 11419.5 bees from treatment colonies. (Tables 1 and 2).

Treatment thresholds by non-treatment bee colonies were observed at 0.83% and 0.94% (acceptable) respectively in November and December 2011, and 2.51% (danger) in January 2022 (Table 3).

Treatment thresholds by treatment bee colonies were observed at 0.43% and 0.66% (acceptable) respectively in November and December 2021. Infestation rates of 1.66% (caution) in January 2022, 2.1%, 3.67%, and 2.56% (danger) from February to April 2022 were recorded (Table 4) respectively.

The total infestation of the bee population of 2006.5 bees, the total dead population of 304 bees, and the highest mortality rate of 53% in January 2022 were recorded from non-treatment bee colonies (Table 5).

The total infestation of the bee population of 11419.5 bees, the total dead population of 1714 bees, and the highest mortality rate of 31.9% and 31.5% in March and April were recorded from treatment bee colonies (Table 6).

According to the meteorological data, the infestation rate of varroa mites was high from January to April 2022 (Table 7).

Table 1 Monthly infestation rate of non-treatment bee colonies during the study period

Months	Bee colony	Population of adult bees and broods	Total sample of bees observed	Total varroa mites collected	Estimate Infestation Bees	Infestation rate (%)
November	5	52500	3500	29	439	0.83
December	5	52500	3500	33	495	0.94
January	5	57750	3500	88	1072.5	2.51
February	-	-	-	-	-	
March	-	-	-	-	-	
April	-	-	-	-	-	
Total	15	162750	10500	150	2006.5	4.23

Table 2 Monthly infestation rate of treatment bee colonies during the study period

Months	Bee colony	Population of adult bees and broods	Total sample of bees observed	Total varroa mites collected	Estimate Infestation Bees	Infestation rate (%)
November	5	52500	3500	15	225	0.43
December	5	52500	3500	23	345	0.66
January	5	57750	3500	58	957	1.66
February	5	126000	7000	147	2646	2.1
March	5	136500	7000	187	3646.5	2.67
April	5	140000	7000	179	3600	2.56
Total	30	565250	31500	609	11419.5	10.93

Table 3 Treatment thresholds by non-treatment bee colony phase

Months	Colony phase	Acceptable	Caution	Danger
November	Population decrease	0.83%	-	-
December	Dorment without brood	0.94%	-	-
January	Dorment with brood	-	-	2.51%
February	Population increase	-	-	-
March	Population increase	-	-	-
April	Peak population	-	-	-

Colony phase

* Acceptable - Futher control not needed (<1-2%)
* Caution - Control may be warranted (1-5%)
* Danger - Control Promptly (>2-5%)

Table 4 Treatment thresholds by treatment bee colony phase

Months	Colony phase	Acceptable	Caution	Danger
November	Population decrease	0.43%	-	-
December	Dorment without brood	0.66%	-	-
January	Dorment with brood	-	1.66%	-
February	Population increase	-	-	2.1%
March	Population increase	-	-	2.67%
April	Peak population	-	-	2.56%

Table 5 The mortality rate of non-treatment bee colonies during the study period

Months	Total no. of bee infested	Total no. of bees dead	Mortality rate (%)
November	439	69	23
December	495	74	24
January	1072.5	161	53
February	-	-	-
March	-	-	-
April	-	-	-
Total	2006.5	304	100

Table 6 The mortality rate of treatment bee colonies during the study period

Months	Total infestation of bees population	Total dead Population of bees	Motality rate (%)
November	225	34	1.98
December	345	52	3.03
January	957	144	8.4
February	2646	397	23.1
March	3646.5	547	31.9
April	3600	540	31.5
Total	11419.5	1714	100

Table 7 Meterological data and infestation rate during the study period

Months	Minimum temperature (ºC)	Miximum temperature (ºC)	Average rainfall (mm)	Humidity (%)	Infestation rate (%)
November	18	28	10	65	0.83
December	11	25	28	70	0.94
January	10	24	8	56	2.51
February	7	24	4	36	2.1
March	8	29	4	30	2.67
April	16	32	42	37	2.56

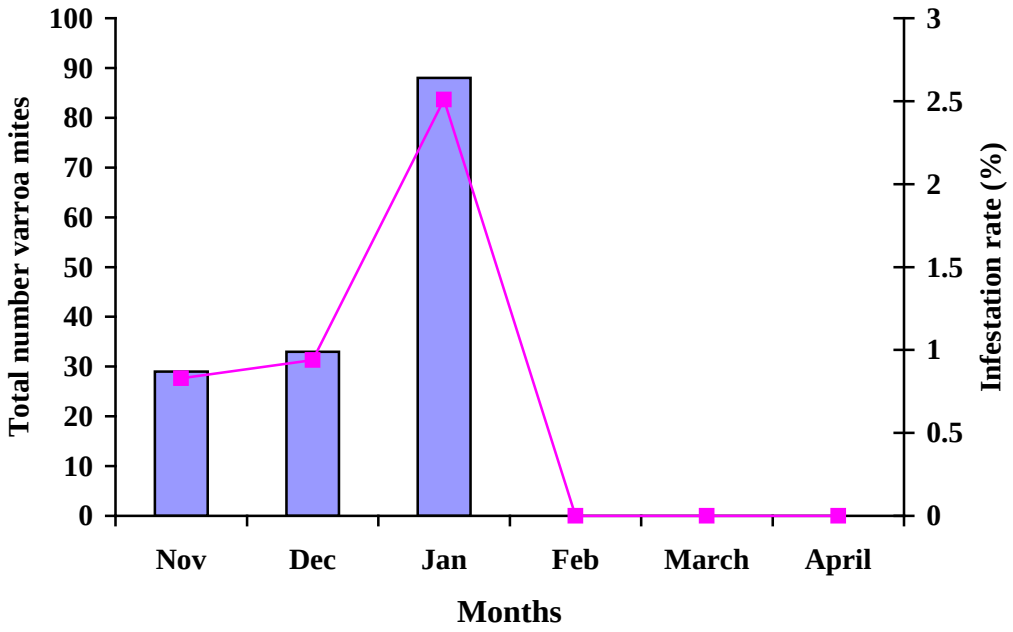

Figure 2 Monthly total number of varroa mites and infestation rate from non-treatment bee colonies

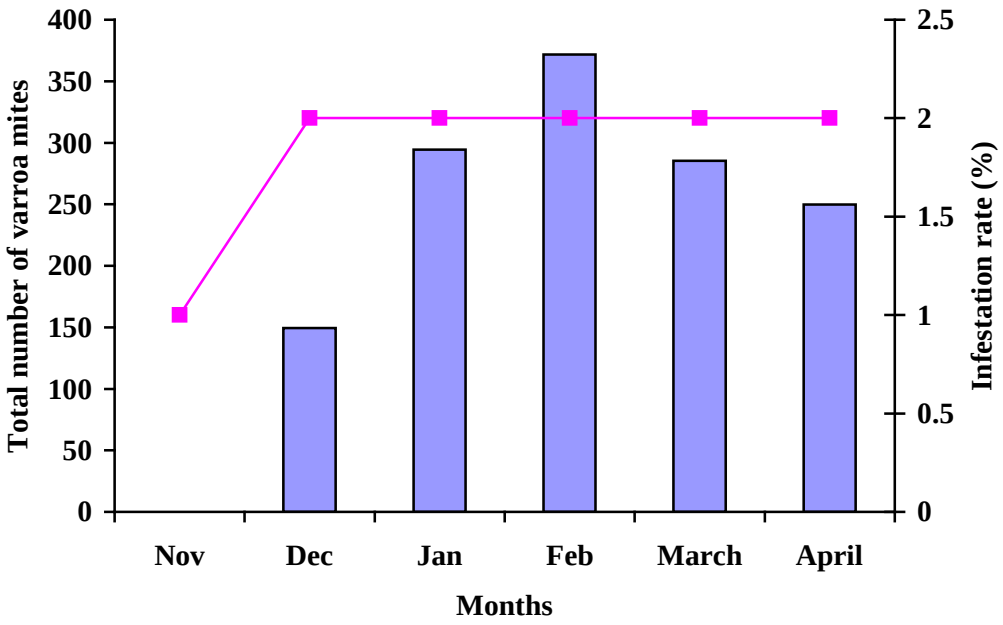

Figure 3 Monthly total number of varroa mites and infestation rate from treatment bee colonies

Plate 1 Varroa mite infestation and treatment thresholds by bee colony

Discussion

The honeybee *Apis mellifera* populations with reduced mite reproductive success may have unique ways of achieving this specific mite-resistant mechanism that could include changes in brood volatiles, adult VSH behavior selectively removing reproducing mites, or even both mechanism control (Barbara Locke, 2015).

Bees on frames containing brood comb had significantly more mites than frames without brood, but the difference is small biologically. Greater levels on brood combs were probably due to mites preferring nurse bees, which tend to stay on brood combs (Pernal *et al*., 2005).

In the present study, a total number of 45 bee colonies with adult bees and broods include non-treatment (15) colonies and treatment (30) colonies. Total population of 162750 bees with total sample of 10500 bees were found with total varroa mites 150 individuals and estimate infested bees 2006.5 individuals in non-treatment colonies. Total population of 565250 bees with total sample 31500 bees were found with total varror mites 609 individuals and estimate infested bees of 11419.5 individuals in treatment colonies. Infestation rate was highest in January, 2022 in non-treatment colonies and highest in January to April, 2022 in treatment colonies.

Mohmood *et al*., (2012) found that (3.2%) oxalic acid treatment had higher effectiveness in controlling *Varroa* than flornic acid and flumethrin strip (Bayvarol). Thus, oxalic acid was used in control treatment. Botreinic *et al*., (2001) concluded that formic acid had significant effectiveness against varroa for honey bee colonies in Iran.

In the present study, treatment threashold by non-treatment bee colony phase was observed at 0.83% and 0.94% (acceptable) in November and December 2021 and (2.15%, danger) in January 2022. Treatment thresholds by treatment bee colony phase was observed at 0.43% and 0.66% (acceptable) in November and December 2021, infestation rate (1.66%, caution) in January 2022 and (2.1%, 3.67% and 2.56%, danger) from February to

April 2022. The results indicated that bees from treatment colonies had more resistance to mites infestation than non-treatment bee colonies.

The causes of the recorded mortality of honeybee colonies remains undetermined, most scientists agree that it is likely due to a combination of several factors ranging from viruses, parasites and diseases, single-source diets, compromised disease, inclement weather and pesticides (Stankus, 2008).

In the present study, the total dead population of 304 bees and the highest mortality rate of 53% in January 2022 were recorded from non-treatment bee colonies. The total dead population of 1714 bees and the highest mortality rate of 3.19% and 31.5% in March and April 2022 were recorded from treatment bee colonies respectively. The results indicated that total infestation of the bee population was correlated with invading of varroa mite disease and their food resources.

Conclusion

The present study emphasized the need for research attention to focus on sustainable solution to the threat of *Varroa* mites for the economic viability of apiculture and agriculture, as well as for honeybee health, conservation, and ecosystem services. Understanding the natural interactions and adaptations between honeybee and *Varroa* mites is an essential for beekeeper. In this study, the mortality rate of bees may be correlated with the infestation of varroa mites, as well as food and the use of insecticide on the plantation.

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ALTERNATIVE PROTEIN SOURCE FOR AQUAFEED PRODUCTION FOR CATFISH, *PANGASIUS HYPOTHALAMUS* (SAUVAGE, 1878)

Kalayar Win Maung[1], Khin Thuzar Win[2], Saw Marlar Than[3], Tin Lay Mon[4], Kay Lwin Tun[5]

Abstract

Silkworm pupae, a waste of the silk industry, are produced in large quantities in Kayin State and Mandalay region of Myanmar and are often discarded to the surrounding environment or used as a fertilizer for plant growth. In the present study, silkworm pupae were used as an alternative animal protein to replace fishmeal used as experimental feed for catfish, *Pangasius hypothalamus*. The feeding trial had three treatments: (1) silkworm pellet (SWP) with 26% of crude protein; (2) commercial floating pellet (CFP) with 32% protein, and (3) commercial sinking pellet (CSP) with 28% protein. Silkworm pellet (SWP) was formulated and produced using a small extruder in the laboratory. Total 180 fingerlings of catfish divided into six groups were stocked in glass aquariums providing two replication tanks for each treatment. The fish were cultured for 3 months from October to December 2019 in the laboratory condition. Among the three different diets, the highest growth rate was observed in CFP with (3.9 %/ day) and the second highest growth rate was recorded in SWP with (3.7 %/ day). Feed Conversion Ratio (FCR) of SWP, CFP and CSP were 3.37, 2.8 and 4.05 respectively. The weight gains (WG) of CFP, SWP and CSP were 10.57, 9.36 and 8.43, respectively at the end of experiment. Only 3% of mortality was found in the tank treated with CFP. This study revealed that the silkworm had very high protein content (52%) and it could be considered as an alternative dietary supplement for catfish.

Keywords silkworm pellet, floating pellet, sinking pellet, *Pangasius hypothalamus*

Introduction

In the compound fish feed, fish meal is primarily used as a protein supplement (Fisheries Centre Research Reports, 2016). About 56% of fish meal was used to feed farmed fish, 20% was used in pig feed, 12% in poultry feed, and 12% in other uses, which included fertilizer. (Miles *et al*., 2015).

Fishmeal can be prepared from almost any kind of seafood, but is generally produced from wild-caught, small marine fish that comprise a high ratio of bones and oil, and are usually supposed to be not appropriate for direct consumption of human. The fish caught for fishmeal drives exclusively are termed "industrial". Fish meal was produced from by catch and byproducts of extras made during processing of various seafood products intended for human consumption (Miles *et al*., 2015).

Small fish will be lost in the trophic level because of high demand of fishmeal manufactured so we need to maintain small fish for sustainability in natural water bodies in Myanmar. Using cultured insect meal instead of fishmeal in the preparation of fish feed is one of the important requirement to develop the sustainable aquaculture. Nowadays, potential inset meals have been considered to be used in aquaculture sector.

Sericulture has been accomplished in many developed and developing countries in the world for many years ago. Among those countries, Japan, Korea, Brazi, China, Thailand, India, Turkey, Iran, and Myanmar were well-known silk industry (Barber,1992). Since 7th century, sericulture was started and recent sericulture was known in 1952 (Barber, 1992). In Myanmar, silkworm (*Bombyx mori*) rearing has been practiced from the earliest time in rural zone and it is still completed as a cottage type in a traditional way. The spent pupae are produced in large quantities and are a major by-product in silk production (Datta, 2007). Eight kilogram (8 kg) of wet pupae can produce one kilogram (1 kg) of raw silk (Patil *et al*., 2013). Pupae are waste material often rejected in the open environment or used as fertilizer in silk production in

Karen State and Mandalay Division in Myanmar. It can be used as one of the ingredients for making fish feed.

The by-product pupa has high nutrition level especially for the source of protein (Hiroyuki *et.,al*, 2010). If we can substitutes by-product pupae in aqua feed production, the natural fish resource can be managed in terms of sustainable fisheries production.

In the present study, the nutritional value of spent pupae was studied and aimed to understand basic nutritional value of by-product**.** Then, aqua feed for catfish was produced using by pupa.

Materials and Methods

Study site and study period

The experiment was conducted in the Aquatic Bioscience Laboratory at the University of Yangon in Myanmar for six months during September 2019 - February 2020.

Experimental fish and design of fish tank

Catfish (*Pangasius hypothalamus*) (Sauvage, 1878) was selected for the present study. A total of 180 fingerlings of the same batch (BW 7.6±1.58g) were purchased from the hatchery of Department of Fisheries at Hlawgar in Yangon Region. The fish were acclimatized into laboratory condition for one week before the experiment and divided into six groups.

Six glass tanks (1m x 0.5m x 0.5m) with 122 liters/tank of water volume were prepared and set up aeration system to maintain dissolved oxygen in the tanks before experiment. All of the fish were starved for 24 hours before the experiment. After 24 hours, random collected 30 fingerlings were weighed and measured for initial data, which were then stocked in each glass tank (Plate. 1). The experimental feed were introduced into the study fish tank with 5% of body weight and two times per day (10:00 AM and 4:00 PM). The water exchange was done with two days interval and water quality parameters such as temperature, pH, dissolved oxygen and ammonia were analyzed on initial day and subsequently at fortnightly interval by standard methods. The fishes in all treatment were measured and recorded the weight after 30 days intervals to analyze the growth performance of the study fish.

Analysis of nutritional value

The experimental fish feed was prepared before the experiment. The spent pupae were dried in oven with temperature (100 ºC). Dried pupae were grinded into powder and their nutritional values such as crude protein, fat, carbohydrate, moisture, ash and fiber were measured at the laboratory in UMFCCI (Union of Myanmar Federation of Chambers of Commerce and Industry) (Table 1).

Preparation of silkworm feed

The silk worm pallet was produced based on the feed formula for catfish. All ingredients (silkworm powder, wheat flour, cassava, vitamin C, carboxymethyl cellulose, butylated hydroxytoluene, guar gum and fish oil) were mixed by the ratio of protein percentage for this experiment (Table 2). Silkworm pellet was produced by a handmade pellet machine (Plate 1). Three different kinds of experimental feed such as silkworm pellet (SWP) with 26% of protein, Floating pellet (CFP) with 32% protein, sinking pellet for control feed (CSP) with 28% protein. Two replications for each experiment were conducted simultaneously.

Growth Parameters and data analysis

The growth performance of experimental fish weight gain (WG), specific growth rate (SGR), percent weight gain (PWG), feed conversion ratio (FCR) and gross conversion efficiency were calculated according to the following formulas.

Weight Gain

The body weight of *Pangasius hypothalamus* (Sauvage, 1878) fingerling was obtained initially and thereafter at thirty days interval up to completion of the experiment i.e. 60th days.

The weight gain (g) was calculated as given below:

Weight gain (WG)

Final weight (g) - Initial weight (g)

Specific Growth Rate (SGR)

$$SGR\ (\%) = \frac{\text{Ln (final weight in grams)} - \text{Ln (initial weight in grams)}}{\text{Experimental days}} \times 100$$

Percent weight gain (PWG)

$$PWG\ (\%) = \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{Initial mean weight (g)}} \times 100$$

Feed Conversion Ratio (FCR)

$$FCR\ (\%) = \frac{\text{Weight of food intake (g)}}{\text{Weight gain of fish (g)}}$$

Table 1 Laboratory analysis of nutritional value of silkworm powder

Test Parameter	**Test Method**	**(%) of compound**
Moisture	AOAC-2000(930.15)	7.27
Ash	AOAC-2000(942.05)	4.04
Crude Protein	AOAC-2000(920.152) (Kjeldahl Method)	52
Crude Fibre	AOAC-2000(978.10) Fiber Cap Method	0.83
Crude fat (Ether Extract)	AOAC(Buchi Soxhlet Method)	29.62
Carbohydrate	By Difference	6.24
Energy value (kcal/100g)		502

Table 2 Experimental feed ingredients with silkworm powder meal

Ingredients	**Percent of composition (%)**
Silkworm	39.49
Wheat flour	48.26
Cassava	7.4
Vitamin C	2
Carboxymethyl cellulose(CMC)	0.33
Butylated Hydroxytoluene (BHT)	0.02
Guar gum	0.5
Fish oil	2

(A) Measuring of fish

(B) Weighing of fish

(C) Experimental tank

(D) Dried Silkworm

(E) Powdered silkworm

(F) Preparation of silkworm paste

(G) Extruding of fish pellet

(H) Cutting of pellet

(I) Silk worm fish pellet

Plate 1 Study species and making fish feed pellet using silkworm

Results

Preparation of formulated fishmeal

The proximate composition of silkworm pupae was shown in Table 1 and it contained 29.62, 0.83, 7.27, 4.04, and 6.24 of crude protein, crude fat, crude fiber, ash and carbohydrate respectively. The energy content was estimated to be 5.02 kcal /g.

The composition of experimental feed ingredients was shown in Table 2. The silkworm fish feed (SWP) was formulated with 26% of protein. The protein contents of CFP (commercial floating pellet) and CSP (commercial sinking pellet) were 32% and 28%, respectively.

Different diet comparison of SGR and FCR

The data on specific growth rate, survival and feed conversion ratio are presented in Table 3. Among three different diets, the highest growth rate was observed in floating pellet (CFP) with SGR (3.9±0.11). Feed Conversion Ratio (FCR) was recorded as (2.84±0.36) from two replications. The lowest growth rate of this experiment was observed in commercial sinking pellet with SGR (3.5±0.15%), the feed conversion ratio FCR was recorded as (4.05±0.95) from two replications. (Table 3)

Table 3 Mean initial body weight, FCR and SGR of three different diets

	SWP	**CFP**	**CSP**
Initial weight	8.05 ± 0.63	7.2 ± 0.56	8.5 ± 1.69
Final weight	17.41 ± 0.57	17.77 ± 0.16	16.93 ± 0.89
SGR	3.72 ± 0.007	3.9 ± 0.11	3.5 ± 0.15
WG	9.36 ± 0.05	10.57 ± 0.73	8.43 ± 0.8
FCR	3.37 ± 0.16	2.84 ± 0.36	4.05 ± 0.95
PWG	116.66 ± 9.9	147.66 ± 21.8	102.15 ± 29.8
Survival rate (%)	100	97	100

Note: SWP = Silkworm pellet, CFP= Commercial floating pellet,
CSP= Commercial sinking pellet

In the experiments, highest and second highest weight gains were resulted in fish treated with commercial floating pellet and silkworm pellet respectively. The lowest weight gain was found in control (commercial sinking pellet) (Figure 1).

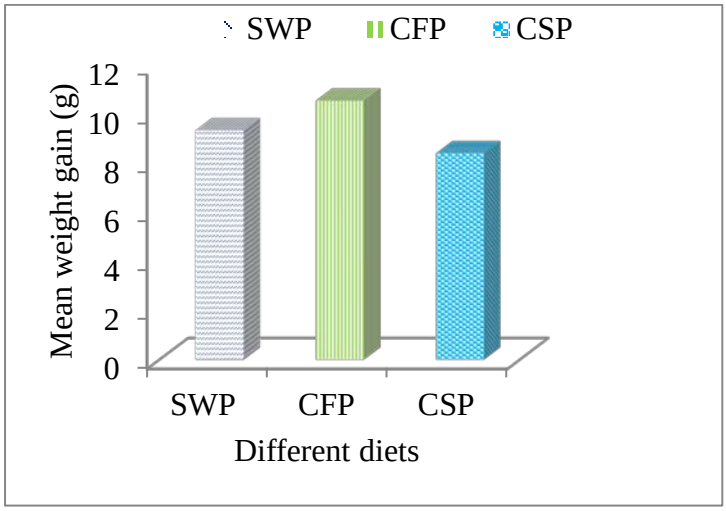

Figure 1 Mean weight gain of three different diets

The weight of fish in all experiments gradually increased during the study period. In the end of experiment, the weight of fish was 17.41±0.57, 17.77±0.16 and 16.93±0.89 in silkworm pellet (SWP), commercial floating pallet (CFP) and commercial sinking pellet (CSP) respectively (Figure 2).

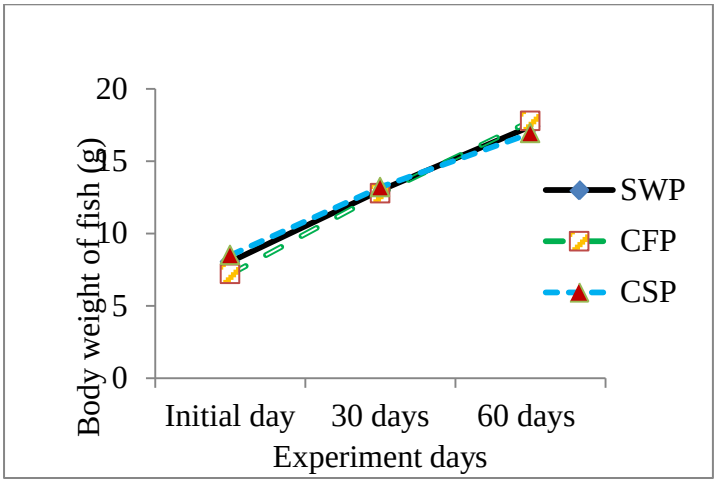

Figure 2 Comparison of mean initial and final weights

The lowest FCR values with the highest SGR % were recorded in fish feed with commercial floating pellet (CFP). The highest FCR values with lowest SGR % were recorded in fish feed with commercial sinking pellet (CSP). The weight of fish fed with the silkworm pellet (SWP) was followed by that of the commercial floating pellet (Figure 3 & 4).

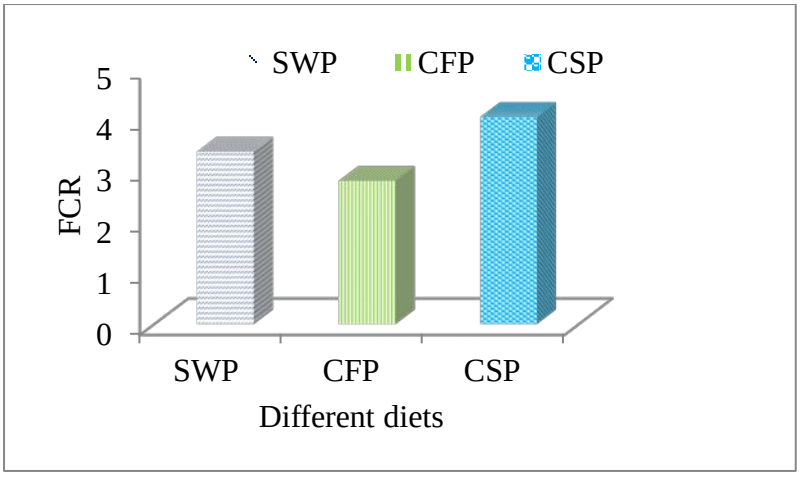

Figure 3 Comparison of feed conversion ratio (FCR) of three different diets

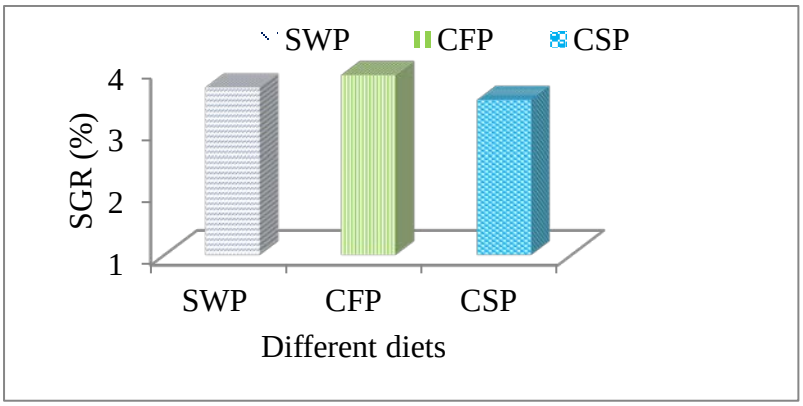

Figure 4 Comparison of specific growth rate % (SGR) of three different diets

Survival rates of three different diets were not differ during the study period except 97% in replicate 1 of commercial floating pellet as shown in table (4).

Table 4 Survival rate of each tank with three different diets during the study period

Different feed	Number of fish (initial)	Number of fish (30days)	Number of fish (60days)	Survival rate (%)
SWP	30	30	30	100
CFP	30	30	29	97
CSP(control)	30	30	30	100

During the experiment, pH, DO, Ammonia and temperature were recorded fortnightly and water exchange had regularly done two times per week. The results of water parameter in the tanks were shown in table (5).

Table 5 Water parameter range of three different diets during the study period

Different diets	Ammonia	pH	DO	Temp
SWP	0.2-0.6	5.5-6.5	6 - 7	22-23.8
CFP	0.6-1	5.5-6.5	6 - 7	22-23.8
CSP (control)	0.6-1	5.5-6.5	6 - 7	22-23.8

Discussion

Aquaculture is the fastest increasing area of world human food production and has a yearly increase of about 10% (FAO, 1997). The use of insects as a source of protein for fish nutrition was a relatively new approach (Bondari and Sheppard, 1981). Various development stages of insects have been used to feed fish and farm animals. Hickling (1962) noted that silkworm pupae have been an important component of carp diet in Japan and China (Newton *et al*., 1977). Therefore, efficient feed formulation should be made by utilizing the knowledge on the nutritional requirements and availability of local feed ingredients, diet palatability, acceptability and digestibility capacity of fish.

During the study period, the three different diets were used in the experiments. This study found that all treatments had increasing weight gain throughout the duration of the experiment. That proved that the catfish reacted favorably to all of the diets. Among them, fish fed commercial floating pellet containing 32% of protein had the highest growth and weight gain. The silkworm pellet containing 26% of protein had the second highest growth rate and the lowest growth rate 28% of protein content. The experimental fish feed with silkworm as the sole animal protein source contained only 26% crude protein. However, growth and FCR was comparable to commercial feeds containing either 32 or 28% crude protein. In contrast, previous research studies with different fish species recommend dietary inclusion levels of silkworm pupae meal in the diet as follows: 30-50% for major and minor carps, 5-15% for trout, 50-60% for masher, 75-100% for catfish, 30-40% for ornamental fishes and 5-20% for shellfishes. These inclusions assured to give better growth performance compared to sole fishmeal as the protein source.

During the study period, the silkworm pupae diet we employed in our research contained only 26% protein. Abdullo *et al.* (2015) studied that effect of the replacement of fish meal with silkworm pupa protein on the growth of *Clarias gariepinus* fingerlings, and concluded that a 50:50 ratio of silkworm pupae and fish meal was appropriate. The authors studied that the growth rate was higher in the group fed diets containing a mixture of fishmeal and silkworm pupae, while it was lower in the group fed diets containing only fishmeal or silkworm pupae at 100%. These findings demonstrated that feeding silkworm pupae in part place of fishmeal resulted in higher growth performance than feeding fishmeal alone. According to Faturati *et al.* (1986), and Akiwanda *et al*. (2002), feed with a protein concentration of 39–41% is optimal for feeding catfish. So, the current findings were insufficient to justify replacing fishmeal with silkworm pupa protein in the diet.

It is advised to replace 50% of the fish meal in the diet with silkworm pupae in order to promote appropriate growth and protein utilization. Our research revealed that this protein can be used in aquafeed, and that doing so will improve the rationality of silkworm rearing and supply high-quality sources of animal protein for aquafeed production. The findings of our experiment point out that silkworm pupa protein can be used as an alternative to fish meal in the diet of catfish.

Conclusion

Insect meal is one of the best protein sources for partial or total replacement of fishmeal in aquaculture feeds. This is mainly due to good amino acid and fatty acid profiles. Moreover, insects are natural food sources for fish. The present study revealed that, the SWP was very rich source of proteins, lipids and minerals so could be used as an alternative dietary supplement in fish feed. According to the experiment, fish fed with silkworm pellet had a comparable growth rate, and relatively low FCR was found by the control (sinking pellet). Therefore, silkworm pellet was suitable for fish culture to reduce feed cost and increase yield and income. When silkworm pupae are locally available, fish farmers can utilize the pupae as an excellent local feed ingredient to replace fishmeal in the pelleted feed and reduce feed cost.

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ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM* STRAINS FROM THE ROOT NODULES OF TWO SELECTED LEGUMES*

Phoo Wint Yee Thaw[1]

Abstract

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). The present study was conducted to isolate and characterize the rhizobia from the root nodules of *Vigna mungo* (L.) Hepper (Black gram) and *Vigna unguiculata* subsp. *unguiculata* (L.) Walp. (Cowpea). The experiment was performed at the Microbiology Laboratory, Department of Botany, University of Yangon in 2022. The two isolated bacterial strains were given named as (PVMR from black gram.) and (PVUR from cowpea). Both isolates on YEMA medium showed white, circular, entire margin, convex elevation, produced mucoid and translucent colonies. The microscopic examination revealed that both isolates are gram negative and rod shaped. In two bacterial strains: PVMR was identified as *Rhizobium* sp. and and PVUR was identified as *Bradyrhizobium* sp. based on the results of authentication test. Biochemical characterization of both strains showed positive reaction with catalase, methyl red test, nitrate reduction, and urea test, while negative reaction was observed in citrate utilization, gelatin hydrolysis, hydrogen sulphide production, indole, Triple Sugar Iron Agar (TSI), and Vogas Proskauer test. In antibiotic resistance test, PVMR strain resistance to only Penicillin and sensitive to Amoxicillin, Chloramphenicol, Tetracycline, whereas, PVUR strain resistance to Amoxicillin, Chloramphenicol, Penicillin but sensitive to Tetracycline. The plant inoculation assay indicated the improvement of plant growth and nodules formation in the treatments inoculated with two rhizobial isolates compared with non-inoculation treatments.

Keywords root nodules, *Rhizobium*, *Bradyrhizobium*, black gram, cowpea

Introduction

Nitrogen is one of the most important elements in atmosphere (approximately 80%), that is used to make various products that plant require for their development. Nitrogen deficiency is a limiting factor to plant growth and has significant ecological and agricultural implications. The extensive use of synthetic nitrogen fertilizers to get high yield is not only expensive but also a threat to the environmental balance and contributes to global warming (Vitousek, 1997). Therefore, eco-friendly and cost-effective agro-technologies to increase crop production are required including the process of biological nitrogen fixation, which can be done by certain soil microbes both symbiotically and as well as non-symbiotically. The ability to fix atmospheric nitrogen comes from the symbiotic relationship between legumes and rhizobia (Howard and Rees, 1996).

Biological nitrogen fixation (BNF) is a natural process where rhizobia and leguminous plants with nodules in their root systems are able to convert the nitrogen gas into a form that is usable for plant life. The symbiotic relationships between leguminous plants and rhizobia have a great importance to agricultural production and reduces the requirement for nitrogenous fertilizer (Dilworth and Parker, 1969; Hunter *et al*., 2007). This association between the host plant (legumes) and rhizobia is mutually beneficial. *Rhizobium* is a well-known group of bacteria that acts as the primary symbiotic fixer of nitrogen. Bacteria of family Rhizobiaceae, including six genera namely *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium* are symbiotic and effectively convert atmospheric nitrogen to usable forms which is utilized by the host (Okazaki *et al*., 2004). According to the growth rate

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* Best Paper Award Winning Paper in Botany (2022)

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(generation time) on laboratory media, rhizobia, are broadly classified into two groups, fast grower and slow grower. Fast growers (generation time < 6 hrs) refer to those rhizobia associated with bean and pea whereas, slower growers (generation time > 6 hrs) refer to those rhizobia associated soybean and cowpea (Jordan and Allen., 1974; Paudyal, 2002).

Leguminous plants possess a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae. Among the legume crops, black gram and cowpea are economically important pulse in Myanmar. Black gram (*Vigna mungo* L. Hepper) is one of the main leguminous crops that provide chief source of food. It is rich in proteins and contains amino acids higher quantities than any other cereals and pulses. Black gram fulfills major part of nitrogen requirement by symbiotic nitrogen fixation with the help of bacterium called Rhizobia (Pareek et al, 1978). Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most ancient crops and an important source of food, income and livestock feed as well as forms a major component of tropical farming systems due to its ability for improvement of soil fertility through nitrogen fixation and as a cover crop (Sanginga *et al*., 2003). In addition, Antova *et al*., 2014 stated that cowpea has a unique ability to fix atmospheric nitrogen under stressed conditions. The cultivation of cowpea stimulates the proliferation of rhizobia in a field for their ability to enhance soil rhizobia populations (Mulongoy & Ayanaba, 1986).

Nitrogen fixing leguminous plants not only supports plant growth but also improve soil nitrogen status for associated crops. The rhizobial inoculants are now widely used in various parts of the world because they are inexpensive, environment-friendly, and easy to use without side effects and improve crop production (Tena *et al*., 2016). Therefore, rhizobium inoculation has become a popular agronomic practice to provide adequate amounts of nitrogen to leguminous plants, instead of the use of nitrogenous fertilizer. For this reason, the present research was aimed to isolate rhizobial strains from the root nodules of two different selected legume plants, to conduct the authentication test for rhizobial isolates which is differentiate from other contaminating microbes, to identify the rhizobial strains using some biochemical tests, to determine the antibiotic susceptibility test of isolated rhizobial strains and to evaluate the plant growth parameters of two rhizobial strains by plant inoculation assay.

Materials and Methods

1. Sample collection and isolation of bacteria from root nodules

The root nodules of *Vigna mungo* (L.) Hepper (Black gram) were collected from Hinthada Township, Ayeyarwaddy Region and *Vigna unguiculata* subsp. *unguiculata* (L.) Walp. (Cowpea) was collected from Hmawbi Township, Yangon Region. The collected root nodules were washed in running tap water to remove the adhering soil particles. The healthy and undamaged nodules were detached from the roots. For the surface sterilization, the nodules were washed with distilled water and sterilized with 70% ethanol for 30 seconds, followed by 1% of sodium hypochlorite solution for 2-3 minutes. Then, rinsed thoroughly with sterilized distilled water (3-5 times) to remove the chemicals. The sterilized nodules of different legumes were crushed with sterile glass rod or forceps in the microcentrifuge tube containing 0.5 ml of N- saline (0.85% NaCl). Then, one loopful of the nodule suspension was streaked on petri plates containing yeast extract mannitol agar (YEMA) medium supplemented with 0.0025% (w/v) congo red as an indicator. The plates were incubated for 3-5 days at room temperature. The purified cultures were maintained on YEMA agar slants, stored at 4°C in refrigerator for further study (Singh *et al*., 2008, Vincent, 1970).

2. Morphological and Microscopical characterization

The colony morphology of bacterial isolates including colony color, form, elevation, margin, mucosity, opacity was determined by observing the colonies on YEMA plates with congo red indicator after incubation period at room temperature. The microscopical characterization was conducted by gram staining.

Gram Staining

The pure cultures of bacterial strains were selected for more specific identification of the colonies. For gram staining, 24 hours old culture was evenly smeared on a clean slide and gently heated to fix by passing over a Bunsen burner. First, stained with crystal violet for 1 minute and second, immersed in Gram's iodine for one minute. Then, ethanol was applied for 30 sec and the counter stain safranine was applied as the final step. Followed by a gentle wash with distilled water after finishing each step. The slides were air dried and examined under the microscope. Gram-negative bacteria retain the pink/red colour while Gram-positive bacteria retain the crystal-violet (Somasegaran and Hoben, 1994).

3. Authentication Tests

Five different authentication (confirmatory) tests such as growth on YEMA with Congo red, Bromothymol blue test, Glucose- peptone agar test, Keto-lactose test, and Hoffer's alkaline test were performed to confirm the isolates as rhizobia.

(i) Growth on YEMA with Congo red

The rhizobial isolates was streaked on CR-YEMA medium (congo red yeast extract mannitol agar) and observed for absorption of congo red dye (Somasegaran *et al*., 1994).

(ii) Bromothymol blue Test

The bromothymol blue test was performed to differentiate between fast and slow growers of *Rhizobium* species (Vincent,1970).

(iii) Growth on Glucose Peptone Agar (GPA)

GPA test was performed to determine the capability of the *Rhizobium* strains to utilize glucose as the sole carbon for its growth medium (Singh *et al*., 2008).

(iv) Keto-lactose Test

The isolates were streaked on the medium and incubated for 2-3 days. Then, Benedict's reagent was added on the plates and kept at room temperature for 1-2 hours (Bernaertz and Daley, 1963).

(v) Hoffer's alkaline Test

The test was conducted to determine the difference between *Agrobacterium* which grows at higher pH level and *Rhizobium* which unable to do so. The isolates were inoculated in Hoffer's alkaline broth and observed the bacterial growth after 24 - 48 hours (Hofer, 1935).

4. Biochemical characterization

Different Biochemical characterization test such as catalase test, citrate utilization test, gelatin hydrolysis test, hydrogen sulphide production test, indole test, methyl red (MR) test, nitrate reduction test, Triple Sugar Iron Agar (TSI) test, urea test, and vogas proskauer (VP) test was done for the identification of *Rhizobium* strains (MacFaddin 2000, Koser, 1923, Sadowsky *et al*., 1983, Vincent,1970)

5. Determination the Antibiotic Susceptibility

The two isolated bacterial strains were test for antibiotic sensitivity by disc diffusion on YEMA agar medium. As described in Lupwayi and Haque (1994), the stock solution of each antibiotic was prepared by dissolving 2g of each antibiotic in 100 ml of water. 200 µl of actively grown cultures of each *Rhizobium* was spread on the YEMA medium using a sterilized cotton swab. Antibiotic discs with the different concentration (30µg/ml for Chloramphenicol and Tetracycline) (10µg/ml for Amoxicillin and Penicillin) were placed equidistantly at the center of plates and incubated overnight at room temperature. The sensitivity or the resistances of *Rhizobium* isolates to antibiotics were determined by observation of absence or presence of growth around the discs. The isolates which showed growth around a particular antibiotic are resistant to that corresponding antibiotic, whereas the isolates whose growth is inhibited by a particular antibiotic seem to be sensitive to that antibiotic.

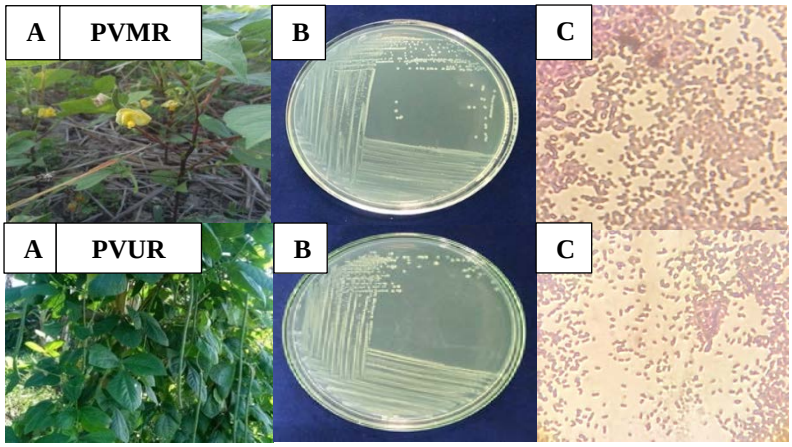
6. Evaluation the effect of two *Rhizobium* strains on plant growth

The plant inoculation experiment, including all the process (inoculum and seed preparation, inoculation test and experimental design for plant assay) were performed. The soil was sterilized by autoclaving at 121°C for 15 minutes. The two *rhizobium* isolates were grown in 50 mL falcon tube containing 20 mL of YEMA broth and incubated in a shaking condition at 150 rpm for 3 days at room temperature (Somasegaran and Hoben 1985). The similar sized seeds of cowpea and black gram were surface sterilized with distilled water and placed on the sterilized paper for 2-3 days. The good germinated seedlings were selected and transferred to the plastic cup (top) containing autoclaved soil and each cup containing 3 seeds were inoculated with 1 ml of bacterial solution with different concentration. The nitrogen-free nutrient solution B & D (Broughton and Dilworth, 1970) were supplied into the plastic cup (bottom) for all treatments. The three inoculation treatments were performed: 1. no inoculation (control); 2. 10^{-1} cfu/ml, and 3. 10^{-5} cfu/ml respectively. These cups were kept in a place full of sun light for proper growth. Plant weight, plant height, and nodule number of both plants were evaluated after 15 days of post-inoculation (Vincent, 1970).

Results

1. Morphological and Microscopical characterization of isolated bacteria

The two bacterial strains (one from each legume) were isolated from the root nodules of two selected legumes plant. The two strains were designated name as PVMR for *Vigna mungo* L. (Black gram) and PVUR for *Vigna unguiculata* subsp. *unguiculata* L. (Cowpea). Colonies of both isolates showed similar morphology and produced white, circular, entire margin, convex elevation, and mucoid colonies when grown on YEMA plates (Figure. 1B). Gram's staining of the isolates was confirmed by microscopic observations and both isolates were found to be gram negative, and rod shaped (Figure. 1C).



PVMR= *Vigna mungo* L.; PVUR= *Vigna unguiculata* subsp. *unguiculata* L.

Figure 1 (A) Habit of plants; (B) Rhizobial colonies on YEMA medium; (C) Gram staining of isolates under the microscope

2. Authentication tests

Table 1 Authentication tests of two *Rhizobium* strains

Confirmatory Tests	**Results**	
	PVMR	**PVUR**
Growth on Congo red medium	White	White
Bromothymol Blue test	Yellow/ Fast grower	Blue/ Slow grower
Growth glucose peptone agar (GPA)	No growth	No growth
Production of ketolactose test	Negative	Negative
Hoffer's alkaline test	No growth	No growth

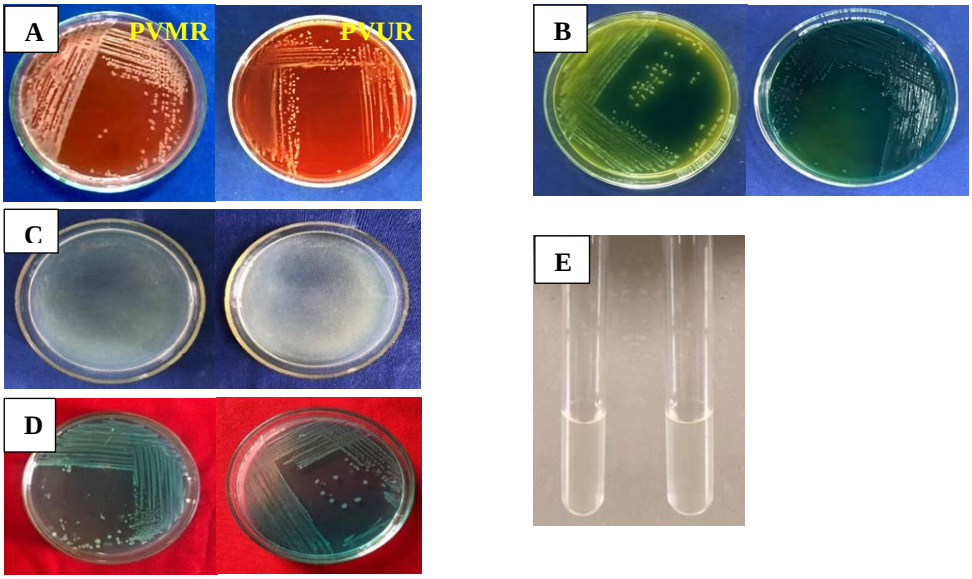


Figure 2 (A) *Rhizobium* colonies on CR-YEMA; (B) Bromothymol Blue test; (C) Glucose Peptone Agar (GPA) test; (D) Keto-lactose test; (E) Hoffer's alkaline Test

3. Biochemical Characterization

Table 2 Biochemical Characterization of two *Rhizobium* strains

Biochemical Tests	Results	
	PVMR	PVUR
Catalase test	+	+
Citrate utilization test	-	-
Gelatin hydrolysis test	-	-
Hydrogen sulphide production test	-	-
Indole test	-	-
Methyl red (MR) test	+	+
Nitrate reduction test	+	+
Triple Sugar Iron (TSI) test	-	-
Urea Test	+	+
Vogas Proskauer (VP) test	-	-

+ (positive test), - (negative test)

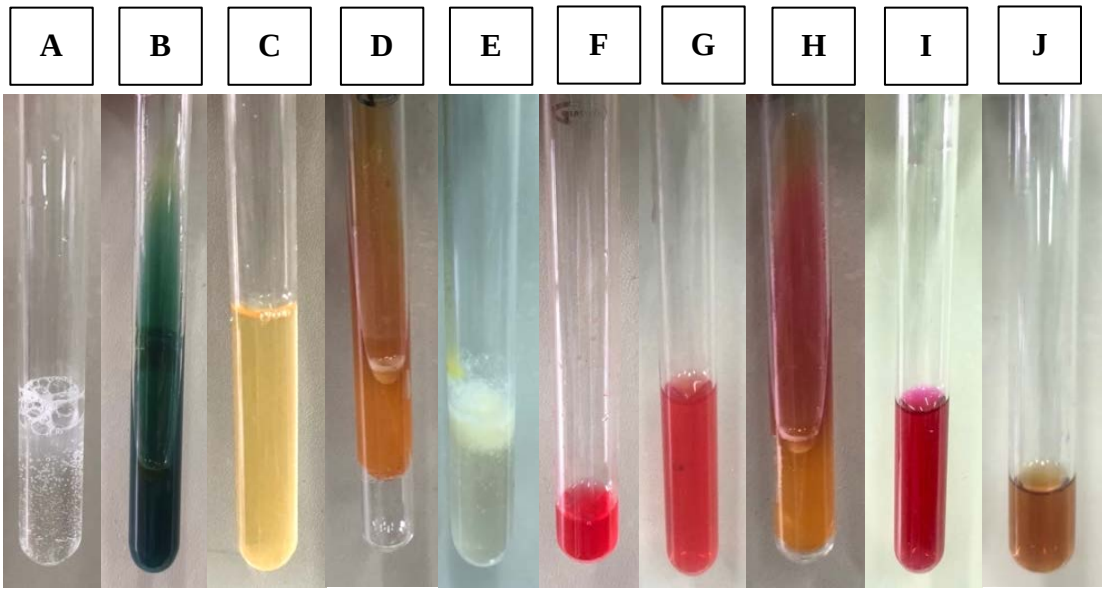
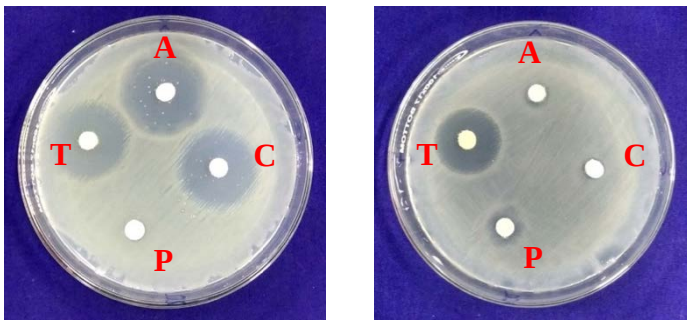


Figure 3 Biochemical tests of two *Rhizobium* strains (A) Catalase test; (B) Citrate utilization test; (C) Gelatinase hydrolysis test; (D) Hydrogen sulphide test; (E) Indole test; (F) Methyl red (MR) test; (G) Nitrate reduction test; (H) Triple sugar iron (TSI) test; (I) Urease test; (J) Voges Proskauer (VP) test

4. Determination on the Antibiotic Susceptibility



PVMR **PVUR**

Figure 4 Antibiotic Susceptibility test of two *Rhizobium* strains (PVMR and PVUR) by disc diffusion test. Amoxicillin (A), Chloramphenicol (C), Penicillin (P), and Tetracycline (T)

5. Evaluation the effect of two *Rhizobium* strains on plant growth

The effect of two rhizobial isolates on the plant growth of cowpea and black gram was evaluated at 15 days post inoculation. The two treatments (10^{-1} cfu/ml and 10^{-5} cfu/ml) inoculated with two rhizobial isolates indicated the highest plant height and plant weight compared with the uninoculated treatment (control) in both selected plants (Figure. 5, 6).

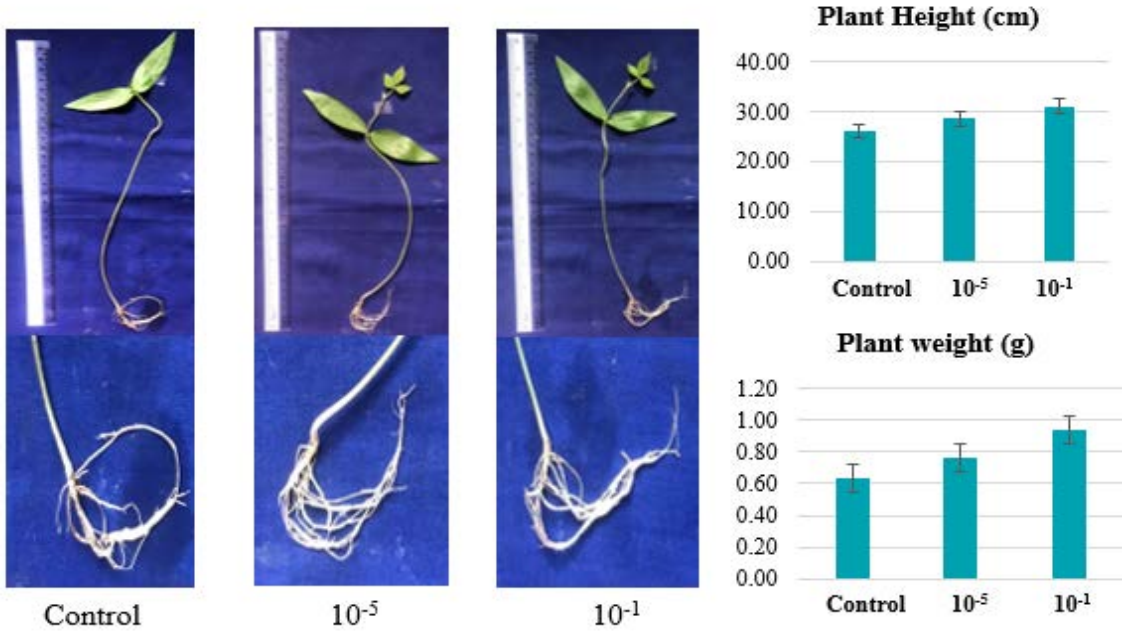


Figure 5 Comparison of plant growth parameters (plant height and plant weight) of black gram, inoculated with no-inoculation (control), 10^{-5} cfu/ml and 10^{-1} cfu/ml of rhizobial isolate PVMR strain at 15 days after sowing. The histograms at each treatment are significantly different at 15 days, $P > 0.05$ (t-test).

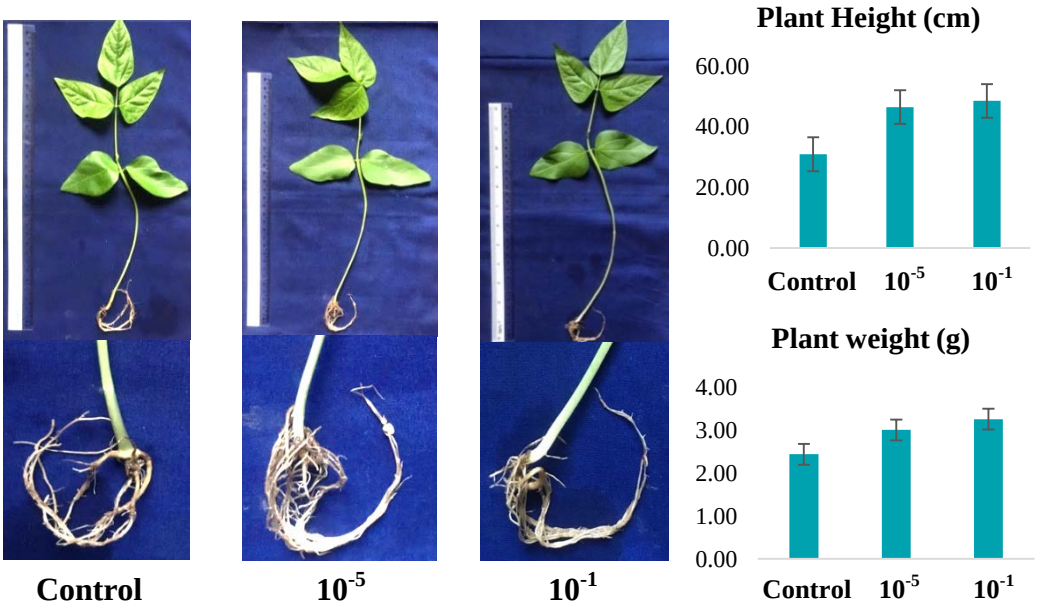


Figure 6 Comparison of plant growth parameters (plant height and plant weight) of cowpea, inoculated with no-inoculation (control), 10^{-5} cfu/ml and 10^{-1} cfu/ml of rhizobial isolate PVUR strain at 15 days after sowing. The histograms at each treatment are significantly different at 15 days, $P > 0.05$ (t-test).

Discussion

In the present study, the two *Rhizobium* strains ('PVMR' from black gram and 'PVUR' from cowpea) were isolated. The colonies of both isolated strains showed white, circular, entire margin, convex elevation, and mucoid colonies production on YEMA medium and microscopic examination revealed that both isolates were rod shaped and gram negative (Figure 1). The morphological and microscopical characteristics resembles with the study carried out by different researchers (Jordan and Allen 1974 and Rasool *et al*., 2015, Keyser *et al*., 1982 and Gauri *et a*l., 2011). The authentication test to confirm the isolates as rhizobia and to differentiate them from other contaminating microbes were conducted and the results of five confirmatory tests was shown in Table 1 and Figure 2.

The results indicated that the colonies of both strains did not absorb the Congo red color on CR-YEMA media (congo red yeast extract mannitol agar) and which is differentiate *Rhizobium* from *Agrobacterium* and other bacterial contaminants (Trinick, 1982). In bromothymol blue (BTB) agar plates, PVMR strain from black gram showed the yellow color indicating the acid production and strain PVUR from cowpea showed the blue color which is indicting the alkali production. Datta *et al*., (2015) showed the fast- growing rhizobial strains isolated from *Vigna mungo* (black gram). Similarly, Shahzad *et a*l., (2012) and Andrews (2017) stated slow-growing strains isolated from *Glycine max* and *Vigna unguiculata* L. From these observations, the present results suggested that PVMR strain might be *Rhizobium* sp. and PVUR strain might be *Bradyrhizobium* sp.

In the GPA test, both isolates showed either poor or no growth on GPA medium after one day indicating the features of rhizobia. The similar observation was reported by Vincent *et al*., (1970). In Keto-lactose test, there is no yellow zone around the colonies after the addition of Benedict's reagent which is the characteristic of *rhizobium*. The negative results on Hofer's

alkaline medium were obtained in the current study which is indicating the *Rhizobium* species normally cannot grow in this medium. These results of both strains similar with the findings of Deshwal and Chaubey (2014) and Deka and Azad (2006).

Moreover, the different biochemical characterization test of two rhizobial isolates (PVMR and PVUR) was performed and results shown in Table 2 and Figure 3. In catalase test, the appearance of bubbles showed the positive result and this agreed with (MacFaddin, 2000). The result of citrate utilization showed the negative result and agreed with (Lupwayi and Hague,1994). Paudyal, 2002 stated that the positive test for the catalase activity and negative test for citrate utilization revealed that is 100% of *Rhizobium* strain and these findings were agreed with the current study. The negative gelatinase activity of *Rhizobium* was observed for both strains and similar with (Hunter *et al*., 2007). There is no hydrogen sulphide production was observed in the current study which is close agreed with (Kumari *et al*. 2010).

The indole test indicated the negative result for both strains which remaining yellow or be slightly cloudy within seconds of adding the reagent and similar result was observed in Shahzad *et al*., (2012). The positive results of methyl red (MR) test agreed with the studies of Raju *et al*., (2017). For nitrate reduction test, a red color indicates a positive and the similar result was agreed with Kumari *et al*. (2010). In TSI test, alkaline (red) slant and acid (yellow) butt showed that only glucose fermentation has taken place and similar results were reported by Kucuk *et al*., (2006). In the current study, both isolated strains showed positive test for urease and similar finding was reported by Gauri *et al*., (2011). The result of Voges-proskauer (VP) showed the negative which has the similar observations with Elsheikh and Wood (1986).

In antibiotic susceptibility test, PVUR (Brady*rhizobium* sp.) and PVMR (*Rhizobium* sp.) showed the different resistance and sensitive results on four antibiotics. PVMR strain indicated that sensitive to Amoxicillin, Chloramphenicol, Tetracycline and which resistance to Penicillin only. In contrast, strain PVUR showed resistance to Amoxicillin, Chloramphenicol, Penicillin but sensitive to Tetracycline (Figure 4). Similarly, Hungaria *et al.,* (2000) stated that the *Rhizobium* isolates were sensitive to tetracycline, kanamycin and streptomycin. Prasuna (2014) found a strain that was resistant to many antibiotics (chloramphenicol, erythromycin, kanamycin, neomycin and penicillin G) and these results were in close agreement with the present findings for both strains.

The plant growth parameters including plant height, plant weight and nodulation efficiency of two rhizobial isolates indicated the positive effects on the two selected plants (black gram and cowpea) at 15 days post inoculation. The improvement of plant growth, leaves number and root structure were observed in the treatment inoculated with two rhizobial isolates compared with the non-inoculation treatment (Figure. 5 and 6). Interestingly, the small nodules formation was recorded in the treatment inoculated with 10^{-1} cfu/ml (PVUR strain) in cowpea while no nodulation was observed in the PVMR strain (black gram). Similarly, a significant increase of nodule number and improved plant height in cowpea was observed when inoculated with *Bradyrhizobium* and close agreement with current study Egamberdiyeva *et al*., 2004, Ndungu, 2017 and Stephen Kyei Boahen *et al*., 2017). According to the report of Maurya *et al*., (1993), and Salam *et al*., (2004), the application of *Rhizobium* significantly increases the plant height, branches/plant and dry matter production in black gram and similar findings found in current study.

Conclusion

The current study indicated that the two isolated strains were the true *Rhizobium* species according to the results of morphological and microscopical characterization, authentication and biochemical test. The rhizobial strain (PVMR) isolated from black gram considered as belonging to the *Rhizobium* group due to fast growing whereas the strain (PVUR) isolated from the cowpea might be *Bradyrhizobium* group which showed slow growing. Based on the results of antibiotic susceptibility test, it can be suggested that these antibiotics resistant strain (PVUR) can survive antibiotic stressed conditions and help to increase rhizobial survivability in the soil and prevent susceptible rhizobial population due to lethal antibiotic compared with less resistance strain (PVMR). In addition, the enhancement of plant height and plant weight were observed from the plants inoculated with both strains after 15 days. Remarkably, the nodulation performance was recorded from the PVUR strain. Therefore, it can be concluded that these findings may give a prospect to do extensive research by using highly performed isolates and also play an important role in sustainable agriculture system. However, it is still necessary to conduct further screening the different plant growth promoting traits and nitrogen fixing ability of both strains as well as to explore the application as potential biofertilizer instead of synthetic fertilizer under laboratory and field conditions

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PRELIMINARY STUDY ON PHYTOCHEMICAL ANALYSIS, ANTIMICROBIAL ACTIVITIES AND ELEMENTAL COMPOSITION OF PLANTAGO MAJOR L.

San San Myint[1], Si Si Thein[2]

Abstract

Plantago major L. is a small perennial herb belonging to the family Plantaginaceae under the order Lamiales. In this research, preliminary phytochemical analysis, antimicrobial activities, elemental composition and medicinal uses were studied. Phytochemical analysis was performed with standard test tube method. Antimicrobial activity of isolated organic compound was determined by Agar well diffusion method. The elemental composition of the whole plant powders was determined by using Energy Dispersive X-ray Fluorescence. In these plant, alkaloids, carbohydrates, flavonoids, polyphenols, phenolic compounds, lipophilic, saponin, α-amino acids, glycosides, tannins and terpenoids were presented. The antimicrobial activities of various extracts from the whole plant of *Plantago major* L. were tested against on six selected microorganisms. According to the results, ethyl acetate extract showed better antimicrobial activity as compared to other extracts. Calcium is found the highest percentage present in this plant.

Keywords *Plantago major* L., phytochemical, antimicrobial, agar-well, elements.

Introduction

Plantago major L. is one of Myanmar indigenous folk medicinal plant. Medicinal plants have curative properties due to the presence of various complex chemical substances of different composition, which are found as secondary plant metabolites in one or more parts of plants. These plant metabolites, according to their composition are grouped as alkaloids, glycosides, corticosteroids, essential oils, etc. (Narayan *et al*., 2003). The previous study affirmed the benefits of this plant in the field of health because of several compounds such as polyphenols, alkaloids, tannins and flavonoids. Plantaginaceae family consists of 3 genera and 255 species (Dassanayake, 1996). In Myanmar, this family consists of about one genera and two species (Kress *et al*., 2003). The selected plant commonly known as Ah-gyaw-paung-ta-htaung or Se-kyaw-gyi in Myanmar and Great plantain in English. *Plantago major* L. widely distributed in Kayah State. The seeds are used as laxative, anti-inflammatory and carminative. The used of the leaves are diuretic, astringent and to treat wounds, insect stings and skin diseases are widespread all over the world (Padua *et al*., 1999). In Thailand, the whole plant or leaves of *Plantago major* L. used as diuretic and antipyretic. In Myanmar, the decoction of the leaves is used as tonic for neurasthenic. The powder of dried whole plants is used for the treatment of diuretic and hypertension. The boiled young leaves are eaten as a salad to cure hypertension (Khin mar Kyu *et al*., 2020). The purpose of this research is to evaluate the phytochemical, elemental and antimicrobial properties of *Plantago major* L. using by relevant methods.

Materials and Methods

The sample of *Plantago major* L. were collected from Bardo ward, Loikaw Township, Kayah State, during the flowering period from March to June 2022. The identification of species was made by Kress *et al*., (2003) and Dassanayake, (1996). All part of the plants were dried under shades for two weeks, grinded to get powder and stored in air-tight containers for the

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phytochemical, elemental analysis and antimicrobial activity experiments. Phytochemical tests were carried out to detect the present or absence of organic constituents of this plant, using by Harbone methods, (1993). For antimicrobial activities, the whole plants were extracted by using methanol, ethanol, ethyl acetate, normal hexane and water. These extracts were tested against on six microorganisms (*Bacillus pumilus, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhimurium)*. These examinations used Agar-well diffusion methods Collins, (1965). The phytochemical tests and antimicrobial activity experiments were carried out in the Chemistry Department of Loikaw University. The determinations of elemental contents were measured at Department of Chemistry, Monywa University.

Results

Morphological characters of *Plantago major* L.

Perennial herbs. Leaves emerge from the short stem, simple, petiolate, petiole long and slender, base expanded, sheathing at base, leaf blade broad-ovate, tips obtuse, margin entire or irregularly obtuse-dentate, glabrous on both surfaces, 3 to 5 parallel-veined, exstipulate. Inflorescence raceme, erect spike. Flowers small, bracteates, ebracteolate, sessile, complete, bisexual, regular, actinomorphic, 4- merous, cyclic, hypogynous. Sepals (4), synsepalous, sepaloid, persistent, inferior. Petals (4), synpetalous, tubular, petaloid (creamy yellow), inferior. Stamens 4, apostamenous, filament long, anthers dithecous, extrorse, versatile, longitudinal dehiscence. Ovary superior, bicarpellary, syncarpous, bilocular, axile placentation, 2- ovules in each locule in T.S, style long and slender, hairy, stigma simple. Fruits circumscissile capsule, seeds smooth, subglobose and dark-brown. Fig.(A,B,C and D).

A. Habit B. Leaves C. Inflorescence and flower D. Fruits and seeds

Figure 1. Morphological characters of *Plantago major* L.

Preliminary Phytochemical Analysis of *Plantago major* L. the whole plant

The phytochemical analysis of *Plantago major* L. plants indicated the presence of alkaloids, carbohydrates, flavonoids, polyphenols, phenolic, lipophilic groups, saponins, α-amino acids, glycosides, tannins and terpenoids. The results were shown in table 1.

Table 1 Preliminary phytochemical analysis of *Plantago major* L.

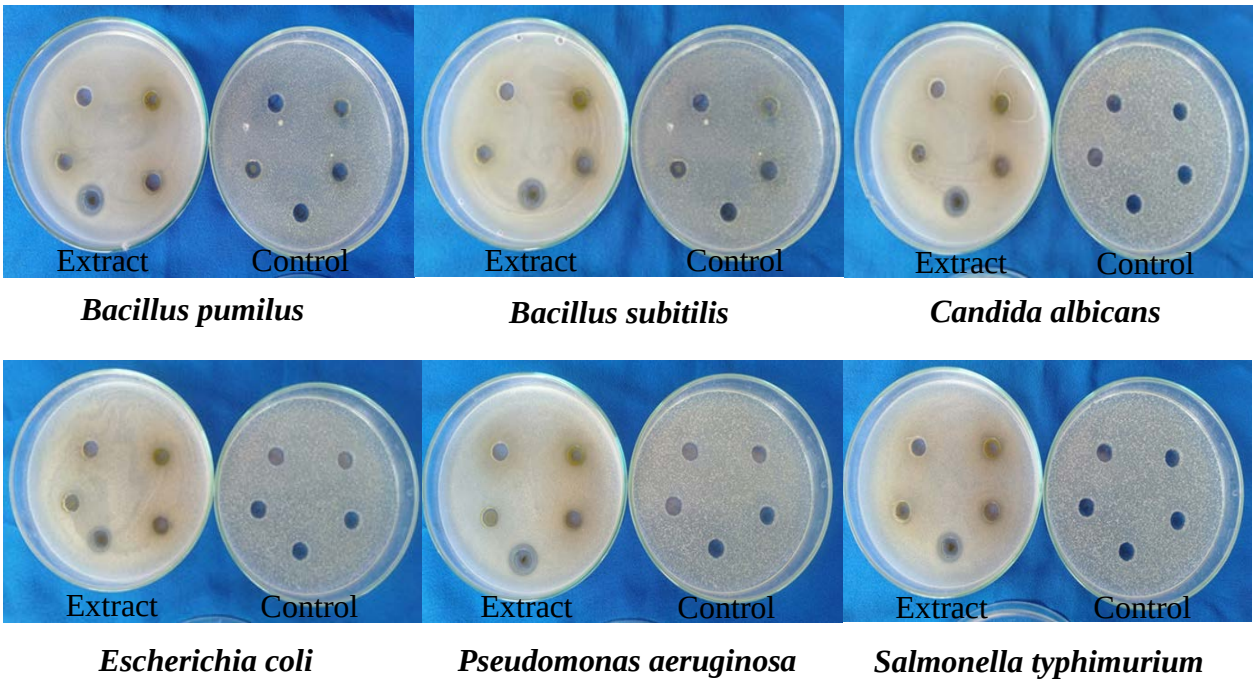
No.	Test	Extract	Test reagent	Observation	Result
1.	Alkaloid	1% HCl	Dragendroff's reagent Wagner's reagent Mayer's reagent Hager's reagent	Reddish brown Deep brown Creamy white ppt. Yellow	+ + + +
2.	Carbohydrate	H_2O	10%α-naphthol,Conc:H_2SO_4	Brown ring	+
3.	Flavonoids	EtOH	Mg,Conc:HCl,1%KOH	Brownish yellow	+
4.	polyphenol	EtOH	1%$FeCl_3$,1%$K_3Fe(CN)_6$	Dark green	+
5.	Phenolics	H_2O	1%$FeCl_3$,1%$K_3Fe(CN)_6$	Bluish green	+
6.	Lipophilic	H_2O	0.5 M KOH	Yellow	+
7.	Saponins	H_2O	$NaHCO_3$	Frothing	+
8.	α amino acid	H_2O	Ninhydrin	Purple	+
9.	Glycoside	H_2O	10%lead acetate	White ppt.	+
10.	Tannins	H_2O	1%$FeCl_3$	Bluish green	+
11.	Terpenoids	H_2O	$CHCl_3$,Conc:H_2SO_4	Reddish brown	+

(+) = present, (-) = absent

Antimicrobial Activities Determination of *Plantago major* L. the whole plant

According to antimicrobial activity screening, methanol extract showed 10.00 - 10.21mm against five tested microorganism. Ethanol extract showed 10.00 - 10.55mm against five tested microorganisms. Ethyl acetate extract showed 11.45 - 13.63mm against six tested microorganisms. Normal hexane and aqueous extracts of *Plantago major* L. not showed the activity on all microbes. These results were shown in table 2 and fig 2.

Figure 2 Antimicrobial Activities of various Solvents Extracts of *Plantago major* L.



Bacillus pumilus ***Bacillus subitilis*** ***Candida albicans***

Escherichia coli ***Pseudomonas aeruginosa*** ***Salmonella typhimurium***

Table2. Antimicrobial Activities of Various Solvent Extracts from *Plantago major* L.

No.	Solvent	Organisms					
		Bacillus pumalis	***Bacillus subtilis***	***Candida albicans***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Salmonella typhimurium***
1.	H_2O	-	-	-	-	-	-
2.	MeOH	10.00mm	10.10mm	10.00mm	-	10.21mm	10.10mm
3.	EtOH	10.50mm	10.44mm	10.21mm	-	10.00mm	10.55mm
4.	EtOAc	13.23mm	12.20mm	11.45mm	12.26mm	13.63mm	12.31mm
5.	n-Hexane	-	-	-	-	-	-

Agar well - 8 mm, 100µL/well

10mm ~ 14mm (low activity), 15mm ~ 19mm (medium activity), 20mm ~ above (high activity)

Elemental composition of *Plantago major* L.

According to the EDXRF analysis, *Plantago major* L. found that calcium 1.208 %, potassium 1.095 %, silicon 0.391 %, sulfur 0.244 %, iron 0.024 %, titanium 0.006 %, zinc 0.003 %, copper 0.002 %, strontium 0.001 %, manganese 0.001 % and rubidium 0.001%. The results were shown in table 3 and figure 3.

Figure 3 Elemental composition of *Plantago major* L.

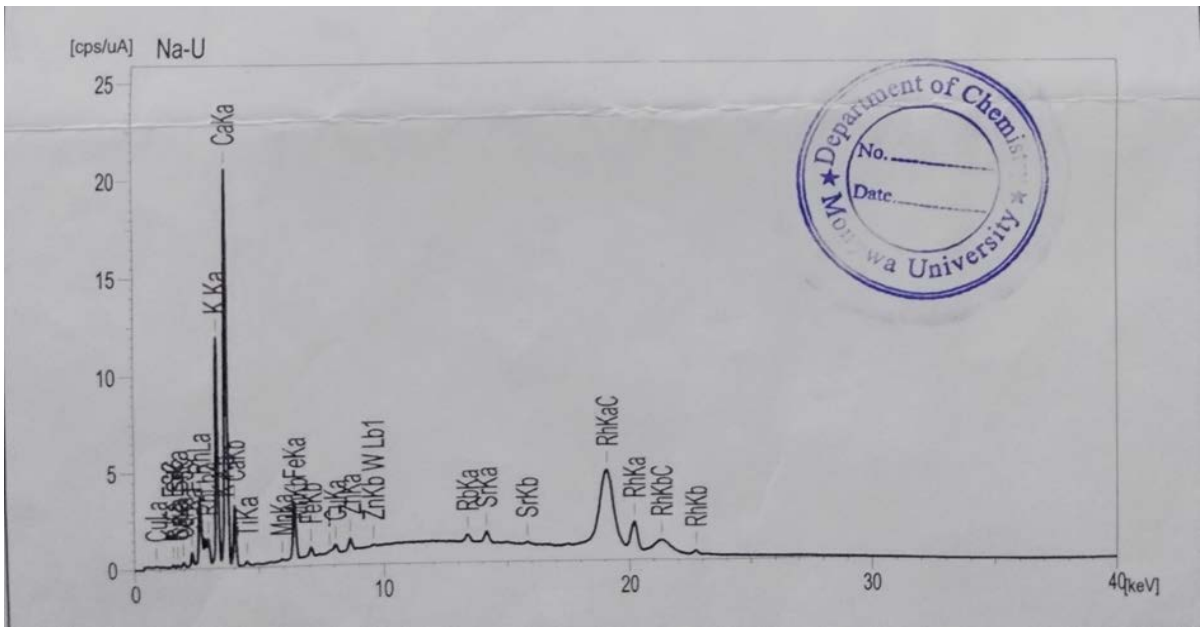


Table 3 Elemental analysis of *Plantago major* L. by using EDXRF

No.	Element	Symbol	Quantitative results
1.	Calcium	Ca	1.208 %
2.	Potassium	K	1.095 %
3.	Silicon	Si	0.391 %
4.	Sulfur	S	0.244 %
5.	Iron	Fe	0.024 %
6.	Titanium	Ti	0.006 %
7.	Zinc	Zn	0.003 %
8.	Copper	Cu	0.002 %
9.	Strontium	Sr	0.001 %
10.	Manganese	Mn	0.001 %
11.	Rubidium	Rb	0.001 %

Discussion and Conclusion

Plantago major L. is an old medicinal plant. The morphological characters of selected studied specimens were agreed with those mentioned by Kress *et al*., (2003) and Dassanayake., (1996). Thus studied plant was confirmed as *Plantago major* L.

In present research, phytochemical analysis of *P.major* showed the presence of alkaloids, carbohydrates, flavonoids, polyphenols, phenolic compounds, lipophilic, saponins, glycosides, tannins and terpenoids. These finding agree with those mentioned by Vandana *et al*, (2017) and Dewi *et al*, (2019).

In present antimicrobial activities, ethyl acetate extracts was against shown on six selected microorganisms. Ethyl acetate extract of *Plantago major* L. showed low activity 13.63mm against *Pseudomonas aeruginosa* and 12.31mm against low activity on *Salmonella typhimurium*. Methanol and ethanol extract were also low activity showed on *Bacillus pumilus, Bacillus subtilis, Candida albicans, Pseudomonas aeruginosa* and *Salmonella typhimurium*, except *Escherichia coli*. Thus, ethyl acetate extract showed better antimicrobial activity as compared to other extracts. This could be due to the present of greater amount of active antimicrobial components which are more soluble on organic solvent (ethyl acetate) than other solvents. According to the elemental analysis, calcium is found the highest percentage, potassium, silicon, sulfur and iron are also found the moderate percentage and titanium, zinc, copper, manganese, strontium and rubidium are small percentage present in this plant. Calcium helps muscles to contract normally, and regulate blood pressure. Potassium was crucial for human as well as animal nutrition and foods the good sources of the minerals. In conclusion, these results suggest that extract of *Plantago major* L. possess essential compounds, antimicrobial and elemental properties therefore it can be used as active ingredients for food and medicines.

Acknowledgement

My heart felt sincere gratitude and thanks to Dr Si Si Thein, Professor and Head, Department of Botany, Loikaw University, for her encouragement during my research. I would like to express my grateful thank to Dr Hlaing Myint Thu, Associate Professor, Department of Chemistry and Loikaw University for his valuable suggestions. My special thanks are also due to Dr Thet Thet Mar Win, Professor, Department of Botany, and University of Yangon for her supervision, guidance and encouragement.

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STUDY ON MORPHOLOGICAL AND HISTOLOGICAL CHARACTERS OF *BIXA ORELLANA* L.

Pyae Sandi Win[1] Swe Swe Aye[2] Thida Oo[3]

Abstract

The seeds of *Bixa* produce one of the dyes most frequently used worldwide, not only in food products but also in textile, paint and cosmetic industries. The present study was conducted to investigate the morphological and histological study of *Bixa orellana* L. According to the results, it was found that the plants were small tree (6.0-8.0 ft), leaves simple, pedicellate, inflorescence terminal branched panicles with 5-15 flowered, flowers actinomorphic, hypogynous, style thickened upward, stigma bi-lobed, parietal placentation, red-brown seed pods covered in soft pines, bi-valved, seeds are obovoid and angular, red-dotted. The distinguished histological characters of *Bixa* revealed that anomocytic stomata present on both surface, druses-shaped crystal and secretory cavities occurred in lamina, petiole, midrib and cortex of stem as well as in roots. Bixin pigment producing cells are commonly found in surface view of petiole. Testa of seeds is composed of thin walled epidermal cells which are filled with pigment bixin whereas tegmen region is made up of colorless sclerenchymatous layers. Starch grain are present in endospermic cells. The histological characters of *Bixa orellana* L. could provide the diagnosis of genus *Bixa*.

Keywords Morphological., Histological., *Bixa orellana* L.

Introduction

The Bixaceae family is one of the smallest plant families, consisting of one genus, *Bixa*. There are five species grouped under a single genus, and the most common species is *Bixa orellana* L. (Srineeraja, 2015).

Bixa orellana L. is known as Annatto or Po-thidin in Myanmar, Kam-ngor, Kham-saet, Kham-ngo in Thai, Hong-mu in China, Hiryu-sida, Okenoki in Japan, Kunyit-jawa, Kesumba, Jarak-belanda in Indonesia and Sotis, Echuete in Philippines.

Bixa orellana L.is widely known for its dye, food coloring agents, dyeing the cloth and painting the skin. (Kala *et al*; 2015). Ayurveda practitioners in India use Annatto as an astringent, mild purgative and are considered as a good remedy for treating dysentery and kidney diseases. Traditional healers in Colombia have also used annatto as an antivenom for snake bites (Dunham and Allard, 1960).

However, the scientific investigations on this plant is still lacking in Myanmar. Therefore, the aim of the present study is to identify and examined the histological characters of *Bixa orellana* L. from Loikaw Township, Kayah Division.

Materials and Methods

Morphological study of *Bixa orellana* L.

The plants used in this research were collected from Loikaw Township (North latitudes 19˚42' 4" and East Longitudes 97˚ 12' 2"), during the flowering and fruiting period from June to November, 2020-2021. The collected specimens were identified by using standard literatures such as Backer, 1963; Hooker, 1885; Kirtikar and Basu, 1973; Flora of China, 1995; Flora of Hong Kong, 2007; Kress *et al*., 2003.

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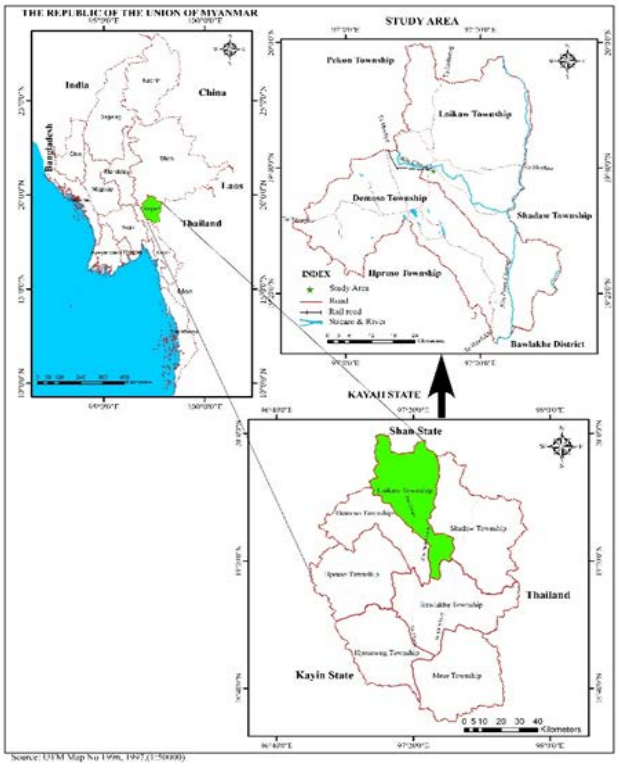


Figure 1 Location map of collection site, Loikaw Township

Histological study of *Bixa orellana* L.

Microscopical study of leaves, stems, roots, fruits and seeds were also examined by using free hand sections according to the method of Esau (1963); Kokate (1994) and Trease and Evans (2002). Then the cut sections were examined by light microscope. Free hand sections were taken, stained and mounted in Safranin Reagent and chloralhydate (50%) solution. The photographs were taken by a Camera Attached Microscope. The following reagents were used for microscopy and histological examination.

1. Chloral hydrate solutions as clearing agent
2. Phloroglucinol was followed by concentrated hydrochloric acid for lignin
3. Acetic acid or 10% sulphuric acid for calcium oxalate crystals,
4. Iodine solution B.P for starch.

Numerical values of leaves of *Bixa orellana* L.
Stomatal Index.

Stomatal index per square millimeter were also investigated according to Trease and Evans (2002).

The average number of stomata per square millimeter of epidermis is termed the stomatal number. The percentage proportion of the ultimate divisions the epidermis of a leaf which have been converted into stomata is termed the Stomatal Index.

$$S.\ I = \frac{S}{E+S} \times 100$$

S. I = Stomatal Index
S = number of stomata per unit area
E = number of ordinary epidermal cells in the same unit area

Diagnostic characters of powdered seeds of *Bixa orellana* L.

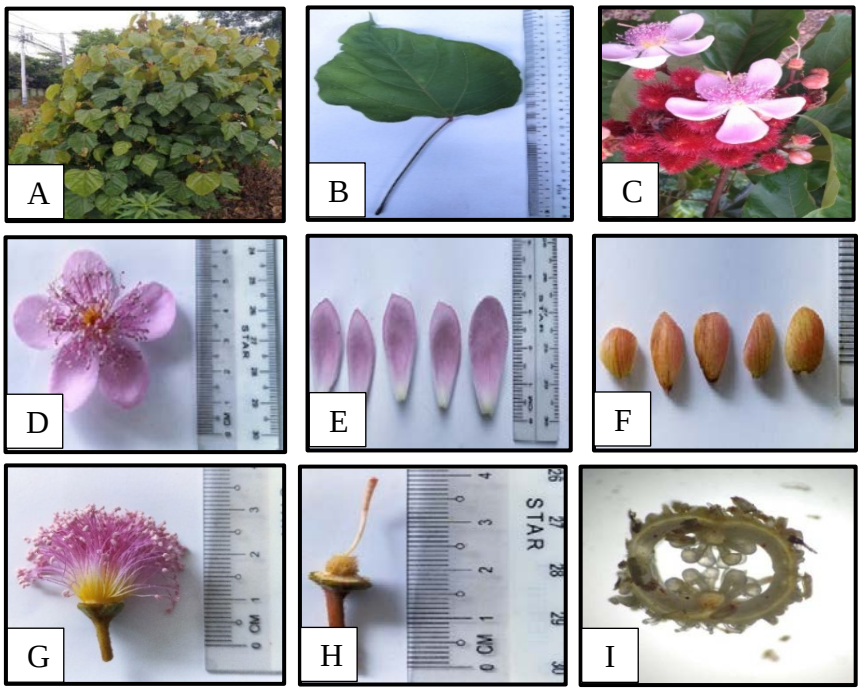
The diagnostic characters of the powder and sensory characters were examined by using the powdered seed.

Results

Scientific name : *Bixa orellana* L.
Family : Bixaceae
Myanmar names : Po – thidin, Thidin
English names : Annatto, Lipstick – tree
Flowering period : June to November

Taxonomic Description

Perennial, small tree, about 6 - 8 ft high; bark dark brown. Leaves simple, alternate, stipulate, petiole 4.5 - 9.5 cm long, laminae ovate, the tips acute, the margins entire, the base cordate, reticulate and netted venation, glabrous, 5.6 - 18.5 cm long, 3.7 - 13.7 cm wide. Corymbose panicles 5 - 15 flowered, scaly, peduncles 3.2 – 5.5 cm long. flowers 3.5 – 5.4 cm in diameter, purplish - pink, pedicel 2.8 – 3.2 cm long, 0.8 – 1 mm wide, bisexual, actinomorphic, 5 merous, hypogynous. Sepals 5, concave, reddish- brown, 0.3 - 0.5 cm long, more or less fleshy. Petals 5, unequal, large and conspicuous, obovate, imbricate, purplish-pink, 2.5 - 3.3cm long, 1.3 - 1.6 cm wide. Stamens numerous, slightly united at the base, filaments filiform, with yellow base and purplish-pink apex, 0.8 - 2.0 cm long, anther dithecous, basifixed. Stamens numerous, slightly united at the base, filaments filiform, with yellow base and purplish-pink apex, 0.8 - 2.0 cm long, anther dithecous, basifixed. Fruits capsules, loculicidally 2-valved, ovoid, 4.7 - 5.5 cm long, 3.8 - 4.5 cm wide, reddish-brown, covered with soft pines. Seeds many, smooth, angular, with flattened apex, orange- red, 0.3 - 0.5 cm long, 0.2 - 0.3 cm wide



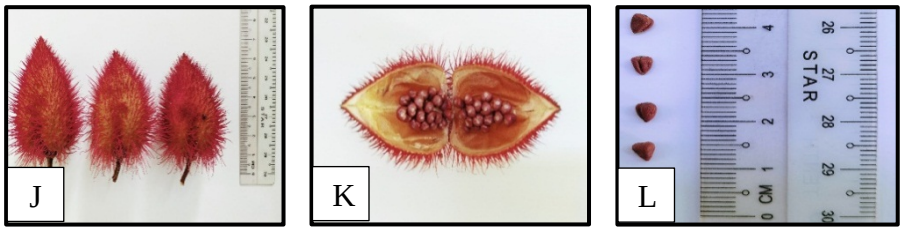


Figure 2 Morphological Characters of *Bixa orellana* L.

(A) Habit	(B) Leaf	(C) Inflorescence	(D) Flower as seen
(E) Calyx	(F) Corolla	(G) Androecium	(H) Gynoecium
(I) T.S of ovary	(J) Fruits as seen	(K) Opened fruit	(L) Seeds as seen

Histological Characters of *Bixa orellana* L.

Lamina

In surface view of lamina, the epidermal cells of both surfaces are parenchymatous, thin-walled, irregular shape. The anticlinal walls of lower epidermal cells are wavier than the upper epidermal cells, stomata are present on both surfaces, the guard cells are reniform in shape with abundant chloroplast. Calcium oxalate crystals occur along the veins of lower surface.

In transverse section, the single layer of epidermal cells is covered by fairly thick cuticle. The mesophyll layer comprises a single layer of palisade mesophyll cells and 2-3 layers of spongy mesophyll cells. The druses –shaped calcium oxalate crystals occur below the upper epidermal layer. The vascular cylinder is collateral type, xylem composed of vessel elements, fibers, tracheid, xylem parenchyma and phloem composed of sieve tube, companion cell and phloem parenchyma.

Midrib

In surface view of midrib, the epidermal cells are thin walled, parenchymatous and polygonal shaped. The bixin pigment producing cells and calcium oxalate crystals are also occurred.

In transverse section, the upper region is convex and inner is concave. The thick wall epidermal cells are compactly arranged. The hypodermis layer lies next to the epidermis and consists of 3-5 layers of collenchymatous cells, 5-7 layers of parenchymatous cells whereas 2-4 layers of collenchymatous cells and 7-10 layers of parenchymatous cells are occurred in abaxial region of transverse section of midrib. The vascular bundle is surrounded by sclerenchymatous sheath. The xylem towards centre and the phloem towards periphery. Moreover, the lysigenous cavities and calcium oxalate crystals occurred in adaxial and abaxial region of midrib.

Petiole

In surface view of petiole, the epidermal cells are thin-walled, rectangular in shape. The bixin pigment producing cells and calcium oxalate crystals are occurred in surface view of petiole.

In transverse section of petiole, single layer of epidermal cells are barrel in shaped. The cortex region is made up of 2-3 layered of collenchymatous cells and 5-8 layered of parenchymatous cells and become larger towards central region. The vascular bundle is concentric and amphicribral type. The lysigenous cavities and crystals are found in the cortex region as well as in the region of petiole.

Stem

In surface view, the epidermal cells are thick wall and rectangular shape. In transverse section, the outline of stem is rounded. The phellem is 1-2 layers, phllogen consists of 2-5 layered of cells. The phelloderm is composedof 2-3 layers of parenchymatous cells. The central vascular bundles are found as collateral type. The pith is present and made up of parenchymatous cells.

Root

In surface view, the epiblema cells are rectangular in shape. In transverse section of root, the epiblema cells are thin-walled and the cortex region consists of thin walled, isodiametric parenchymatous cells, the sclereids are occurred as patches in cortex region. The innermost layer of cortex is endodermis. The vascular bundle shows radial arrangement, xylem elements show exarch condition and the phloem structure is similar to those of stem.

Seed

In surface view, the epidermal cells are polygonal and their walls are straight. In transverse section, the seed coat is differentiated into two regions. The testa region is composed of thin wall epidermal cells, 1-2 layers thick which are filled with pigment bixin. Next layer is single layer of closely packed, colorless palisade cells and the tegmen is composed of two regions. The outer region is the pigment zone and contain 1-2 layers of sclerenchymatous cell. Next to this layer is 2-3 layers of colorless sclerenchymatous layer and made up of osteosclereids. The endosperm is composed of parenchymatous cells which were densely filled with starch grain.

Diagnostic characters of powdered *Bixa orellana* L.

The powdered seeds were orange red in color, the odor was pungent, the taste was slightly nutty and the texture was fine. It consists of fragment of fiber and reticulate vessel, fragment of osteosclereids and group of starch grains.

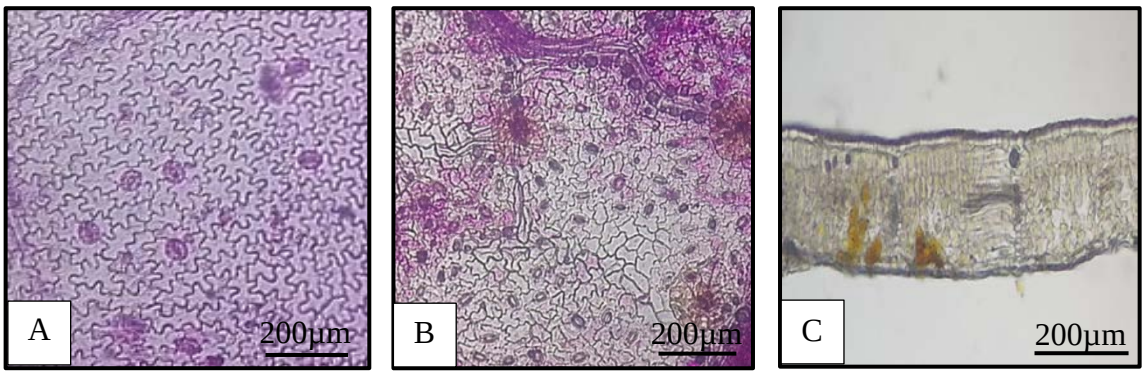


Figure 3 Internal Structures of lamina of *Bixa orellana* L., (A) Upper surface of lamina, (B) Lower surface of lamina, (C) Transverse section of lamina

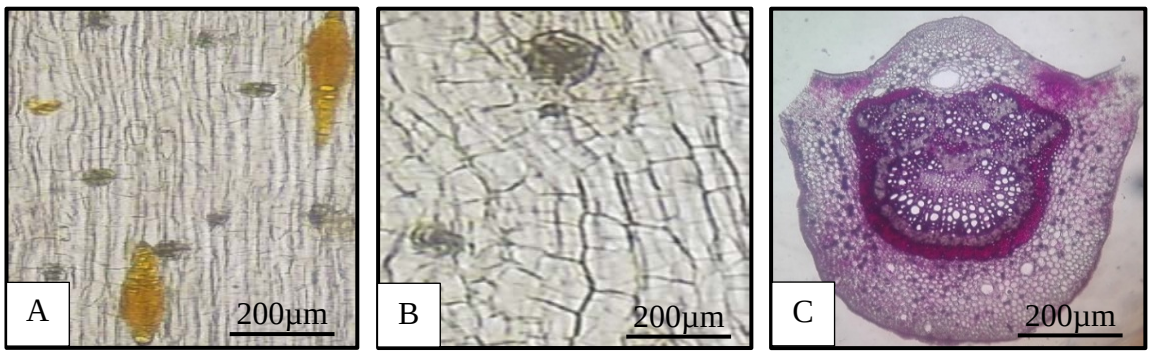


Figure 4 Internal Structures of midrib of *Bixa orellana* L. (A) Surface view of midrib, (B) Surface view of midrib showing a calcium oxalate crystal, (C) Transverse section of midrib

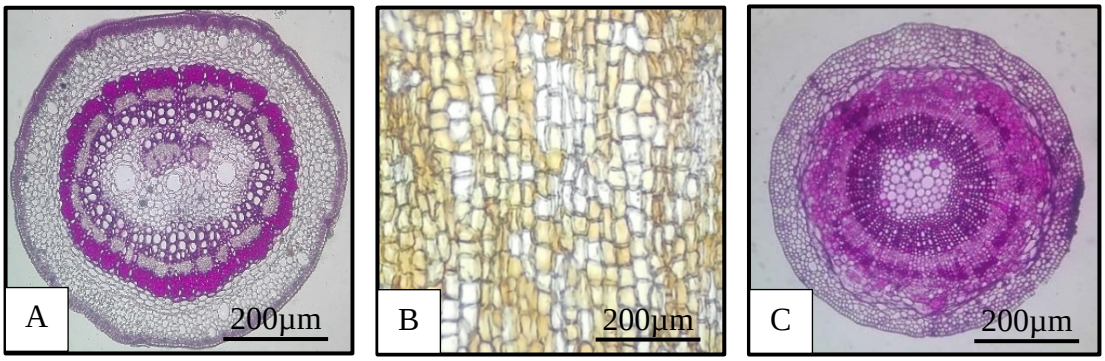


Figure 5 (A) Transverse section of petiole, (B) Surface view of stem (C) Transverse section of stem

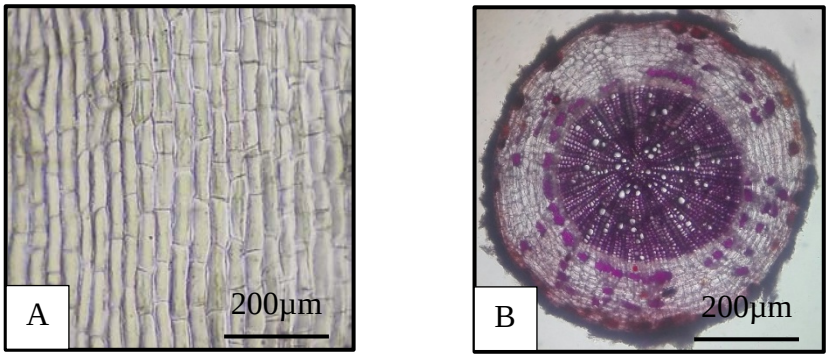


Figure 6 (A) Surface view of root, (B) Transverse section of root

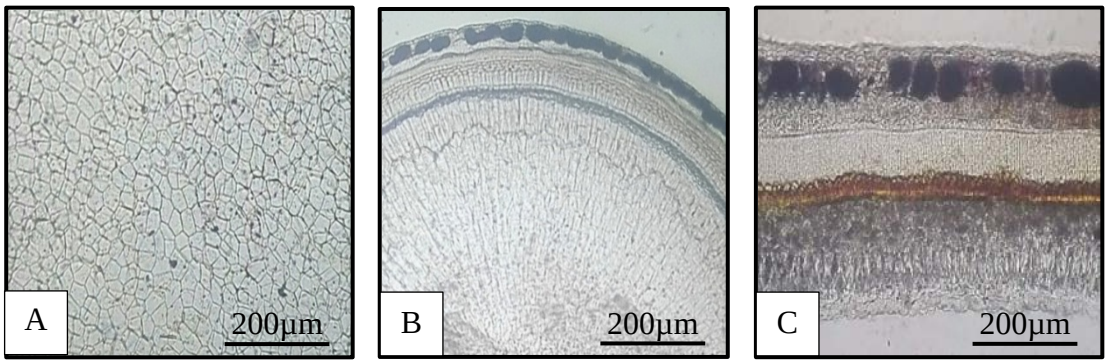


Figure 7 Internal Structures of Seed of *Bixa orellana* L., (A) Surface view of seed coat, (B) A portion of T.S of seed coat, (C) An enlarge cellular portion of testa

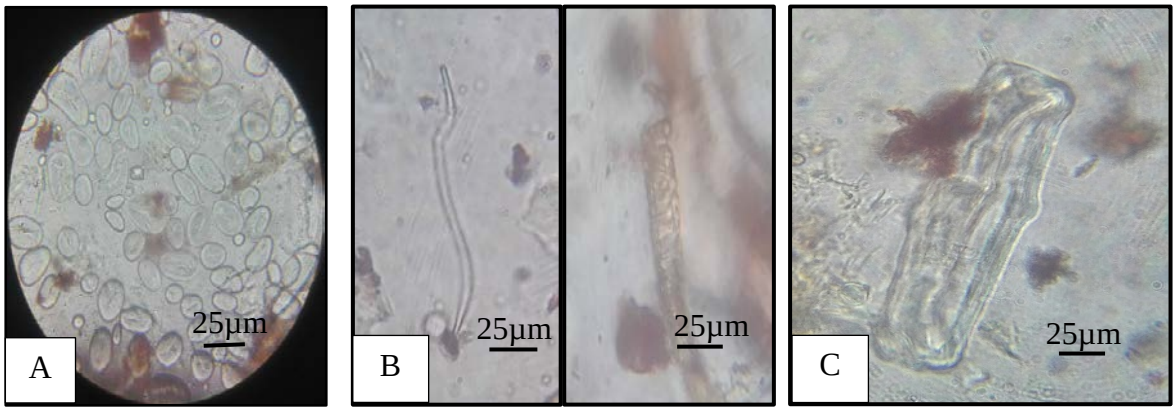


Figure 8 Diagnostic Characters of Powdered Seeds of *Bixa orellana* L., (A) Starch grains treated with iodine, (B) Fragment of fibre and reticulate vessel, (C) Fragment of osteosclereids

Discussion and Conclusion

In this research, the morphological and histological characters of *Bixa orellana* L. (Family –Bixaceae) have been undertaken.

In Myanmar, there is only one genus and one species in family Bixaceae (San Khin, 1970 and Kress *et al*., 2003).

Bixa orellana L. is a perennial small tree, leaves simple, petiole erect, flowers 10-20 in corymbose panicles, sepals and petals 5, free, imbricate, stamens many, anthers 2-celled, ovary superior, unilocular, parietal placentae, stigma bilobed, capsule 2-valves and seeds many. These characters are in agreement with Hooker (1894), Kirtikar (1935), Backer (1963) and Dassanayake (1981).

In the present study, anomocytic stomata present on both surface, druses-shaped crystal and secretory cavities occurred in lamina, petiole, midrib and cortex of stem as well as in roots.

Metcalf and Chalk (1950) described that the lamina of genus *Bixa* had anomocytic stomata that occurred on both surfaces, secretory cavities and cluster crystals of calcium oxalate present in the mesophyll and in the peripheral tissue of the petiole.

In transverse section of seeds, the reddish-brown pigment is found in testa region of seed coat of Annatto. The color of seed coat in *Bixa orellana* L. varies from orange to reddish brown, due to the carotenoid content, which is mostly found in the aril.

Bixa seeds has a- bixin, b-bixin, a- norbixin and b-norbixin carotenoids. Moreover, the minor apocarotenoid pigment are also present. (Reith 1971, Mercadante *et al*.,1999).

In conclusion, *Bixa orellana* L. in Bixaceae family could be identified based on microscopically characteristics of leaves, stems, roots and seeds.

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PHYTOCHEMICAL ANALYSIS AND ANTIMICROBIAL ACTIVITIES OF *NYCTANTHES ARBOR-TRISTIS* L.

Htay Htay Myint[1], Kathy Myint[2], Phyo Phyo Win[3]

Abstract

The plant *Nyctanthes arbor-tristis* L. or Night jasmine belongs to the family Oleaceae. The plant is known as Seik-Phalu in Myanmar. It grows in tropical and subtropical region. The specimens were collected from Banmaw University Campus, Kachin State, during the flowering and fruiting periods. The morphological characters, preliminary phytochemical test and antimicrobial activities were studied. The plant used in traditional medicines as stomachic, carminative, intestinal astringent, expectorant, in biliousness, piles, various skin diseases and hair tonic. In morphological study, the plant was perennial small tree, quadrangular branches, opposite and decussate, simple leaves and fragrant flowers. Preliminary phytochemical test revealed the presence of alkaloid, α-amino acid, glycoside, flavonoids, reducing sugar, phenolic compound, saponin, starch, tannin and carbohydrate. The different solvent extracts were prepared for antimicrobial activities tests and their inhibitory zones were also evaluated. The antimicrobial activities were carried out by agar well diffusion method on seven types of test microorganisms. Methanol extract of leaves showed significant antimicrobial activity against *Bacillus pumilus, Bacillus subtilis* and *Staphyloococcus aureus*. Ethanol extracts of leaves showed the significance against on *Bacillus pumilus and Xanthomonas oryzae*. Watery extracts showed moderate activities on *Bacillus pumilus* and *Bacillus subtilis*, petroleum ether extracts exhibited against only on *Bacillus subtilis* and chloroform extracts of leaves showed the best activities on *Candida albicans*.

Keywords *Nyctanthes arbor-tristis* L., Phytochemical test, Antimicrobial activities

Introduction

Natural products and traditional medicines are of great importance. They have own advantages, such as chemical structures, biological activities and antimicrobial activities. Since prehistoric times, humans have used natural products, such as plants, animals, microorganisms, and marine organisms for the treatment of diseases. According to fossil records, the human use of plants as medicines may be traced back of least 60,000 years (Shi *et al*., 2010).

The plant *Nyctanthes arbor-tristis* L.or Night jasmine belongs to the family Oleaceae. *Nyctanthes arbor-tristis* L. is native to India. It is also widely distributed in Bangladesh, Indo-Pak subcontinent and South-East, tropical and sub-tropical South East Asia. It grows in the Indo-Malayan region and distributed across Terai tracts as well as Myanmar, Ceylon and Thailand (Kirtikar and Basu, 1975). The common names of the plant are night jasmine, coral jasmine (English) and Sihau(Hindi). It has highly fragrant and brilliant flowers. The flowers start falling after midnight and by the day break. The generic name Nyctanthes has been coined from two Greek words Nykhta (Night) and Anthos (flower).

The plants are used as traditional medicine for women related problems, such as provoke menstruation, for treatment of scabies and other skin diseases and hair tonic (Jain and Pandey, 2016). The bitter leaves juice is used to expel roundworms and threadworms in children, chronic fever, malarial fever, obstinate sciatica and rheumatism. The leaves decoction is used for the treatment of arthritis and malaria. The young leaves are used as female tonic and in alleviating gynecological problems (Bhalakiya and Modi, 2019).

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The aim and objectives of the present research were to verify the morphological characters of *Nyctanthes arbor-tristis* L., to know the phytochemical constituents, medicinal uses and to examine the antimicrobial activities of leaves.

Materials and Methods

Botanical Studies

The specimens used in this research were collected from Banmaw University Campus, Kachin State. They were collected especially during the flowering and fruiting period from July to October in 2021. The collected fresh specimens of the plants were identified by using literatures of Lawrences, 1964; Backer, 1965; Hundley and Chit Ko Ko, 1987; Dassanayake and Clayton, 2000 and Kress *et al*., 2003.

Chemical Studies

The collected leaves of *Nyctanthes arbor-tristis* L. were washed with distilled water thoroughly and dried at room temperature under the shade for 2-3 weeks. The dried samples were pulverized by grinding with a blender to get fine powder and stored in air tight container. For preliminary phytochemical test, the powered leaves were tested for alkaloids, α-amino acid, carbohydrates, flavonoids, glycoside, phenolic compound, reducing sugar, saponin, starch and tannin.

Preliminary Phytochemical Test of *Nyctanthes arbor-tristis* L. Leaves

The preliminary phytochemical tests were carried out according to Vogel, 1956; British Pharmacopoeia 1968, Marini Bettolo *et al*., 1981; Robinson 1983 and Central Council for Research in Unani Medicine, 1987.

Test for Alkaloid

One gram of powdered sample was boiled for about 20 minutes with 20ml of 10% HCl and filtered. The filtrate tested with Wagner's reagent. The precipitate formed an addition of the reagent indicated the presence of alkaloid (Robinson, 1983).

Test for α-Amino acid

One gram of powdered sample was boiled with 10ml of distilled water for 20 minutes and then filtered. And then, a few drops of each filtrate was spotted on a filter paper using a capillary tube, allowed it to dry and sprayed with ninhydrin reagent. The filter paper was dried at room temperature and then kept it in over at 110°C for a few minutes after which the purple colour appears due to the presence of α-amino acids (Marini Bettolo *et al*., 1981).

Test for Glycoside

One gram of powdered sample was heated in a glass test tube with 10ml of distilled water on the water-bath for 20 minutes. The mixture was filtered and 10% basic lead acetate solution was added drop-wise to the filtrate. Pale yellow precipitate was observed which showed the presence of glycoside (Marini Bettolo *et al*., 1981).

Test for Carbohydrate

One gram of powdered sample was boiled with 10 ml of distilled water for 20 minutes and filtered. The filtrate was introduced into a test tube and a few drops of 10% α-naphthol was added and shaken. The test tube was then inclined at an angle of 45° and concentrated sulphuric acid was added slowly along the side of the inner tube. A red ring was formed between the two layers, showing the presence of carbohydrate (Marini Bettolo *et al.*, 1981).

Test for Flavonoids

Dried powdered sample one gram was extracted with methanol and filtered. When the methanolic extract was treated with a few drops of concentrated hydrochloric followed by a small piece of magnesium. The solution was boiled for a few minutes. The appearance of dark green colour indicates the presence of flavonoid (Central Council for Research in Unani Medicine, 1987).

Test for Reducing Sugar

One gram of powdered sample was boiled with dilute sulphuric acid and filtered. The filtrate was then neutralized with dilute sodium hydroxide solution. When the resulting solution was treated with Benedict's solution, it furnished yellow precipitates, indication the presence of a reducing sugar (Marini Bettolo *et al.,* 1981).

Test for Starch

One gram of dried powdered sample was boiled with 10 ml of distilled water for about 20 minutes. It was then filtered and two drops of iodine solution were added to the filtrate. Reddish brown precipitate was formed which indicate the presence of starch (Central Council for Research in Unani Medicine, 1987).

Test for Saponin

One gram of powdered sample was boiled with 10 ml of distilled water for about 20 minutes and filtered. The filtered and the filtrate shaken vigorously with distilled water for a few minutes. Marked forthing which lasted for about half an hour to take place, indicating the presence of saponin (Marini Bettolo *et al*., 1981).

Test for Tannin

One gram of powdered sample was boiled with 10ml of distilled water for about 20 minutes and filtered. The filtrate was treated with a few drops of 1% ferric chloride solution. If a bluish black or yellowish brown colour resulted indicating the presence of tannins (Marini Bettolo *et al*., 1981).

Test for Phenolic compound

One gram of powdered sample was boiled with 10 ml of distilled water for 20 minutes and filtered. The filtrate was treated with neutral 5% ferric chloride solution, it gave dark green colour, indicating the presence of phenol groups (Marini Bettolo *et al*., 1981).

Antimicrobial Activities of Different Solvent Extracts of *Nyctanthes arbor-tristis* L. Leaves

The antimicrobial activity test on different solvent extracts of powered sample was done by agar well diffusion method described in Medicinal Microbiology (Cruickshank, 1975).

Extraction of crude drugs

Five grams of powder was soaked with 50 ml of different solvents such as methanol, ethanol, acetone, water and pet-ether for about three days and thoroughly shake. The mixture was filtered and evaporated.

Cultivation of Test Organisms

Bacillus pumilus (IFO 905771), *Bacillus subtilis* (IFO 90571), *Candida albicans* (NITE 09542), *Micrococcus luteus* (NITE 83297), *Pseudomonas* sp. (IFO 94307), *Staphylococcus aureus* (ATCC 12877) and *Xanthomonas oryzae* were used. They were inoculated into the nutrient broth and transferred into nutrient agar media.

Preparation of Plates for Antimicrobial Activities Test

The antimicrobial activities were performed by agar-well diffusion method. Nutrient agar was prepared according to method described by Cruickshank, 1975. Nutrient agar was boiled and 20-25 ml of the medium was poured into a test- tube and plugged with cotton wool and autoclaves at 121 °C for 15 minutes. Then, the tubes were cooled down to 30-35°C and poured into sterilized petridishes and 0.01 ml of spore suspension were also added into the dishes. The agar was allowed to set for 30 minutes after with 8 mm plate agar well was made with the help of sterilized cork borer. After that, about 0.1ml of sample was introduced into the agar-well and incubated at 37°C for 24-48 hrs. The inhibition zone showed around the agar-well indicating the presence of anti-microbial activity. The extent of antimicrobial activity was measured from the zone of inhibition diameter. The results were shown in Table 2, Figures 4 - 5.

Results

Morphological Characters of *Nyctanthes arbor-tristis* L.

Scientific name	-	*Nyctanthes arbor-tristis* L.
Myanmar name	-	Seik-Phalu
English name	-	Night Jasmine
Family	-	Oleaceae
Flowering and fruiting period	-	July to October
Parts used	-	Leaves

Perennial small tree, about 5 m in high. Stems cylindrical, woody with grey or greenish-white bark; branches quadrangular. Leaves simple, opposite and distichous, petiolate, exstipulate; lamina ovate-lanceolate, 8.3-14.3 x 2.7-8.6 cm, rounded at the base, entire or with a few large distant teeth at the margin, acuminate at the apex, dark green above and pale green beneath, coriaceous, hirsute. Inflorescences axillary or terminal cluster cymes, 3-7 flowered. Flowers white with orange-red center, about 3.4 cm across at anthesis, bracteate, ebracteolate, sessile, complete, bisexual, regular, actinomorphic,6-merous, cyclic, hypogynous, delicate fragrant. Calyx 6 lobes, narrowly campanulate, greenish yellow, ribs present, glabrous with and cilliate without; tube about 0.9cm long; lobes obscurely. Corolla salverform, glabrous; tube 1-1.1 cm long, orange colour, lobes unequally obcordate, cuneate,1-1.3 cm long, white with an orange-red center, glabrous. Stamens 2, epipetalous, inserted near the top of the corolla tube; filaments very short, adnate to the corolla tube, bright orange, glabrous; anthers oblong, about 0.2 cm long, pale yellow, dithecous, dorsifixed, dehiscence longitudinal slit. Ovary superior, globoid, about 0.15 cm in diameter, yellowish green, glabrous, carpels 2, bicarpellary, syncarpous, bilocular with one ovule in each locule,basal placentas; style terete, about 0.2 cm long, white, glabrous; stigma bilobed. Fruits capsule, obcordate, 1.5-2.5x1.2-1.7cm, one seeded.

Habit

Ventral View of Leaves

Dorsal View of Leaves

Inflorescence Flowers L.S of Flower Fruits

Figure 1 Morphological Characters of *Nyctanthes arbor-tristis* L.

Preliminary Phytochemical Test of *Nyctanthes arbor-tristis* L. Leaves

The results of preliminary phytochemical test of air-dried powdered leaves from *Nyctanthes arbor-tristis* L. indicated that alkaloid, α-amino acid, glycoside, flavonoids, reducing sugar, phenolic compound, saponin, starch, tannin and carbohydrate were found to be present. Among them, the amount of precipitate from glycoside was highest than the other tests.

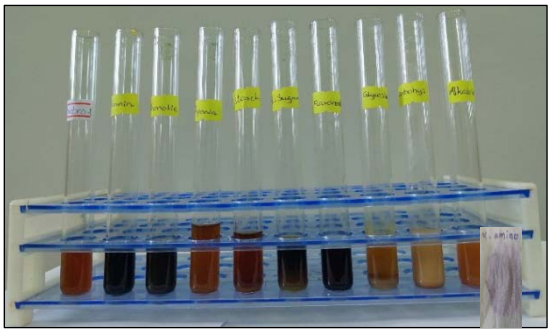


Figure 2 Phytocehmical Test of *Nyctanthes arbor-tristis* L. Leaves

Table 1 Preliminary Phytochcmical Test of Leaves of *Nyctanthes arbor-tristis* L.

No.	Test	Extract	Test reagent	Observation	Results
1.	Alkaloid	10% HCl	Wagner's reagent	Brown ppt	+
2.	α-amino acid	H_2O	Ninhydrin reagent	Purple	+
3.	Glycoside	H_2O	10% lead acetate solution	Pale yellow ppt	+
4.	Carbohydrates	H_2O	10% α-naphthol (H_2S0_4Conc:)	Red ring	+
5.	Flavonoids	MeOH	H_2S0_4 Conc: + Magnesium	Dark green	+
6.	Reducing sugar	H_2O	Benedict's solution	Furnished Yellow	+
7.	Starch	H_2O	Iodine solution	Reddish Brown ppt	-
8.	Saponin	H_2O	Distilled water	Foaming	+
9.	Tannin	H_2O	1% $FeCl_3$ solution	Bluish Black	+
10.	Phenolic compound	H_2O	5% $FeCl_3$ solution	Dark Green	+

Key to the table (+) = present (-) = absent (ppt) = precipitate

Antimicrobial Activities of Different Solvent Extracts of *Nyctanthes arbor-tristis* L. Leaves

In this study, ethanol extracts of leaves showed the significant against on , *Bacillus pumilus and Xanthomonas oryzae, moderate against on Bacillus subtilis, least against on Candida albicans, Staphyloococcus aureus and gave the negative results on Micrococcus luteus* and *Pseudomonas* sp. Methanol extract of leaves showed significant against on *Bacillus pumilus, Bacillus subtilis,* and *Staphyloococcus aureus,* moderate against on *Xanthomonas oryzae* and negative results on all other test organisms. Watery extracts showed moderate activities on *Bacillus pumilus, Bacillus subtilis* and gave the negative results on all other test organisms. Ethyl acetate extracts of leaves exhibited negative results on *Candida albicans* and *Micrococcus luteus,* moderate activities exhibited on other test organisms. Pet-ether extracts exhibited against only on *Bacillus subtilis.* Acetone extract revealed the negative result on *Micrococcus luteus,* moderate against on *Candida albicans* and the best activities on all other test organisms. Chloroform extracts of leaves showed the moderate activities on *Bacillus pumilus, Bacillus subtilis* and the best activities on all other test organisms.

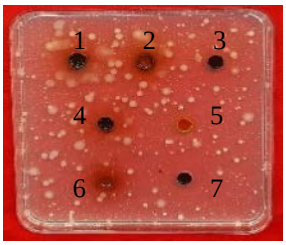
Table 2 Inhibition Zone Exhibited by Different Solvent Extract of *Nyctanthes arbor-tristis* L. Leaves

No.	Microorganisms	Inhibition Zones (mm)						
		H_2O	MeOH	EtOH	EtOAC	$CHCl_3$	P.E	Ace
1.	*Bacillus pumilus*	15	20	20	15	15	-	18
2.	*Bacillus subtilis*	10	18	18	15	15	12	20
3.	*Candida albicans*	-	-	12	-	22	-	15
4.	*Micrococcus luteus*	-	-	-	-	18	-	-
5.	*Pseudomonas* sp.	-	-	-	12	20	-	20
6.	*Staphyloococcus aureus*	-	17	15	14	18	-	17
7.	*Xanthomonas oryzae*	-	15	20	13	17	-	18

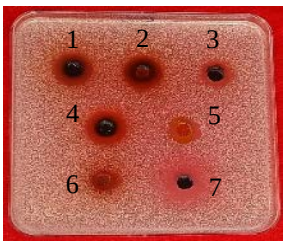
Key to the Table

MeOH - Methanol EtOH - Ethanol P.E - Pet-ether $CHCl_3$ - Chloroform

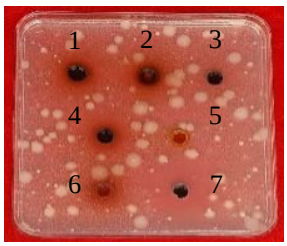
Ace - Acetone H_2O - Watery EtOAC -Ethylaceate Agar well = (8 mm)



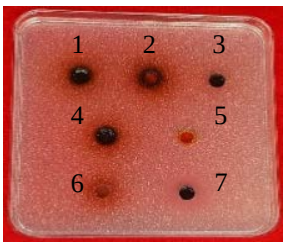
Bacillus pumilus



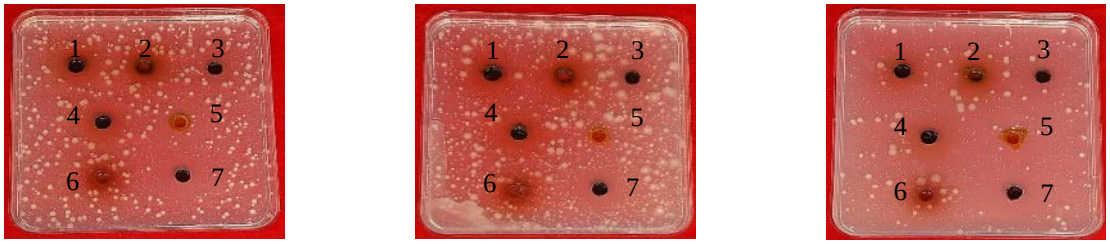
Bacillus subtilis



Candida albicans



Micrococcus luteus



Pseudomonas sp. *Staphyloococcus aureus* *Xanthomonas oryzae*

1. Ethanol Extract 2. Methanol Extract 3. Ethylaceate Extract 4.Acetone Extract
5. Pet-ether Extract 6. Watery Extract 7. Chloroform Extract

Figure 3 Antimicrobial activities of leaves from *Nyctanthes arbor-tristis* L.

Discussion and Conclusion

Herbal medicines, also referred to as botanical medicine or phytomedicine. According to "The World Health Organization", 80% of people in some Asian and African countries rely on herbal medicines for their primary health care. Pharmacologist, botanists, microbiologists and chemists have been used various plants for treatment of infectious diseases. Plants are rich in a variety of secondary metabolites. The quality control of herbal medicine became important as the use of a variety of medicinal plants. Therefore, the plant materials are the most important for human health care.

Nyctanthes arbor-tristis L. is perennial small tree, about 5 m in high. Stems cylindrical, woody with grey or greenish-white bark; branches quadrangular. Leaves simple, opposite and distichous, petiolate, exstipulate; lamina ovate-lanceolate, rounded at the base, entire or with a few large distant teeth at the margin, acuminate at the apex, dark green above and pale green beneath, coriaceous, hirsute. Inflorescences axillary or terminal cluster cymes,3-7 flowered. Flowers white with orange-red center, across at anthesis, bracteate, ebracteolate, sessile, complete, bisexual, regular, actinomorphic, 6-merous, cyclic, hypogynous, delicate fragrant. Calyx 6 lobes, narrowly campanulate, greenish yellow, ribs present, glabrous with and cilliate without; tube about 0.9cm long; lobes obscurely. Corolla salverform, glabrous; tube orange colour, lobes unequally obcordate, cuneate, white with an orange-red center, glabrous. Stamens 2, epipetalous, inserted near the top of the corolla tube; filaments very short, adnate to the corolla tube, bright orange, glabrous; anthers oblong, pale yellow, dithecous, dorsifixed, dehiscence longitudinal slit. Ovary superior, globoid, yellowish green, glabrous, carpels 2, bicarpellary, syncarpous, bilocular with one ovule in each locule , basal placentas; style terete, white, glabrous; stigma bilobed. Fruits capsule, obcordate, one seeded.

In Ayurvedic medicine, *Nyctanthes arbor-tristis* L. leaves were used for the treatment of sciatica, chronic fever, rheumatism, internal worm infections, laxative, and diuretic. Leaf juice is mixed with honey and given thrice daily for the treatment of cough. Paste of leaves is given with honey for the treatment of fever, high blood pressure and diabetes (Meshram *et al*., 2012).

In this research, the preliminary phytochemical analysis of the extracts of leaves contained alkaloid, α-amino acid, glycoside, flavonoids, reducing sugar, phenolic compound, saponin, starch, tannin and carbohydrate were found to be present. These data were agreed with Jain and Pandey, 2016. They stated that the presence of secondary metabolites such as tannins, saponins, alkaloids, flavonoids, steroids, phenolic compounds, reducing sugar and carbohydrate. Rani *et al*., 2012 stated that alkaloids, phenolics, tannins, flavonoids, glycosides and saponins.

In the antimicrobial activities test, ethanol extracts of leaves showed the significant against on *Bacillus pumilus and Xanthomonas oryzae ,moderate against on Bacillus subtilis, least against on Candida albicans, Staphyloococcus aureus and gave the negative results on Micrococcus luteus* and *Pseudomonas* sp. Methanol extract of leaves showed significant against on *Bacillus pumilus, Bacillus subtilis*, and *Staphyloococcus aureus,* moderate against on *Xanthomonas oryzae* and negative results on all other test organisms .Watery extracts showed moderate activities on *Bacillus pumilus, Bacillus subtilis* and gave the negative results on all other test organisms. Ethyl acetate extracts of leaves exhibited negative results on *Candida albicans* and *Micrococcus luteus*, moderate activities exhibited on other test organisms. Pet-ether extracts exhibited against only on *Bacillus subtilis.* Acetone extract revealed the negative result on *Micrococcus luteus,* moderate against on *Candida albicans* and the best activities on all other test organisms. Chloroform extracts of leaves showed the moderate activities on *Bacillus pumilus, Bacillus subtilis* and the best activities on all other test organisms. Priya and Ganjewala, 2007, Shrivastava and Bharadwaj, 2018 stated that methanolic extract of leaves exhibited significant antibacterial activity against *Staphylococcus aureus, Staphylococcus epidemis, Samonella typhi, Samonella paratyphi.* Meshram *et al.,* 2012 stated that chloroform extract showed both antibacterial and antifungal activity, the petroleum ether and ethanol extracts possess only antibacterial activity.

The present study was undertaken to investigate the preliminary phytochemical analysis of leaves, to check the secondary metabolites and antimicrobial activities of *Nyctanthes arbor-tristis* L., help in other experimental analysis, to distribute the knowledges and advandages of traditional medicines for local people.

Acknowledgements

We would like to give our sincere gratitude to Professor Dr Thida Oo, Professor and Head of the Department of Botany, University of Yangon, for her permission to provide the research facilities and available references for this research. We are greatly indebted to Dr Thet Thet Mar Win, Dr Khin Min Min Phyo, Dr Ni Ni Tun, Professors, Department of Botany, University of Yangon, for their valuable suggestions in this research.

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ISOLATION AND IDENTIFICATION OF PROTEOLYTIC FUNGI FROM BEAN CROP SOIL AND PADDY MILL SOIL

Phyo Wai Khaing[1], Nu Yin Aye[2]

Abstract

The present study, eleven fungi were isolated from bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and twelve fungi were isolated from paddy mill soil in Daik-U Township. Twenty-three fungi were screened by visible plate technique using Potato Dextrose Agar medium (Supplement with 1% Casein). Among all isolated fungi, eight proteolytic fungi were showed clear zone around the fungal colony on Skim Milk Agar (SMA) medium for the preliminary study of proteolytic fungi. Although four proteolytic fungi (V2,V5, D4 and D12) exhibited prominent enzyme activity about (2.5cm for five days, 3.5cm for four days, 2.2cm for four days and 2cm for six days) respectively based on the ratio of diameter of the clearing zone and colony. These four fungi were selected for identification of their colony morphology and spore formation. In the identification of four selected fungi, two *Penicillium* sp. V2 and V5 were isolated from bean crop soil and one *Penicillium* sp. D4 and one *Cladospordium* sp. D12 were isolated from paddy mill soil.

Keyword *Penicillium* sp. and *Cladospordium* sp., Protease fungi

Introduction

Proteolytic enzymes are also termed as peptidases, proteases and proteinases, which are able to hydrolyze peptide bonds in protein molecules. Proteases are generally classified as exopeptidases and endopeptidases. Exopeptidases cut the peptide bond proximal to the amino or carboxy termini of the protein substrate and endopeptidases cut peptide bonds distant from the termini of the protein substrate (Amer Ali Mahdi., 2019). Cells of every living organism consist of a chemical substance that possesses the ability to catalyze or speed up a biochemical reaction and acts as biocatalysts which are known as enzymes. Enzymes have better catalytic efficiency, adjustable activity and high specificity when compared to catalysts of chemical or synthetic origin. Proteases are obtained from diverse groups of organisms such as plants, animals and microorganisms, but commercially viable proteases are obtained from microorganisms, especially bacterial and fungal species.

Fungi are considered GRAS (Generally Regarded as Safe) organisms as compared to other microorganisms because they fulfill the criteria of industrial demands such as efficient growth on culture media in short duration and continuous supply of desired products. Fungi also secreted a large variety of proteases, lipases and amylases that play an important role in physiological processes such as germination, as defensins against other pathogens or for nutritional requirements for development. Secretions of fungal enzymes occur from the cells present at the top of hyphae. These secreted enzymes can be used for industrial preparations of valuable products. Fungal proteases have been widely studied due to their wide diversity. Proteases have been isolated from different fungi such as *Aspergillus flavus, Penicillium janthinellum, Neurospora crassa*, etc. Fungal proteases can be isolated through the fermentation process exhibiting high catalytic and specificity for the substrate Salazar-Cerezo *et al*., 2020.

Proteases are considered the most useful and powerful enzymes as they break down complex protein compounds into amino acids and peptides. Around 60% of global enzyme usage is accounted for by protease enzymes. Alkaline protease are the most industrially used or exploited enzymes. More than 3000 different enzymes have been identified and a lot of them

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being utilized in biotechnological and industrial applications. The protease enzyme constitutes two-thirds of the total enzymes used in various industries including meat tenderization, detergents, cheese making, dehairing, baking, contact lens cleaners, waste management and silver recovery Gupta *et al*., 2002 and Amer Ali Mahdi., 2019.

Materials and Methods

Collection of soil samples

The soil samples were collected from bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and paddy mill soil in Daik-U Township. The dust and dead matter on the upper layer of the solid were removed and it was dug down to 6 inches deep. Then the soil sample was put into the laboratory using sterilize plastic bags for further analysis.

Isolation of fungal strains (Atlas, 1993)

The fungal strains were isolated on Potato Dextrose Agar (PDA) medium (potatoes - 200g, casein - 100g, dextrose - 15g and agar - 20g at pH 7.0) was chosen as growth medium used then the media were boiled until the agar well dissolved. The pH of the media was adjusted before autoclaving and then the media were sterilized by autoclaving at 121ºC (15psi) for 15minutes. After cooling down, chloramphenicol were added to the medium and then the medium was poured into sterilized petridishes.

Preparation of samples by serial dilution methods (Waksman, 1927)

The soil samples were used to isolate the different colonies of fungi by serial dilution method. 1g of soil samples was suspended in 100mL and through mixed well for 15minutes with the vortex to obtain 10^{-2} dilution (1:100) and 1mL of sample solution was mixed with 9mL of sterilize distilled water in a sterilize test tube to make 10^{-3}, 10^{-4}, 10^{-5} respectively.

Isolation of pure culture from plate (Collins, 1964 and Atlas, 1993)

After incubation period by pour plate method, the different types of fungal colonies were stored into respectively separate agar slants. For further study, the slant were repeatedly sub-cultured by streaking method to obtain pure culture and then pure cultured fungi were kept at room temperature for further studies of colony morphological characters and spore formation.

Preliminary screening of protease producing fungi (Pelczar and Chan, 1972)

The isolated strains were screened for preliminary protease enzyme production on Skim Milk Agar (SMA) medium by using streak plate method. Skim Milk Agar (SMA) medium were used for screening of protease production by fungi (Namasivayam and Nirmala, 2013, Ayob and Simarani,2016). The isolates pure culture isolates were streaked on the Skim Milk Agar (SMA) medium and were incubated at room temperature for 1 to 7days. Then the appearance of clear zone in the medium around the colony were indicates protease activity. The zone diameter were measured in cm and results are recorded (Warcup, 1950 and Abe *et al*., 2015). The enzyme index (EI) expressed as R/r, which R is the degradation zone diameter and r is the colony diameter according to the method of (Hankin and Anagnostakis, 1975, Abe *et al*., 2015). The species that exhibits maximum clear zone selected for further identification.

Identification and characterization of protease enzyme

The four selected fungi were identified with their colony morphological and spore formation according to the method of Barnett (1960) and Dube (1983).

Results

Isolation of fungi

In the present investigation, the bean crop soil in Vegetable and Fruit Resources Development Centre, Hlegu Township and paddy mill soil in Daik-U Township were used for the isolation of fungi by using serial dilution of pour plate method at room temperature for five days. Protease producing fungi eleven and twelve strains isolated from the bean crop soil and paddy mill soil respectively on Potato Dextrose Agar (Added 1% Casein) medium. All isolated fungi were designated as shown in following Table (1).

Table 1 Designated of all isolated fungi by using Potato Dextrose Agar (Supplement with 1% Casein) medium

No.	Sources	Isolated Strains
1.	Bean crop soil	V1 to V11
2.	Paddy mill soil	D1 to D12

Preliminary production of proteolytic enzyme on Skim Milk Agar (SMA) Medium

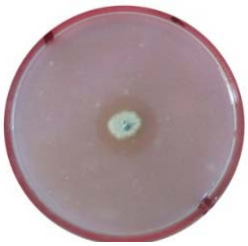
In this investigation, all isolated strains were screened for protease production ability on Skim Milk Agar (SMA) medium by streak plate method. The clear zone formation around the fungal growth was identified as the positive protease produce which may be due to hydrolysis of casein. Among all isolated fungi 4 strains from the bean crop soil and 4 strains from paddy mill soil were showed clear zone on Skim Milk Agar (SMA) medium for the studied of preliminary protease producing fungi as shown in Figure (1 to 8). These result were calculated based on the method of (Hankin and Anagnostakis,1975, and Abe *et al*.,2015). Among 8 proteases positive activity, more prominent of the 4 strains were selected for further investigation. In the bean crop soil, V5 exhibited the high of enzyme activity about 3.5cm for four days and also V8 were showed the lower of enzyme activity about 1.2cm for four days as shown in Table (2). In paddy mill soil, D12 showed the prominent of enzyme activity about 2cm for six days and also D11 shown the poor enzyme activity about 1.9cm for three days based on the ratio of diameter of clearing zone and colony respectively were selected for further investigation.

Selection of most potent proteolytic activity of fungi

In this study, 8 proteolytic fungi 2 strains from bean crop soil and 2 strains from paddy mill soil were selected for further investigation based on their prominent clear zone than the other strains. And then, the four selected fungi were identified based on colony morphology and spore formation according to the method of Barnett, 1960 and Dube, 1933.

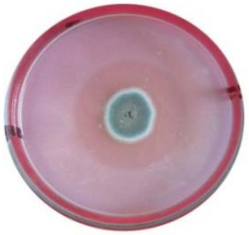
Table 2 Enzyme index of the 8 positive isolated from bean crop soil and paddy mill soil according to the (Hankin and Anagnostakis, 1975, and Abe *et al*., 2015)

Isolated fungi	Clear zone diameter (cm)							Colony diameter (cm)							Enzyme index (EI) (cm) = Clear zone diameter / Colony diameter						
	Incubation hours																				
	24	48	72	96	120	144	168	24	48	72	96	120	144	168	24	48	72	96	120	144	168
V2	1.3	2	2.7	3	3.7	-	-	0.6	0.9	1.1	1.3	1.5	-	-	2.2	2.2	2.4	2.3	2.5	-	-
V5	0.7	2	2.7	2.8	-	-	-	0.5	0.6	0.7	0.8	-	-	-	1.4	3.3	3.8	3.5	-	-	-
V8	1.3	2	2.2	2.3	2.5	3	3.5	0.8	1.2	1.6	1.8	2	2.5	3	1.6	1.7	1.4	1.3	1.2	1.2	1.2
V11	1	1.2	2	-	-	-	-	0.7	0.7	0.8	-	-	-	-	1.4	1.7	2.5	-	-	-	-
D4	1.5	2	2.3	2.7	-	-	-	0.8	1	1.1	1.2	-	-	-	1.9	2	2.1	2.2	-	-	-
D10	1	1.5	2	2.6	-	-	-	0.6	0.8	1	1.2	-	-	-	1.6	1.8	2	2.1	-	-	-
D11	0.8	1.3	1.7	-	-	-	-	0.5	0.7	0.9	-	-	-	-	1.6	1.8	1.9	-	-	-	-
D12	1	1.5	2	2.5	3	3.2	-	0.5	0.7	0.9	1.2	1.5	1.6	-	2	2.1	2.2	2.1	2	2	-

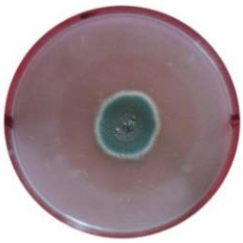


One day old culture

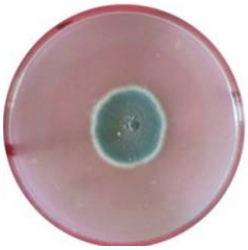
Two days old culture



Three days old culture



Four days old culture



Five days old culture

Figure 1 Proteolytic activity of isolated V2 on Skim Milk Agar medium for one to five days old culture

One day old culture Two days old culture Three days old culture Four days old culture

Figure 2 Proteolytic activity of isolated V5 on Skim Milk Agar medium for one to four days old culture

Oneday old culture Two days old culture Three days old culture Four days old culture

Five days old culture Six days old culture Seven days old culture

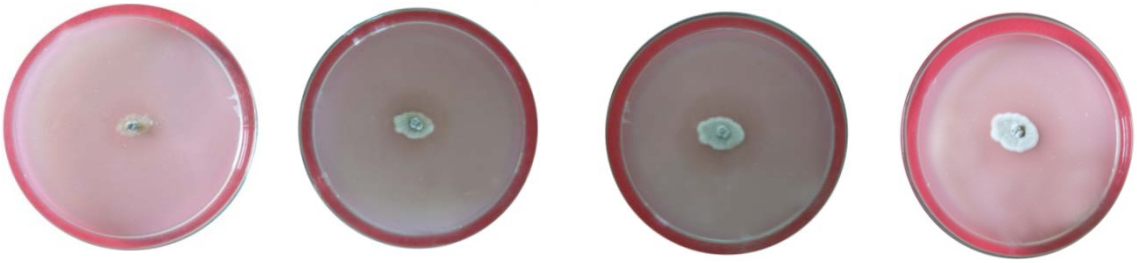
Figure 3 Proteolytic activity of isolated V8 on Skim Milk Agar medium for one to seven days old culture

One day old culture Two days old culture Three days old culture

Figure 4 Proteolytic activity of isolated V11 on Skim Milk Agar medium for one to three days old culture

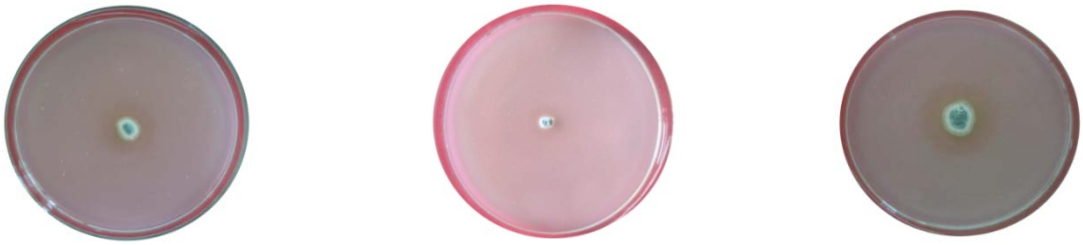
One day old culture Two days old culture Three days old culture Four days old culture

Figure 5 Proteolytic activity of isolated D4 on Skim Milk Agar medium for one to four days old culture



One day old culture **Two days old culture** **Three days old culture** **Four days old culture**

Figure 6 Proteolytic activity of isolated D10 on Skim Milk Agar medium for one to four days old culture



One day old culture **Two days old culture** **Three days old culture**

Figure 7 Proteolytic activity of isolated D11 on Skim Milk Agar medium for one to three days old culture

One day old culture **Two days old culture** **Three days old culture**

Four days old culture **Five days old culture** **Six days old culture**

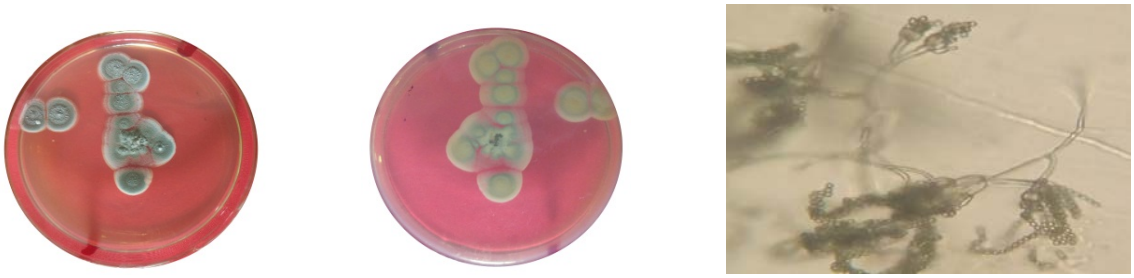
Figure 8 Proteolytic activity of isolated D12 on Skim Milk Agar medium for one to six days old culture

Morphological characteristics of genus level on prominent fungi

In this investigation, morphological characters of 4 selected strains were carried out for identification. In this study of investigation, isolated V2 belong to *Penicillium* sp., isolated V5 belong to *Penicillium* sp., isolated D4 belong to *Penicillium* sp. and D12 belong to *Cladosporium* sp. according to the method of Barnett,1960 and Dube,1933as shown in Figure (9 to 12).

Microscopical characters of V2

The mycelium color is green color inside and white color periphery in surface view and yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like, conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, V2 may be *Penicillium* sp**.** as shown in Figure (9).

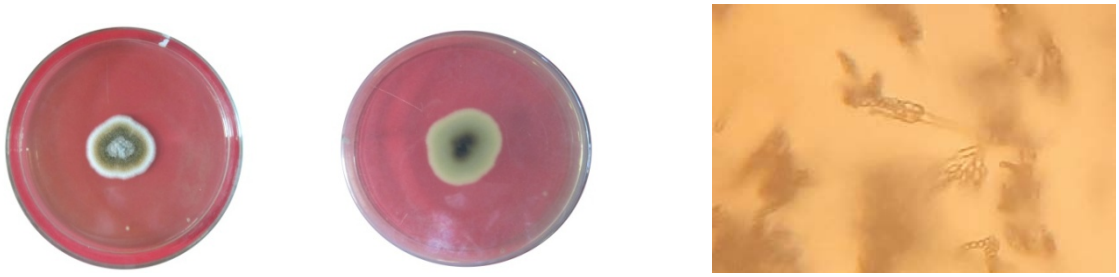


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 9 Cultural characters and morphological character of isolated fungi V2 from bean crop soil

Microscopical characters of V5

The mycelium color is yellow brwon color inside and white color periphery in surface view and pale yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like,conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, V5 may be *Penicillium* sp**.** as shown in Figure (10).

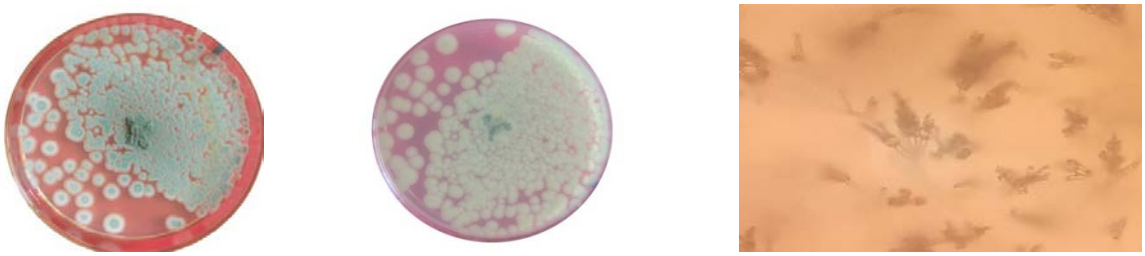


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 10 Cultural characters and morphological character of isolated fungi V5 from bean crop soil

Microscopical characters of D4

The mycelium color is pale green color inside and white color periphery in surface view and pale yellow color on reverse view. Conidiospores arising from the mycelium singly, branched near the apex to form a brush-like,conidia-bearing apparatus, conidia brightly colored in mass, 1-celled, ovoid, produced basipetally. According to these microscopical characteristics, D4 may be *Penicillium*sp**.**as shown in Figure (11).

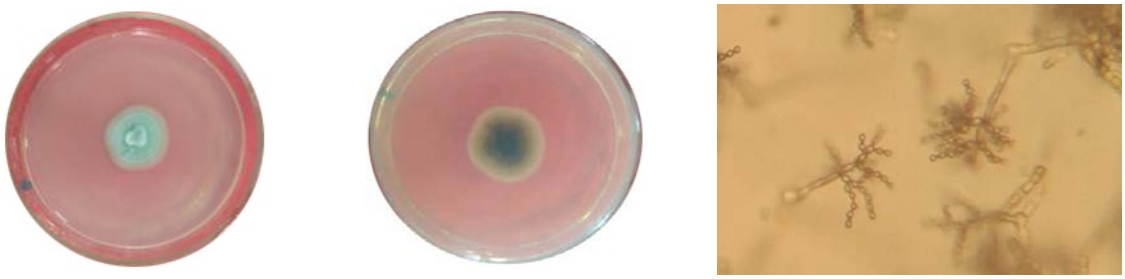


Surface view **Reverse view** **Micrograph of *Penicillium* sp. (X400)**

Figure 11 Cultural characters and morphological character of isolated fungi D4from paddy mill soil

Microscopical characters of D12

The mycelium color is green color inside in surface view and pale brown color on reverse view. Pigment present. Conidiophores dark, branched variously near the apex, clustered, conidia dark, 1 or 2 celled, ovoid to cylindrical, some typically lemon-shaped. According to these microscopical characteristics, D12 may be *Cladosporium*sp. as shown in Figure (12).



Surface view **Reverse view** **Micrograph of *Cladosporium* sp. (X400)**

Figure 12 Cultural characters and morphological character of isolated fungi D12 from paddy mill soil

Discussion and Conclusion

A total of twenty-three fungi were isolated from bean crop soil (Vegetable and Fruit Resources Development Centre, Hlegu Township) and paddy mill soil (Daik-U) Township on Potato Dextrose Agar medium (supplement with 1% Casein). Among all isolated fungi, eleven fungi from bean crop soil and twelve fungi from paddy mill soil were study for preliminary protease producing fungi on Skim Milk Agar (SMA) medium. In the 8 proteolytic fungi, V2 (2.5cm) for four days, V5 (3.5cm) for four days from bean crop soil and D4 (2.2cm) for four days, D12 (2cm) for six days from paddy mill soil were selected based on larger clear zone of hydrolysis (halo) as shown in Table (2) and Figure (1 to 8).

The maximum protease activity of four selected fungi were subjected to colony morphology and spores formation to identify probably fungi species and our fungi isolate were found out to be of three *Penicillium* sp. (V2, V5 and D4) and one *Cladosporium* sp. (D12) as shown in Figure (9 to12). Vaishali Choudhary and P.C. Jain, 2012 were reported that the valuable for protease production observed in the garden soil, crop field soil and poultry farm soil. Vamsi Krishna *et al*., 2009 and Benluvankar *et al*., 2016 were stated that a high protease activities secreted by soil fungi observed in *Penicillum*sp. Sawao *et al*., 1971 were also reported that the acid protease of *Cladosporium* sp. as a good strains for the producing of protease

enzyme. The investigation of present research was focused on isolation; screening and identification of protease producing fungi were showed significant ability to degrade the proteolytic material in present plantation site. The selected proteolytic strains from this study were be continuing for further study to examine the optimal experimental conditions for production of protease enzyme.

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STUDY ON THE REPRODUCTIVE GROWTH AND YIELD EVALUATION OF VARIOUS CORN CULTIVARS (*ZEA MAYS* L.) IN AYEYARWADDY AND YANGON REGIONS

Thiri Shwe Yee[1], Aye Aye Mu[2]

Abstract

The field experiment with seed priming technique and organic fertilizer treatments was carried out at the Maubin University, Ayeyarwaddy Region from January 2022 to May 2022 using the seven various cultivars seeds of *Zea mays* L. (Corn). In this experiment, study on the total of seven cultivars of corn was collected from two different localities within Ayeyarwaddy and Yangon Regions. According to the experiment, the first germination percentage (Pan – pyaung) are the best in germination percentage, (A- Kaut gyi) and (Y- Sat kyar) are the lowest germination percentage although local varieties have a germination percentage of over 90%. In the second germination percentage, except Pan, the other varieties are more than 95% and Pan is the lowest. The experiment showed that the reproductive growth of (Y - Nga chaik) is the highest reproductive growth and (Pan - pyaung) plants is the lowest. (Y - Lay tan) and (Y- Sat kyar) are the medium reproductive growth. Among them, (Y- Nga chaik) is better than other varieties. (Y- Nga chaik) is weather resistant and has good yield for this region. So, it is a local variety that should be planted. Therefore, it is recommended that the (Y- Nga chaik) is strong and resistant local variety. Because it is economically beneficial of the farmers, it should be cultivated.

Keywords germination percentage, reproductive growth, varieties and yield evaluation

Introduction

Corn was originally domesticated in Mexico by native peoples about 9,000 year ago. Corn is also called maize, cereal plant of the grass family (Poaceae) and edible grain. Maize is the third most important cereal crop species, after wheat and rice, grown throughout a wide range of climates. Corn is cultivated mainly in the country's site of Shan, Chin states, Sagaing, Magway and Mandalay regions as a seasonal crop in monsoon and winter (Thandar Soe, 2019). It is among the ten most important world crops by value. Next to rice, maize stands as the second most important cereal crop in Myanmar, which is used as human consumption, animal feed for livestock farming and as one of the major agricultural products for export (Huang, *et al*., 2006). Hence more production of corn is needed through expansion of cultivable area and increased production per unit area (Thandar Soe, 2019). Therefore, corn becomes an important crop for growing population around the world. Corn is one of the most important crop around the world. Corn is generally a crop of warm climates with adequate moisture and annual crop (Denmead & Shaw 1960). Corn grows best in deep, well-aerated, warm, loam soils rich in organic matter, and with a high nitrogen, phosphorus and potassium content (Jugenheimar, 1992). Corn can be grown on soils with pH values ranging from 5.5 to 8.0 and does best at neutral pH (Garki, *et al*, 2004). Raising two to twenty feet high, corn stalks can have anywhere from eight to forty-eight leaves and multiple ears (Goldsworthy *et al*, 1974). Corn is a crop which is very sensitive to water stress (Pandey *et al*, 2000; Abdelgadir, 2002; Cakir, 2004; Kuscu & Demir, 2012).

The price of corn which was grown in dry season was higher than that of rainy season. In addition, growing maize during dry season increased annual production (Global Agricultural Information Network [GAIN], 2018). It will be needed to apply water using effective irrigation methods. Its importance as a major food in many parts of the world, corn is inferior to other cereals in nutritional value. It is usually planted at the end of winter and harvested during the

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summer, although in certain regions it is grown all year around (Usoh *et al*, 2017). The present study was conducted to investigate the collection of various local corn varieties in the Ayeyarwaddy and Yangon Regions. The objectives of this study were: the germination percentage of various varieties of corn, the growth and yield evaluation of various varieties of corn and to preserve the local corn cultivars.

Materials and Methods

Collection of corn cultivars

Three cultivars of *Zea mays* L. corn varieties were collected from Ayeyarwaddy Region are Nga chaik, Wat si and Kaut gyi. And then, four cultivars of corn varieties were collected from Yangon Region are Nga chaik, Lay-tan, Sat kyar and Pan. All the cultivars varieties were collected from locally grown for many decades using traditional methods.

Time and Experimental Site

The experimental site was carried out using the seeds of corn in the farm of Maubin University, Ayeyarwaddy Region from January 2022 to May 2022.

Soil Analysis

Soil samples from 30 cm depth of soil surface were randomly taken from ten places of planting area. The collected soil samples were analyzed at laboratory of Land Use Division, Department of Land Use, Myanmar Agriculture Service (MAS), Insein Township, Yangon Region.

Seedling establishment

Seeds were germinated in small tray filled with soil, sand and rice husk. The seed of seven different cultivars were soaked in water for three days. One centimeter holes were made in the prepared medium. The germinating of corn seed was sown in the tray for one week. Then the seedlings of corn in the tray were transplanted to the prepared land.

Soil Preparation

The soil from cultivation field was thoroughly plowed by machine and cleaned to remove hard soil balls, stones and garbage's. The hole in the depth of 8 cm was dug and mixed with humus, fungicide and pesticide. Seven days after germination, the seedlings of corn were transplanted to the prepared field.

Transplanting Method

The germinating seedlings were sown directly in the prepared field plots. The seedling was sown in rows, the space between each row was three feet, space between each plant was one feet and space between each block was nine feet. The total cultivation area was 117 ft × 37 ft. There were seven blocks and each block containing three plots which represents one cultivars. Each plot has ten plants, the total of 210 plants were cultivated in the prepared soil.

Experimental layout design

T_1 = (Y- Pan-pyaung) (control)
T_2 = (A –Nga chaik)
T_3 = (A – Wat si)
T_4 = (A – Kaut gyi)
T_5 = (Y – Nga chaik)
T_6 = (Y – Lay tan)
T_7 = (Y – Sat kyar)

Cultural management practices

Weeding was done once in a week interval. Supplementary weeding was carried out in small scale when necessary. The whole plot was watered at ten days' intervals. Irrigation system is two times in one-week interval after brace root starts to emerge.

Data collection of reproductive characters

The data were collected weekly. A total of 15 plants from each cultivar were selected for evaluation. Reproductive characters such as ear number per plant, ear length, ear girth, kernel number per ear, kernel weight per ear, kernel row number per ear, kernel 100 weights per ear, husk number per ear and cob weight per ear were measured and recorded.

Germination percentage Test

Germination percentage was determined by using (Soupe, 2009). The calculation of percentage in Germination was as follows:

$$\text{Germination percentage (\%)} = \frac{\text{Number of Total Germinated Seeds}}{\text{Total Number of Seeds Tested}} \times 100$$

Results

Soil analysis

The soil analysis of the cultivation area was carried out to evaluate the production based on soil composition. The results of soil analysis showed that soil texture was loam, the pH (4.42) and extremely acid, moisture content (9.58 %) and low organic carbon content (1.90 %). Humus content was medium (3.27 %). However, the nitrogen (0.25 %) and potassium contents (0.45 meq/100gm) were medium phosphorous (0.22 ppm) was low and available nutrient potassium oxide content (21.23 mg/100gm) was high (Table 1).

Table 1 Analyzed result of the cultivate soil

Moisture (%)	pH	Texture				Organic carbon (%)	Humus (%)	Total N_2 (%)	K(meq /100gm)	Available P(ppm)	Available K_2O mg/100gm
		Sand	Silt	Clay	Total (%)						
9.58	4.42	37.58	19.38	43.04	100	1.90	3.27	0.25	0.45	0.22	21.23
	(extremely acid)		(loam)			(low)		(medium)	(medium)	(low)	(high)

Figure 1 Soil preparation of experimental area

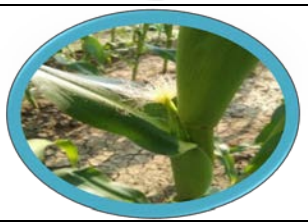
Figure 2 Seedlings were grown in the tray

Figure 3 Transplanting plant of seven corn field varieties

Figure 4 Growth of corn in cultivated Soil plot

Development stages of corn varieties

Development stages of corn were carried out by using organic fertilizers (cow dung). After the germination of corn put it in the tray for 7 days. After sowing on the ground: 5.5 cm after 14 days, 21.6 cm after 28 days, 37.2 cm after 42 days and 53.4 cm after 56 days. Early male tassel was observed in all treatments at 70 days after fertilizers treatments. Ear development continued and the mature was observed at 84 days after treatments. The female inflorescence, with young silk observed at 98 days after treatments. Stalks, ears and silk early fruit set was started at 112 days after treatments and female inflorescence, with young silk for harvest was resulted at 126 days after treatments. Therefore, it was noted that the mature maize ear on a stalk took 126 days after treatments (Figure 5).

Stage of vegetative growth	DAS	Stage of reproductive growth	DAS
	7 DAS		70 DAS
	14 DAS		84 DAS
	28 DAS		98 DAS
	42 DAS		112 DAS

Stage of vegetative growth	DAS	Stage of reproductive growth	DAS
	56 DAS		126 DAS

DAS = Days After Sowing

Figure 5 Development stages of corn varieties

Reproductive characters of seven corn varieties on ear length and ear girth

Reproductive characters of seven corn varieties on ear length and ear girth are shown that the reproductive characters of ear length and ear girth (Pan-pyaung) are the highest and (A - Kaut gyi) are the lowest (Table 2 and Figure 6).

Table 2 Reproductive characters of seven corn varieties on ear length and ear girth

No.	Reproductive Characters (cm)	Pan-pyaung	A - Nga chaik	A - Wat si	A – Kaut gyi	Y - Nga chaik	Y - Lay tan	Y - Sat kyar
1.	Ear length(cm)	19.6	18.6	17.4	16.2	19.4	18.1	16.7
2.	Ear girth(cm)	17.0	12.5	13.0	12.0	15.8	15.9	14.0

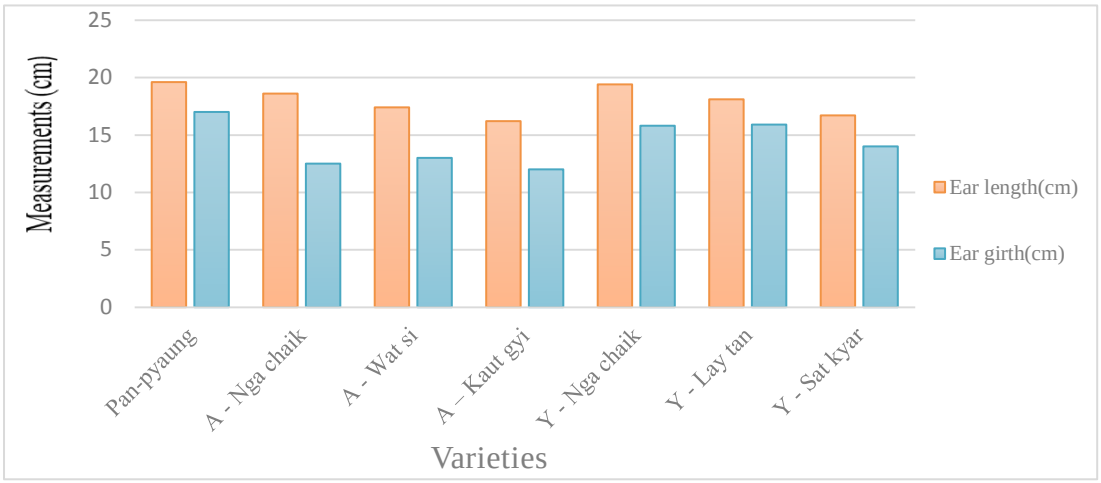


Figure 6 Reproductive character of seven corn varieties on ear length and ear girth

Reproductive characters of seven corn varieties on ear number, husk number, row number and seed number

Reproductive characters of seven corn varieties on ear number, husk number, row number and seed number are shown that the reproductive characters of ear number (A- Nga chaik), (A- Wat si), (A- Kaut gyi), (Y- Nga chaik), (Y- Lay tan) and (Y- Sat kyar) are the highest and (Pan- pyaung) is the lowest. Husk number (Y- Nga chaik) is the highest and (Pan- pyaung) is the lowest. Row number (Y- Nga chaik) is the highest and (Pan- pyaung) is the lowest. Seed number (Y- Nga chaik) is the highest and (A- Wat si) is the lowest (Table 3 and Figure 7).

Table 3 Reproductive characters of seven corn varieties on ear number, husk number, row number and seed number

No.	Reproductive Characters	Pan-pyaung	A - Nga chaik	A - Wat si	A – Kaut gyi	Y - Nga chaik	Y - Lay tan	Y - Sat kyar
1.	Ear number	1	2	2	2	2	2	2
2.	Husk number	10	18	17	21	22	20	20
3.	Row number	14	24	21	24	25	23	21
4.	Seed number	440	457	326	522	543	428	482

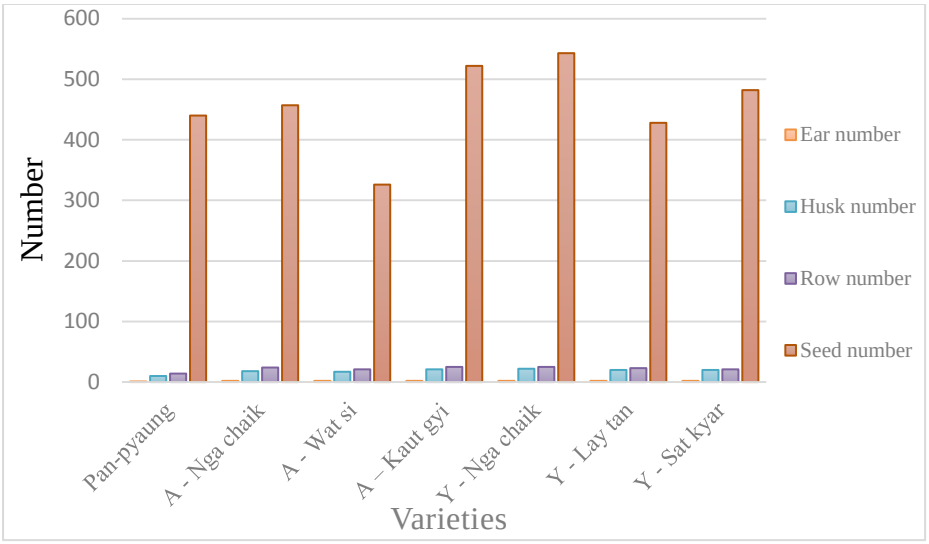


Figure 7 Reproductive character of seven corn varieties on ear number, husk number, row number and seed number

Reproductive characters of seven corn varieties on kernel weight, kernel 100 weight and cob weight

Reproductive characters of seven corn varieties on kernel weight, kernel 100 weight, and cob weight are shown that the reproductive characters of kernel weight (Y- Nga chaik) is the highest and (A- Wat si) is the lowest. Kernel 100 weight (Y- Nga chaik) is the highest and (Pan-pyaung) is the lowest and cob weight (Y- Lay tan) is the highest and (Pan-pyaung) is the lowest (Table 4 and Figure 8).

Table 4 Reproductive characters of seven corn varieties on kernel weight, kernel 100 weight and cob weight

Weight (g)	Varieties						
	Pan-pyaung	A - Nga chaik	A - Wat si	A – Kaut gyi	Y - Nga chaik	Y - Lay tan	Y - Sat kyar
Kernel weight	479.7	321.3	232.7	286.5	482.3	370.3	420.7
Kernel 100 weight	38.0	55.1	72.1	64.9	78.7	70.8	63.6
Cob weight	41.0	61.9	85.0	56.3	76.6	95.0	66.3

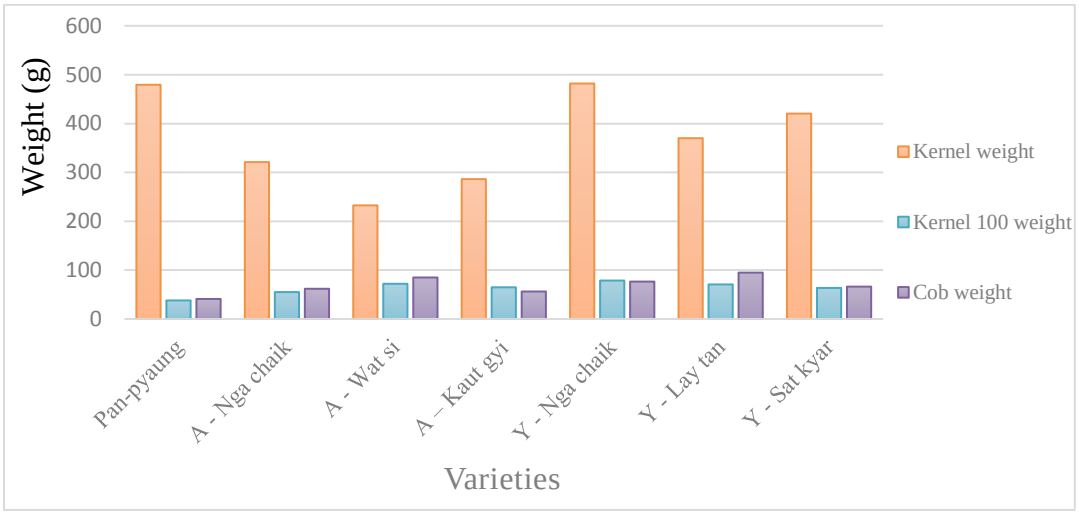


Figure 8 Reproductive character of seven corn varieties on kernel weight, kernel 100 weight and cob weight

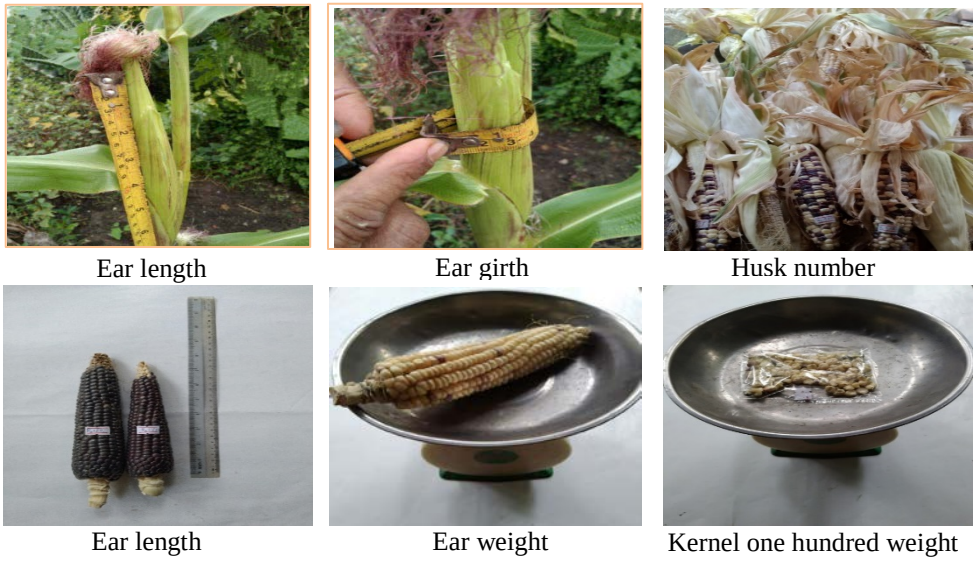


Figure 9 Data collection of reproductive characters corn varieties

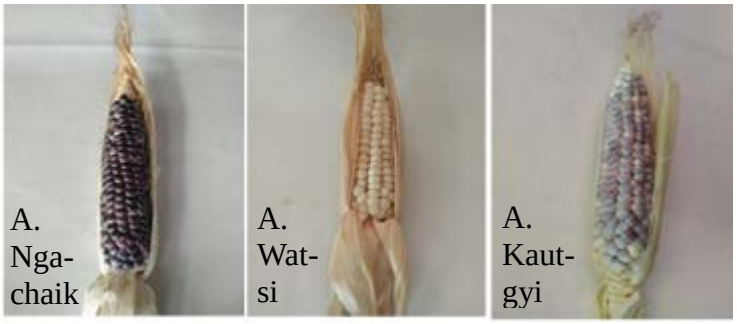


Figure 10 Harvested of seven corn varieties (Ayeyarwaddy Region)

Figure 11 Harvested of seven corn varieties (Yangon Region)

Comparison of the first and second germination percentage of seven corn varieties

Seeds of the seven cultivars were soaked in water and germination rates for individual cultivars were recorded at two different times. (Pan) variety germination in the first test was (98%) and (38%) germination in the second test. (A- Nga chaik) variety germination in the first test was (90%) and (97%) germination in the second test. (A- Wat si) variety germination in the first was (92%) and (98%) germination in the second test. (A- Kaut gyi) variety germination in the first was (64%) and (96%) germination in the second test. (Y- Nga chaik) variety germination in the first test was (94%) and (99%) germination in the second test. (Y- Lay tan) variety germination in the first was (96%) and (98%) germination in the second test. (Y- Sat kyar) variety germination in the first was (86%) and (97%) germination in the second test. Therefore, in the case of second germination, except for (Pan- pyaung), it is found that the other varieties are more than the first germination (Table 5 and Figure 12).

Table 5 Comparison of the first and second germination percentage of seven corn Varieties

No.	Varieties	Column-1	Column-2
		First germination percentage test	Second germination percentage test
1.	Y - Pan-pyaung	98%	38%
2.	A - Nga chaik	90%	97%
3.	A - Wat si	92%	98%
4.	A - Kaut gyi	64%	96%
5.	Y - Nga chaik	94%	99%
6.	Y - Lay tan	96%	98%
7.	Y - Sat kyar	86%	97%

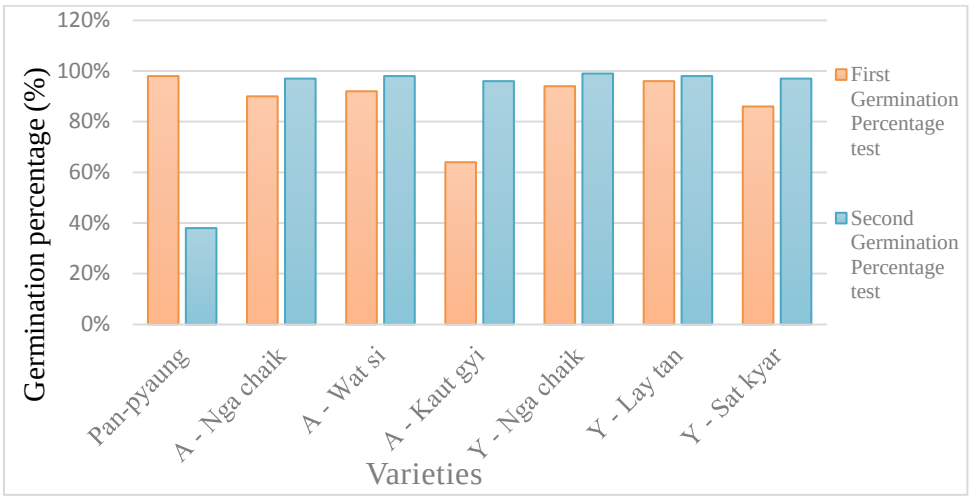


Figure 12 Germination percentage of seven corn varieties

Discussion and Conclusion

The experiment was conducted from January 2022 to May 2022, using cow dung fertilizer. According to soil analysis showed that soil texture was loam, the pH of 4.42 and extremely acid, moisture content of 9.58 % and low organic carbon content 1.90 %. Humus content was medium 3.27 %. However, the nitrogen (0.25 %), and potassium contents (0.45 meq/100gm) were medium, phosphorous (0.22 ppm) was low and available nutrient potassium oxide content (21.23 mg/100gm) was high. Denmead & Shaw (1960) reported that the optimum pH range for a corn is 5.0 to 6.2. Any soil pH below this level is considered low. Corn health may be visibly affected by low pH, but it is usually manifested as a nutrient deficiency. Now, the transplanted soil is more acidic than normal soil. Even though it is very acidic, it can be seen that these types of plants can grow in these areas. So, the local varieties can be grown on any kind of soil and are hardy. Although the soil in which the varieties are grown in these regions is very acidic, it is found that they can grow. Cultivated corn usually prefers full sun, most levels of moisture, and fertile loamy soil. It will be needed to apply water using effective irrigation methods. Maize is a crop which is very sensitive to water stress agreement with respective references, (Abdelgadir, 2002). Humus is better than the other fertilizer for fruit quality and yields are moderate. Therefore, it can be use suitable for business agricultural plantation. Reduce the cost by natural fertilizer application and these plants are strong and it is also resistant to diseases.

The result of the experiment showed that the first germination test, (Pan-pyaung) was highest percentage but (A - Kaut gyi) and (Y - Sat kyar) were lowest percentage in germination rate. In the second germination test, (Y -Nga chaik) was highest percentage but (Pan-pyaung) was lowest percentage in germination rate. In other varieties of germination rate is more than 95%. The authors reported that the kernel moisture content is an important determinant of differences in germination rate between freshly harvested. The percentage of germination required for certification is high in a crop like corn (90%), these results were in agreement with the findings of (Austin & Longden, 1967).

At the second germination rate, the Yangon Region has better germination rate and can be grown in any region. So, it saves the cost of purchasing the species. Farmers should focus on cultivating their own local varieties with suitable germination rates. (Abbas, *et al.*, 2005) reported that the irrigation water is available, corn can grow during dry season even in central dry zone. Most farmers used furrow irrigation method. In addition, growing corn during dry season increased annual production. The agronomic characters of (Y – Nga chaik) has the highest reproductive growth and (A- Kaut gyi) plants has the lowest. The yield was found to be almost identical except the Pan - pyaung. In the reproductive phase, seasonal changes such as high temperatures and heavy rains reduce the rate of nutrient uptake, affecting yields.

The results of the experiment showed that the seven husk numbers of corn varieties, (Y- Nga chaik) is the highest and (Pan - pyaung) is the lowest and the other is the moderate husk numbers. Tight husks have been shown to reduce earworm damage in corn husk trials (Archer *et al.*, 1994 and Wiseman *et al.*, 1977), presumably by acting as a physical barrier to entry into the developing ear. The purpose of counting the husk number was to increase the number of buds on a plant as the number of insects in a plant increased.

In conclusion, (Y- Nga chik) found that the reproductive part was the best in the husk number, row number, seed number, kernel weight and kernel 100 weight. Among them, (Y- Nga chaik) is better than other varieties. The ear number of (Y- Nga chaik) is high due to the high number of nodes. The reason for counting the number of nodes is that the output depends on the good or bad due to the ears coming out from between these nodes. Climate changes such as untimely rains; we experienced hypothermia and gusty winds two times. Therefore, the yield is

found to be lower than normal. Due to these conditions, the output rate is affected. So, (Y- Nga chaik), it also grows on highly acidic soil and is resistant to adverse weather conditions than other varieties.

As a follow up to this research, hybrids and local varieties will be hybridized to produce new varieties with good quality and yield in the future. The nutritional value of hybrid varieties and local varieties can also be compared through experimental work. A variety of yields can also be realized by growing in different soils and climates. The above points are intended for further study in the future.

Acknowledgements

I would like to express my sincere thanks to Dr Ni Ni Oo, Rector of Maubin University, for her permission to perform this study. I am thankful to Dr Than Than Nu, Professor and Head, Department of Botany, Maubin University for her invaluable support and suggestions. I am also greatly indebted to Dr Naw Blute Tser, Professor, Department Botany, Maubin University, for her advice and valuable guidance. I would like to express my gratitude to my supervisor Dr Aye Aye Mu, Associate Professor, Department of Botany, Maubin University, for her invaluable overall guidance, moral support and suggestions cooperation during my study.

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ISOLATION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI FROM SIX MEDICINAL PLANTS

Theingi Aye[1], Moe Moe Aye[2] and Win Yu Yu Naing[3]

Abstract

The six plants samples were collected at Pathein Township, Ayeyarwady Region. The isolation was undertaken by Surface sterilization method using LCA medium. Thirty endophytic fungi were isolated from six different plants. These endophytic fungi were isolated by using Low Carbon Agar (LCA) medium for first culture and Potato Glucose Agar (PGA) medium for pure culture. Isolated fungi were given as temporary names TFO-01 to TFO-30. The colours of all isolated fungi rouge from white, green, black, dark green, yellowish green, pale cream, whitish gray, dark gray, whitish cream, greenish gray to gray. In the study of antimicrobial activity, fungus TFO-16 exhibited the highest activity against *Candida albicans* and TFO-15 against *Saccharomyces cerevisiae*. TFO-15 also showed antibacterial activity against *Salmonella typhimurium* and *Escherichia coli*. Among these two fungi, fungus TFO-15 was selected for further investigation.

Keywords Endophytic fungi, Antimicrobial activity

Introduction

Endophytes are microorganisms that live in the intercellular spaces of stems, petioles, roots, and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed (Strobel and Long, 1998). An endophyte is an endosymbiont, often a bacterium or fungus, that lives within a plant for at least part of its cycle without causing apparent disease.

Most of the endophytes isolated from plants are known for their antimicrobial activity. Endophytes isolated from medicinal plants showed bioactivity for broad spectrum of pathogenic microorganisms. Most of the bioactive compounds isolated from endophytes are known to have functions of antibiotics, immunosuppressants, anticancer agents, biological control agents, and so forth.

Many endophytes have the potential to synthesize various bioactive metabolite may, directly or indirectly be used as therapeutic agents against numerous diseases. The emergence of antibiotic-resistant bacteria such as *Salmonella typhimurium* (Penicillin-resistant bacterium) calls for inventive research and developing strategies (Ezhil *et al*, 2011).

The aim and objectives of this research were to isolate endophytic fungi from medicinal plants and to study the antimicrobial activities of endophytic fungi.

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Materials and Methods

Table 1 Plants samples used for the isolation of endophytic fungi

No.	Sources	Family
1.	*Mangifera indica* L.	Anacardiaceae
2.	*Averrhoa carambola* L.	Oxalidaceae
3.	*Cajanus cajan* Millsp.	Fabaceae
4.	*Talinum triangulare* (Jacq.) Willd.	Talinaceae
5.	*Sauropus androgynus* (L.) Merr.	Phyllanthaceae
6.	*Jatropha podagrica* Hook.	Euphorbiaceae

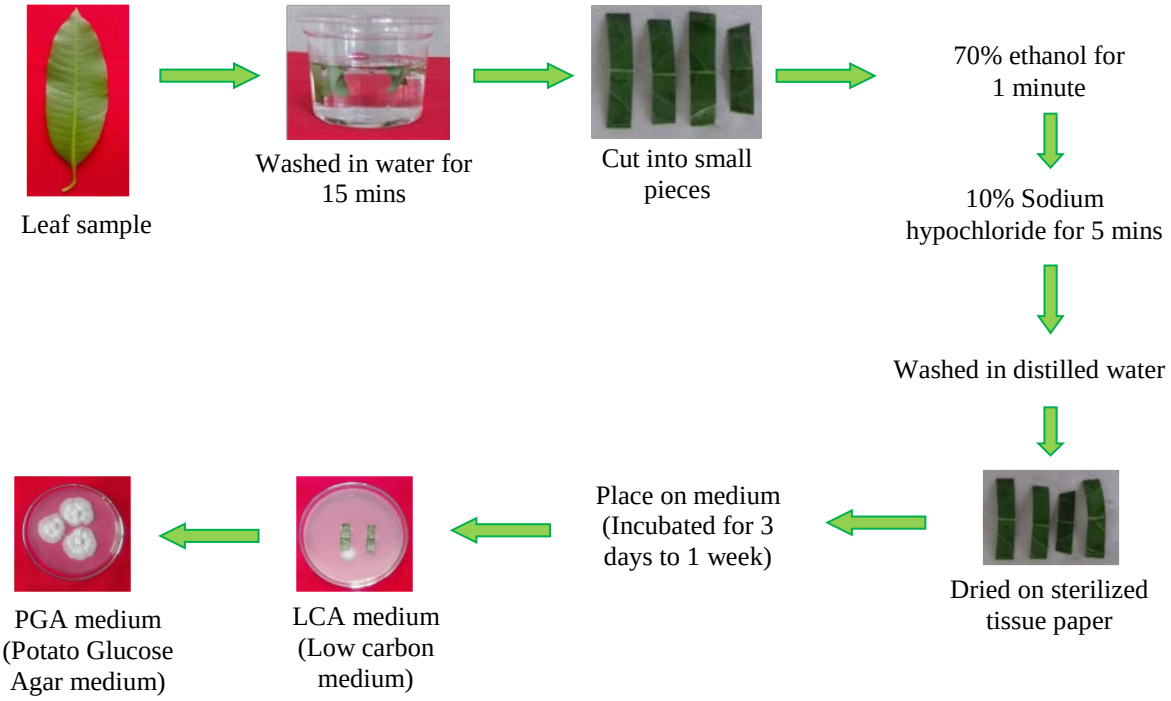


Figure 1 Procedure of Surface sterilization Method (NITE, 2004)

Medium Used for the isolation of fungi (NITE, 2004)

LCA medium

Glucose	1.0 g
Yeast extract	0.2 g
K_2HPO_4	1.0 g
$MgSO_4\,7H_2O$	0.2 g
$NaNO_3$	2.0 g
KC*l*	0.2 g
Agar	1.8 g
DW	100 mL
pH	6.5

PGA medium

(Potato Glucose Agar Medium)

Potato	20 g
Glucose	1.5 g
Agar	1.8 g
DW	100 mL
pH	6.5

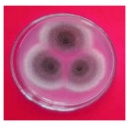
(after autoclaving chloramphenicol (20mg/L) was added to the medium)

Study on the Antimicrobial Activities

Preliminary study for antimicrobial activities by paper disc diffusion assay was carried out by the method of NITE (2004). Six kinds of test organisms *Saccharomyces cerevisiae*, *Candida albicans*, *Micrococcus luteus*, *Pseudomonas fluorescens*, *Escherichia coli* and *Salmonella typhimurium* were used in paper disc diffusion method.

Procedure for Antimicrobial Activity Test (NITE, 2004)

1. Isolated fungi were incubated for 7 days at 25ºC on PGA agar plates.



2. A piece of agar with fungi was inoculated into the tube containing 10 mL fermentation medium.

3. The tube with fungus was incubation at 25ºC for 5 days to ferment for the activity of antimicrobial metabolites.
4. Sample (20 µL) was put on paper disc and antimicrobial activities were investigated using eight kinds of test organisms.

Fermentation Medium

(NITE, 2004)

Glucose	2.0 g
glycerol	1.5 g
Yeast Extract	1.0 g
Polypeptone	0.6g
Malt Extract	0.4g
$MgSO_4.7H_2O$	0.001 g
K_2HPO_4	0.001 g
$CaCO_3$	0.1 g
DW	100 mL
pH	6.5

Assay Medium

Glucose	1.0 g
Peptone	0.3 g
Agar	1.8 g
DW	100 mL

Results

Scientific Name - *Mangifera indica* L.
Famil - Anacardiaceae
Myanmar Name - Tha-yet
English Name - Mango

Scientific Name - *Averrhoa carambola* L.
Famil - Oxalidaceae
Myanmar Name - Zaung-ya
English Name - Star fruit

Scientific Name - *Cajanus cajan*(L.) Millsp.
Famil - Fabaceae
Myanmar Name - Pe-Zin-Gon
English Name - Pigeon-pea

Scientific Name - *Talinum triangulare* (Jacq.) Willd.
Family - Talinaceae
Myanmar Name - Unknown
English Name - Water leaf

Scientific Name - *Sauropus androgynus* (L.) Merr.
Family - Phyllanthaceae
Myanmar Name - Kyet-tha-hin
English Name - Sweet leaf

Scientific Name - *Jatropha podagrica* Hook.
Family - Euphorbiaceae
Myanmar Name - Da-bin-shwe-htee
English Name - Buddha belly plant or gout plant

Table 2 Isolated fungi from six medicinal plant samples by Surface sterilization Method

No.	Sample	Surface sterilization Method	
		Total	**Isolated No**
1.	*Mangifera indica* L.	5	TFO-01,02,03,04,05
2.	*Averrhoa carambola* L.	4	TFO-06,07,08,09
3.	*Cajanus cajan* Millsp.	5	TFO-10,11,12,13,14
4.	*Talinum triangulare* (Jacq.) Willd.	5	TFO-15,16,17,18,19
5.	*Sauropus androgynus* (L.) Merr.	6	TFO-20,21,22,23,24,25
6.	*Jatropha podagrica* Hook.	5	TFO-26,27,28,29,30

Habit Leaves Inflorescence

Figure 2 Habit of *Mangifera indica* L.


Habit


Leaves


Inflorescence

Figure 3 Habit of *Averrhoa carambola* L.


Habit


Leaves


Inflorescence

Figure 4 Habit of *Cajanus cajan* (L.) Millsp.


Habit


Leaves


Inflorescence

Figure 5 Habit of *Talinum triangulare* (Jacq.) Willd.


Habit

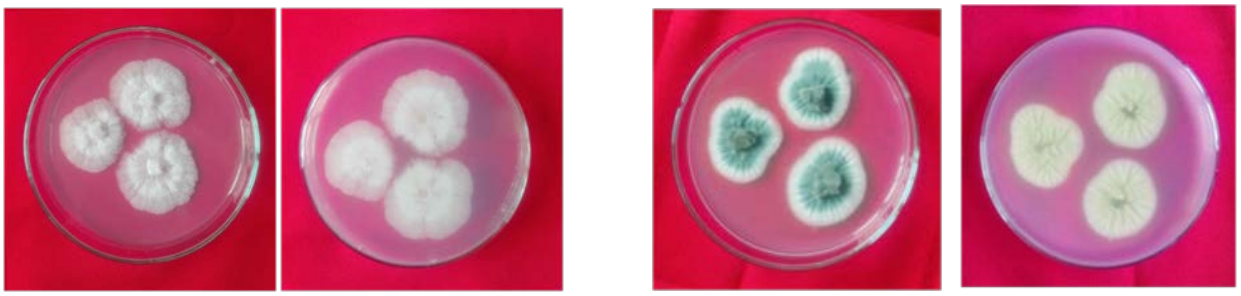

Leaves


Inflorescence

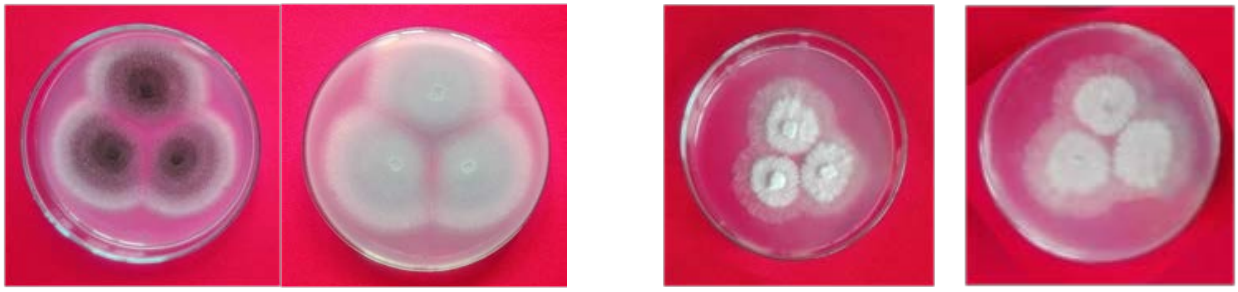
Figure 6 Habit of *Sauropus androgynus* (L.) Merr.

Habit Leaves Inflorescence

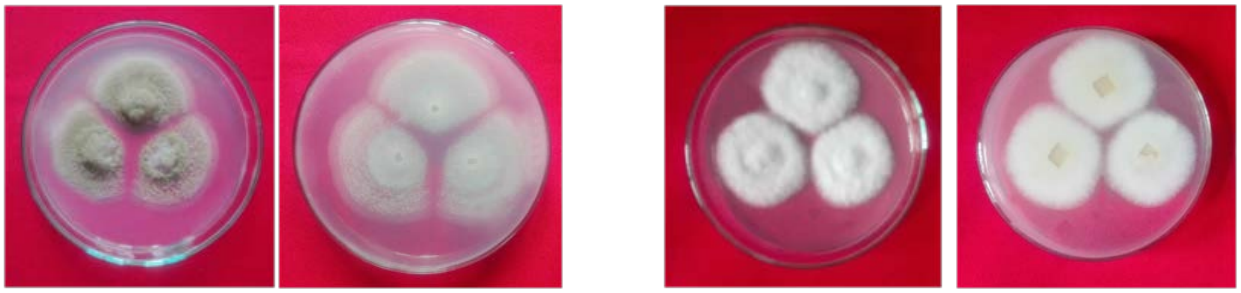
Figure 7 Habit of *Jatropha podagrica* Hook.



Front view TFO-01 Reverse view Front view TFO-02 Reverse view



Front view TFO-03 Reverse view Front view TFO-04 Reverse view



Front view TFO-05 Reverse view Front view TFO-06 Reverse view

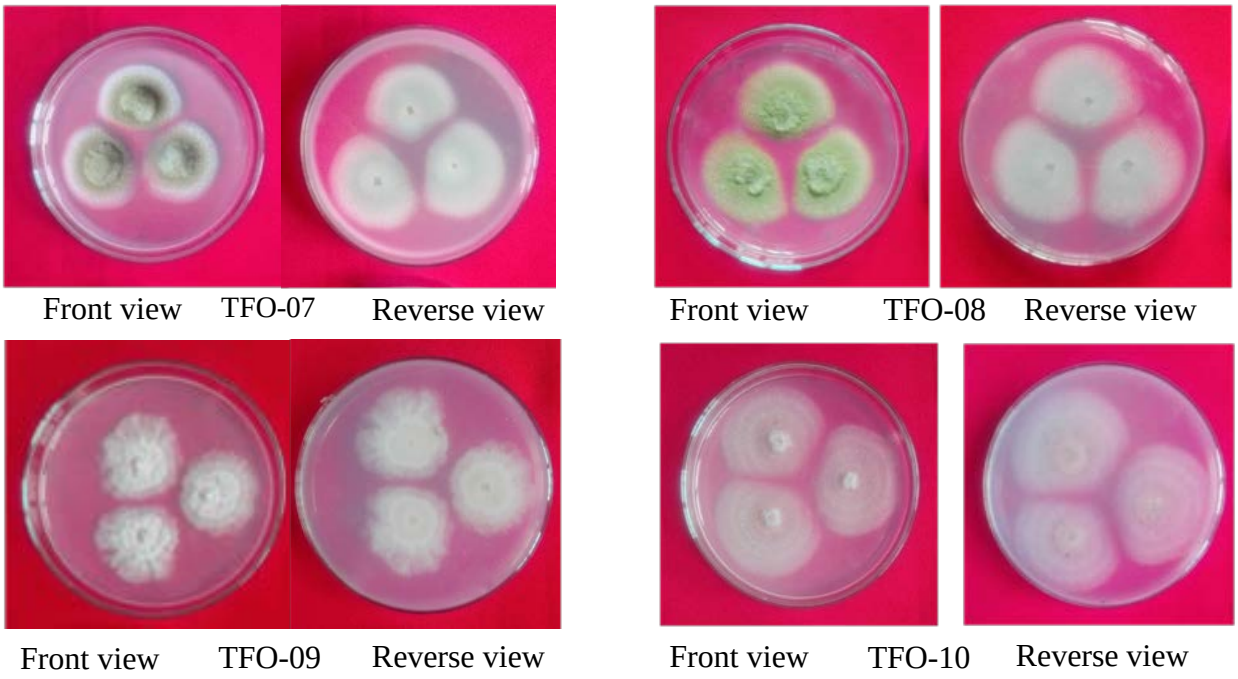
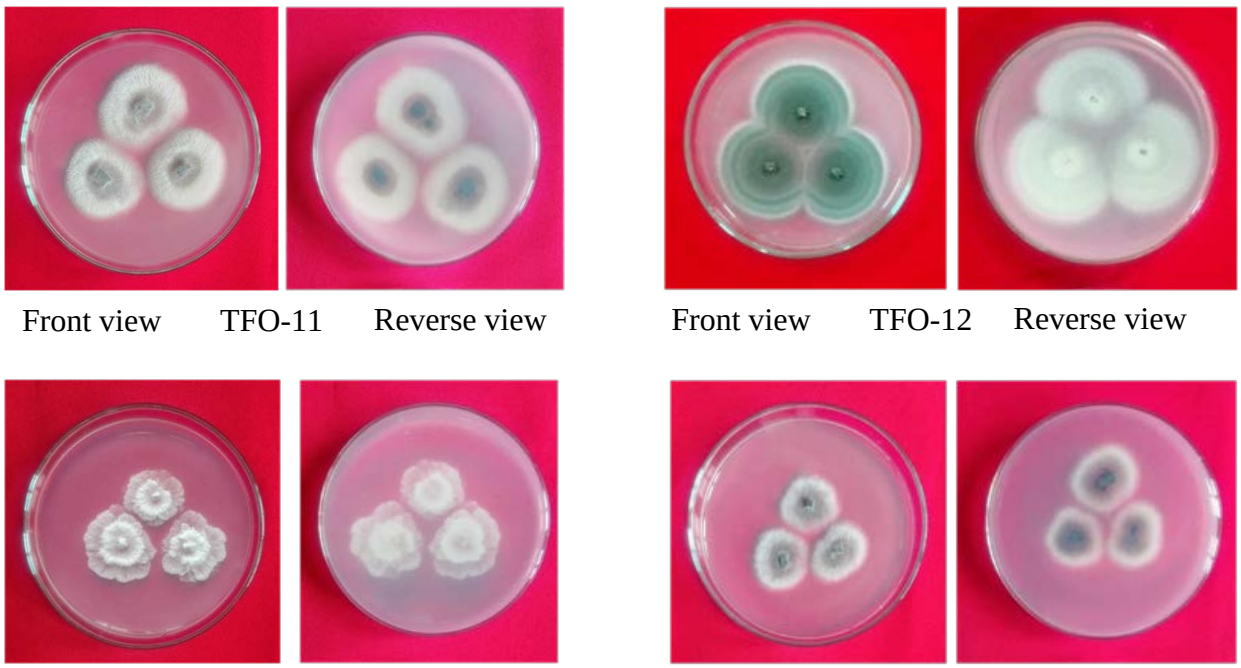
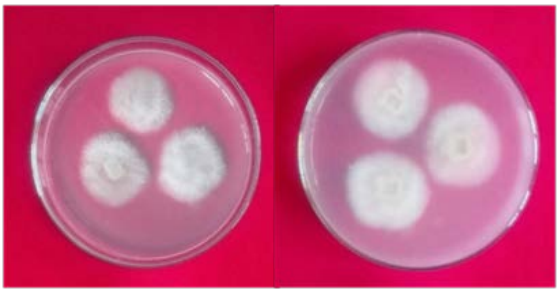


Figure 8 Morphology of fungi TFO-01 to TFO-10 (7 days old culture on PGA medium)

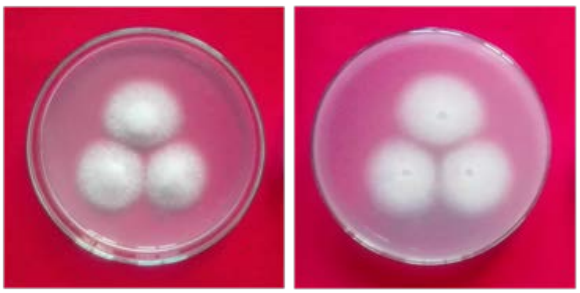




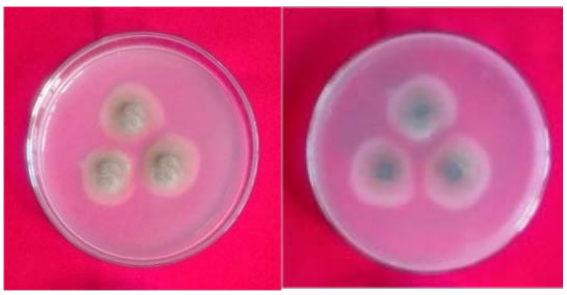
Front view TFO-15 Reverse view

Front view TFO-16 Reverse view

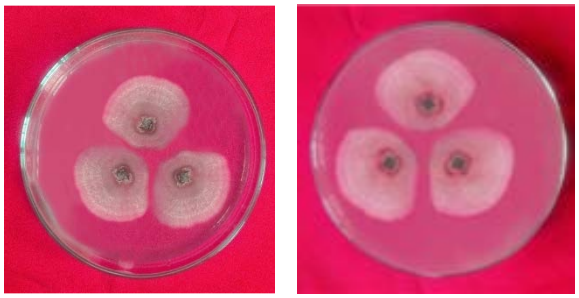
Front view TFO-17 Reverse view



Front view TFO-18 Reverse view



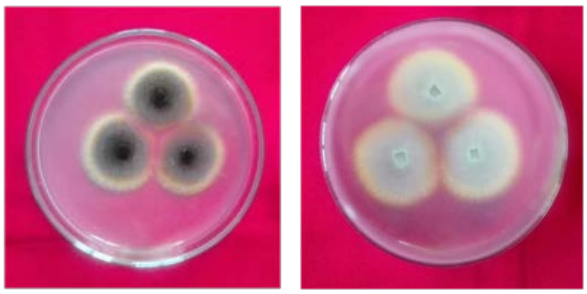
Front view TFO-19 Reverse view



Front view TFO-20 Reverse view

Figure 9 Morphology of fungi TFO-11 to TFO-20 (7 days old culture on PGA medium)

Front view TFO-11 Reverse view



Front view TFO-12 Reverse view

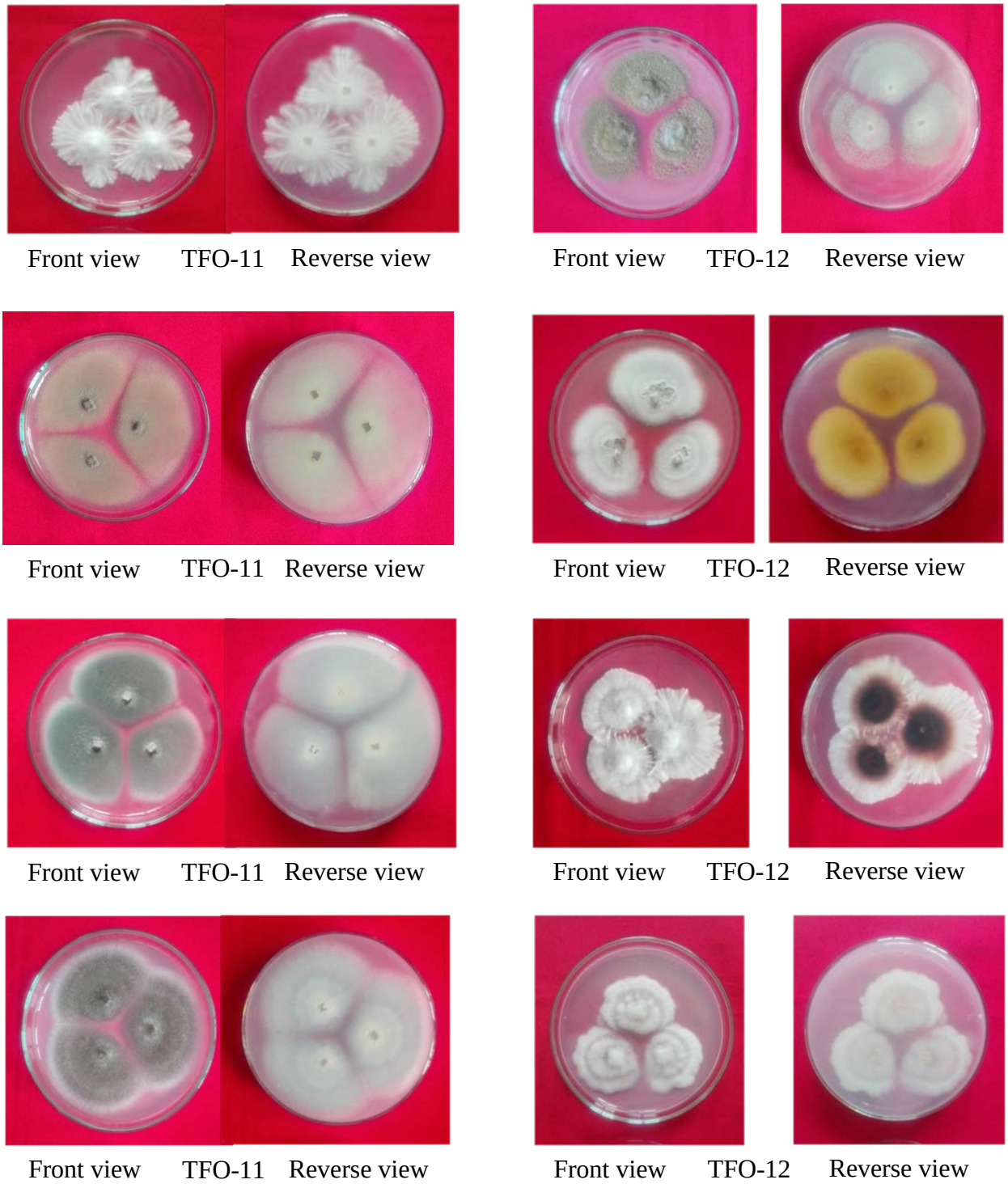


Figure 10 Morphology of fungi TFO-21 to TFO-30 (7 days old culture on PGA medium)

Table 3 Antimicrobial activities of isolated fungi (At 7 days fermentation)

Isolated fungi	Test Organisms and Inhibitory Zone (mm)					
	1	**2**	**3**	**4**	**5**	**6**
TFO-01	17.60	-	16.94	13.40	-	
TFO-02	18.16	13.06	17.14	-	-	-
TFO-03	17.48	20.88	14.17	-	-	-
TFO-04	16.42	-	16.46	-	-	-
TFO-05	14.03	13.35	16.33	-	-	-
TFO-06	20.65	11.09	16.46	-	-	-
TFO-07	15.05	-	15.67	12.36	-	19.38
TFO-08	22.86	12.75	17.14	-	15.25	20.25
TFO-09	23.65	15.75	14.17	-	22.06	23.73
TFO-10	24.64	-	16.46	-	-	21.00
TFO-11	24.63	-	16.33	-	12.00	20.40
TFO-12	21.82	12.05	15.31	-	15.12	21.15
TFO-13	13.36	-	-	-	-	-
TFO-14	20.63	21.32	-	-	18.15	-26.40
TFO-15	24.35	22.85	-	-	26.14	26.40

Table 4 Antimicrobial activities of isolated fungi (At 7 days fermentation)

Isolated fungi	Test Organisms and Inhibitory Zone (mm)					
	1	**2**	**3**	**4**	**5**	**6**
TFO-16	20.57	26.15	-	-	20.00	-
TFO-17	19.86	21.45	-	13.26	16.35	-
TFO-18	17.34	20.92	18.03	-20.08	15.40	-
TFO-19	15.75	15.05	-	-	14.35	-
TFO-20	16.30	-	19.36	-	15.00	-
TFO-21	20.36	-	18.37	-	13.65	-
TFO-22	20.18	-	17.03	12.85	13.00	-
TFO-23	27.65	-	18.63	-	15.15	-
TFO-24	18.07	-	18.03	-	16.52	-
TFO-25	12.07	23.54	17.16	14.50	-	-
TFO-26	-	-	16.82	24.00	-	-
TFO-27	-	-	19.46	27.30	-	-
TFO-28	-	-	19.70	24.50	-	20.05
TFO-29	-	-	19.45	13.00	-	-
TFO-30	10.00	-	18.35	25.05	-	-

Table 5 Higher Antimicrobial activities of isolated fungiTFO-15 and TFO-16

Ser. No	**Isolated fungi**	***S. cerevisiae***	***E. coli***	***S. typhimurium***	***C. albicans***
1.	TFO-15	26.14 mm	24.35 mm	26.40 mm	
2.	TFO-16	-	-	-	26.15 mm

1 = *Escherichia coli* 2 = *Candida albicans*

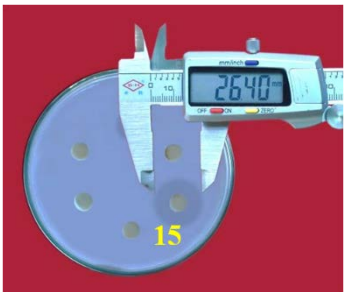
3 = *Micrococcus luteus* 4 = *Pseudomonas fluorescens*

5 = *Saccharomyces cerevisiae* 6 = *Salmonella typhimurium*

(Paper disc = 8 mm)

Against
Saccharomyces cerevisiae

Against
E. coli



Against
Salmonella typhimurium

Figure 11 Antimicrobial activities of fungus TFO-15

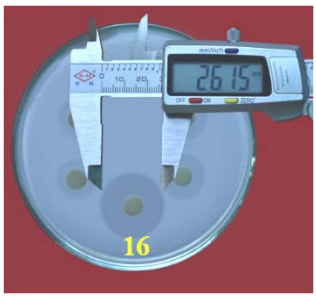


Figure 12 Antifungal activities of fungus TFO-16 on
C. albicans

Discussion and Conclusion

In the isolation of fungi, six plants were collected at Pathein Township. Endophytic fungi were isolated by the method of Surface sterilization Method (NITE, 2004). In this study thirty endophytic fungi were isolated from six different plants.

Five fungi were isolated from *Mangifera indica*, four fungi were isolated from *Averrhoa carambola*, five fungi were isolated from *Cajanus cajan*, five fungi were isolated from *Talinum triangulare* (Jacq.) Willd. Six fungi were isolated from *Sauropus androgynus*, five fungi were isolated from *Jatropha podagrica*.

In the study of antimicrobial activities, it was found that fungus TFO-16 exhibited the highest activity against *Candida albicans* and TFO-15 against *Saccharomyces cerevisiae*. TFO-15 also showed antibacterial activity against *Salmonella typhimurium* and *E. coli.*

Therefore, fungus TFO-15 was selected for further investigations such as identification, fermentation and purification of antibacterial metabolite.

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I would like to express my gratitude to Dr Than Tun, Rector, Pathein University, for his various guidance, suggestion and permissions to do the research. I am also grateful to thanks Dr Myint Myint Khaing, Dr Nay Aung and Dr Nilar Kyu Pro-rectors, Pathein University, for their suggestion and advices. I wish to express most sincere gratitude to Dr Moe Moe Aye, Professor and Head, Department of Botany, Pathein University, for her guidance, invaluable suggestions and comments offered in writing this research. I also wish to express my thank to Dr Khin Soe Soe, Professor, Department of Botany, Pathein University, for her encouragement and suggestion for this paper.

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FERMENTATION CONDITIONS FOR THE ANTIBACTERIAL METABOLITES FROM ENDOPHYTIC FUNGUS YF-16

Win Yu Yu Naing[1], Moe Moe Aye[2] and Theingi Aye[3]

Abstract

In the course of the isolation of fungi, twenty-nine fungi were isolated from five plant samples collected at Kangyidaung Township. The isolation of fungi was undertaken by surface sterilization method using LCA medium. Among them showed higher activity than other fungi. Therefore, these fungi were selected for further investigation. In the screening program for antimicrobial activity against *Staphylococcus aureus.* In the screening of antibacterial activity possessing microorganisms, isolated fungus YF-16 was highest activity reached at 3 days fermentation with 84hrs age and 10% size of inoculum. The selected fungus YF-16 was highest activity showed at FM2 medium.

Keywords Age and Size, Antibacterial activity, Fermentation

Introduction

Fermentation is a process which is usually influenced by different environmental parameters (Emily *et al*, 2009, Singh *et al*, 2017). Age and size of inoculum, temperature and pH are usually considered as most important parameters for microbial growth and production of value added products. Inoculum age and size also plays an important role in metabolite production (Xiaobo *et al*, 2006). The growth of microorganisms in the starter culture with respect to the time duration is crucial by using the inoculum (Emily *et al*, 2009).

Medium optimization is still one of the most critically investigated phenomenon that is carried out before any large scale metabolite production, and possess many challenges too. For designing a production medium, the most suitable fermentation conditions (e.g., pH, temperature, agitation speed, etc.) must be identified and optimized accordingly. Further, by optimizing the above said parameters, maximum product concentration could be achieved (Gupte and Kulkarni, 2003; Franco-Lara *et al*., 2006; Xiaobo *et al*, 2006; Wang *et al.*, 2011).

Therefore, the effects of carbon and nitrogen for the fermentation was investigated to produce antibacterial metabolite against *S. aureus*. Microbial fermentation has several advantages over using parts of the plants for the production of drugs and bioactive substances as this can easily be carried out in tank fermenters, providing unlimited supply of drugs and negating the requirement of plant parts. Moreover, different stronger derivatives of the drugs can be obtained by altering the culture conditions. Plants and microbes produce secondary metabolites with various biological activities that can treat various diseases (Radji, 2005).

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Materials and Methods

Investigation on Factors Affecting for the fermentation

1. Age and Size of Inoculum for the fermentation

2. Medium optimization for the fermentation

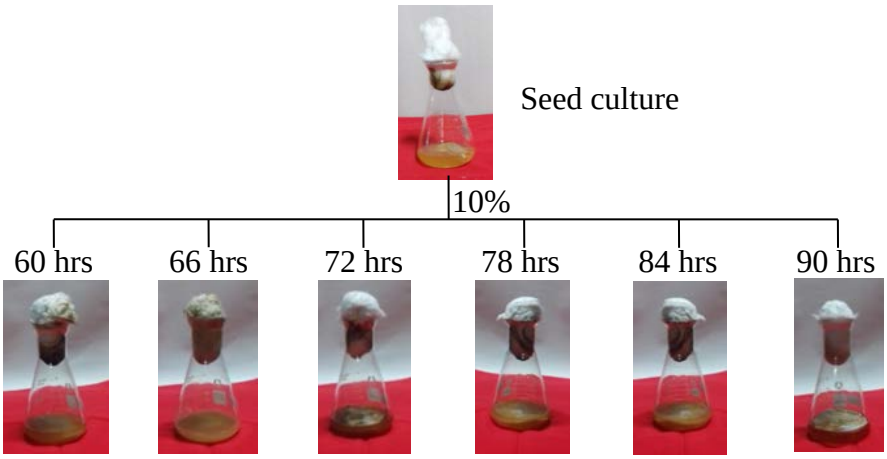
Study on Age and Size of Inoculum for the Fermentation

Fermentation is a process which is usually influenced by different environmental parameters (Emily *et al*, 2009). Inoculum age and size also plays an important role in metabolite production. Therefore, on the basis of the results of microbial growth kinetics, age of inoculum (60, 66, 72, 78, 84 & 90 hrs) with 10% seed culture was conducted for the fermentation to produce the metabolite. In the investigation of sizes of inoculum, 2.5 %, 5.0%, 7.5 %, 10.0%, 12.5 % and 15.0% were utilized with 84 hrs for YF-16 ages of culture.

Seed Medium (NITE, 2005)		**Fermentation Medium (NITE, 2005)**	
Glucose	1.0 g	Glucose	1.0 g
Glycerol	1.0 g	Glycerol	1.2 g
Yeast extract	0.8 g	Peptone	0.6 g
Polypeptone	0.7 g	$NaNO_3$	0.8 g
K_2HPO_4	0.001g	$MgSO_4.7H_2O$	0.001 g
$MgSO_4.7H_2O$	0.001g	K_2HPO_4	0.001 g
DW	100 mL	DW	100 mL
pH	6.5	pH	6.5

Assay Medium

Glucose	1.0 g
Yeast extract	0.2 g
DW	100 mL



Fermentation flasks with seed medium

Figure 1 Procedure for the study on the effects of YF-16 ages of inoculum

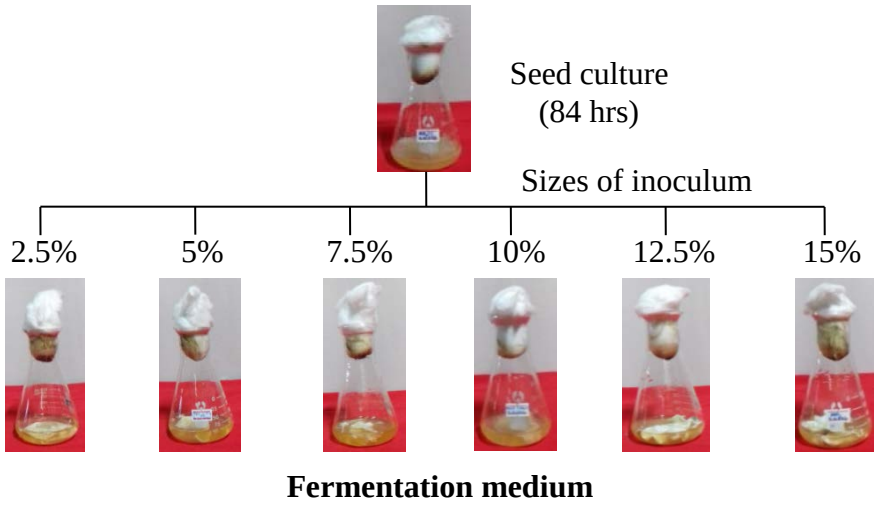


Figure 2 Procedure for the study on the effects of size of seed culture

Medium Optimization for fermentation and production of Antibacterial Metabolite

Fermentation was undertaken with suitable conditions of 84 hrs age of inoculum and 10 % sizes of inoculum with five different fermentation medium.

NITE (2005)

Seed medium	
Glucose	1.0 g
Yeast extract	0.5 g
Polypeptone	0.3 g
K_2HPO_4	0.001 g
$MgSO_4.7H_2O$	0.001 g
DW	100 mL
pH	6.5

Assay medium	
Glucose	1.0 g
Yeast extract	0.2 g
DW	100 mL

Fermentation Media (NITE 2005)

FM-1		**FM-2**		**FM-3**	
Glucose	1.0 g	Glucose	1.0 g	Glycerol	1.0 mL
Yeast extract	0.8 g	Sucrose	1.0 g	Yeast extract	0.8 g
Polypeptone	0.8 g	Yeast	1.5 g	Peptone	1.0 g
Glycerol	1.0 mL	K_2HPO_4	0.001 g	Tapioca powder	1.2 g
K_2HPO_4	0.001 g	DW	100 mL	K_2HPO_4	0.001 g
DW	100 mL	pH	6.5	DW	100 mL
pH	6.5			pH	6.5

FM-4

Glycerol	1.5 mL
Sucrose	1.8 g
Fish cake	1.8 g
K_2HPO_4	0.001 g
DW	100 mL
pH	6.5

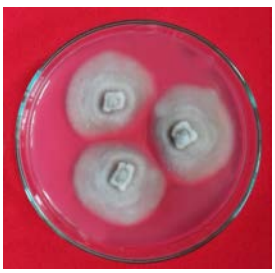
FM-5

Glucose	1.5 g
Molasses	1.5 g
KNO_3	0.5 g
Yeast extract	0.8 g
K_2HPO_4	0.001 g
DW	100 mL
pH	6.5

Results

Table 1 Fungi isolated from five medicinal plant samples by Surface sterilization Method

Sample	Surface Sterilization Method	
	Total Isolated Fungi	**Fungi No**
Clerodendrum indicum L. Kuntz	8	YF-02,02,03,04,05,06,07,08
Plumbago zeylanicaL.	7	YF-09,10,11,12,13,14,15
Sesbania grandiflora (L) Poret.	5	YF-16,17,18,19,20
HIppobroma longiflora (L) G Dong	4	YF-21,22,23,24
Dregea volubilis(L.f) Bth.ex.hook	5	YF-25,26,27,28,29
Total Isolated Fungi	29	



Front view

Reverse view

Front view - gray, Review - gray, Spore color - gray

YF-16 was isolated from leaf of *Sesbania grandiflora* (L.) Poiret.

Figure 3 Morphology of fungus YF-16 (7 days old culture on PGA medium)

Table 2 The effects of age of inoculum for the fermentation

Seed Culture (hrs)	Activity (mm)
60	25.73
66	27.10
72	29.18
78	30.06
84	33.51
90	29.76

These activities are three days fermentation from initial of fermentation.

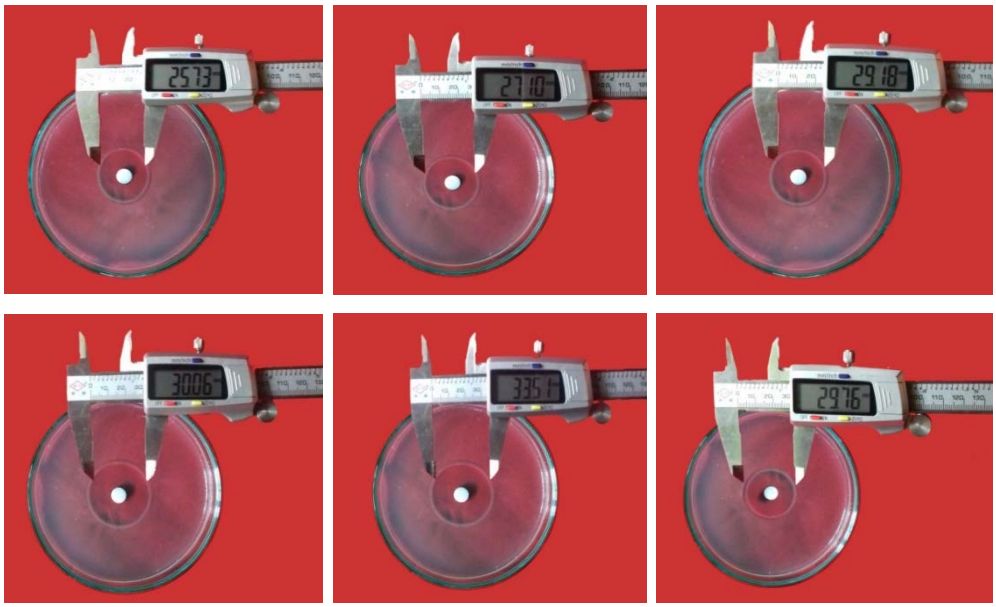


Figure 4 The Effects of Ages of Inoculums on Fermentation by YF-16 against *Staphylococcus aureus*

Table 3 The effects of size of inoculum for the fermentation

Seed Culture (%)	Activity (mm)
2.5	27.60
5.0	29.18
7.5	30.13
10.0	30.86
12.5	25.79
15.0	22.66

These activities are three days fermentation from initial of fermentation.

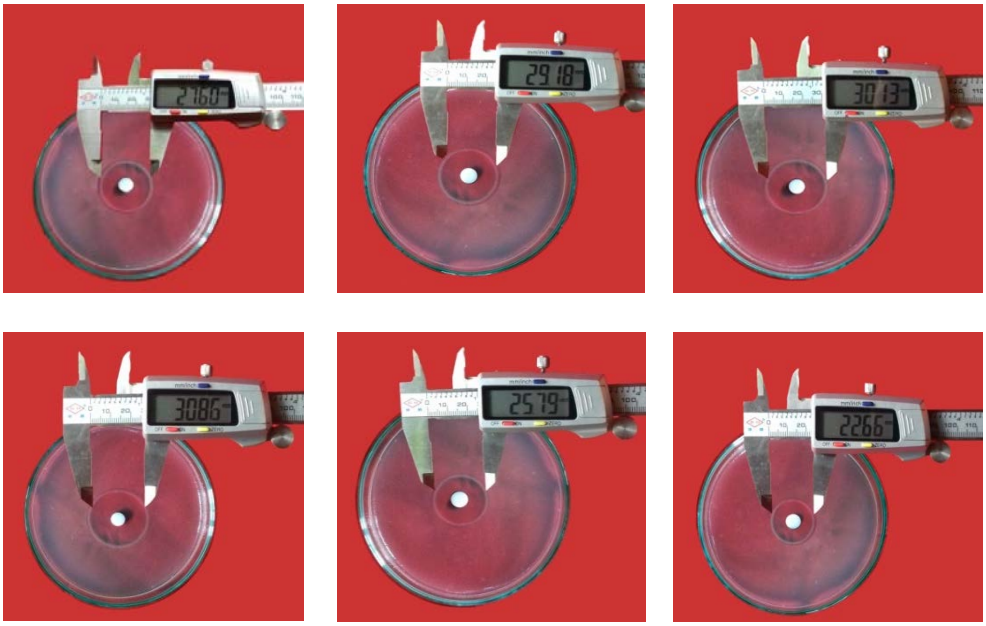


Figure 5 The Effects of Sizes of Inoculums on Fermentation by YF-16 against *Staphylococcus aureus*

FM-1 FM-2 FM-3 FM-4 FM-5

Figure 6 The media for the fermentation of antibacterial activity against *Staphylococcus aureus*

Table 4 The effect of media for the fermentation antibacterial activity against *Staphylococcus aureus*

Fermentation media	Antibacterial activity (clear zone, mm)
FM-1	31.11
FM-2	37.30
FM-3	26.53
FM-4	29.86
FM-5	30.68

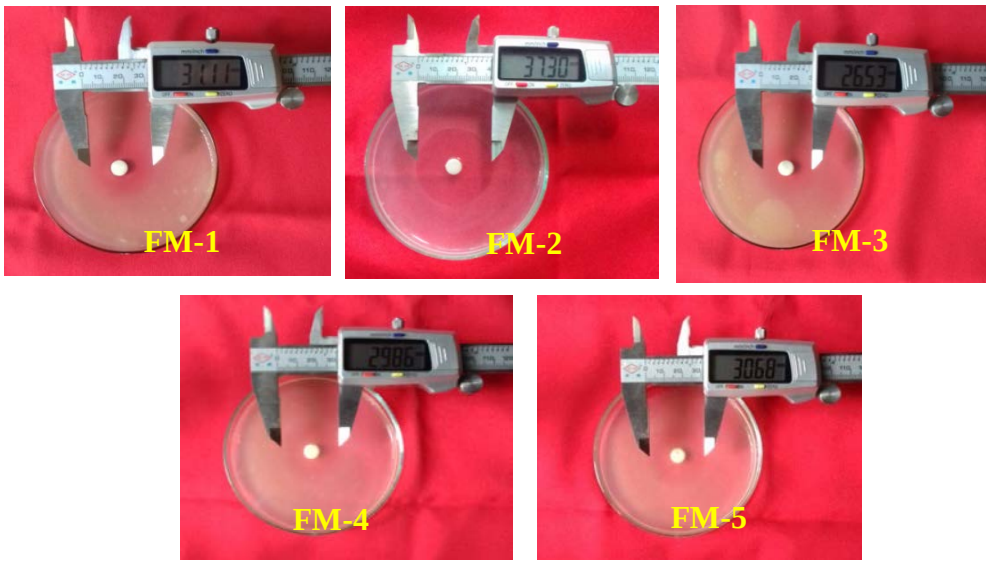


Figure 6 The effect of media for the fermentation of YF-16 Antibacterial activity against *Staphylococcus aureus*

Discussion and Conclusion

In the course of the isolation of fungi, twenty nine fungi were isolated from five plant samples collected at Kangyidaung Township. Eight fungi were isolated from *Clerodendrum indicum* L. Kuntz, seven fungi were isolated from *Plumbago zeylanica* L., five fungi were isolated from *Sesbania grandiflora* (L.) Poiret. Engl. and four fungi were isolated from *Hippobroma longiflora* (L.) G. Don., Five fungi were isolated from *Dregea volubilis* (L. f) Bth. ex.hook. Among them, fungus YF-16 exhibited the highest antibacterial activity against *S. aureus* because the test organism, Staphylococcus aureus is Methicilline (& other antibiotics) resistant bacterium that this fungus was selected for further investigations. In the study of time course of fermentation to produce the metabolite, the highest activity reached at 3 days fermentation as against *S. aureus* (33.51 mm) with 84hrs age and (30.86 mm) 10% size of inoculum. In this study FM2 medium is the highest activity for the production of metabolites. The proper cultivation (Ages) and transfer (Sizes) of inoculum is crucial for the production of both primary and secondary metabolites.

According to above mentioned results, time course of fermentation, extraction and purification of metabolite and bio-chemical properties will be undertaken.

Acknowledgements

I would like to express my gratitude to Dr Than Tun, Rector, Pathein University, for his various guidance, suggestion and permissions to do the research. I am also grateful to thanks Dr Myint Myint Khaing, Dr Nay Aung and Dr Nilar Kyu Pro-rectors, Pathein University, for their suggestion and advices. I wish to express most sincere gratitude to Dr Moe Moe Aye, Professor and Head, Department of Botany, Pathein University, for her guidance, invaluable suggestions and comments offered in writing this research. I also wish to express my thank to Dr Khin Soe Soe, Professor, Department of Botany, Pathein University, for her encouragement and suggestion for this paper.

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THE STUDY ON WILD ORCHIDS AT YEE-AYE RESERVED FOREST OF KALAW TOWNSHIP IN SOUTHERN SHAN STATE (PART - 3)

Moe Sandar Shein[1]

Abstract

The present work was concerned with the study on wild orchids of natural habitat in Yee Aye Reserved Forest in Kalaw Township. The Yee Aye Reserved Forest is situated in Kalaw Township of Taunggyi District and also the southern west part and 5.5 miles distance from Kalaw city. In this recent study 4 genera and 10 species were recorded from study area. The Yee Aye Hill wetland located the centre of Yee Aye Reserved Forest. Most of the wild orchids were collected around the area of this Hill wetland. Epiphytic genera namely ***Dendrobium, Eria, Flickingeria*** **and** ***Sunipia,*** were recorded the habits of orchids in nature. The collected specimens were classified, identified and described with color photographs of their natural habitats and inflorescence. The morphological characters have been emphasized and artificial keys from the tribe to the species have been constructed and GPS location system.

Keywords Wild Orchids, Yee Aye Reserved Forest, Hill wetland, Epiphyte, artificial keys.

Introduction

The family Orchidaceae are largest family among Angiospermae, Manocotyledonae. Some botanist estimated about 35000 orchids among flowering plants. Orchidaceae grow well throughout the world (Seidenfaden, 1992). Myanmar is situated in Southeast Asia and is also part of Indo-Burma biodiversity hotspot, with high species richness and diversity. Botanical explorations have sharply decreased in Myanmar since 1950, leading to large gap of knowledge on flora of Myanmar (Kress et al. 2003). Now The study area is Kalaw Township in Taunggyi district of Southern Shan State. Kalaw Township is located on the east by Shwe Nyaung Township, on the west by Thazi Township, on the south by Pin Laung Township, on the north by Pindaya Township, and it lies between North latitude 20°25′-21˚0′ and East latitude 96°20-97˚10′. The invest gold area is Yee Aye reserved forest in the recent study. Which forest is Hill evergreen forest type Hill evergreen forest where the wild Orchids have grown on the various plants which are (Thit-ya) ***Shoreaob longifolia*** Klall., (Thit-el) ***Castanea sativa*** **Mliler** (Pyirn ma) ***Lagerstromia speciera*** Pers (Pyin-Ka-doe). ***Pyliadolabri formis*** Benth and (In-pin) ***Dipterocarpus obtusifolius*** Teysm. ex. Miq. Yee Aye reserved forest situated in Northern East of Pin Laung Township and North by Shwe Nyaung Township and Southern West part between Kalaw and Tharzi Township 5.5 miles far from Kalaw city. The area of these forest is about 1952 acres and altitude of 1465 meters and lies between North latitude 20°36′ and East longitude 96°31′. Genus ***Dendrobium, Eria, Flickimgeria*** and ***Sunipia*** have been found in this area.

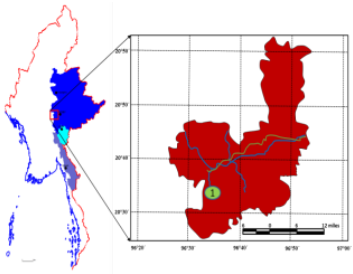
In this study, (1) Subfamilies belong to (1) Tribes (3) Subtribes (4) genera and (10) species have been collected from this study area including epiphyte and lithophytes. The aim of the study was to know of Myanmar wild orchids and to access the presence distribution of wild orchids in Yee Aye reserved forest. To fulfill this aims to classified and descried the collected wild orchids systematically.

[1] Department of Botany, Maubin University

Methodology

The specimens were collected from Kalaw Township of Taunggyi District. All these specimens were colorful photographed to record their actual habitat and the nature of inflorescence in 2021-2022. All these collected specimens were classified according to Dresseler's classification (Dresseler (1927) and identified by Seidenfaden (1992) Grant: (1966), Nantiya Vaddhanaputi (2006), Hooker, J.D. (1954), Seidenfaden and Smitch (1965), Dassanayake, N.D. (1981), Flora of China Vol. 25 (2013) and Flora of Thailand Vol. XI & XII. Part I&II (2014) methods.

Location of Study Area



Result

This classification of Sbufamily in the study is in accordance with Dresseler (1927)and the key below is cited from Seidenfafen and Wood (1992) described in The orchids of indochina." In this paper (1) subfamily, (1) tribes, (1) subtribes (4) genera and (10) species have been collected from study area.

Arrangement of the Subfamily;Tribe;Subfamily;Genus and Species

Class :Liliopsida
(Monocotyledoneae)
Subclass :Orchidales
Family :Orchidaceae
Subfamily : Epidendroideae
Tribe : Epidendreae
Subtribe : Errinnae
Genus : (1) ***Eria***
Subtribe : Dendrobibiinae
Genus : (2) ***Dendrobium***
: (3) ***Flickigeria***
Subtribe : Sunipiinea
: (4) ***Sunipiea***

Subfamily Epidendroideae

In this recent study four genus *Eria, Dendrobium, Flickingeria* and *Sunipiea* under the Subtribe of Tribe Epidendreae in subfamily Epidendroidea.

Key to the Subtribe of Tribe Epidendreae

1. Pollinia 8 withdistinct visidium----------***Eriinae***

1. Pollinia 2-4 without viscidium----------2.

2. Pseudobulb several internode. Inflorescenceupper axillary or terminal .----------
----------***Dendrobiinae***

2. Pseudobulb withoutl internode. Inflorescence arising base of the internode----------
----------***Sunipiinae***

Key to the Genus of Subtribe Dendrobibiinae

1. Pseudibulb slender with many internode unbranched. Leaves not solitary. Inflorescence arising on the node with many flowers.----------***Dendrobium***

1. Pseudobulb swollen, branched. Leaves arising from the top of the pseudobulb with one leaf .Inflorescence with one or two flowers on adaxile.----------
----------***Flickingeria***

Key to the Species of Genus *Dendrobium*

1. Pseudobulb dwarf----------2

1. Pseudobulb long----------3

2.Pseudobulb fusiform, brownish black. Flower reddish orange.lip triangular with distinct veins----------(1)***D.seidenfaden***

2. Pseudobulb ovoid and 4 angles. Flower various colors----------4

3.Pseudobulb cylindric and stout----------6

3.Pseudobulb various shape----------7.

4.Pseudobulb 4 angle with furrow, dwarf crowded. Inflorescence arising on the node, peduncle short. Flower golden yellow, delicate----------(2) ***D.junkaisai***

4. Pseudobulb ovoid, smooth. Inflorescence erect, arising from the base of the pseudobulb. Flower greenish yellow with recurved lip----------(3) ***D.gregulus***

5. Pseudobulb erect, club shaped, stout. Inflorescence suberect with many showy flowers. Flower golden yellow, fragrant. Lip orbicular, excurved, densely pubescent on adaxilly,yellow with reddish brown patch each side of mesochile----------
----------=-------(4)***D.suavissimum***

5. Pseudobulb suberect, cylindric with many swollen nodes. Inflorescence with 2-3 flowers. Flower white with purplish tipped. Lip white with golden yellow at the base in middle and purplish red tipped----------(5)***D.pendulum***

6. Flowers large, white with distinct vein. Lip Qudangular ,white with small yellow patch on the mesochile----------(6)***D. formosum***

6. Flower medium, creamy white with purple tinpped. Lip orbicular, orange with purplish red on tipped.----------=-----(7)***D.crystallinum***

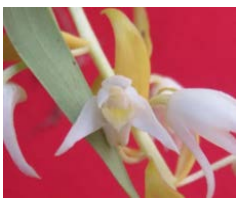
I. Subfamily Epidendoideae

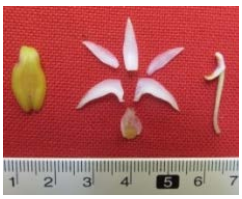
Subfamily	Tribe	Subtribe	Genus	Species	Myanmar name
Epidendroideae	Epidendreae	Eriinae	*Eria*	*acervata*	နတ်သမီးပန်း
		Dendrobiinae	*Dendrobium*	*seidenfadenii*	သင်္ကန်းသစ်ခွ
				jenkensii	သင်းခွေချပ်
				gregulus	None
				suavissimum	အာမဲမောက်ခမ်းဝါ
				findlayanum	ချိုချဉ်သစ်ခွ
				formosum	ငွေတူကြီး
				crystallinum	စက္ကူပန်း
			Flickingeria	*sp*	None
		Sunipiinae	*Sunipia*	*racemosa*	သဇင်အချိတ်

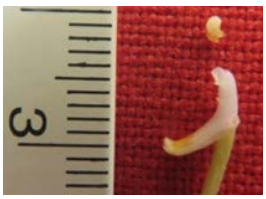
In this recent study only one genus *Eria* was collected under Subtribe Eriinae.

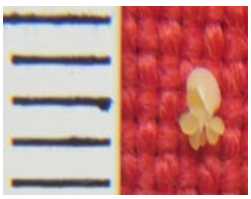
1.*Eria acervata* Lind.l


Habit


Inflorescence


Segment of flower


collum


Pollinia

***Eria acervata* Lind.1**

Epiphyte, evergreen, tuff. Pseudobulb 2-4.00 cm long and 1.5 cm wide with node, erect crowded. Leaves oblong acute about 8.00 cm long and 1.5 cm wide. Inflorescence erect, arising from the new leafy shoot at the base of the pseudobulb, about 6.9.00 cm long and 2.00 m wide. Flowers white, fragrant with district bract, pedicel pale green 1.7 cm long and 1.00 cm wide not opened widely about1.00 cm across. Dorsal sepal oblong acute ,1.5 00 cm long and 0.4.00 cm wide, lateral sepals oblong acute, 1.20 cm long and 0.5 cm wide. Petals oblong acute, about 1.2.00 cm long and 0.3 cm wide. Labellum tri-lobes, side lobes narrowly embracing the column 0.7 cm long and 2.00 mm wide with 2 distinct yellow keels on each side, mid lobe ovate acute incurved, pale yellow, about 4.00 cm long and wide. Coloum short, 7.00 mm long and 2.00 mm wide, white. Operculum cap shaped, white. Pollinia 8, rounded, pale yellow with not stipe.

Myanmar Name- Nat Thamee Pan.

Occurrence -Kalaw, Yee Aye reserved forest, N 20˚ 36′ 44″ E 96˚ 32′ 1.5″

Distribution - Nepal eastwards to Tibet and China (Seidenfaden, 1982) .and Flora of Thailand, 2014.

Ecology - Epiphyte, on the trunk, Alt-1442 m, Hill evergreen forest.

Flowering Period-April –May

In this recent study (7) species of genus ***Dendrobium*** were recorded.

2.1. *Dendrobium Seidenfadenii* Seng & Backer

Habit

labellum

Anther cap & pollina

pollina

fruit

***Dendrobium Seidenfadenii* Seng & Backer**

Epiphyte, dwarf. Pseudobulbs clavate, erect, brownish black with white sheath, about 6.00-8.00 cm long and 0.8-1.00 cm wide leaves oblong acute, brown, about 5.00 cm long and 0.8 cm wide. Inflorescence with one two or flowers, suberect. Flower orange with reddish brown stripe non-resupinnate, about 4.00 cm across, pedicle orange about 1.7 cm long - 2.00 mm wide, bract ovate acute, pale brown 4.00 mm long 3.00 mm wide. Dorsal sepal oblong acute orange with reddish-brown stripe about 2.8 cm long and 4.00 mm wide, lateral sepals oblong acute, about 2.7 cm long and 4.00 mm wide. Petals oblong acute, smaller than the sepals about 2.8 cm long 3.00 mm wide. Labellum tripallate, convolute at the base, orange with reddish purple veins on the upper surface, 3 distinct keels, two lateral keels long and middle one short on the hypochile. Column short, 5.00 mm long and 2.00 mm wide. Operculum yellow, globose. Polinia 4, oblong, yellow about 1.5 mm long 1.00 mm wide.

Myanmar Name	-Thinguan Thit Khwa
Occurrence	- Kalaw, Yee Aye reserved forest, N 20˚ 36′ 15.2″ E 96˚ 32′ 6.5″
Distribution	- Myanmar, Thailand, (Seidenfaden, 1992) and Flora of Thailand ,2014.
Ecology	- Epiphyte, on the trunk, Alt-1212 m, Hill evergreen forest. Flowering Period- March – April

2.2. *Dendrobium Jenkensii* (Wallich ex Lindley) Brieger

Habit

Inflorescence

Flower

2.2 *Dendrobium Jenkensii* (Wallich ex Lindley) Brieger

Callista aggregata Kuntze var.
Dendrobium aggregatum Raxburg var. Jenkensii (Walhich x lingley)
D.marseillei Gagnepain

Epiphyte, dwarf, evergreen. Pseudobulb oblong, tetragonal, compressed, clustered with 1 leaved, 4 angled, two or three nodes. Leaf solitary, oblong, retuse, about 2-3 in, in height. Inflorescence lateral with 2 or 3 flowers, arising from the top of the psendoubulb, short peduncle, floral bract oblong acute. Flower 2-3 golden yellow flowers with slender pedicel, pedicel and ovary 3.5-5.00 cm. Dorsal sepal oblong elliptic, obtuse with 5 veins, about 10-15 mm x 6 mm wide, lateral sepals narrowly ovate elliptic with 5 veins, apex obtuse, mentum 4.00mm wide.

Petals orbicular with 5-veined about 15.00mm long and 10.00 mm wide. Lip reniform retuse, spreading and large, slightly pubescent on the wide lip in adaxial, entire ,erose. Column short and stout, 6.00mm, pale green. Pollinia 4 in mass.

Myanmar Name - Yadana Shwe Ket Lay

Occurrence - Kalaw, Yee Aye reserved forest, Se Si, Hopone Township N 20˚ 35′ 12″ E 96˚ 31′8.13″ Distribution - Myanmar Grant, B (1966). S-Yunnan, Bhutan, NE India, N Laos, Myanmar, N Thailand, N Vietnam. (flora of Chin,Val. 25. (2014).NE India, Burma, Thailand and China, Seidenfaden (1992).

Ecology - Epiphyte, on the trunk, Alt-1212 m, Hill evergreen forest. Flowering Period- March – April

2.3. *Dendrobium gregulous Seidenfaden 1985*

Habit Inflorescence Flower Segment of flower

Dendrobium gregulous Seidenfaden 1985

Aporum heterocaulan (Guillaumin) Rauschert `1983
D.heterocaulan (Guillaumin) 1965

Epiphyte, dwarf plant. Pseudobulbs crowded, cluster, rounded acute covered by scale like sheath, about 1.00 cm across, golden yellow leaves. Inflorescence erect,4-6 flowers arising from the top of the leafless pseudobulb, peduncle yellow ,3.5 cm long, floral bract ovate acute, 2.00 mm, membranous. Flower non resupinate, pale greenish yellow with red purple veined lip, pedicle and ovary,7.00mm long and 2.00mm wide, slender, bract obovate. Dorsal sepal oblanceolate, pale green with 4 veined, mentum projection forward, acute 1.5mm long and 1.2mm wide, lateral sepals obliquely ovate triangular, acute, sulphur yellow, apex acute incureved, mentum projection, acute, about 6.00.00mmlong and 4.00mmwide. Lateral petals oblanceolate, smaller than the sepals,7.00mmlong and 2.00mm wide, sulphur yellow. Lip trilobed, broadly ovate acute on outline, side lobes erect, large, rounded, sulphur yellow with distinctly red purple veined, margin with, undulate, green callus on the mesochile, apex acute. Colum pale yellow with red atripe o margom. Anther cap yellow. **(Note- Threatened Species, http:// tropicos.org)**

Myanmar Name - Nil
Occurrence - Myanmar, Yee Aye Reserved forest, Kalaw , N 20˚ 36′ 12″ E 96 ˚32′12.5″
Distribution - Southern Thailand (www. Orchidspecies.com)
Ecology - Epiphyte, on the trunk, Alt-1242 m, Hill evergreen forest.
Flowering Period- March – April

Flowering period, September-October

2.4. *Dendrobium chrysotoxum* Lindl. in Bot.Reg.1847. var.*suavissimum* Rchb.f.


Flower


Infloresce


Flower


collum


Pollinia & Anther cap


pollinia

***Dendrobium chrysotoxum* Lindl. in Bot.Reg.1847. *suavissimum* Rchb.f.**

***D. clavatum*,Roxb.Fl.ind.iii, 471**

Epiphyte. Pseudobulb, erect, club shaped, stout, furrow, about 16.00 cm and 2.5 cm wide, golden yellow in mature. Leaves oblong acute, 3-5 leafs in subterminal, 14.00 cm long and 3.5 cm wide, leathery, contracted in base. Inflorescence slightly pendulous, arising from upper portion of the stem with many flowers, pedencle green and stout with sheath,14.00 cm long and 0.5 cm wide floral bracts ovate acute, membranous. Flower golden yellow with reddish brown spot on each side at the base with fimbriate labellum, very fragrant about 3.5 cm across with pale yellow pedicel about 4.00 cm long and 0.2 cm wide and small bract. Dorsal sepal oblong obtuse, yellow, veined about 20 mm long,8mm wide, lateral sepals oblong obtuse, 23.00mm long and 9.00 mm wide, yellow, veined, mentum subglobose. Petals ovate, yellow, about 22.00mm long ,15.00 mm wide, margin slightly dentate, veined. Labellum orbicular, curved at the tip, about 20.00mm long, 25.00 mm wide, densely pubescent on adaxilly, margin undulate, yellow with reddish brown patch on mesochile, convolute at the base. Colums short, 5.00 mm long, 2.00 mm wide, yellow, with reddish brown stripe. Operculum yellow two pointed and protruding about 2.00 mm across glabrous. Pollinia 4, oblong, yellow, 2 in part, stripe absent.

Myanmar Name- Shwe Tu Mouk Kham Mae

Occurrence - Kalaw, Yee Aye reserved forest, N 20˚ 36′ 12″ E 96 ˚32′12.5″
Distribution - NE India, Myanmar, Thailand and China (Seidengaden, 1982) and Flora of Thailand, 2014. NE India, Laos, Myanmar, Thailand and Vietnam, (Flora of China.Vol.25, 2013).
Ecology - Epiphyte, on the trunk, Alt-1312 m, Hill evergreen forest.
Flowering Period- April –May

2.5. *Dendrobium findlayanum* Par.&Rchb.f.


Habit


Inflorescence


Flower

***Dendrobium findlayanum* Par.& Rchb.f.**

Epiphyte. Stem long, club-shaped, large upward and tapering, about 20-30.00cm long and1.5-2.00cm wide. Leaves oblong lanceolate, acute both surfaces glabrous. Inflorescence raceme, lateral upper portion of the pseudobulb 2-3 flowers per node. Flower lavender, resupinate , slightly fragrant, about 5.00cm across . Dorsal sepals linear lanceolate acute, lavender, fleshy, with faintly purple tip about 2.5cmlong and 8.00 mm wide, two lateral sepals

larvenda, linear lanceolate acute, forming a mentum, mentum lavenda obutuse. Petals 3, two lateral petals oblong ovate about 2.5cm long and 1.5 cm wide, slightly undulate, lavender with faintly purple tip. Lip broadly rounded convolute at the base and slightly acute at the tip, lavender with deeply yellow in centre and purple at pointed end, finely pubescent on the upper surface. Colum short with stipe. Anther white, terminal, 2 celled .Pollinia 4 in pairs.

Myanmar Name - Cho Chin Thitkwa
Occurrence - Kalaw, Yee Aye reserved forest, N 20˚ 38’ 32″ E 96˚ 33′ Distribution
- Native on the mountain between Burma and Thailand (R.E.Holttum1964), Burma (Grant B, 1966), Myanmar, Thailand, (Seidenfaden, 1992).
Ecology - Epiphyte, on the trunk, Alt-1224 m, Hill evergreen forest.
Flowering Period- February-April

2.6. *Dandrobium formosum*, Roxb in Wall. Cat 1998. Var. berkleyic





Habit Inflorescence Flower Labellum

6. *Dandrobium formosum*, Roxb in Wall. Cat 1998. Var. berkleyic

***D. infundibulum*. Ruhb.y**

Epiphyte, ever green species. Stem pendulous, stoutish terete covered by sheath pubescent about 30-35.00 cm long and 0.8 cm wide. Leaves ovate, emerginate obliquely, thiny, coriocuous about 30- 40.00 cm long and 25-30.00 cm wide amplexicaul. Inflorescence 3.5 flowers on upper portion of the old deciduous stem, peduncle short with basal sheath. Flower fragrant, 3.00 in across, large, spreading, white with yellow patch lip, pedicel and ovary 4.00 cm x 0.2 cm, bract ovate. Dorsal sepal oblong lanceolate, acuminate, white with veined about (2.5 x 0.5cm) laterals sepals uniquely with dorsal sepal. Petals suborbicular, undulate, cuspidate, larger than the sepals, white with veined, mentum conical. Lip trilobed, broadly obovate, entire, mid lobe dilated, retuse, apiculate with 2 tubercled ridges and disk golden, about (2.5 x 2.00cm). Column stout, pale green about 5.00 mm long and 3.00 m wide. Anther cap conic.

Myanmar Name - Ngwe Tu Gyi
Occurrence - Kalaw,Yee Aye reserved forest, N 20˚ 38’ 32″ E 96˚ 33′
Distribution - Tropical Himalaya, Nepal, Bhutan, Myanmar, Andamon Island (Grant B, 1966), NE India Myanmar, Thailand, Yunman (Seidenyaden, 1992).
Ecology - Epiphyte, on the trunk, Alt-1224 m, Hill evergreen forest.
Flowering Period - April –May

2.7. *Dendrobium crystallinum* Rchb.f.







Inflorescence Segment of Flower collum Anther Cap & pollinia

7. *Dendrobium crystallinum* Rchb.f.

Epiphyte. Stem erect and stout, cylindric about 50-70.00 cm long and 8.00 cm wide, unbranch, greenish yellow in mature. Leaves oblong lanceolate acute, leathery, about 10-16.00 cm long and 1.5-1.8 cm wide, dorsiventrally veins. Inflorescences 2-3 flowers on upper portion of the old stems, peduncle short with basal sheaths, floral bracts pale white, membranous, ovate acute. Flower showy with yellowish orange lip with purple margin, 4.00 cm across, pedicle and ovary 3.5 cm long 2.00 mm wide, bract ovate acute,1.2 cm long and 5.00 mm wide. Dorsal sepal oblong lanceolate, 3.00 cm long 1.00 cm wide, veined, lateral sepals oblong lanceolate 3.00 cm long and 8.0 mm wide base obliquely, mentum conical. Petals oblong acute, veined, slightly undulate, 2.5 cm long and1.5 cm wide, creamy white with purple tint. Lip suborbicular, pubescent in both surfaces, 3.00 cm long 2.7 cm wide, creamy white with yellow in center and purple tint, veined. Colum 8.00 mm long and 5.00 cm wide, short , pale purple stripe. Operculum long, white with hairs, 5.00 cm long and 3.00 m wide protuding. Pollinia oblong curved, yellow, hard, 2.00 mm long and1.0 mm wide.

Myanmar Name- Setku Pan Thidkwa

Occurrence - Kalaw, Yee Aye reserved forest, N 20’ 36’ 2.12’ E 96 31’
Distribution - Myanmar, Thailand, China (Seidenfaden, 1992) and Flora of Thailand 2014.Cambodia Laos, Myanmar, and Thailand, Vietnam. (Flora of China ,Vol.25)
Ecology - Epiphyte, on the trunk, Alt-1217 m, Hill evergreen forest.
Flowering Period- March –April

3.1. Flickingeria nodosa (Dalzell) Seidenfaden






Habit & Inflorescence Flower Segment of flower Collum and anther cap

Flickingeria nodosa (Dalzell)Seidenfaden

Epiphyte. Rhizome creeping, internode about 2.5-3.5 cm long and 3-4 cm wide. Stem up erect and pendulous, branched. Pseudobulbs elongated fusiform with light furrow, greenish about 4-5.oo cm long and 1.00-1.2 cm wide, covered by sheath in young. Leaves shortly petiole, ovate to oblong, leathery, apex obtuse about 11.00 cm long and 1.5.00 cm wide. Inflorescence usually only flowered, adaxial, flower lasting half a day, peduncle very short with several thinly sheath. Flower white with baby pink lip, thinly textured, lasting half a day,1.5 cm across, pedicle and ovary pale yellow, about 1.00 cm long and 1.00 mm wide, bract ovate acute scale like leaf, dull white 2.00 mm long and1.00 mm wide. Dorsal sepal oblong lanceolate, acuminate, about 12.00mm long and 4.00 mm wide, milky white, reflexed, lateral sepals obliquely ovate lanceolate, base obliquely wide, creamy white with pale yellow base, larger than the dorsal sepal, mentum perpendicular to ovary, obtuse, about 3.00mm. Petals narrowly oblong, acuminate, the same color in sepals, smaller than the sepals. Lip trilobed, side lobed erect, subobovate, entire, about 6.00 mm apart, pale pink with red sport, mid-lobe flabellate in outline, margin undulate criped two half in apex, disk with two reddish purple lamellae extending from the base to apex of midlobe. Coloum stout with foot, about 7.00mm.

Note- (New record for Myanmar)

Myanmar Name- Nil

Occurrence - Kalaw, Yee Aye reserved forest, N 20’ 35’ 12’ E 96 33’
Distribution - Myanmar, 2022. Southern India. (http:// orchidspecies.com)
Ecology - Epiphyte, on the trunk, Alt-1242 m, Hill evergreen forest.
Flowering Period- October

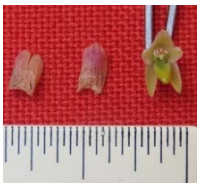
4.1. *Sunipia racemosa* (J.E.Sm.) Tang & Wang


Habit


Inflorescence


Segment of Flower


Flower


Bract &Flower

***Sunipia racemosa* (J.E.Sm.) Tang & Wang**

***Lone siamemsis* Rolfe,Kew Bull.1908:413**

Rhizome stout, creeping. Pseudobulb well-spaced on rhizome,4.00cm in part, soft, obliquely ovate, 3.5 cm long and 1.8 m wide. Leaves blade oblong, obtuse, leathery with distinct petiole, retuse, 12.00 cm long 1.2 cm wide. Inflorescence racing nodding with long peduncle with 20-30 flowers, 35.00 cm long, peduncle 18.20 cm log and erect with node and large several bract. Flower small, pale green with green lip, 8.00 mm long 5.00 mm wide with short pedicel and enclosed in floral bracts, pedicel 2.00 mm long 1.00 mm wide, bract pale brown, ovate acute about 7.00 mm long 6.00 mm Wide. Dorsal sepal ovate concave, apex acute, pale green, 4.00 mm long 5.00mm wide, thick, lateral sepals obliquely ovate, lower edge connected to each other apex acute, 4.00 mm long 2.00 mm wide pale green, thick. Petals obliquely ovate about 2.00 mm and 3.00mm wide, soft. Lip ligulate, fleshy, 4.00mm long and 2.00 mm wide, green with black spot on hypochile, glabrous, adaxial base with shallow shape. Column stout, short 2.00 mm long 1.00 mm wide. Pollininia top, 4 pollinia, with stripe 1.00 mm long and wide globose, yellow orange.

Myanmar Name- Nil

Occurrence - Kalaw,Yee Aye reserved forest, N 20’ 36’ 12’ E 96 32’
Distribution - Nepal, Sikkim, NE India, Myanmar, Thailand,Yunnan. (Seidenfaden, 1982) and Flora of Thailand 2014.
Ecology - Epiphyte, on the trunk, Alt-1242 m, Hill evergreen forest.
Flowering Period - March - April

Discussions and Conclusions

This paper based on some collected wild orchids specimens. The present list is (1) subfamily, (1) tribe, (3) subtribe, (4) genera and (10) species. The subfamily Epidendroideae includes (1) tribe, (3), subtribe, (4) genera and (10) species. Genus ***Eria, Dendrobium, Flickingeria and Sunipia*** have been collected from study area. In recent study only one genus of ***Eria*** of subtribe Erriinae was recorded in recent study. One species of genus ***Eria***, ***Eria acervata* Lindl.** which possess pseudobulb flask shape, closed, midlobe ovate acute incurved, white with two distinct yellow keels on each side.

Only two genus ***Dendrobium and Flickingeria*** of subtribe Derdrobiinae was recorded in study area Seven species of genus ***Dendrobium*** are ***D. seidenfadenii* Seng&Backer.,** ***D. jenkinsii* (Wallich ex Lindley)** ***Brieger D.gregulus*. *D.suavissimum* Rchb.f. *var. chrysotoxum* Lindl.** ***D. findlayanum*** Par.&Rchb.f. ***D.formosum*** Roxb.ex.Lindl. and ***D.chrystallinum*** Par.&Rchb.f. ***D. seidenfadenii*** contains deep orange flower, tripallte lip with three distinct keels ,two lateral keels are short and middle one is long this character with agree with Seidenfaden ,(1992). ***D. jenkinsii*** has oblong tetragonal closely pseudobulbs, golden yellow flower with deep yellow lip its agree with (Grant.B.1966). ***D. gregulus*** present erect inflorescence with4-6 pale greenish yellow flowers and recurved broadly ovate lip with distinctly reddish purple veined. This species is new record for Myanmar and also threatened species.(http://www.tropicos.org). ***D. findlayanum*** possess pear shape upper swollen internode, pale lavender sepal and petals and orbicular lip with finely pubescent in upper portion and glabrous in base this character agree with Nantiya (2005) . ***D.formosum*** present large flower, white sepals and petals and broadly obovate white lip with dilated retuse yellow blotch in the center and two tubercled ridges and golden disk. ***D.chrystallinum*** has orbicular yellow lip with purple tip and large glassy papillose operculum. These character agree with mention by (Grand, 1966), (Seidenfarden, 1992) and (Henrik, *et. al*, 2014).

One species of genus ***Flickingeria*** is ***Flickingeria nodosa:***possess white flower with pale pink lip, sidelobes pink with reddish purple sport and midlobe undulate crisped with two incurved apical half and two lamellae wavy keels. (Note- This species is new record for Myanmar and also found in only Southern India (http://orchidspecies.com) Only one genus ***Sunipiea*** of subtribe Sunipiinae was recorded in study area. One species of genus ***Sunipiea*** is ***Sunipiea racemosa*** possess pendulous inflorescence with greenish brown many flowers, ligulate fleshy green lip with black sport on hypochile. These character agree with mention by (Grand, 1966) , (Seidenfarden, 1992) and(Chen, *et.al* .Vol.25,2014). In this research paper all collected species are epiphyte. ***Dendrobium gregulus* (Seidenfaden)** regarded as threatened species (http://www.tropicos.org) ***Dendrobium chrystallinum*** Rchb.f**.** ***D. findlayanum*** Par.& Rchb.f. ***D. formosum* Roxb in Wall.** put in Appendix II (IUCN Red list conservation status, controlled trade) and ***Eria acervata*** are widely distributed in Yee Aye reserved forest and are included in Appendix II. Among them two species of ***Dendrobium seidenfadenii, D.findlayanum*** found only in Myanmar and Thailand (Seidenfaden, 1992) . *Dendrobium gregulous, D. seidenfadenii*, *Sunipia racemosa* are new record for Myanmar. *Eria acervata* was Chin, Mandalay and Thaninthari by (Kress *et. al*, 2003). but also found in Shan State in recent study.*D. formosum* was Bago, Chin, Mon, Rakhine, Sagaing, Taninthayi,Yangon by (Kress *et.at*,2003) but also collected in Shan State. Botanical collection are still needed to cover the whole floristic diversity of Myanmar, because botanical exploration have sharply decreased in Myanmar 1950. (Kress *at el*, 2003) and ***Dendrobium gregulous, D. seidenfadenii, Sunipia racemosa*** and ***Flickingeria nodosa*** is also new record for Myanmar and all collected species put in Myanmar Red list in 2022, March. (http://phytokey.pensoft.net)

Myanmar orchids flora have lagged behind being well documented and studied. So the orchidologist will have to find out continuously to get update current wild orchids information and report to government for protection of our living jewels.

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Website

(http://www.tropicos.org)

(http://www.iucnredlist.org)

ISOLATION OF ENDOPHYTIC FUNGI FROM *ANDROGRAPHIS PANICULATA* WALL.*ex* NEES AND THEIR ANTIMICROBIAL ACTIVITIES

Thet Thet Khaing[1], Tin Moe Aye[2], Aye Khaing Oo[3]

Abstract

In this research work, *Andrographis paniculata* plant samples were collected from Magway University Campus, Magway Region. The isolation of endophytic fungi was undertaken by the surface sterilization method and Baiting method. Fourteen fungi was isolated from leaves, stem and root of *Andrographis paniculata.* All isolated fungi were tested by five kinds of test organisms, *Escherichia coli, Pseudomonas fluorescence, Bacillus subtilis, Saccharomyces cerevisiae* and *Candida albicans* by paper disc diffusion assay method. Among them, Fungus TF-02 exhibited only selective antimicrobial activity against *Escherichia coli* and the highest activity. Therefore, this fungus TF-2 was selected for further investigations such as fermentation for the production of antimicrobial metabolite, identification. In the study of microbial growth phase of TF-2 was observed that between 66hrs and 96 hrs ages of inoculums and 5%, 10%, 15%, 20%, 25%, 30% size of inoculums. In the investigation of ages and sizes of culture, 84hrs cultivation and 25% seed culture could be optimized for fermentation. The highest activity reached at 84hrs and 25% seed culture by using five kinds of fermentation medium, FM-4 at 25ºC showed the best activity on test organism. The extraction of compound was extracted from the fermented broth. The antimicrobial metabolite TF-02 was conducted with eluting solvents, Chloroform ($CHCL_3$), Dicholoromethane (CH2CL2), Toluene and Hexane. The eluting solvent, Dichloromethane (CH2CL2) was chosen by antimicrobial fungus TF-02. This fungus TF-02 was identified as *Aspergillus versicolar.*

Keyword Endophytic Fungi, Metabolite, Bioactive

Introduction

The microorganisms that isolated from plant parts are endophytes. Tough the meaning of the term "endophytes" varies depending on the researchers, it can be defined the endophytes as microorganisms living inside the healthy plants. (Scott and Lori 1998). their role in plants growth stimulation, protection, against biotic and abiotic stresses and pest via modulation of growth hormone signalling, higher seed yield and plant hormones. (Miliute *et al*., 2015).

Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substance metabolites, insecticides compound that promote inhibit growth, attractor, repellent etc, secondary metabolites produced from fungi in production, function and specify to a particular fungus. These metabolites were being exploited in different fields of medicine and industry (Kishare *et al.*, 2007).

The research work, endophytic fungi were isolated from *Andrographis paniculata* (Say-Kha-Gyi) plant part collected Magway University Campus. Endophytic fungus TF-02 for the production of antibacterial secondary metabolite against *Escherichia coli*. In the investigation of fermentation of TF-02 showed the ages and sizes of inoculum for the production of antibacterial activity the best for fermentation. Medium optimization studies are usually carried out in the chemical, food and pharmaceutical industries with respect to increase the yield and activity of the desired product. (Shih *et al.*, 2002, Singh and Rai 2012).

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Four kinds of solvent were used for the extraction of bioactive from fungus TF-02. And then bioactive compound was extracted from the fermentation broth of fungus TF-02 using best solvent Dichloromethane. Identification of organisms was an important step in understanding and analyzing biological process. Traditional methods of classification and identification of the organisms were based on morphological, physiological, biochemical developmental and nutritional characteristics (Singh and Rai 2012).

In this study, aim and objectives are to know the knowledge that endophytic fungi can isolated from (Say-Kha-Gyi) plant parts, to find out the antibacterial activity of secondary metabolite from fungus TF-02, to know different ages and sizes of inoculum to optimize the fermentation, to share the suitable fermentation medium for the production of secondary metabolite, to know how extract bio-active compound. From the fermented broth by which solvent systems. To give knowledge of identical the isolated endophytic fungi.

Materials and Methods

Isolation of Endophytic Fungi from *Andrographis paniculata* Parts

Plant parts sample were collected from Magway University campus. Plant parts sample were carried out from 5 to 12 January, 2020. Plant parts sample were transported to the laboratory and processed immediately for the isolation and cultivation of fungi. Plant parts samples used for the isolation of endrophytic fungi and their location, collected data are shown in Table.

Table 1 Plant Parts Sample Collected at Magway University Campus

Plant	Family	Place	Location	Collected Date
Andrographis paniculata Wall ex. Part Use- Leave, stem and root	Acanthaceae	Magway university campus	20º8'13.12"N 94º56'12.66"E	29.12.2019

Figure 1 Habit of *Andrographis paniculata* NEES

Scientific Name - *Andrographis paniculata* Wall.ex Nees. Pl. Asiant Rar 3:116. 1832 *Justici paniculata* Burman, f.FL. Ind.9.1768
Myanmar Name - Say-kha-gyi
English Name - King of Bitter
Family - Acanthaceae

Annual herbs, stems quadragular, glabrous. Leaves simple, opposite and decussate; exstipulate; leaf blades lanceolate. Inflorescences terminal, leafy panicles of second racemose. Flowers bisexual, zygomorphic, pentamerous, hypogynous, white, with purplish spot; bracteate; bracteolate. Calyx 5-partite. Corolla bilabiate, infundibuliform. Stamens 2, anther basifixed. Ovary bilocular, superior. Capsule linear-oblong. Seeds orbicular, glabrolus.

Surface Sterilization Method (NITE 2004)

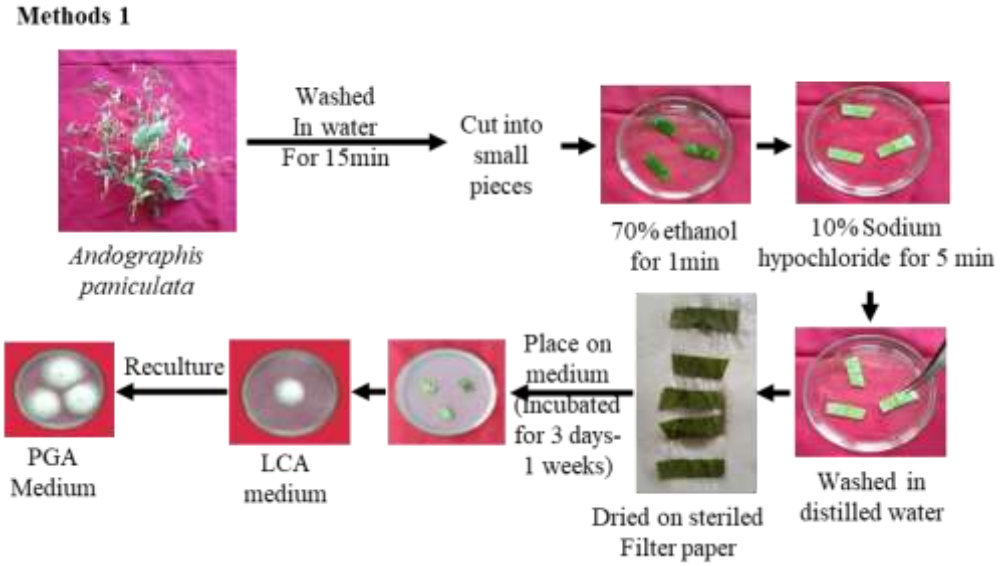


Figure 2 Procedure of Isolation Endophytic Fungi on Leaf, Stem and Root of *Andrographis paniculata*

Baiting Method (NITE-2004)

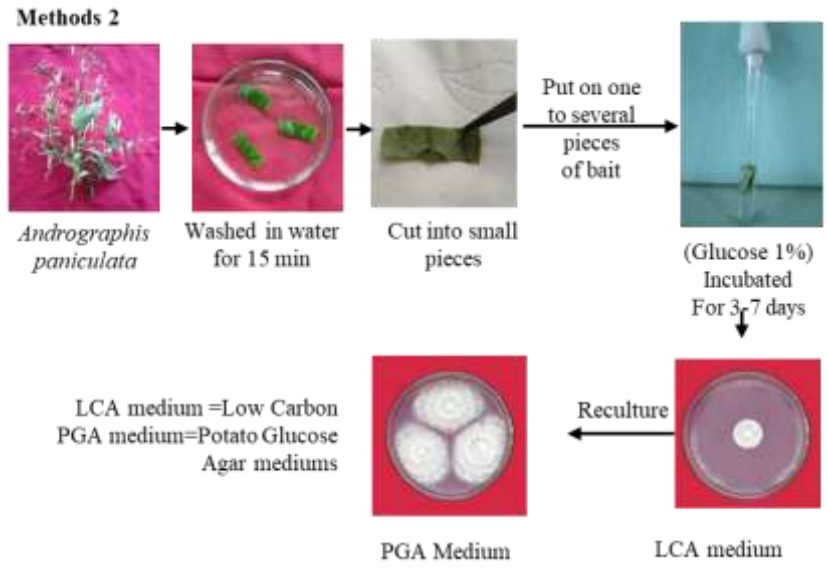


Figure 3 Procedure of Isolation Endophytic Fungi on Leaf, Stem and Root of *Andrographis paniculata*

Medium Used for the Isolation of Fungi

LCA medium
(Low Carbon Agar medium, Ando, 2004)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
DW	100 ml

(after autoclaving chloramphenicol was added to the medium)

PGA medium
(Potato Glucose Agar Medium)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
Polypeptone	0.1 g
Potato+DW	20+80 =100 ml

(after autoclaving chloramphenicol was added to the medium)

Figure 4 Procedure for the Antimicrobial Activity Test

Seed Medium Used for the Endophytic Fungi

Glucose	-	2.0 g
Glycerol	-	1.0 g
Yeast extract	-	1.04 g
$MgSO_4$	-	0.003 g
K_2HPO_4	-	0.002 g
DW	-	100 ml
pH	-	6.5

Fermentation Medium Used For the Endophytic Fungi

Glucose	-	2.5 g
Glycerol	-	1.5 g
Yeast extract	-	1.2 g
Polypeptone	-	0.8 g
K_2HPO_4	-	0.002 g
$MgSO_4$	-	0.003 g
$FeSO_4$	-	0.001 g
DW	-	100 ml
pH	-	6.5

Assay Medium

Glucose	-	1 g
Polypeptone	-	0.1 g
Agar	-	1.8 g
D.W	-	100 ml

Study on the Effects of Age and Size of Inoculum for the Fermentation (Omura, 1985, Crueger and Crueger, 1989)

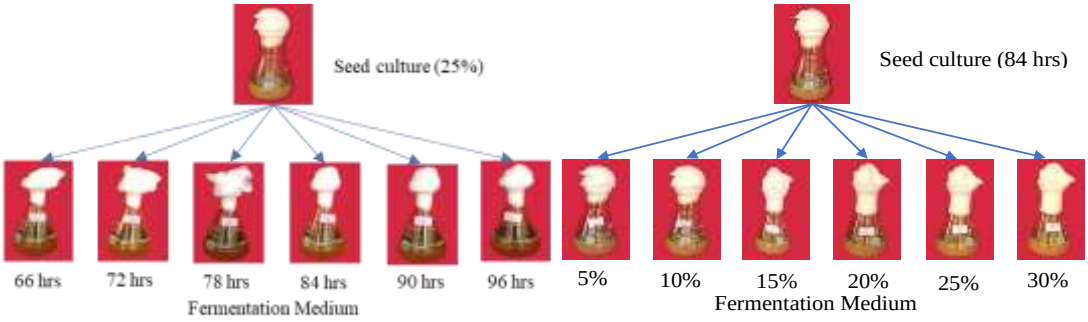


Figure 5 Procedure for the Study on the Effects of Ages and Sizes of Inoculums

Medium Optimization for Fermentation and Production of Antimicrobial Metabolites

Fermentation medium is also important for the production of metabolite (Crueger and Crueger, 1989). Therefore, fermentation was undertaken with suitable conditions of 25% sizes and 84 hrs age of inoculum with five different media.

Fermentation Medium Used for the Endophytic Fungi

FM-1			**FM-2**			**FM-3**		
Glucose	-	2.5 g	Sucrose	-	1.8g	Glucose	-	1.8 g
Glycerol	-	1.5 g	Glycerol	-	1.5g	Tapioca	-	1.5 g
Yeast extract	-	1.2 g	Yeast extract	-	1.6g	Meat extract	-	1.2 g
Polypeptone	-	0.8 g	Polypeptone	-	0.5g	Yeast extract	-	1.2 g
K_2HPO_4	-	0.002 g	K_2HPO_4	-	0.003g	Polypeptone	-	0.8 g
$MgSO_4$	-	0.003 g	$MgSO_4$	-	0.001g	K_2HPO_4	-	0.002 g
$FeSO_4$	-	0.001 g	$FeSO_4$	-	0.001g	$MgSO_4$	-	0.003 g
DW	-	100 ml	DW	-	100 ml	$FeSO_4$	-	0.001 g
						DW	-	100 ml

FM-4			**FM-5**		
Glucose	-	2.0 g	Glycerol	-	2.0 g
Potato powder	-	1.5 g	Tapioca	-	0.8 g
Meat extract	-	0.8 g	Malt extract	-	0.8 g
Yeast extract	-	1.6 g	Yeast extract	-	1.2 g
Polypeptone	-	0.5 g	Polypeptone	-	0.8 g
K_2HPO_4	-	0.003 g	K_2HPO_4	-	0.002 g
$MgSO_4$	-	0.001 g	$MgSO_4$	-	0.003 g
$FeSO_4$	-	0.001 g	$FeSO_4$	-	0.001 g
DW	-	100 ml	DW	-	100 ml

Preliminary Study for the Extraction of Bioactive Compound

Preliminary study for the extraction of bioactive compound was undertaken by the method of Thomashow *et al.*, 2008 and Jain and Pundir (2011).

Purpose: The purpose of their preliminary study to know how to extract the active metabolite from the fermented broth by which solvent system. The solvents used are

1. Chloroform $CHCL_3$
2. Dichloromethane CH_2CL_2
3. Toluene
4. Hexane

The fermented broth (5ml) was added with each solvent (5ml). After shaking till separation two layers, each layer was examined the activity against *Escherichia coli.*

The Macroscopial and Microscopical Character of Endophytic Fungus TF-02

The macroscopial and microscopical were observed by the methods of Domasch, 1993, Taxonomy and significance of black Aspergilla. The morphological and microscopical characters were observed by the methods of Ando and Inaba, 2004. Microscopical characters were studied by microscope. Comparison of these characters with reference keys was undertaken to identify.

Results

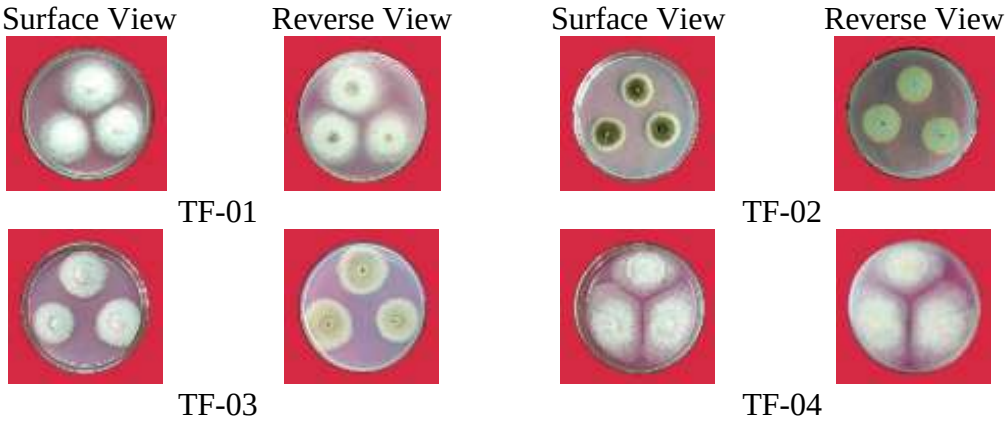
Isolation of Endophytic fungi from *Andrographis Paniculata* Parts Sample

In the isolation of Endophytic fungi, 14 fungi were isolated from *Andrographis paniculata* parts sample collected from Magway University Campus. Fungus TF-01, TF-02, TF-03, TF-04 isolated from leave of *Andrographis paniculata*. Fungus TF-07, TF-08, TF-09, TF-10 from stem of *Andrographis paniculata.* Fungus TF-11, TF-12, TF-13, TF-14 from root of *Andrographis paniculata* respectively. In this study Nine fungi were collected by surface sterilization method (NITE, 2004). Five fungi were collected by Baiting method (NITE, 2004).

Sampling Sites and Plant Parts Collection

Table 2 Isolated Endohytic Fungi from Plant Parts Samples

No.	Plant parts sample	Isolation Method	Number of endophytic fungi	Isolated endophtic
1.	Leaf	Surface sterilization method	TF-01, TF-02, TF-03, TF-04	4
		Baiting Method	TF-05, TF-06	2
2.	Stem	Surface sterilization method	TF-07, TF-08, TF-09	3
		Baiting Method	TF-10	1
3.	Root	Surface sterilization method	TF-11, TF-12	2
		Baiting Method	TF-13, TF-14	2
		Total Isolated endohpytic fungi		14



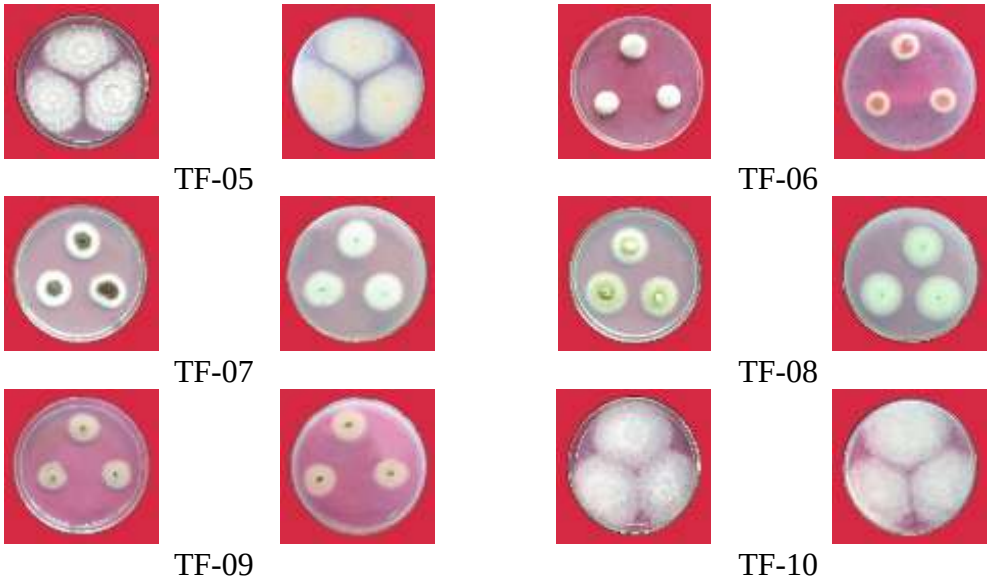


Figure 6 Morphology of Fungus TF-01 and TF-10

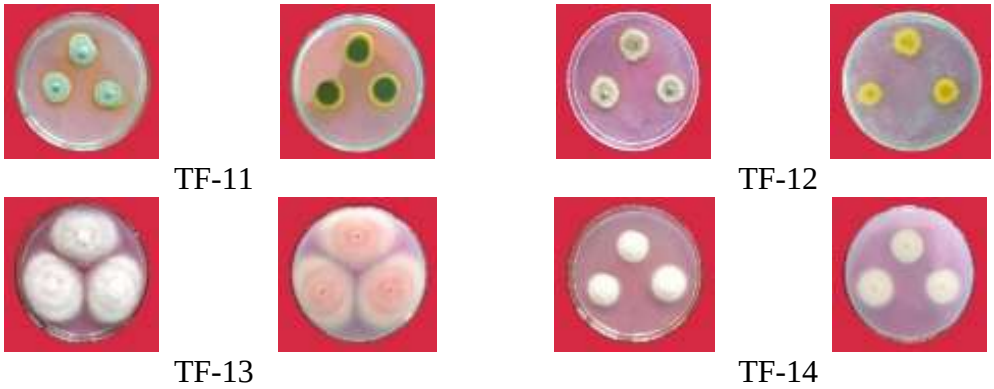


Figure 7 Morphology of Fungus TF-11 and TF-14

Preliminary Study for Antimicrobial Activities by Paper Disc Diffusion Assay NITE, 2004

Table 3 Antimicrobial Activities of Isolated Fungi on Test Organisms

Isolated fungi	Fermation day and inhibitory zone (mm)				
	Pseudomonas fluorescence	***Bacillus subtilis***	***Escherichia coli***	***Saccharomyces cerevisiae***	***Candida albicans***
TF-01	-	19.58	-	-	-
TF-02	-	-	35.32	-	-
TF-03	-	-	-	23.89	-
TF-04	-	-	-	-	28.60
TF-05	16.42	-	19.78	-	-
TF-06	-	23.29	-	-	-
TF-07	-	-	21.57	18.44	-
TF-08	-	-	23.89	-	-
TF-09	24.12	-	16.49	-	-
TF-10	-	-	-	-	21.67
TF-11	-	-	13.85	-	-
TF-12	-	19.21	-	-	-
TF-13	-	-	-	14.54	-
TF-14	-	-	-	-	16.40

Figure 8 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Escherichia coli*

Figure 9 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Bacillus subtilis*

Figure 10 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Pseudomonas fluorescence*

Figure 11 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Saccharomyces cerevisiae*

Figure 12 Antimicrobial Activities of Isolated Fungi TF-01 to TF-14 on *Candida albicans*

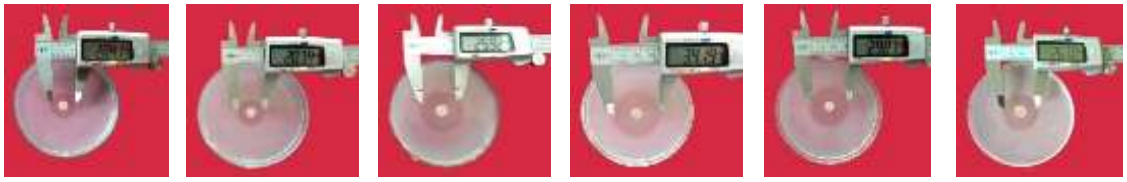
The Effects of Ages and Sizes of Inoculums for the Fermentation

Table 4 The Effects of Ages of Cultures on Fermentation

Growth Hour (hrs)	**Inhibitory Zone (mm) on *Escherichia coli***
66 hrs	20.41
72 hrs	20.74
78 hrs	25.92
84 hrs	34.19
90 hrs	29.87
96 hrs	26.70

Table 5 The Effects of Sizes of Cultures on Fermentation

Culture Times (Sizes of Culture, %)	Antibacterial activity on *Escherichia coli* (Clear zone, mm)
5%	23.83
10%	25.79
15%	27.38
20%	29.49
25%	33.55
30%	23.95



66hrs 72hrs 78hrs 84hrs 90hrs 96hrs

Figure 13 The Effects of Ages of Cultures on Fermentation (4 days)

5% 10% 15% 20% 25% 30%

Figure 14 The Effects of Size of Cultures on Fermentation (4 days)

Medium Optimization for Fermentation and Production of Antibacterial Metabolite

Table 6 Selection of Medium Based on the Results of Antimicrobial Activity

Sizes of Culture (%)	Antimicrobial Activity (Clear Zone, mm)
FM-1	23.07mm
FM-2	26.26mm
FM-3	23.60mm
FM-4	37.69mm
FM-5	36.13mm

FM-1 FM-2 FM-3 FM-4 FM-5

Figure 15 Antimicrobial Activity of Fungus *Aspergillus versicolor* (at Fermentation 5 Days)

Preliminary Study for the Extraction of Bioactive Compound (Jain and Pundi 2011)

Table 7 Activity of Pre-extraction with 4 Solvents System

No.	Solvent	Upper Layer	Lower Layer
1.	Chloromethane $CHCl_3$	21.25mm	16.17mm
2.	Dichloromethane CH_2Cl_2	24.29mm	17.46mm
3.	Toluene	19.31mm	14.26mm
4.	Hexane	21.98mm	10.70mm

In this study, it was found that the highest activity was shown in the solvent Dichloromethane CH_2CL_2.

In this study, it was observed that found that Dichloromethane CH_2CL_2 extract exhibited the highest activity although all extracts showed the activity. Therefore, if was assumed that the bioactive compound can be extracted twice with CH_2Cl_2 from the fermented broth.

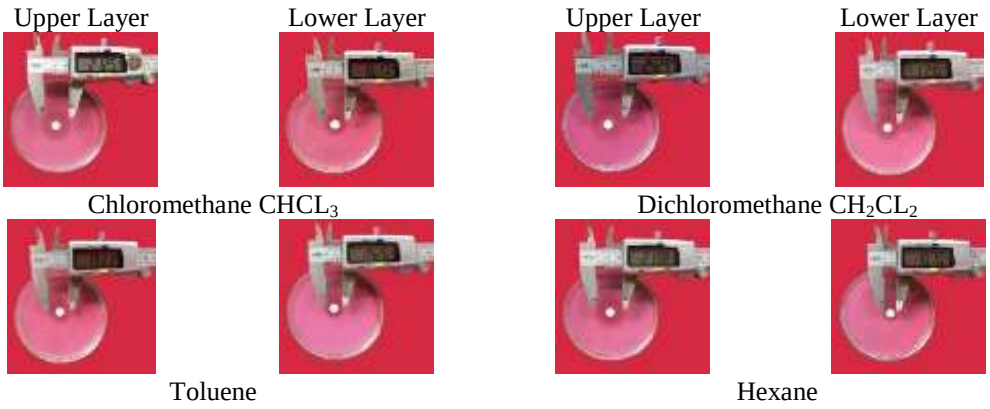


Figure 16 Solvent Based on Antimicrobial Activity

Morphological Characters of Endophytic Fungi TF-02

Colonies are usually fast growing, white yellow and then Shade of Yellow brown, brown to black, mostly consisting of a dense flat or erect conidiophores. Texture is velvety or cottony. Reverse is white, goldish or brown.

(X200) (7 days old culture) **(X200) (7 days old culture)**

Figure 17 Morphology and Photomicrograph of Fungus TF-02

Microscopic Characters of Endophytic Fungi TF-02

Hyphae are long, septate hyphae than appear glassy and transparent conidio -phores, which are specialized hyhphal stakes for asexual reproduction, typically measure 120-700 μm in length. Conidiophores terminate is small vesicles (10-15 μm in diameter), that are biseriate (i.e) with two successive layers of cells interposing the vesicle and conidia. The first layers of cells are called metulae upon which phialides are born. Hulle cells present and globose. The vesicles are variable in shape but often described as spoon shaped. Conidia are spherical approximately 2.5-3.5 μm in diameter and may have smooth or slightly roughened surfaces.

KEY TO GENUS

(Domsch *et al*, 1993; Klich, 2002; Mc Clenny, 2005; Nyongesa *et al*, 2015)

KEY TO GROUP

1. Conidium lacking septum…. ……..Ameroconidium
2. Conidium with 1 septum ….. ……..Didymoconidium
3. Conidium with more than 1 septum and only transverse septa ……... .…….. ……...Phragmoconidium
4. Conidium body subdivided by intersecting septa in more than one plane …….. …….....Dictyoconidium

Ameroconidium

A. Conidiophore not produced
B. Conidiophore not produced or not clear
C. Conidiophores with or without septa develop single, not branched
   1. Conidia holoblastic
   2. Conidia enteroblastic
      i. Phialo-conidium
      ii. Multi-phialides with parallel arrangement
         - *Penicillim*
         - *Aspergillus*
         - *Purpureocillium*
         - *Paecilomyces*

According to references key, TF-02 fungus may be the genus of *Penicillium, Aspergillus, Purpureocillium* and *Paecilomyces*.

Table 8 Comparison of Microscopical characters of *Penicillium, Aspergillus, Paecilomyces, Purpureocillium* and fungus TF-02

Genus of Fungi	Distinct Characters
Penicillium *	Phialides have thicker apices
Aspergillus *	Vesicle present
Paecilomyces *	Phialides are basically swollen, taper towards their apices are slightly apart from each other
Purpureocillium *	Phialides are basically swollen, taper towards their apices are slightly apart from each other
TF-02	Vesicle present

* (Domsch *et al*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al*., 2015)

In the study on the comparison of microscopical characters, fungus TF-02 was designated to the genus *Aspergillus* due to the presence of vesicle.

Discussion and Conclusion

In the present study, *Andrographis paniculata* (Say-Kha-Gyi) collected from Magway University campus from isolation of endophytic fungi. Endophytic fungi were isolated by the method of Surface sterilization method and Baiting method. In this study, Nine fungi were isolated from surface sterilization method and five fungi were isolated from Baiting method. Fungi TF-01, TF-02, TF-03, TF-04, TF-05 and TF-06 were isolated from Say-Kha-Gyi leaves. Fungi TF-07, TF-08, TF-09 and TF-10 were isolated from Say-Kha-Gyi stem. Fungi TF-11, TF-12, TF-13 and TF-14 were isolated from Say-Kha-Gyi root. According to results, more fungi were isolated by surface sterilization method than Baiting method.

For the investigation of antimicrobial activities of isolated endophytic fungi, five kinds of test organisms were used by paper disc diffusion assay method. Fungus TF-02, TF-08 and TF-11 showed that antimicrobial activities against *Escherichia coli.* TF-01, TF-06 and TF-12 showed

that antimicrobial activities against *Bacillus subtilis*. TF-05 and TF-09, showed that antimicrobial activities against *Pseudomonas fluorescence*. TF-03, TF-07 and TF-13 showed that antimicrobial activities against *Saccharomyces cerevisiae*. TF-04, TF-10 and TF-14 showed that antimicrobial activities against *Canidida albicans*. According to the results, Fungus TF-02 was showed the highest antibacterial activity against *Escherichia coli*.

For the optimal age of inoculum 66hrs, 72hrs, 78hrs, 84hrs, 90hrs and 96hrs and for the size of inoculum 5%, 10%, 15%, 20%, 25% and 30% were used. By the results, the age of inoculum 84hrs (34.19mm) and the size of inoculum 25% (33.55mm) at 9 day cultured was best for TF-02 of fermentation. For the selection of fermentation, five kinds of fermentation media FM-1, FM-2, FM-3, FM-4, FM-5 were used for antibacterial activity. Among them, FM-4 showed the best antibacterial activity (37.69mm) at 7 days fermentation. The fermentation study is to know optimum size and age of inoculum and suitable fermentation medium for the production of antibacterial metabolize from fungus TF-02.

The purpose that preliminary study for the extraction of bioactive compound was to know how to extract the bioactive compound from fermented broth. The study for the extraction of bioactive compound from the fermented broth by using four solvents, Chloroform, Dichloromethane, Toluene and Hexane. In this study it was found that the highest activity was shown in the solvent Dicholoromethane. The study of morphological fungus TF-02 on MEA medium at 7 days' culture was excellent growth. In the investigation of identification, fungus TF-02 was identified as *Aspergillus versicolor* (vuill) Tirabosahi 1926 base on its morphological characters, microscopical characters and reference keys.

In conclusion, Endophtic fungus TF-02 were isolated from Say-Kha-Gyi and its was identified as *Aspergillus versicolor*. Endophytic fungus TF-02 was antibacterial activity against *Escherichia coli*. By the literature references, *Escherichia coli* can cause diarrhea was known. Therefore, this isolated endophytic fungus TF-02 will more or less help on against pathogenic microbes *Escherichia coli*.

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I would like to express my special thanks to Professor Dr Khin Maung Oo, Rector, University of Magway for his permission to conduct this research. I sincerely thankful to Dr Sanda Myint, Professor and Head, Department of Botany, University of Magway, for her permission to carry out this paper and for providing me the necessary departmental facilities.

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ISOLATION OF ENDOPHYTIC FUNGI FROM SOME SPECIES OF FABACEAE AND ANTIBACTERIAL ACTIVITY

Tin Moe Aye[1], Thet Thet Khaing[2], Aye Khaing Oo[3]

Abstract

In the study endophytic fungi, isolated from four species of Fabaceae (Myay-pae, Pe-sin-gon, Pe-Lun and Pe-nauk) collected at Kanpyar Village, Magway Region were undertaken by direct inoculation method. In this investigation, eleven different kinds of fungi TF 1-11 were isolated from collected four species of Fabaceae. TF 1-3 were isolated from Myay-pae, TF 4-6 were isolated from Pe-sin-gon, TF 7-9 were isolated from Pe-Lun, TF 10 and TF-11 were isolated from Pe-nauk. According to the present result indicated that, the surface view and reverse view of endophytic fungi. Five kinds of test organisms (*Escherichia coli*, *Agrobacterium tumefaciens*, *Staphylococcus aureus, Pseudomonas fluorescens,* and *Bacillus subtilis*) were used in paper disc diffusion method. In the study of antagonistic property of isolated fungi, it was observed that fungus TF-7 exhibited highest activity on *Agrobacterium tumefaciens*. Therefore, this fungus TF-7 was selected for further investigations. In the fermentation study, it was also found that the optimal ages are 96hrs (24.21 mm) and optimal sizes are 25% (21.91 mm) seed culture at 7 days fermentation. Four fermentation medium (FM 1-4) were used in the fermentation study and it was found that FM-2 showed highest antibacterial activity on the medium. In the preliminary study for the extraction of active compound with four solvents system (Hexane, Chloroform, Dichloromethane and Toluene) were used it was found that chloroform provided highest antibacterial activity on *Agrobacterium tumefaciens*. The selected isolated fungus TF-7 was identified as *Trichoderma hamatum*

Keyword endophytic fungi, microorganism, metabolite.

Introduction

Endophytes are microorganisms that are present in living tissue of various plants (root, fruit, stem, seed, leaf etc) establishing mutual relationship without apparently any symptom of diseases. The endophytic fungi play important physiological and ecological roles in their host life. Endophytic fungi are a good source of antibiotics. Endophytic fungi are also capable to produce antimicrobial metabolites. Endophytic fungi are ubiquitous symbiotic to slightly parasitic microorganisms that live within plant tissues for all or part of their life cycle. *Trichoderma* is a genus of fungi in the family Hypoceraceae that is present in all soils, where they are the most prevalent culturable fungi. Many species in this genus can be characteristized as opportunistic avriulent plant symbionts. The ability of several *Trichoderma* species to form mutualistic endophytic relationships with several plant species. Trichoderma species are economically important for their production of industrial enzymes, antibiotics and their action as biocontrol agents against plant pathogens based onvarious mechanisms such as the production of antifungal metabolites, competition for space and nutrients and mycoparasitism (Howell, 2003).

Materials and Methods

Outstanding Morphological characters of each species was carried by using Dassanayake (1991)

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Table 1 Four species of Fabaceae are collected at Kanpyar Village.

No.	Collected Plants	Scientific Name
1.	Myay pea	*Arachis hypogaea* L.
2.	Pe-sin- gon	*Cajanus cajan* L.
3.	Pe-lun	*Vigna unguiculate* L.,
4.	Pe-nauk	*Vigna radiatus* L

Figure 1 Kanpyar Village Map

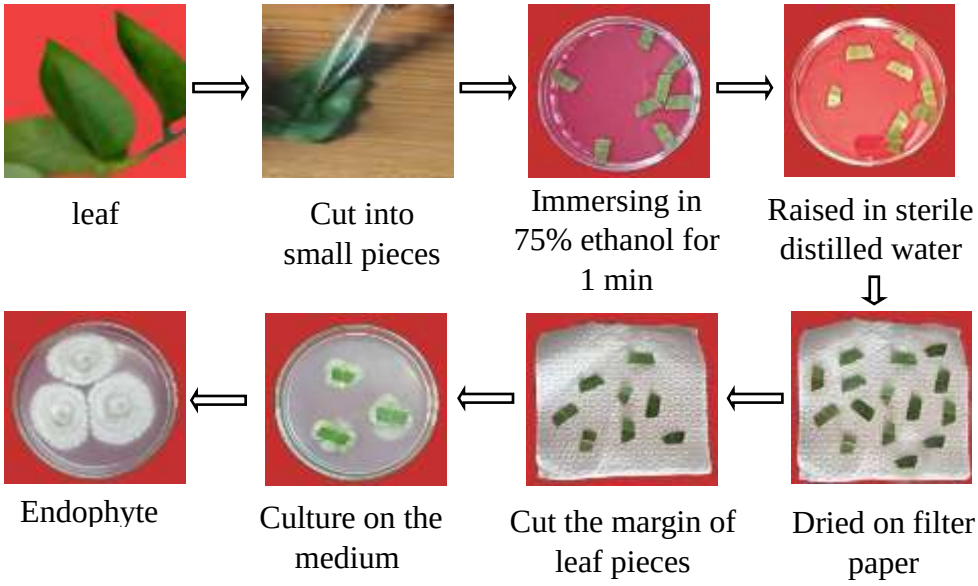


Figure 2 Isolation procedure for endophytic fungi

Medium Used for the isolation of fungi

PGA medium
(Potato Glucose Agar Medium)

Glucose	0.5 g
Yeast	0.1 g
K_2HPO_4	0.001 g
Agar	1.8 g
Poly peptone	0.1g
Potato+Dw	100mL

(after autoclaving chloramphenicol was added to the medium.)

LCA medium
(Low Carbon Agar medium, Ando, 2004)

Glucose	0.2 g
Sucrose	0.2 g
K_2HPO_4	0.1 g
$MgSO_4 7H_2O$	0.05 g
KNO_3	0.1 g
KCL	0.05 g
Agar	1.8 g
DW	100 mL
pH	6.5

(after autoclaving chloramphenicol was added to the medium.)

Study on the Antibacterial Activity of Isolated Fungi

Assay Medium

Glucose	1.0g
Yeast extract	0.2g
Agar	1.8g
DW	100mL
pH	7.0

Seed Medium

Glucose	1.0g
Yeast extract	0.2g
NZ amine type A	0.3g
K_2HPO_4	0.001g
DW	100mL
pH	7.0

Study on the Effect of Age and Size of Inoculum of the Fermentation (Omura, 1985, Crueger and Crueger, 1989)

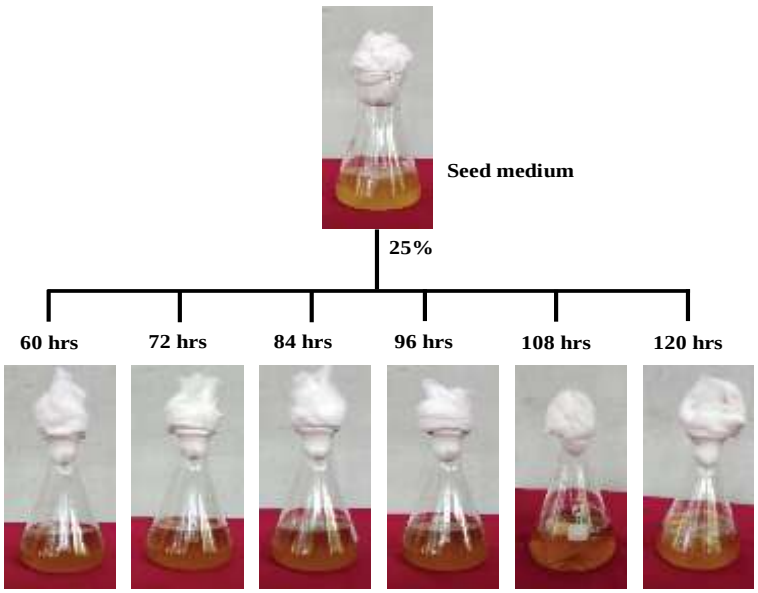


Figure 3 Procedure for the study on the effects of ages of seed culture

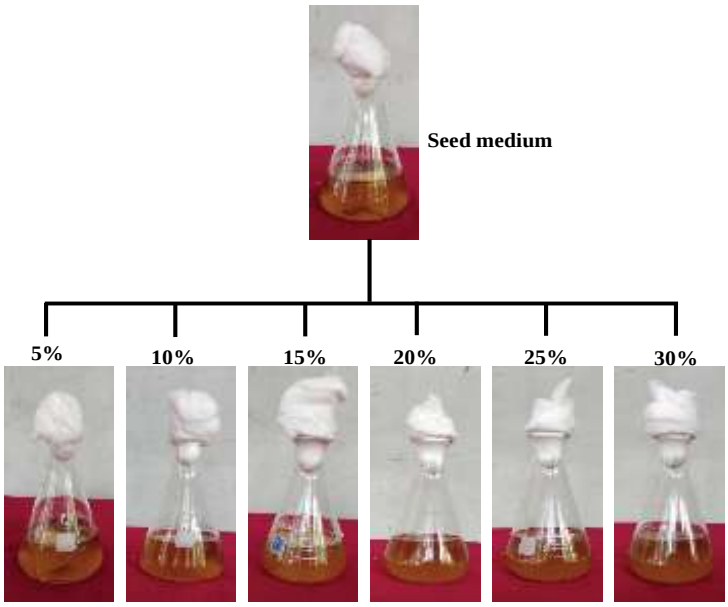


Figure 4 Procedure for the study growth of Fungus TF-07

Preliminary study for the extraction of metabolite (Jain and Pundir, 2011)

In the studies of extraction of metabolite from TF-07, four kinds of solvents (Chloroform, dichloromethane, toluene and hexane). Were utilized and checked with paper disc by assay method.

Identification of Fugus TF-07

The selected fungus TF-07 was cultured on Glucose Yeast Agar (GYA) medium. After 5 days cultured, colony were observed for macroscopic character and microscopic character. The selected fungus was identified followed by Domsch *et al*.,1993.

Results

Isolation of Endophyte Fungi

Scientific Name - *Arachis hypogea* L.
Family - Fabaceae
Myanmar Name - Myay-pe
English Name - Groundnut

Figure 5 Habit of *Arachis hypogaea* L. (Myay-pe)

Annual herb. Stem subteret. Leaves alternate, pinnately compound. Inflorescence axillary, cymose. Sepals (5), pale green, deciduous. Petals 5, papilionaceous, pale yellow,

deciduous. Stamens (10), Monadelphous. Ovary monocarpellary, marginal placentation. Pods oblongoid.

Scientific Name - *Cajanus cajan* L.
Family - Fabaceae
Myanmar Name - Pe-sin-gon
English Name - Red gram

Figure 6 Habit of *Cajanus cajan* L. (Pe-sin-gon)

Annual shrubs. Stem branches. Leaves alternate, trifoliate, pinnately compound. Inflorescence terminal or axillary, cyme. Sepals 5, valvate, green. Petals 5, papilionaceous, white, deciduous. Stamens 10, diadelphous (1+9). Ovary monocapallary, marginal placentation. Pods oblongoid.

Scientific Name - *Vigna unguiculata subsp cylindrica* (L). Walpers *Phaseolus cylindricus* L.,
Family - Fabaceae
Myanmar Name - Pe-lun
English Name - Cow Pea

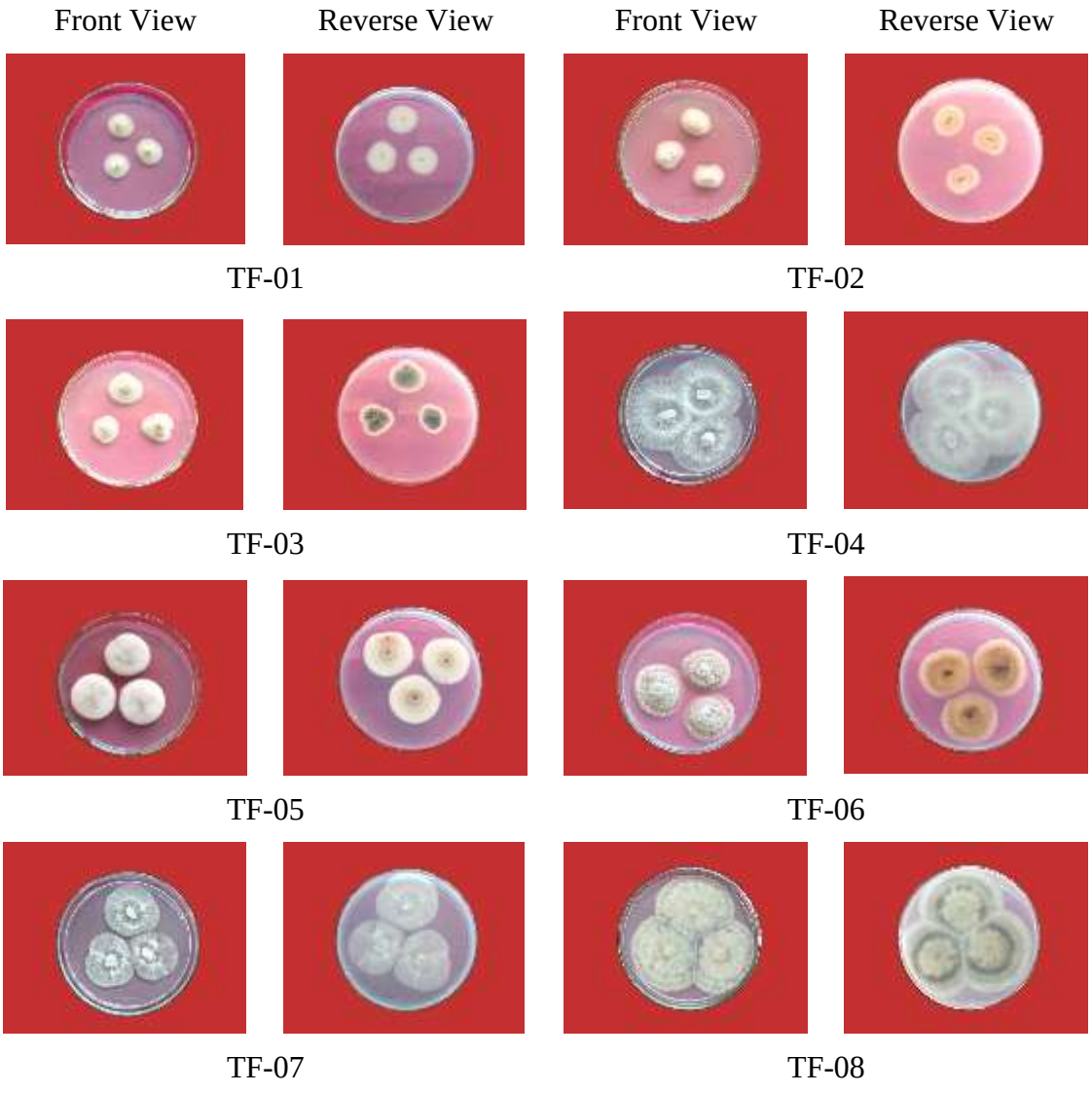
Figure 7 Habit of *Vigna unguiculate* L. (Pe-lun)

Annual herbs, stems subglabrous, stipulate. Leaves trifoliate compound, leaflets ovate-rhomboid. Inflorescences axillary racemes, Flowers blue to purple, papilionaceous; Calyx campanulate; standard suborbicular; wings subdeltoid, blue to purple, keel white stamens 10, diadelphous. Ovary superior, unilocular. Legumes terete. Seeds oblong or reniform.

Scientific Name - *Vigna radiatus* L.
Family - Fabaceae
Myanmar Name - Pe-nauk
English Name - Mung bean

Figure 8 Habit of *Vigna radiatus* L. (Pe-nauk)

Annual erect herb. Stem cylindrical angular. Leaves alternate, trifoliate, pinnately compound. Inflorescence axillary raceme. Sepals 5, valvate, sepaloid, persistent. Petals 5, paoilionaceous, greenish-white. Stamens 10, diadelphous (1+9). Ovary monocapallary, marginal placentation. Pods long and cylindrical.



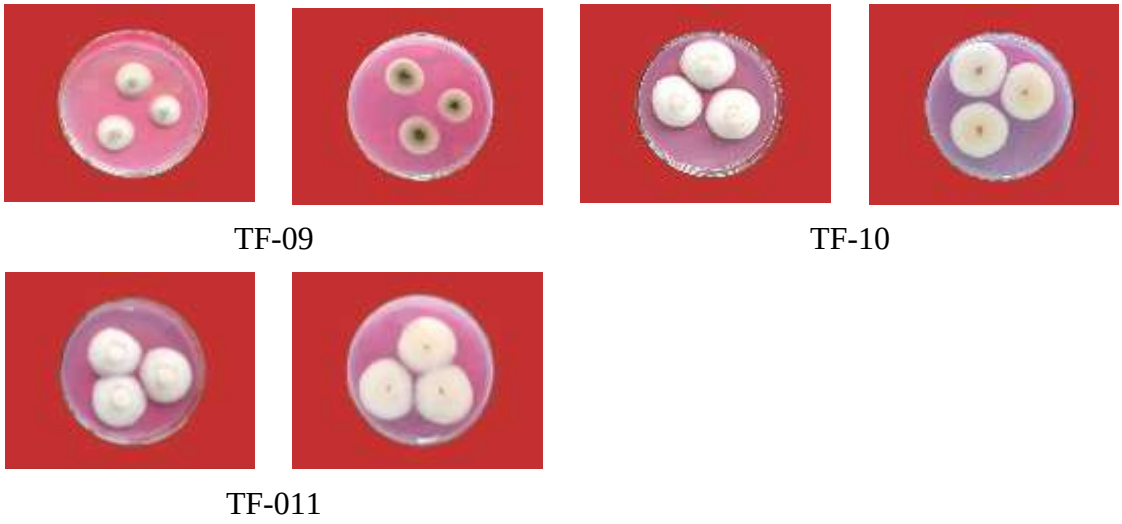


Figure 9 Morphology of fungus TF-01 to TF-11 (7 days old culture on PGA medium)

Table 2 Antibacterial Activities of Isolated Fungi on Test Organisms

Used plants	Isolates	*Escherichia coli*	*Agrobacterium tumefaciens*	*Bacillus subtilis*	*Pseudomonas fluorescens*	*Staphylococcus aureus*
Myay Pea	TF-01	-	18.43	-	-	-
	TF-02	-	16.43	-	-	-
	TF-03	-	12.91	12.96	-	-
Pe-sin-gon	TF-04	-	-	11.00	-	-
	TF-05	-	11.78	-	-	-
	TF-06	-	-	14.98	-	-
Pe-Lun	TF-07		21.39	-	10.89	12.34
	TF-08	-	-	-	-	-
	TF-09	-	12.70	-	-	-
Pe-nauk	TF-10	-	-	-	-	-
	TF-11	-	13.06	-	-	-

Figure 10 Antibacterial activities of isolated fungi TF-07 on *Agrobacterium tumefaciens*

Table 3 The Effect of Ages of Inoculum on Fermentation

Culture Time (hrs)	Activity (Clear zone, mm)
60	18.48mm
72	23.86mm
84	24.06mm
96	**24.21mm**
108	23.49mm
120	20.98mm

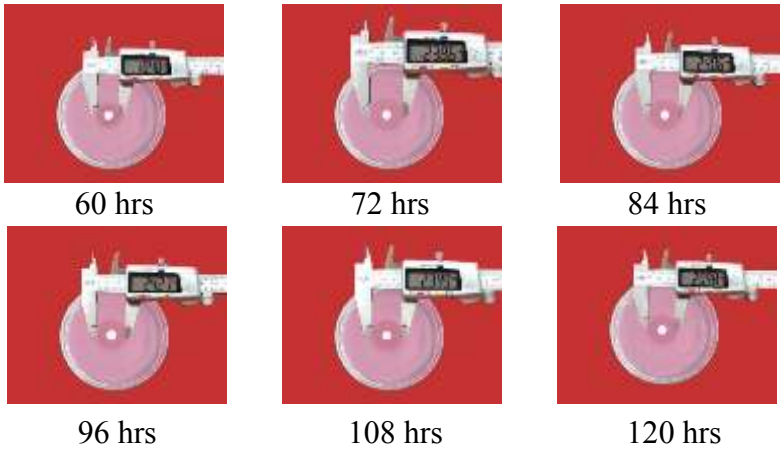


Figure 11 The Effects of Ages of Inoculums on fermentation

Table 4 The Effect of Sizes of Inoculum on Fermentation

Size of inoculum (%)	Antibacterial Activity (Clear zone, mm)
5%	18.02mm
10%	18.78mm
15%	19.70mm
20%	21.69mm
25%	**21.92mm**
30%	18.69mm

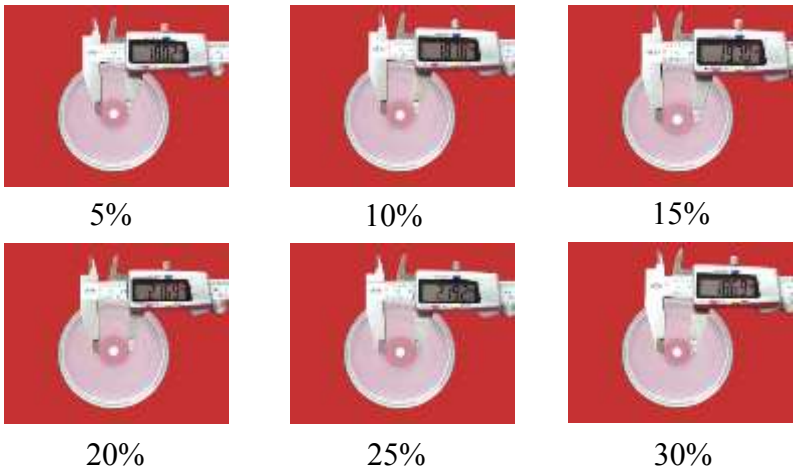


Figure 12 The Effects of Sizes of Inoculums on fermentation

Table 5 Selection of Medium Based on the Results of Antibacterial Activity

Fermentation Medium	Inhibitory Zone (mm)
FM-1	11.56mm
FM-2	20.91mm
FM-3	11.84mm
FM-4	18.89mm
FM-5	12.48mm

According to the results of antibacterial activity, fermentation medium FM-2 was selected for the production of antibacterial metabolite.

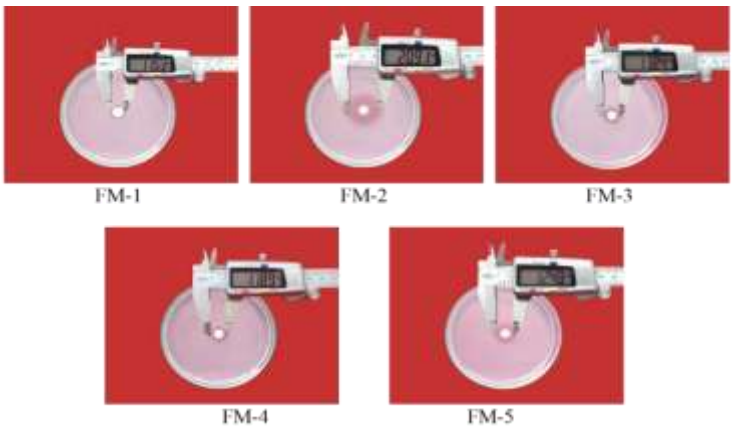


Figure 13 Medium Based on the Results of Antibacterial Activity.

Table 6 Activity of pre-extraction with 4 solvent system

Solvents Used	Activity (mm)	
	Upper Layer	Lower Layer
Hexane	-	18.27
Chloroform	18.42	-
Dichloromethane	18.35	-
Toluene	-	15.39

In this study, it was observed found that Chloroform extract exhibited the highest activity (Table & Figure)

Figure 14 Activity of pre-extraction with 4 solvent system.

Identification of Fungus TF-07

Figure 15 Micrograph of *Trichoderma hamatum* (x 200)

KEY TO GENUS

(Domsch *et al.*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al.*, 2015)

KEY TO GROUP

1. Conidium lacking septum…. ……..… Ameroconidium
2. Conidium with 1 septum ….. ……. Didymoconidium
3. Conidium with more than 1 septum and only transverse septa ……..…. Phragmoconidium
4. Conidium body subdivided by intersecting septa in more than one plane .....Dictyoconidium

Ameroconidium

A. Conidiophore not produced
B. Conidiophore not produced or not clear
C. Conidiophores with or without septa develop single, not branched
D. Cpnidiophores with septa develop single and branched

1. Conidia holoblastic
2. Conidia enteroblastic
    i. Phialo-conidium
    ii. Annello-conidium
        - *Paecilomyces*
        - *Penicillium*
        - *Trichoderma* and
        - *Verticillium*
        - According to references key, fungus TF-07 may be the genus of *Paecilomyces, Penicillium, Trichoderma* and *Verticillium.*

Table 7 Comparison between fungus TF-7 and *Paecilomyces, Penicillium, Trichoderma* and *Verticillium*

Genus	Characters
Paecilomyces	Long Phialides
Penicillium	Long Phialides
Trichoderma	Phialides not long and not crowded
Verticillium	Long Phialides
TF-07	Phialides not long and not crowded

According to this comparison (Domsch *et al*, 1993), it was considered that fungus TF-7 may be the group of *Trichoderma*.

Key to the species for Genus *Trichoderma*

(Domsch *et al*, 1993; Klich, 2002; McClenny, 2005; Nyongesa *et al*, 2015)

1. Conidiophores long and thick, side branches mostly short and thick, crowed, short and plump phialide, colonies white ……..…….. 2

Conidiophore and side branches long and slender, phialides not crowed, colony yellowish, bright dull to dark green ……..…..…..... 5

2. Sterile hyphae elongation absent, conidia globose, hyaline…..…….. *T. piluliferum*

   Sterile hyphae elongation present, conidia not globose ……..... 3

3. Conidia green, short ellipsoidal ……. *T. saturnisporum*

   Conidia smooth-walled or finely punctuated……. 4

4. Conidia hyaline, small, 2.4-3.8 x 1,8 x 2.2 mm …… *T. polysporum*

   Conidia white or green, small, 3.8-6.0 x 2.2-2.8 mm…… *T. hamatum*

Based on the references key (Domsch *et al*, 1993; Klich, 2002; Mc Clenny, 2005; Nyongesa *et al*, 2015), and characters, the fungus TF-07 was identified as *Trichoderma hamatum* (Bonord.) Bain. 1906.

Kingdom:	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Hypocreaceae
Genus	Trichoderma
Species	*T. hamatum*

Discussion and Conclusion

In the isolation of endophytic fungi, eleven fungus was isolated from the four kinds of Fabaceae plants from Kanpyar Village, Magway Region.

Among them isolated endophytic fungi TF-01, TF-02, TF-03 were isolated from Myay pea, TF-04, TF-05, TF-06 were isolated from Pe-sin-gon, TF-07, TF-08, TF-09 were isolated from Pe-lun and TF-10, TF-11 were isolated from Pe-nauk. Isolated endophytic fungi TF-07 were coinvestigated their antibacterial activities by using paper disc on assay medium for five test organisms.

Isolated fungi, antibacterial activities were excellent growth on *Agrobacterium tumefaciens*. To observe the age of inoculum, six different hours of 60hrs, 72hrs, 84hrs, 96hr, 108hrs, 120hrs and for the size of inoculums 5%, 10%, 15%, 20%, 25% and 30% were used respectively.

In conclusion, the present study described the fungi *Trichoderma hamatum* from some species of Fabaceae collected at Kanypyar Village, Magway Region.

Acknowledgements

I would like to express my gratitude to Dr Khin Maung Oo, Rector, University of Magway for her permission to conduct the present research work.

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OPTIMIZING FERMENTATION CONDITIONS OF SELECTED SOIL FUNGUS *PENICILLIUM RUGULOSUM* AGAINST *ESCHERICHIA COLI*

Aye Khaing Oo[1], Moe Moe Aye[2], Tin Moe Aye[3]

Abstract

In this research work, eighteen fungi were isolated from nine soil samples collected in Magway Township Magway Region, by using chemical treatment dilution and soil dilution methods. All isolated fungi were tested with ten kinds of test organisms were using paper disc diffusion assay method. In the study of fungus KFO-06, *Penicillium rugulosum* showed the highest antibacterial activity on *Escherichia coli*. Therefore, this fungus was selected for further investigation. In the study of carbon and nitrogen utilization of *Penicillium rugulosum* for the growth, tapioca powder was excellent for carbon source and peanut cake was excellent for nitrogen source. However, it was observed that the highest antibacterial activity with obtained by using glycerol as carbon source and yeast extract as nitrogen source. Six fermentation media were used for fermentation study and it was found that FM-I showed the highest antibacterial activity on test organism *Escherichia coli* than other medium. In the fermentation studies 72 hrs seed cultures and 15% size of inoculums were optimized for the fermentation.

Keywords Fermentation, Optimization, Antibacterial activity

Introduction

Microorganisms play a major role in soil ecosystem along as fungi, bacteria, protists, small invertebrates and plants, through complex, tropic interactions. They are very important functional group of soil organisms (Abigail *et al.*, 2005). One gram of soil may harbor up to 10billion microorganisms of possible thousands of different species. Soil and marine environments contain thousands of unknown microbial species, many of them fungi. Soil microorganism are involved in many biogeochemical processes (Arnold, 2001). Fermentation procedure must be developed for the cultivation of microorganisms under optimal conditions and for the production of desired metabolites or enzymes by the microorganisms (Yamane and shimizu, 1984). The aim of the present investigation was to find out the effects of age and size of inoculums, carbon and nitrogen sources for the production of antibacterial metabolites.

Materials and Methods

Effect of Carbon and Nitrogen Sources for the Growth of Fungus KFO-06, *Penicillium rugulosum*

Carbon sources (each 1.0g) such as glucose, sucrose, glycerol, soluble starch, fructose, molasses, potato powder and tapioca powder were used. Above carbon sources with basal medium was Yeast extract 0.2 g, Polypeptone 0.3 g, K_2HPO_4 0.001g, $MgSO_4$.$7H_2O$ 0.001g, $CaCO_3$ 0.02g, DW 100mL, pH 6.5.

Nitrogen sources (each 0.5g) such as Yeast extract, NZ amine type A, Polypeptone, Meat extract, KNO_3, Rice bran, Peanut and Fish cake were utilized. Above nitrogen sources with basal medium was Glucose 1.5g, Glycerol 0.5g, K_2HPO_4 0.001g, $MgSO_4$ $7H_2O$0.001g, $CaCO_3$ 0.02g, DW 100mL, pH 6.5.

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The Microbial Growth Kinetic of Fungus KFO-06

Microbial growth kinetic of fungus KFO-06 was investigated by the methods of Crueger and Crueger, 1989; Phay, 1997; Theobald *et al*., 2000; Singh, 2008. Seven days old culture (agar plate) was inoculated into the seed culture 100 ml medium (Glucose 2.0%, Yeast extract 0.5%, Polypeptone 0.5%, pH 6.5). The cultured was performed for 120 hrs at 25·C. Dried Cell Weight (DCW%) was measured 12 hrs intervals (Omura,1985; Phay,1997; Theobald *et al*., 2000; Singh, 2008).

Effect of Ages of Inoculums for the Fermentation

The fungus 7 days old culture was transferred into seed medium. Seed cultures of 66 hrs, 72 hrs, 78 hrs, 84 hrs, 90 hrs and 96 hrs incubation was inoculated into the conical flasks containing fermentation medium. The procedure for the study on the effects of ages inoculums was shown in figure 1. After that, seed culture was checked in 6 hours intervals for the ages.

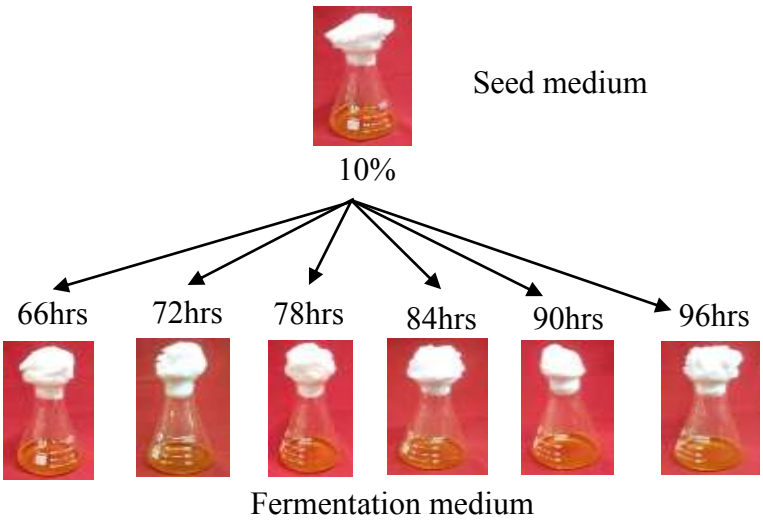


Figure 1 Procedure for the effects of ages of inoculums for the fermentation

Effect of Sizes of Inoculums for the Fermentation

In the study of sizes of inoculums, 5%, 10%, 15%, 20%, 25%, 30% of 72hrs seed culture were utilized for the fermentation. Fermentation was carried out 8 days and antibacterial activity was tested by paper disc diffusion assay (Omura,1985, Crueger and Crueger 1989). Figure 2.

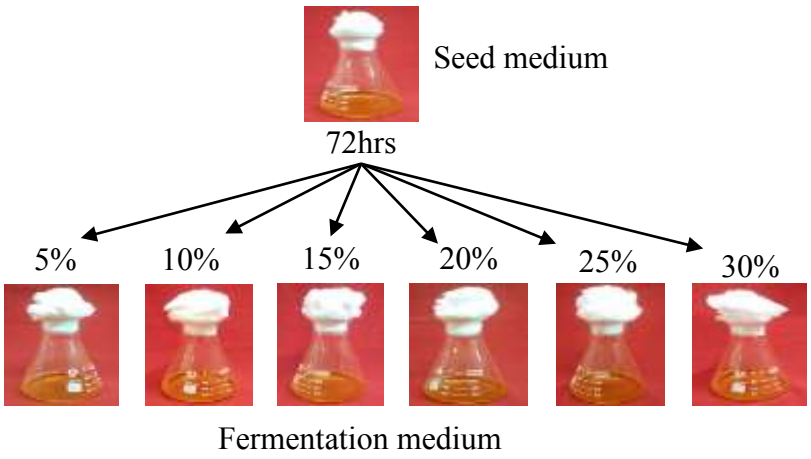


Figure 2 Procedure for the effects of sizes of inoculums for the fermentation

Effects of Carbon and Nitrogen Sources for the Fermentation

In the study of the effects of carbon and nitrogen sources for the fermentation. Carbon sources with basal medium Yeast extract 1.0%, Polypeptone 0.7%, $K_2HPO_47H_2O$ 0.001g, $FeSO_4$ 0.001g, $CaCO_3$ 0.03g, DW 100ml, pH 6.0 and nitrogen sources with basal medium Glucose 2.0g, Glycerol 1.0g, $K_2HPO_47H_2O$ 0.001g, $FeSO_4$ 0.001g, $CaCO_3$ 0.03g, DW 100ml, pH 6.0 were utilized with 72 hrs. age and 15% size of seed culture at 25·C for 8 days.

Medium Optimization for Fermentation and Production of Antibacterial Substance

In the investigation of the effects of fermentation medium for the production of metabolites six kinds of media were utilized with 72 hrs age and 15% size of seed culture at 25·C for 8 days depending on the utilization of carbon and nitrogen sources.

NITE (2004)

Seed Medium		**Assay Medium**		**Fermentation Medium**	
Glucose	1.5 g	Glucose	1.0 g	Glucose	1.0 g
Glycerol	0.5 mL	Yeast extract	0.3 g	Glycerol	1.5 mL
Yeast extract	0.8 g	Agar	1.8 g	Malt extract	0.4 g
Polypeptone	1.0 g	DW	100 mL	Yeast extract	1.0 g
K_2HPO_4	0.001 g	pH	6.5	Polypeptone	0.6 g
$MgSO_4.7H_2O$	0.001 g			MgSO4.7H2O	0.001 g
DW	100 mL			K2HPO4	0.001 g
pH	7			CaCO3	0.1 g
				DW	100 mL
				pH	6.5

Fermentation Media (NITE, 2005)

FM-1		**FM-2**		**FM-3**	
Glucose	1.0 g				
Glycerol	1.0 mL	Glucose	1.0 g	Glycerol	1.0 mL
Yeast extract	0.8 g	Sucrose	0.5 g	Yeast extract	0.8 g
Polypeptone	0.8 g	Yeast extract	1 .0 g	Peptone	0.8 g
K_2HPO_4	0.001 g	K2HPO4	0.001 g	K2HPO4	0.001 g
DW	100 mL	DW	100 mL	DW	100 mL
pH	6.5	pH	6.5	pH	6.5

FM-4		**FM-5**		**FM-6**	
Tapioca power	1.0 g	Glucose	0.5 g	Glycerol	1.0 mL
Glycerol	1.0 g	Molasses	1.5 g	Soluble starch	1.0 g
Sucrose	0.5 g	KNO3	0.5 g	Yeast extract	0.8 g
Fish cake	1.0 g	Yeast extract	1.0 g	Polypeptone	1.0 g
K_2HPO_4	0.001 g	K_2HPO_4	0.00 1g	K_2HPO_4	0.001 g
DW	100 m	DW	100 mL	DW	100 mL
pH	6.5	pH	6.5	pH	6.5

Results

Effects of Carbon and Nitrogen Sources for the Growth of Fungus KFO-06, *Penicillium rugulosum*

In the investigation, the selected fungus KFO-06 were used carbon source such as, Sucrose, Glycerol, Tapioca powder, Potato powder, Glucose, Soluble starch, Molasses and Fructose. Nitrogen sources such as KNO_3 Fish cake, Yeast extract, Meat extract, Polypetone, NZ amine type A, Peanut cake and Rice bran were utilized. The excellent growth of the fungus was found on carbon sources such as Tapioca powder, nitrogen sources such as Peanut cake. It was found that the good growth of KFO-06 on carbon source was sucrose, Glycerol, Glucose, Soluble starch, Molasses and Fructose and nitrogen source was KNO_3 Fish cake, Yeast extract, Meat extract, Polypetone, NZ amine type A and Rice bran. Carbon source of Potato powder gave poor growth for fungus KFO-06. These results are shown in Tables 1 and 2, Figures 3 and 4.

Table 1 Morphological Characters of Fungus KFO-06 on Various Carbon Sources

Carbon sources	Growth
Sucrose	Good
Glycerol	Good
Tapioca powder	Excellent
Potato powder	Poor
Glucose	Good
Soluble starch	Good
Molasses	Good
Fructose	Good

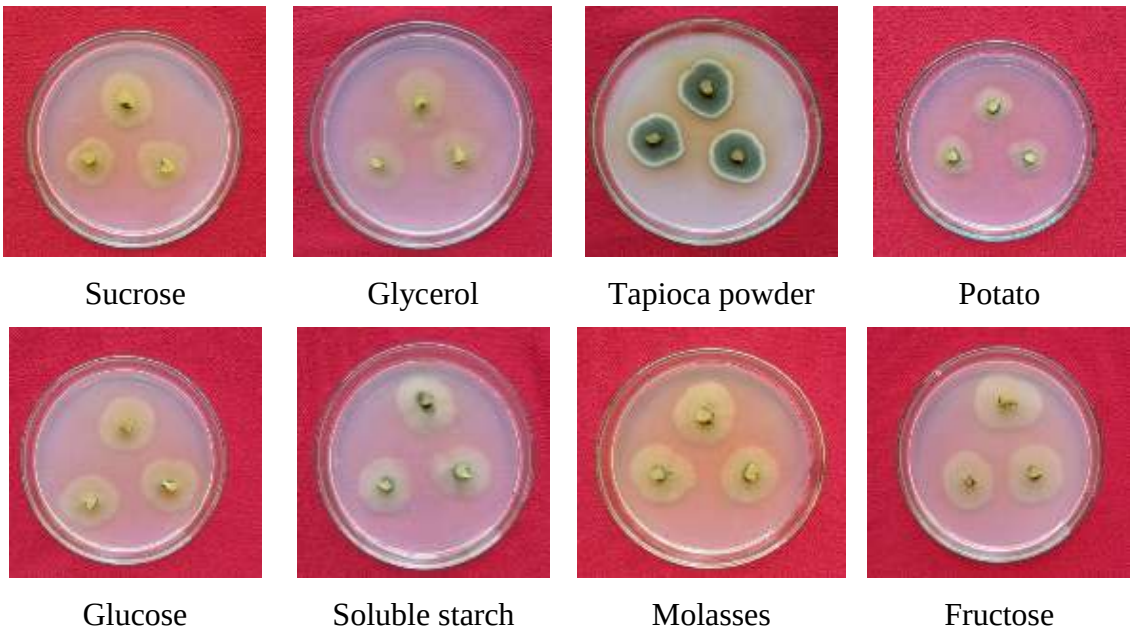


Figure 3 Morphological characters of fungus KFO-06 on various carbon sources (7-days old culture)

Table 2 Morphological Characters of Fungus KFO-06 on Various Nitrogen Sources

Nitrogen sources	Growth
KNO_3	Good
Fish cake	Good
Yeast extract	Good
Meat extract	Good
Polypeptone	Good
NZ amine type A	Good
Peanut cake	Excellent
Rice Bran	Good

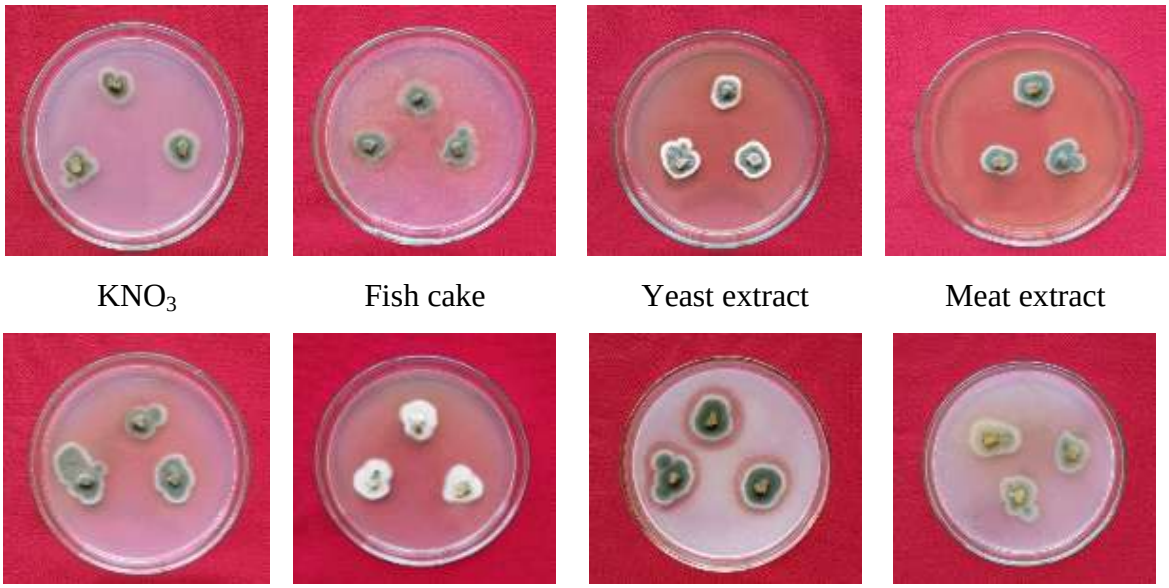


Figure 4 Morphological characters of fungus KFO-06 on various nitrogen sources (7-days old culture)

The Microbial Growth Kinetics of *Penicillium rugulosum*

In the microbial growth kinetics study, as shown in Figure 5, it was found that the lag phase was between 48 hrs and 66 hrs. Growth phase was between 66 hrs and 96 hrs. It was considered that growth phase (66 hrs to 96 hrs) is to be conducted to optimize the age of inoculum for the fermentation. Therefore, the effect of age of inoculums (66 hrs to 96 hrs) was continuously investigated for the fermentation.

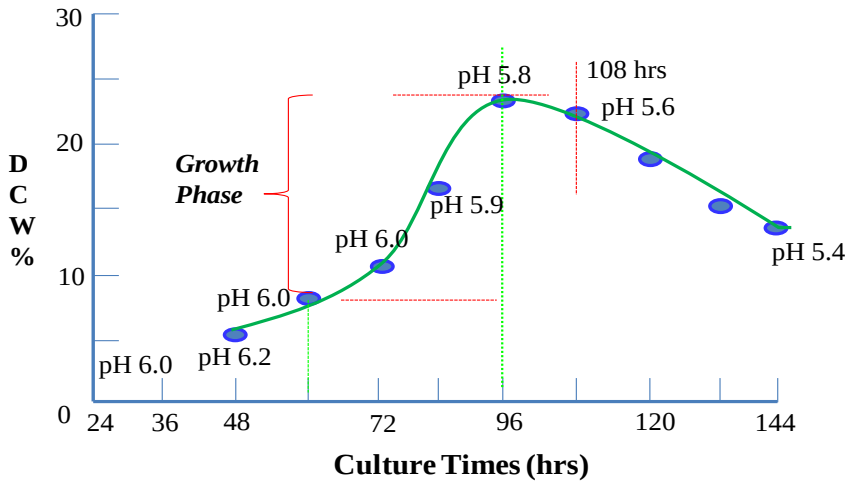


Figure 5 Microbial growth kinetics of *Penicillium rugulosum*

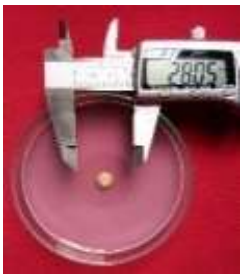
Ages of Inoculums for the Fermentation

In the investigation of the age of inoculums, six different hours of 66 hrs, 72 hrs, 78hrs, 84 hrs, 90 hrs and 96 hrs were used and the results showed that the inhibitory zone of 23.15mm, 28.05mm, 24.03mm, 22.31mm, 20.75mm and 19.18 mm, respectively against *Escherichia coli*. It was observed that 72hrs seed culture showed the best activity on *Escherichia coli* than others seed culture. Therefore, 72 hrs seed culture was selected for the fermentation as shown in Table 3 and Figure 6.

Table 3 The Effects of Ages of Inoculum for KFO-06 against *Escherichia coli*

Culture time (hrs)	Activity (Clear zone, mm)
66	23.15
72	28.05
78	24.03
84	22.31
90	20.75
96	19.18

66 hrs



72 hrs

78 hrs



84 hrs **90 hrs** **96 hrs**

Figure 6 The effects of ages of inoculum on the fermentation of fungus KFO-06

Based on the result, 72 hrs age of seed culture showed the inhibitory zone 28.05mm on *Escherichia coli.*

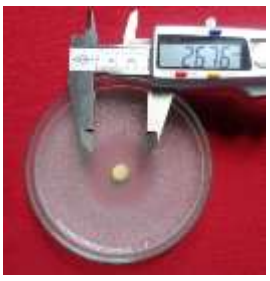
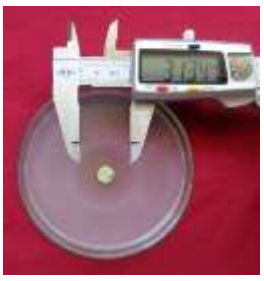
Sizes of Inoculums for the Fermentation

In the study, seed culture 5%, 10%, 15%, 20%, 25% and 30% six conditions (six flasks) were employed to determine the size of inoculums for the fermentation. The results showed the inhibitory zone of 19.16mm, 26.76mm, 31.34mm, 27.00mm, 22.42mm and 18.46mm, respectively against *Escherichia coli.* It was found that 15% size of inoculums gave the best activity on *Escherichia coli.* (Table 4, Figure 7). According to the results, it was determined that 15% inoculum was proper condition to carry out the fermentation.

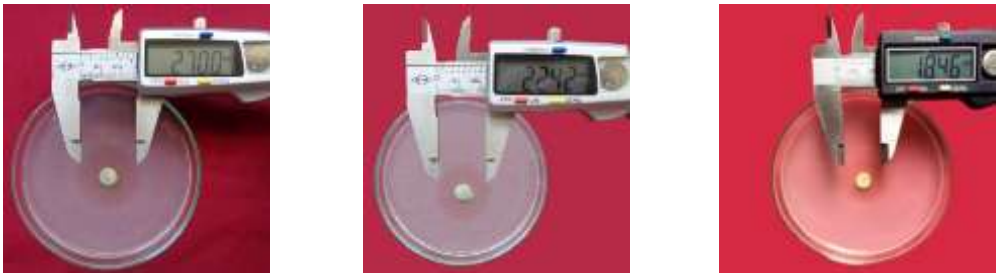
Table 4 The Effects of Sizes of Inoculum for KF-06 against *Escherichia coli* (activity at 5 days fermentation)

Sizes of Culture, %	Antibacterial activity (Clear zone, mm)
5 %	19.16
10 %	26.76
15 %	31.34
20 %	27.00
25%	22.42
30%	18.46

According to the results, 15% size of inoculum was selected for fermentation.

5% **10%** **15%**



20% **25%** **30%**

Figure 7 The effects of sizes of inoculum for KFO-06 against *E.coli*

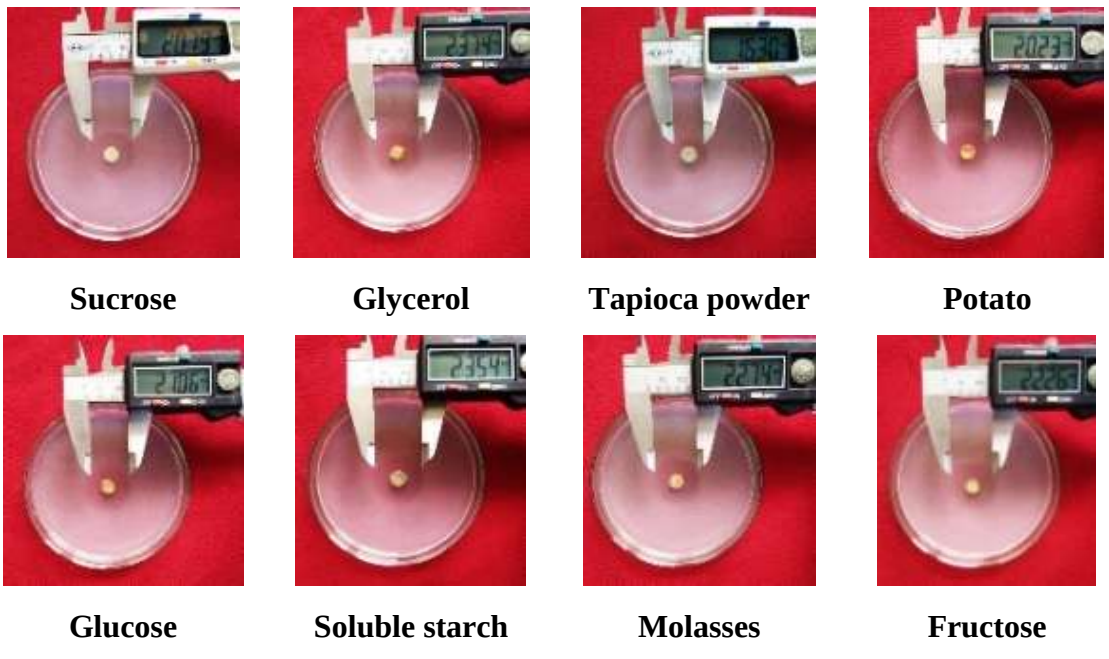
According to the result, 15% size of inoculum gave the best activity on *Escherichia coli*.

The Effects of Carbon and Nitrogen Sources for the Fermentation

The effects of carbon and nitrogen sources for the fermentation study were shown in (Table 5 and Figure 8) and (Table 6, Figure 9), respectively.

Table 5 The Effects of Carbon Sources on the Fermentation

Carbon sources	Inhibitory zone (mm)
Sucrose	20.79
Glycerol	**23.74**
Tapioca powder	16.30
Potato powder	20.23
Glucose	21.06
Soluble starch	23.54
Molasses	22.74
Fructose	22.26



Sucrose **Glycerol** **Tapioca powder** **Potato**

Glucose **Soluble starch** **Molasses** **Fructose**

Figure 8 Antibacterial activity of KFO- 06 on various carbon sources

Table 6 The Effects of Nitrogen Sources on the Fermentation

Nitrogen sources	Inhibitory zone (mm)
KNO_3	10.16
Fish cake	11.33
Yeast extract	**22.31**
Meat extract	18.62
Polypeptone	17.00
NZ amine type A	18.46
Peanut cake	12.56
Rice Bran	9.86

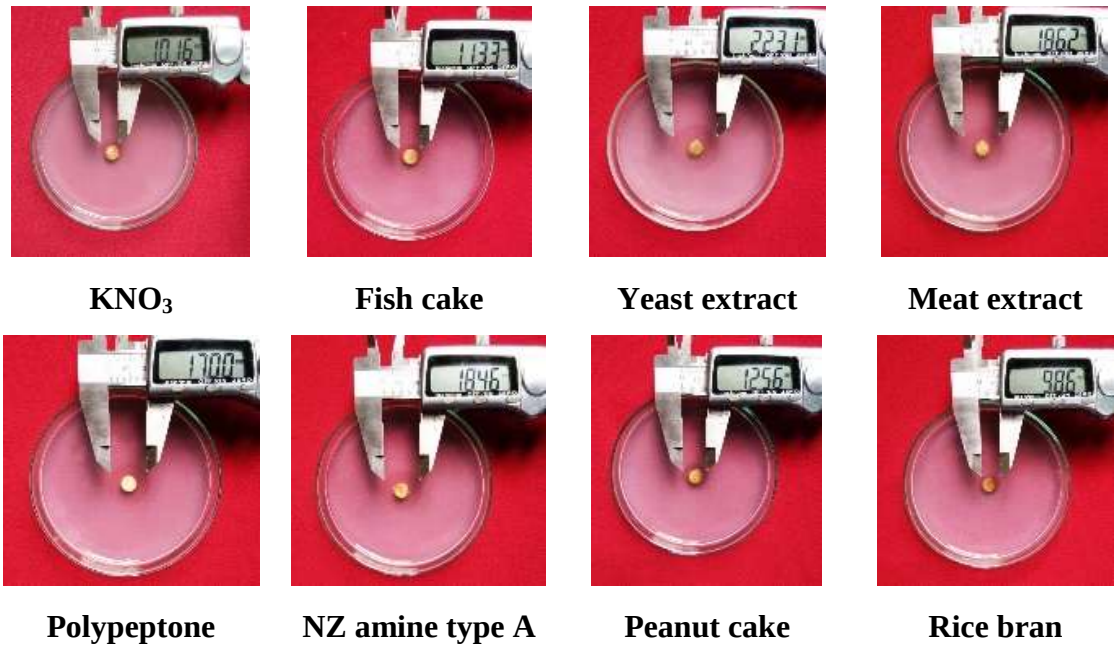


Figure 9 Antibacterial activity of KFO- 06 on various nitrogen sources

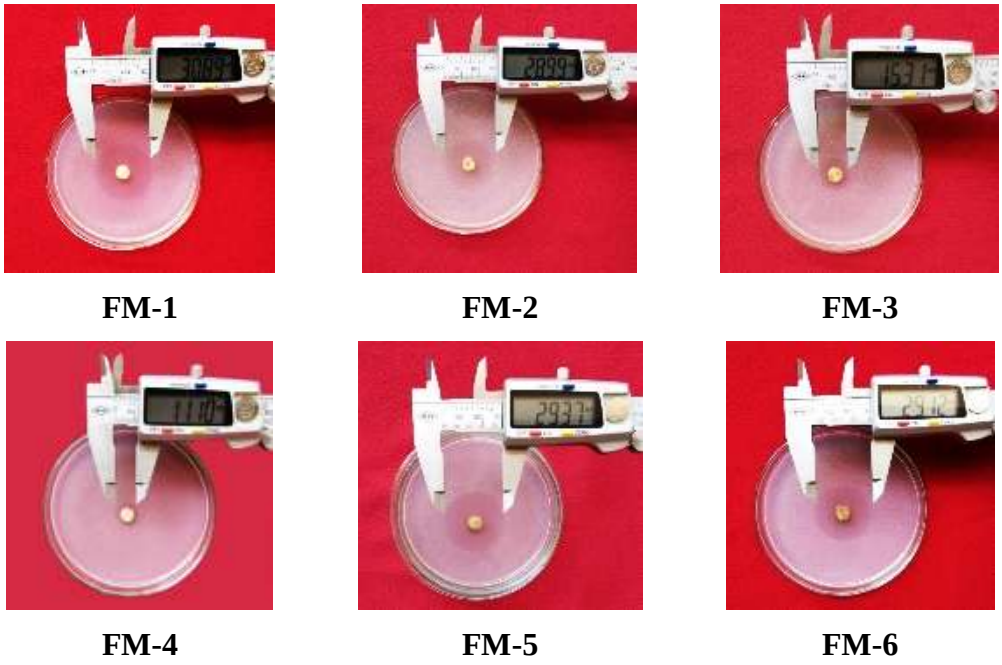
Medium Optimization for Fermentation and Production of Antibacterial Metabolites

In the study of medium optimization six kinds of fermentation media were used. It was observed that in the six kinds of fermentation of the inhibitory zone of FM-1showed 30.89 mm, FM-2, 28.99 mm, FM-3, 15.31mm, FM-4, 11.10 mm, FM-5, 29.37mm and FM-6 29.12mm inhibitory zone respectively. It was determined that FM-1 showed the best activity on *Escherichia coli* (Table 7 and Figure 10). Therefore, FM-1 was selected for the production of antibacterial metabolite.

Table 7 Selection of Medium Based on the Results of Antibacterial Activity

Fermentation media	Inhibitory zone (mm)
FM-1	**30.89**
FM-2	28.99
FM-3	15.31
FM-4	11.10
FM-5	29.37
FM-6	29.12

According to the results, FM-1 is the highest activity on *E. coli.*



FM-1 **FM-2** **FM-3**

FM-4 **FM-5** **FM-6**

Figure 10 Antibacterial activity of KFO- 06 on various fermentations

Based on the results, FM-1 gave the best activity on *E. coli* than other medium.

Discussion

The balance of nutrients (carbon and nitrogen sources) are crucial for the fermentation to produce the metabolite (Omura, 1985). Fermentation medium is also important for the production of metabolite (Crueger and Crueger, 1989). In the study of KFO-06 carbon and nitrogen containing media were investigated, tapioca powder was excellent; glucose, glycerol, sucrose, soluble starch, molasses and fructose were good and potato powder was poor for carbon source. Satish, 2008 reported that glucose was found to be the suitable carbon source for growth of microorganisms. NZ amine type A, yeast extract, KNO_3, fish cake, polypeptone, rice bran and meat extract were good and peanut cake was excellent for nitrogen sources.

In the microbial growth kinetic study, it was found that the lag phage was between 48 hrs and 66 hrs. Growth phase was between 66 hrs and 96 hrs. It was considered that growth phase (66 hrs to 96 hrs) is to be conducted to optimize the age of inoculum for the fermentation.

Therefore, the effect of age of inoculums (66 hrs to 96 hrs) was continuously investigated for the fermentation.

The fermentation studies, the growth phase was between 66 hrs to 96 hrs. It was observed that the growth of fungus KFO-06 declined after 96 hrs. The results obtained in this study show that organic nitrogen sources such as yeast extract support rapid growth and antibacterial productions. Several authors suggested that yeast extract is a good substrate for many microorganisms (Smith *et al.*, 1975). In the studies of the effects of ages and sizes of inoculum for fermentation, it was observed that 72 hr ages and 15% sizes of seed culture were suitable for the fermentation. Six kinds of fermentation media were used for the antibacterial activity of selected fungus KFO-06. According to the results of antibacterial activity, fermentation medium FM-1 was selected for the production of antibacterial metabolite. It was concluded that according to the result, FM-1 was the optimum fermentation medium for fungus KF-06 against *Escherichia coli.* Senthi, 2017 reported that the isolated soil fungi showed maximum antibacterial activity against *Escherichia coli.* It is thus indispensable that optimal effect of fermentation conditions can only produce the best result in metabolite production.

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BOTANICAL AND PHYTOCHEMICAL INVESTIGATION ON THE LEAVES OF *THUNBERGIA LAURUFOLIA* LINDL.

Ye Linn Maung[1]

Abstract

Thunbergia laurifolia Lindl. is known as Nwe-nyo belong to the family Acanthaceae. The plant was collected from Hmawbi Township, Yangon Region, during the flowering and fruiting period from December to May, 2019-2020. In this research, morphological characters of leaves, inflorescence, flower, calyx, corolla, androecium and gynoecium are presented. The plant is perennial herbaceous climber; simple leaves, opposite and distichous; terminal inflorescence with racemose flowers are borne; bisexual, zygomorphic flower. In microscopical characters of leaves, the lamina epidermal cells of anticlinal walls were wavy, both surfaces; stomata were present on lower surface, in this type of diacytic (cross celled) stomata type; in the transverse section of lamina, two layers of palisade mesophyll cell and about 7-10 layers of spongy mesophyll cell were present. In midrib outline, both epidermal cells are barrael-shaped; adaxial side and abaxial side are convex. In petiole outline, both epidermal cells are rounded to barrel-shaped. In the diagnostic characters of leaves powdered samples were observed with sensory characters and microscopical characters. These characters of Nwe-nyo plant were identified by many literatures. Furthermore, the results of phytochemical analysis were various extracts of leaves showed the presence of alkaloid, Glycoside, Phenolic compound, flavonoid, steroid/terpenoid, tannin, saponin, α amino acid, protein, reducing sugar, starch and carbohydrate.

Keywords Botanical investigation of *Thunbergia laurifolia* Lindl. and phytochemical analysis

Introduction

Thunbergia laurifolia Lindl. is a genus of flowering plant of Acanthaceae family, including 200 species and native to tropical region of Africa, Madagascar, Australia and South Africa. *Thunbergia laurifolia* Lindl. species are annual, perennial and shrubs *Thunbergia laurifolia* Lindl. are placed in Acanthaceae for 14 genera are list in Myanmar (Kress,2003). The genus *Thunbergia laurifolia* is made up of about 200 species from warm areas of central and south Africa and Asia (Wagner *et al*., 1999). Flowers of *Thunbergia laurifolia* Lindl. can be divided into 3 types depending on flower color, including while, yellow and purple. The purple type is believed to possess health benefits (Sarawoot Palipoch *et al*., 2013)

The chemical composition of *Thunbergia laurifolia* Lindl., contains number of compounds such as apigenin, coffecic acid, glycosides, flavonoids and phenolic compounds. Many researches have evaluated that these phytochemical substances have the major on diabetes. (Sarawoot palipoch *et al*., 2013 and Piya Kosai *et al*., 2015). phenolic acids such as caffecic acid and gallic acid and alkaloids and flavonoids (Thongsuard *et al*., 2005). The effects of *Thunbergia laurifolia* Lindl., leaves extract significantly decrease the levels of blood glucose.

Thunbergia laurifolia Lindl., is traditional herb in Thailand that has a variety potential benefit such as addiction, detoxification and poisoning, hangovers, liver disease, muscle pain, stomach aches, and ulcers (Govermann *et al*, 2013). *Thunbergia laurifolia* Lindl., is traditionally used for anti-inflammation (Boonyarikpunchai *et al*., 2014), anticancer (Jetawanchaikasem *et al*., 2013). The aqueous leaves extract of *Thunbergia laurifolia* Lindl., showed that contain total phenolic content and total antioxidant capacity (Moe Pwint Phyu *et al*.,2013).

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Materials and Methods

Collection and Identification of *Thunbergia laurifolia* Lindl.

In this research, the species *Thunbergia laurifolia* Lindl. was collected from Hmawbi Township, Yangon Region. They were collected during the flowering and fruiting period from December to May in the year of 2019-2020

For morphological and taxonomical studies, the fresh specimens were identified by using with the help of available literature such Hooker, 1885; Burkill, 1935; Hundley and Chit Ko Ko, 1961; Backer, and Brink, 1963; Lawrence,1969; Dassanayake and Claylon.,1998; Croft, J., Cross, N., Hinchcliffe, S., Lughadha, E.N., Stevens, P.F., West, J.G. and Whitebread, G., 1999; Kress *et al*., W. J, R. A. Defilipps Ellen farr and Yin Yin Kyi., 2003. Herbarium specimens were also prepared and kept in the Botany Department, WYU.

The anatomical characters of fresh leaves were also examined by free hand sections according to the literature of Metcalfe and Chalk (1950) and Trease and Evans (2002). In anatomical studies, chloral hydrate solution was used as cleaning reagent to examine the section cutting. The specimens were recorded with photographs.

Preliminary phytochemical investigation was carried out on the powdered samples of leaves of *Thunbergia laurifolia* Lindl. About 2g of powdered leaves, of *Thunbergia laurifolia* was extracted with 20 ml of methanol on each sample and aqueous solution were subjected for qualitative chemical analysis to detect the presence or absence of alkaloid, glycosides, phenolic compounds, flavonoids, steroids and terpenoids, tannins, saponins, amino acids, protein, reducing sugar, starch and carbohydrates. The experimental procedure was prepared and tested by the methods mentioned in Marni Bettolo *et al*., (1981), Central Council for Research and Unani Medicine, (1987) and Trease and Evans, (2002). The results were shown in Table1.

Result

Morphological Characters of *Thunbergia laurifolia* Lindl., Gard. Chron.260. 1856.

Myanmar Name : Nwe-nyo
English Name : Laurel clock vine,Trumpets vine
Family : Acanthaceae

A perennial herbaceous climber, grow like a twining vine. Leaves usually opposite and distichous pairs along the stem, simple, ovate-oblong and tip acute, up to 1-16 cm long, 2.5 - 5.8 cm broad on leaves. Inflorescence grows on terminal and flowers are borne on hanging racemose, 3 to 23 cm long and 9 to 28 flowers can bear on each peduncle. Flowers are usually opened out into five petals and joined at the base to form tube (yellowish throat and petals are in pale purple color), bisexual, zygomorphic (one of which is longer than the other petals). Calyx 2 lanceolate, tipped with a pointed and it long about 1.2 to 3.9 cm. Corolla-5 lobes, synpetalous, valvate aestivation, begin as approximately 1.8 to 2.3 cm long and 1.5 to 2.5 cm wide. Androecium-stamen 4, didynamous, epipatelous, dithecous, stout, basifixed, longitudinal dehiscence, very hairy on anther and long from 1.2 to 1.5 cm. Gynoecium oval shaped, mostly 3-4 cm long, bicarpellary, axile placentation (disc present), style long about 3 cm and curve at tip, stigmas are flattened and folded form. Fruits elliptical shape or bird’s beak and long about 3.5 cm, consists of two to four seeds in each capsule.

Flowering and fruiting period : Throughout the year
Part Used : Leaves and stem
Uses : The drop of juice leaves is put into ear to treat deafness and it is applied as a poultice on cuts and boils. The leaves extract of this plant is reported as an antidote against poisonous and anti-inflammatory activity.
Specimen examined : Ye' Linn Maung, (2) Quarter, Hmawbi Township, Yangon Region, 17˚ 06' 08" North Latitude, 96˚ 03' 04" East longitude, 22.8 km.

Morphological Characters of *Thunbergia laurifolia* Lindl.

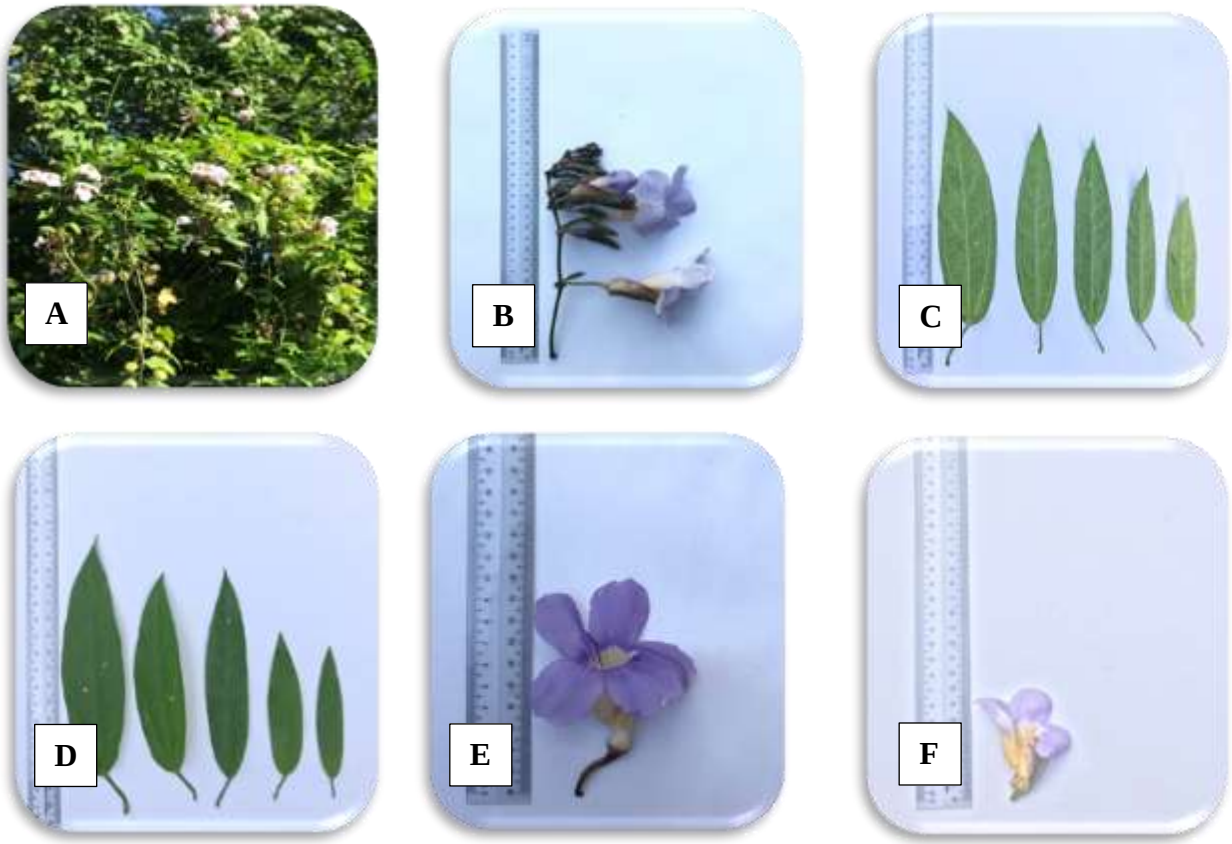

Figure 1 A. Plant in natural habit B. Inflorescence with floral buds C. Leaves (ventral view) D. Leaves (Dorsal view) E. Flower F. L.S of flower

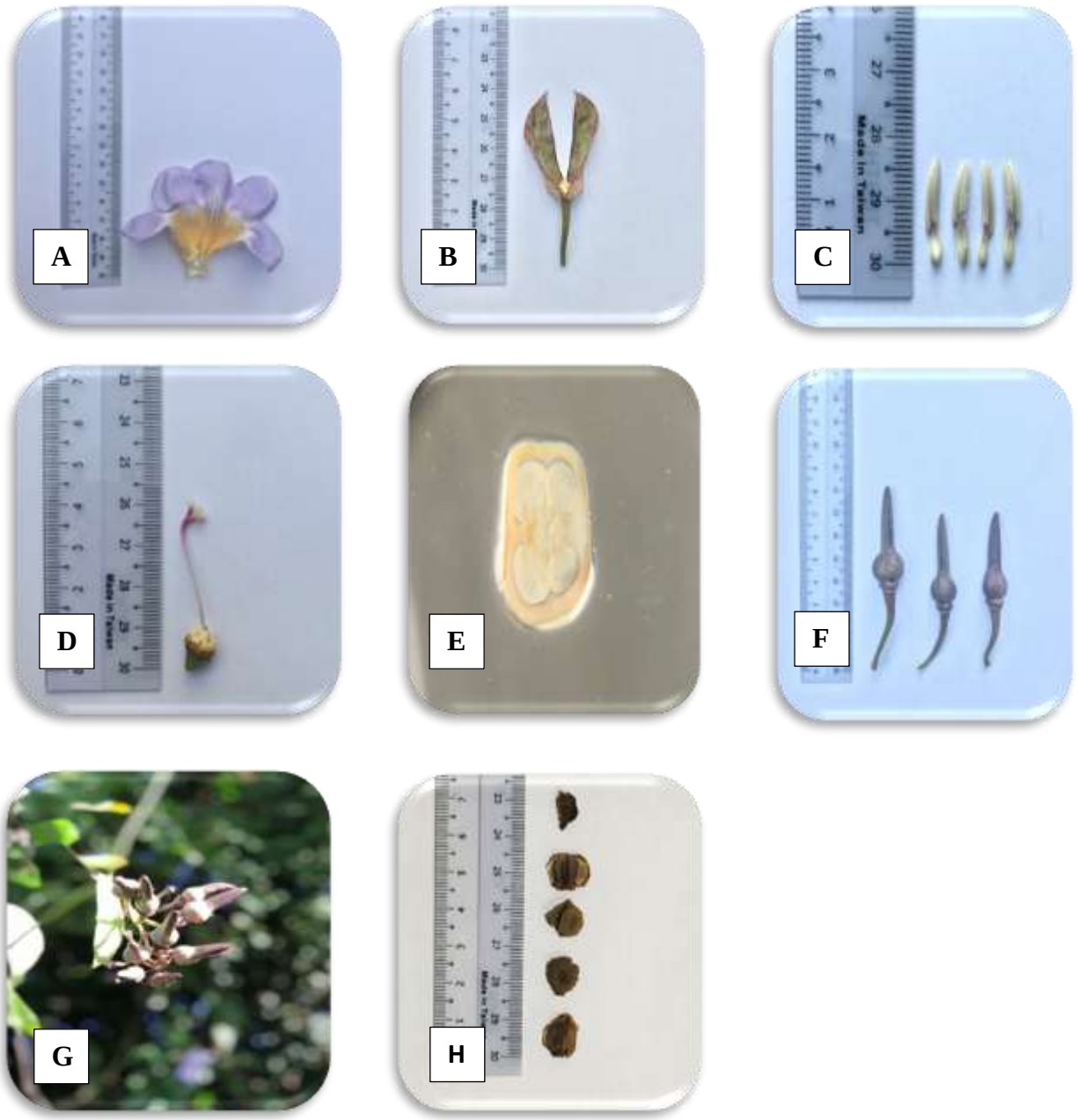

Figure (2) A. Corolla B. Calyx C. Stamens and filament
D. Ovary with disk E. T.S of ovary F. Various sizes of fruits
G. Fruits H. Various sizes of Seeds

Microscopical characters of *Thunbergia laurifolia* Lindl.

Lamina

The lamina of *Thunbergia laurifolia* Lindl. studied is dorsiventral.

In surface view, both upper and lower epidermal cells one-layered of parenchymatous cell walls straight. Stomata present on lower surface, diacytic type. There is no stoma on upper surface.

In transverse section, epidermis one-layered on both sides, compactly barrel-shaped, outer and inner wall straight, thin, smooth, cuticle present on both surface. Epidermis one-layered on both sides, compactly barrel-shaped, outer and inner wall straight. The mesophyll composed of palisade parenchyma at adaxial side and spongy parenchyma at abaxial side. The palisade mesophyll 2 layered, cell vertically elongated, compactly arranged numerous chloroplasts present. Spongy mesophyll 2 layered, irregular to isodiametric in shape, loosely arranged, intercellular spaces large. In mesophyll cells small vascular bundles embedded, rounded. It is not well developed.

Midrib

In surface view, epidermal cells parenchymatous, rectangular walled, cell walls thin and straight, stomata absent.

In transverse section, the adaxial side and the abaxial side convex. The epidermis one-layered, cells collenchymatous, barrel-shaped or rounded. The adaxial collenchymatous cell 5 to 7 layered adaxial, 3 to 12 layered at the abaxial side, oval and polygonal in shape, thicked at the corner. Parenchymatous cells and endodermis are above the vascular bundle. Vascular bundle is collateral type and crescent-shaped Trace of small bundle are present.

Petiole

In surface view, the epidermal cells were thin-walled parenchymatous cells, irregular polygonal in shape. Stomata are present in petiole.

In transverse section, petiole semi-circular in outline with concave surface at the upper side with two wings and convex at the lower side. The cuticle layer was thin. The epidermis one-layered, the cell parenchymatous barrel-shaped.The collenchymatous cell 5 to 12 layered, supporting tissue, the layer at the adaxial and abaxial sides, intercellular spaces present. The sclerenchymatous cells one-layered, the cell barrel-shaped above the vascular bundle. The vascular buldles were collateral type and a large vascular cylinder surrounds the parenchymatous pith, where both the xylem and phloem are discontinuous.

Diagnostic characters of dried leaves from *Thunbergia laurifolia* Lindl.

The fragments of the leaves composed of fragmented reticulate vessel, spiral vessel, tracheids, trichome, pitted vessel and fragment of epidermal cell.

Microscopical characters of *Thunbergia laurifolia* Lindl.

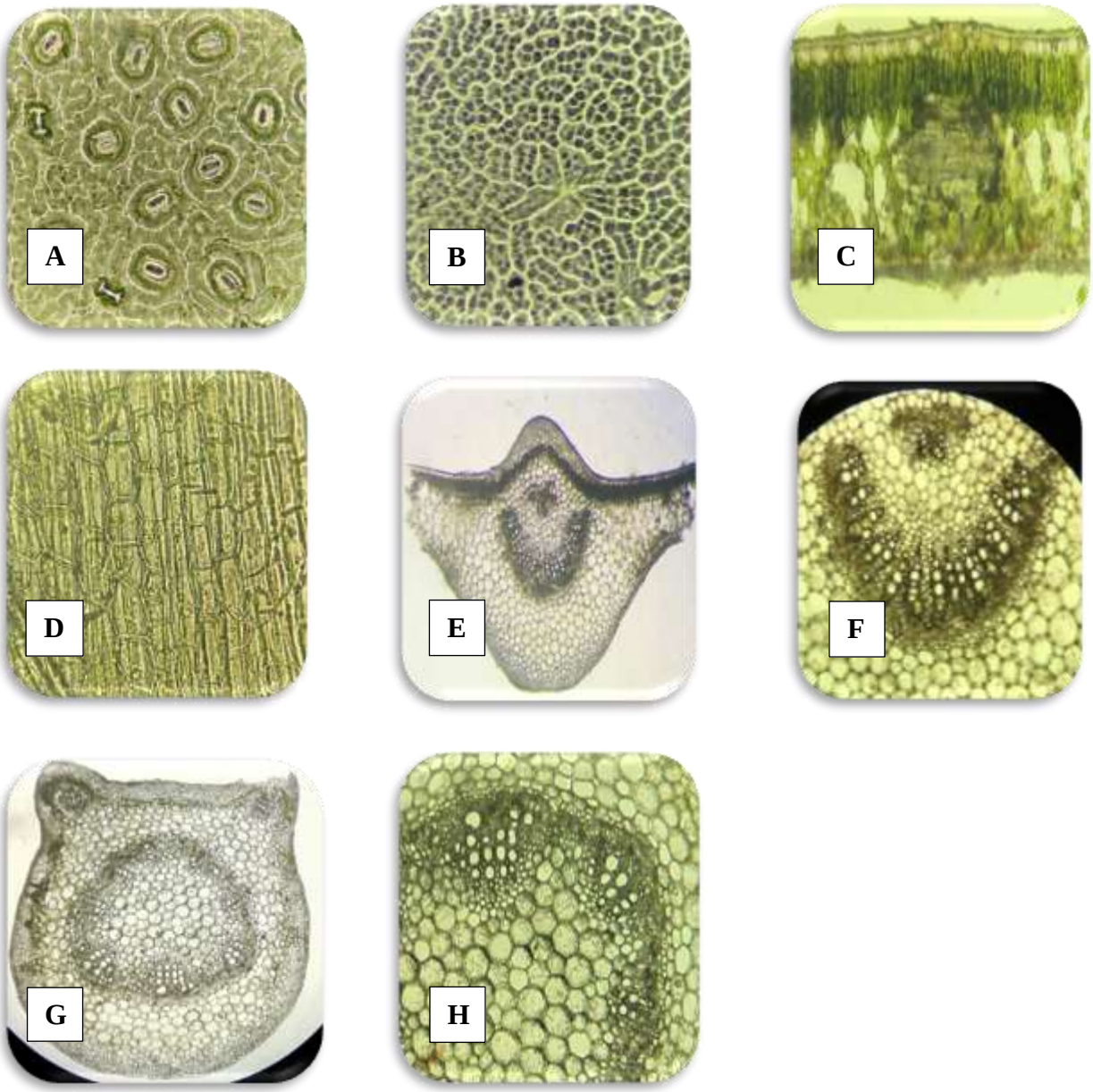

Figure (3)
A. Surface view of lower epidermis with stomata (400X)
B. Surface view of upper epidermal cells (400X)
C. T.S of lamina showing palisade and spongy mesophyll (400X)
D. Surface view of midrib showing epidermal cell and trichome (400X)
E. T.S of midrib showing collenchyma, parenchyma cells and vascular bundle (100X)
F. T.S of midrib showing vascular bundle (400X)
G. T.S of petiole showing collenchyma, parenchyma and vascular bundles (100X)
H. T.S of petiole showing close up view of vascular bundle (400X)

Diagnostic characters of powder samples of leaves, stems and roots of *Thunbergia laurifolia* Lindl.

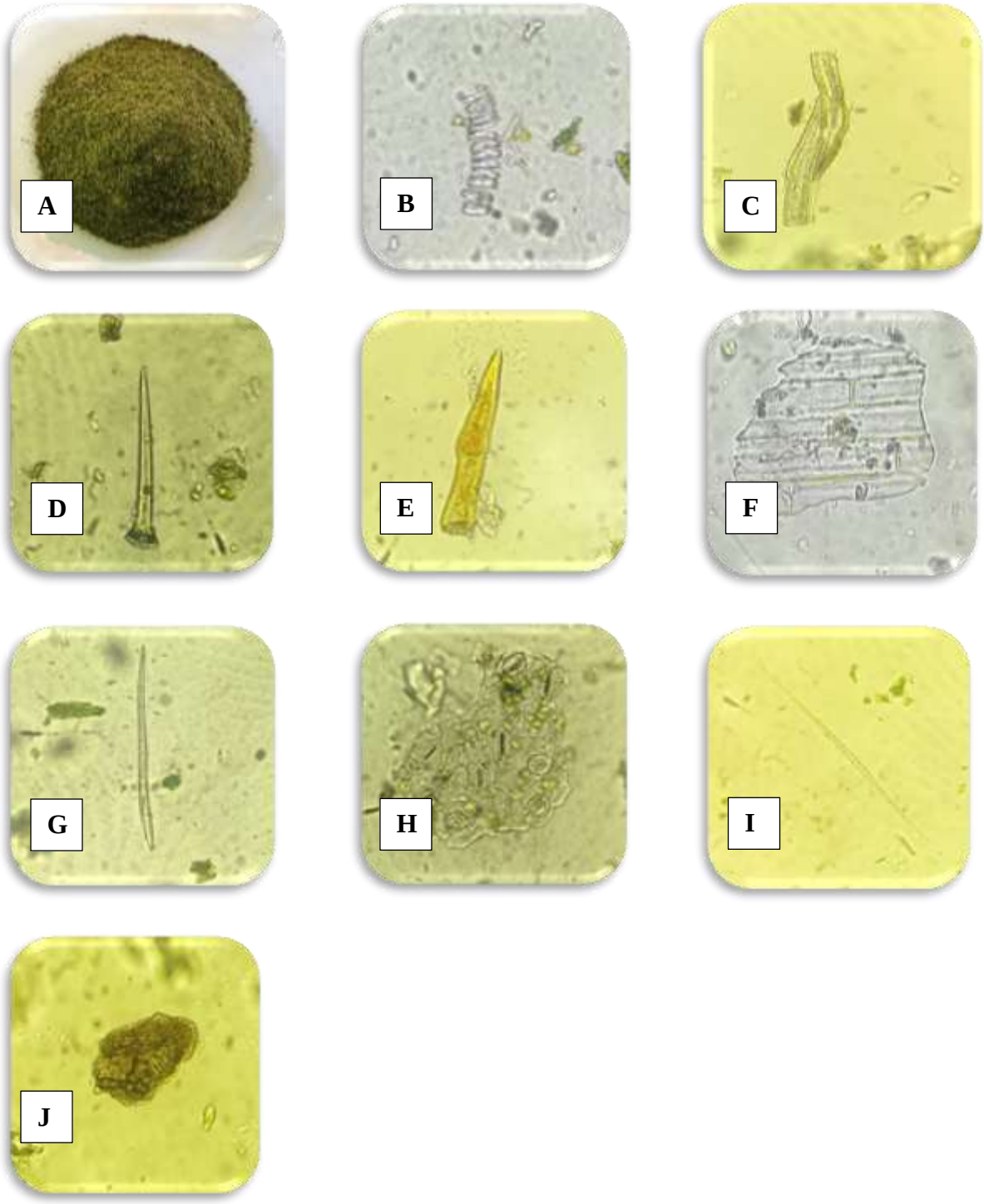

Figure (4) Diagnostic characters of powder samples of leaves

A. Powdered leaves. B. Spiral vessel. (400X)
C. Reticulate vessel element. (400X) D. Unicellular trichome. (400X)
E. Multicellular trichome. (400X) F. Fragment of epidermal cell. (400X)
G. Tracheid (400X) H. Fragment of stomata (400X)
I. Fibre (400X) J. Stone cell

Phytochemical Investigation of leaves of *Thunbergia laurifolia* Lindl.

Preliminary phytochemical tests were carried out on the powdered samples of leaves, of plant ***Thunbergia laurifolia*** Lindl., Nwe-nyo, to determine the presence and absence of chemical compounds, according to standard methods of Central council for research in Unani Medicine (1987) and Trease & Evans (2002).

Preliminary phytochemical tests of leaves indicated that alkaloids, glycosides, phenolic compounds, flavonoids, steroids and terpenoids, tannis, saponin, α-amino acids, proteins, reducing sugar, starch and carbohydrates were present. The result of phytochemical test of leaves were shown in Table 1.

Table 1 Phytochemical Investigation of the leaves *Thunbergia laurifolia* Lindl.

No.	Test	Solvent Extract	Test Reagents	Leaves	
				Observation	Result
1.	Alkaloids	1%HCl	Mayer's reagent	White ppt	+
			Wagner's reagent	White ppt.	+
			Hager's reagent	Yellowish green	+
2.	Glycosides	MeOH	1ml H2O + NaOH	Yellow green	+
3.	Phenolic compound	MeOH	2ml H2O + 10% FeCl3	Blackish	+++
4.	Flavonoids	MeOH	Mg coil + HCl (dil) H2SO4	Crimson	+
5.	Steroids/ Terpenoids	MeOH	CHCl3+ H2SO4(conc)	Green/Reddish brown	+
6.	Tannins	Water	5%FeCl3+ H2SO4(dil)	Yellow brown ppt.	+
7.	Saponin	Water	Shaken with 2ml H2O	Forthing	+
8.	α-amino acids	Water	Ninhydrin reagent	Purple spot	+
9.	Proteins	Water	Millon's ragent (Heated)	Purple ppt.	++
10.	Reducing sugar	Water	1ml H2O and mixture equal part fehling's A and B	Brick red ppt.(Heated)	++
11.	Starch	Water	Iodine	Brown ppt.	+
12.	Carbohydrates	Water	5%α- napthol sol; + H2SO4	Purple ring	++

+ = Present - = Absent ppt. = precipitate

Discussion and Conclusion

In the present study, the species ***Thunbergia laurifolia*** Lindl., is belong to the family Acanthaceae carried out according to Lawrence, (1969), Hundley and Chit Ko Ko (1969).***Thunbergia laurifolia*** is native to India, Burma, and Malaysia. In Myanmar, this plant is known as New-nyo or Kyi-Kan-Hnok-Thi. It is widely distributed, commonly grows in Kachin State, Mandalay and Yangon Region or planted throughout Myanmar. In Myanmar, the plant of ***Thunbergia laurifolia*** Lindl. haven't still been traditionally used for the treatment of disease. In this paper, the collected specimens are identified by using available literature.

In morphological study, these plants are perennial, herbaceous climber and it grows. Leaves are opposite ovate-oblong shaped and tip acute. It grows up to 8 - 16 cm long, 2.5 - 5.8 cm broad on leaves. These characters were in aggrement with those stated by Backer (1963) and Sarawoot (2013).

Inflorescence are on terminal peduncle and flowers are borne on hanging. It can grow from 3 - 23 cm long and 9 - 28 flowers can bear on each infloresence.Flowers are bisexual, zygomorphic and it opens out into five petals and joined at the base to form tube (yellowish throat and petals are in pale purple colour. Calyx are 2, lanceolate, tipped with a pointed and it persistent till long after the fall at the corolla. Corolla are five lobes, synpetalous and valvate aestivation form. Stamens usually 4 and didynamous or rarely 5, epipetalous dithecous, basifixed, longitudinal dehiscence. Ovary oval-shaped, mostly 3 - 4 cm long, bicarpellary, axile placentation (disc present). Styles are long about 3 cm and curved at tip and stigma are flattened and folded form fruit it grows like elliptical shaped or large capsules and consist two to four seeds in each capsule. Seeds flat and winged. These characters are in aggrement with those mentioned by Hooker (1885), Lawrence (1964), Dassanayake (1981) and Flora of Hong Kong (2009).

In microscopical study, the leaf is usually dorsiventral and presence of simple unicellular and multicellular trichomes. Stomata is diacytic type. Leaf is dorsiventral and the mesophyll layer consisting of 2 cell layers thick of regularly-shaped palisade parenchyma and two layer of spongy parenchyma reaching downward to the abaxial epidermis. Lower epidermis consists of one cell layers that covered by cutin, but less than that of upper epidermis and the amount of diacytic stoma in this part are more than that of upper epidermis which are agreed with Jackson (1990), Somporn Putiyanan *et al* (2008) and Cassandra (2010)

In the phytochemical investigation, the species of ***Thunbergia laurifolia*** **Lindl.** of leaves are showed that that the presence of flavonoids, alkaloids, tannins, steroid/terpenoid, saponins, glycosides, carbohydrates, protein, α-amino acids, proteins and phenolic compounds. Among them, abundant phenolic compound compound is observed in leaves. These characters are in agreement with those whose mentioned by Somporn Putiyanan *et al*., (2008) and Moe Pwint Phyu *et al.,* (2013).

In conclusion, the preliminary phytochemical test of leaves extracts showed that the presence of flavonoids, alkaloids, tannins, steroid/terpenoid, saponins, glycosides, carbohydrates, protein, α-amino acid, reducing sugar and phenolic compounds. Among them, phenolic compound presented abundantly in leaves, it plays an essential role not only in the treatment of diseases like diabetes but also in the commercial products as herbal tea. Then, to find out more active components of medicinal values from the leaves of ***Thunbergia laurifolia*** Lindl., it needs further studies for physicochemical properties and antimicrobial activity tests of the leaves.

Acknowledgements

Firstly, I sincerely thank to Dr. Ko Tin, Professor and Head, Department of Botany, West Yangon University, for his assistance and kindly letting me to do this research and for his precious advice. Secondly, I would like to thank to Dr, Yadana, Professor, Department of Botany, West Yangon University, for her generous support. Last but not least, I am exceedingly grateful to my colleagues who gave me encouragement.

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SIGNIFICANT DIFFERENCES MORPHOLOGICAL CHARACTERS OF POACEAE AND CYPERACEAE FAMILIES

Lwin Mar Saing[1]

Abstract

The grass family Poaceae and Sedges family Cyperaceae are belonging to order Poales. (APG IV, 2016) and very similar in features. But these two families are so differing from each other by individual morphological characters. Grass belongs to family Poaceae (Gramineae) and the largest family in monocotyledonous gramineous flowering plants. The name of family Poaceae is derived from types genus *Poa* L. Family Cyperaceae is the third largest family of monocotyledonous gramineous flowering plants and type genus is *Cyperus.* Grass taxonomy is very different and difficult from other flowering plants. Grass floret is spikelet and individual spikelet possess each substances bract (glumes, lodicule) and various modified inflorescences types and underground organs. These reproductive and vegetative morphological characters of Poaceae is more advance evolution trends features than Cyperaceae. This feature agreed in evolutionary trends of morphological characters and APG IV 2016 system. Family Cyperaceae is one spiculose flower and these organized into spikelet (glumes, perianth). Spikelet further arranged into higher order spicate, paniculate or umbellate inflorescence (anthela). Flowers of both families may be perfect or imperfect, usually monoecious. In this research highlights the quite different characters of very similar morphological unit features of grass and sedges and then verify primitive and advance comparison characters between Poaceae and Cyperaceae with systematics science record in region, Myanmar.

Keywords Taxonomy, differences, grass, sedges, morphology, evolution

Introduction

The English word “grass” probably come from the Old Height German word grass (Bor 1960). Grass family Poaceae is widely adaptation in various habitats. Grasses are usually herbaceous, which indicates that they produce a seed, do not develop woody tissue, and die down at the end of a growing season. They are annual or perennial and monocotyledonous leaf sprouts from the seed fruit called grain (caryopsis) which feeds much of the world and they often have jointed, slender, sheathing (wrapping) leaves on alternately arranged in the stem(culm). Grasses can be large, giant grass bamboo, sugarcane, corn, or small like annual bluegrass. Though the grain is valued by humans, grasses have green leaves and stems not digestible for humans that are the main food source for animals. Some grain is fed to livestock but the leaves and stems are the mainstay of animals feed and can be used for building materials, medicines, and biomass fuels. The word Cyperus derived from the Greek word “Kyperas”. English name “Sedge” is “Sword-man ‘'with linear leaves and parallel venation, Stebbins (1956). Cyperaceae is a largely widespread and most suited to damp habitats with a preference for waterlogged often acids soil rich in humus. Cyperaceae grow in wetland, artificial marshes or swamps created for anthropogenic discharge such as wastewater, strong water ran off of sewage treatment in various parts of the world and it serve ecosystem services especially they can play in a particular role in the maintaining and improvement of water quality, Baker (1965). Cyperaceae leaves are three - ranked, meaning that, they arise as through off the points of tringle’s three-ranked condition. It is partially obvious in three- way sedges and their seed are achene. The study species were collected in some area of Kachin State, especially the Mohnyin University Campus and Mohnyin Distinct during vegetative and reproductive time from 2021, December to 2022, May. The survey area located in Group of Ywa-tthit-kone village, Mohnyin Township, Mohnyin Distinct in Kachin State, Myanmar. It is situated in 24° 47' 03" N and 96° 23' 21"E and above the

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elevation nearly about 6000 feet. The morphological characters of grasses comprise vegetative organs of culm (stem), underground organs, nodes, branching and prophyllum, leaves (sheath; blade, ligule) and reproductive organs of (inflorescences; spike, raceme, panicle; true panicle, false panicle, pseudospike and flower or spikelet (glumes, lemma and palea, lodicule, awned), seed (caryopsis). Family Poaceae are divided into 5 Subfamilies, Bambusoideae, Oryzoideae, Pooideae, Chloridoideae and Panicoideae based on the morphological characters of spikelet (flowers) and vegetative structure (Hafliger and Scholz's1981).

Genus Meloncanna in tribe Bambuceae of Sub-family Bambusoideae, genus Oryza in tribe Oryzeae of Sub-family Oryzoideae, genus Arundinella in tribe Arundinellae of Sub-family Pooideae, genus Chloris in tribe Chlorideae of Sub- family Chloridoideae, genus Pennisetum in tribe Paniceae of Sub-family Panicoideae in family Poaceae were recorded. The habit, leaves position, inflorescence variation types and more complexity spikelet structures are advance morphological characters of Poaceae than Cyperaceae in evolutionary trends. In Cyperaceae, vegetative organs; culm (stem), underground organs, leaves(trifoliate) involucre bract, reproductive organ; inflorescence (anthela) (Corymb, capitulum and spike), flower (spikelet, glume and perianth, seed (achene) are unit characters. Family Cyperaceae divided into 4 Sub-families are Sub-family Cyperoideae, Scripoideae, Rhynchoporoideae and Caricoideae (Halfliger and Scholz's1981). Genera of Actinoscripus, Pycerus Eleocharis, Lipocarpha in tribe Cypereae of Sub-family Cyperoideae and genus Scleria in tribe Sclerieae of Sub-family Caricoideae. This research highlights distinct differences morphological characters and evolutionary trends of family Poaceae and Cyperaceae under Order Poales in graminoids clade.

Materials and Methods

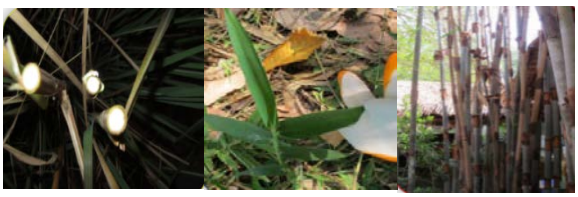
Collection Procedure and Classification, Identification, Verification and Evolutionary Trends

Specimens were collected from some areas of Mohnyin University Campus and Mohnyin Distinct during from 2021, December to 2022, May. The morphological characters of grass and sedges were classified according to Hafliger and Scholz's classification (1981) that based upon the morphological characters. The identification, verification and evolutionary trends were done by using keys, principles of many author citations; Rhind, 1945; Stebbins, 1956; Bor, 1960; Dassanayake et.al, 1968; Hafliger and schalz, 1981; Hundley and Chit Ko Ko, 1987; Willis, 2002, APG IV,2016.

Results

Morphology of Grass and Sedges

Culm Structure of Grass



Culm Structure of Sedges

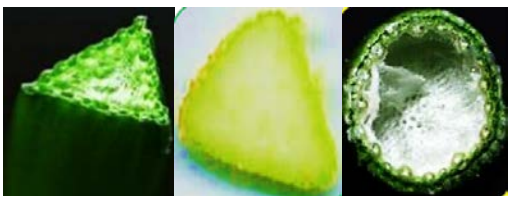


Figure (1) Terete to flattered and giant culm in Poaceae Triangular to terete culm in Cyperaceae

Structure of Grass Spikelet

The spikelet is the unit of inflorescences. It can be differentiated into 1 to 2 flowered and 1 to many flowered spikelet with articulation. The spikelet may be terete, flattened, gibbus and rounded shape. Spikelet comprises glumes. The basally outer 2 glumes are lower empty glume and upper empty glume. The flowering glumes; outer lemma and inner palea arrange the above of empty glumes and this joint portion called rachilla. All glumes may be various modified characters etc. texture, nerved, silky hairs, bristles and awns.

Figure (2) Habits

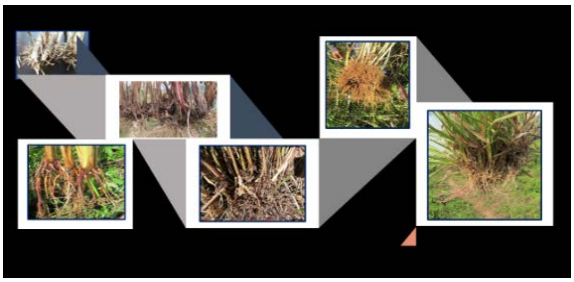


Figure (3) Various Underground Parts

Figure (4) Inflorescences, Spikelet

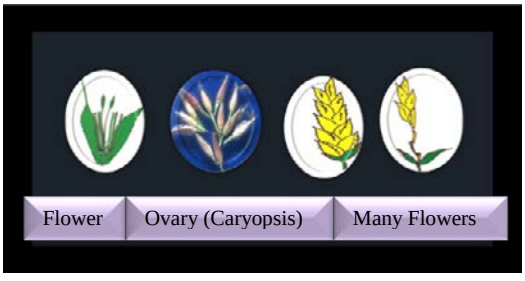


Figure (5)

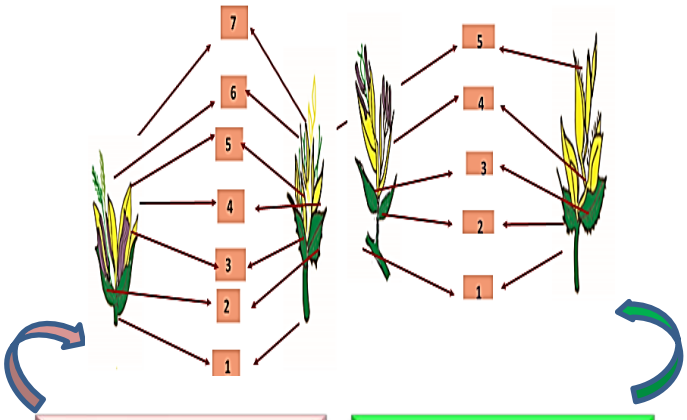


Figure (6) 2-flower spikelet,
1. Pedicel, 2. Lower glume, 3. Upper glume, 4. Lower lemma, 5. Lower palea, 6. Upper lemma, 7. Upper palea

More than 2 flower spikelet
1. pedicel, 2. Lower glume, 3. Upper glume, 4. Lowest lemma, 5. Upper most lemma

Spikelet Structure of Sedges

Cyperaceae spikelet (flowers) are protected by only one empty glume sometime two empty glumes and one flowering glume (sometime present perianth (scales or bristles). In multi-floral spikelet, these glumes may be two - ranked or spirally arranged, depend on the species. The spikelet may be either flattened or circular in crossing - section. In the primitive form, the spikelet axis is not articulated; the glumes are shed in an ascending sequence. In the advanced form the spikelet axis in articulated below each flower or glumes are united to the spikelet axis.

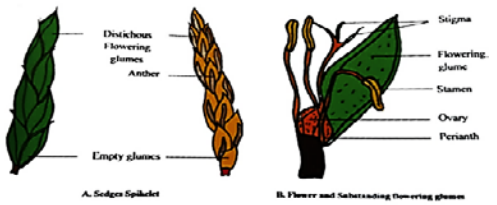


Figure (7) structure of sedges spikelet

Figure (8) Various Habits

Figure (9) Various Underground Parts

Figure (10) Inflorescences, Spikelet

Figure (11) Ovary (Achene)

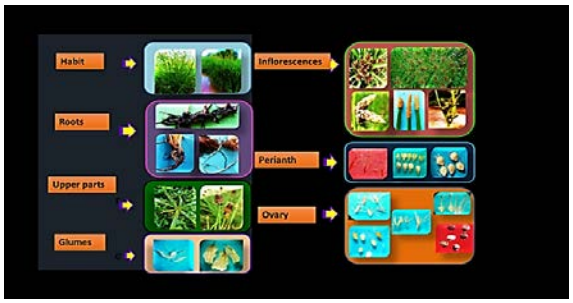


Figure (12) Sedges Morphological Characters

1. Family - Poaceae (Gramineae)
   Scientific Name - *Meloncanna arundina* Darkison (Tribe Bambuseae)
   Sub-family - Bambusoideae
   Myanmar Name - Kayan -wa- gale, Ta- been- daing- gale

Figure (13) 1. Habit, 2. Foliage leaf and branching, 3. Culm sheath; ligule and culm blade

An evergreen tufted moderate sized bamboo, singly from the rhizomes at distance, culm yellowish-green, culm surface puberulous. Culm up to 5 m high, 2.0 - 5.0 cm in diameter, wall up to 2.0 mm thick. Culm-sheath yellowish-green, shining cylindric, rounded and inflated, culm blade subulate acuminate, numerous branches at each node. Foliage leaf auricles with long hairs, branching is densely equally branchlets from node.

2.Family - Poaceae (Gramineae)
Sub-family - Oryzoideae (Tribe Oryzeae)
Scientific Name - *Oryza sativa* L.
Myanmar Name - Sa-ba

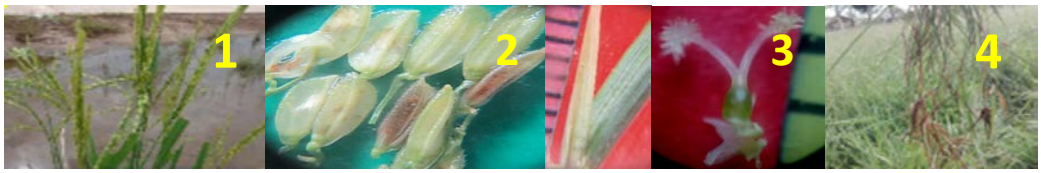


Figure (14) 1. Habit,2. Spikelet, 3. Ligule, 4. Ovary, Roots

Annual. Culm 40-50 cm high, terete. Leaf-sheath lanceolate; ligule membranous, acute, leaf blade linear. Inflorescence opens panicle, rachis scabrous joint of rachis and pedicel silky hairs. Spikelet solitary, lanceolate-oblong, light green to yellowish green, 7.0-8.0 x 2.0-2.5 mm, laterally compressed, pedicel 1.5-3.5 mm long, scabrous, rachilla disarticulating below the glume,1-flowered, bisexual, mature floret with 1-grain. Lower glume narrow-elliptic, Upper glume narrow elliptic. Lemma lanceolate-oblong, chartaceous, margins revolute. Palea lanceolate-oblong, chartaceous, cuspidate at the apex, margins revolute; lodicule 2; stamens 6, oblong, filament slender, ovary ovoid, style 2, stigma 2, plumose.

3.Family - Poaceae (Gramineae)
Sub-family - Oryzoideae (Tribe Oryzeae)
Scientific Name - *Oryza grandiglumis* (Doell) Prodhl.
Myanmar Name - Sa-ba

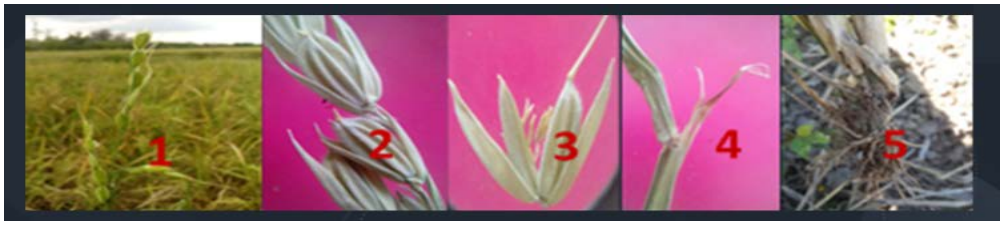


Figure (15) 1. Habit, 2, 3. Spikelet,4. Ligule, 5. Roots

Annual. Culm 60-70 cm high, terete, fibrous roots. Leaf sheath lanceolate, ligule membranous, leaf blade linear. Inflorescence opens panicle, peduncle straight, rachis scabrous, joint of rachis and pedicel with distinct hairs. Spikelet solitary, lanceolate-oblong, light green to yellowish green, 7-10 mm x 3.5- 4.5 mm, laterally compressed, acuminate at the apex, awned, persistent, pedicellate, pedicel 4-7 mm long, scabrous, rachilla disarticulating below the glume, 1-flowered, bisexual, glume equal, as long as the lemma and palea of same texture. Lower glume linear-lanceolate, 8.0-9.0, margins revolute. Upper glume linear-lanceolate, 8.0-9.0 x 2.0-2.2 mm, chartaceous. Lemma ovate-lanceolate, 7.0-7.5 x 4.0-5.0 mm, awn 2.5-3.5 cm long, scabrous, whitish, base of awn with dense hairs, 5-nerved, both margins and back scaberulous, margins revolute. Palea ovate-lanceolate, margins revolute; lodicule 2, distinct; stamens 6, oblong, filament slender, ovary ellipsoid, style 2, stigma 2, plumose.

4.Family - Poaceae (Gramineae)
Sub-family - Pooideae (Tribe Arundinellae)
Scientific Name - *Arundinella pumila* (Hochst) Steut
Myanmar Name - Kaing

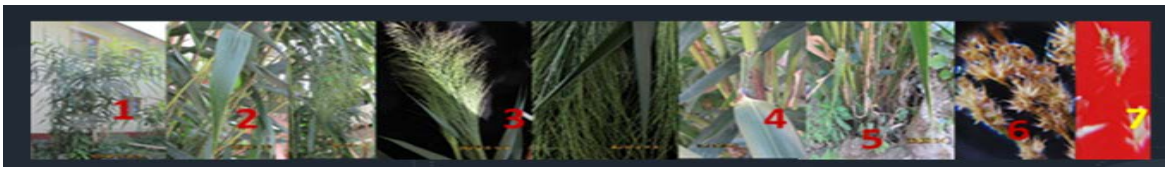


Figure (16) 1. Habit, 2. Leaf blades, 3. Inflorescences, 4. Rhizome, 6. Spikelet, 7.

Strongly reed like tall culm, rhizome system, inflorescences very large open plumose panicle, spikelet cluster, very small, bristle numerous, florets 4 - 6, lowest to middle floret perfect, the upper most neuter, inflorescences are used for cleaning material.

5.Family - Poaceae (Gramineae)
Sub-family - Chlorodoideae (Tribe Chlorideae)
Scientific Name - *Chloris Pilosa* Schmach
Myanmar Name - Unknown

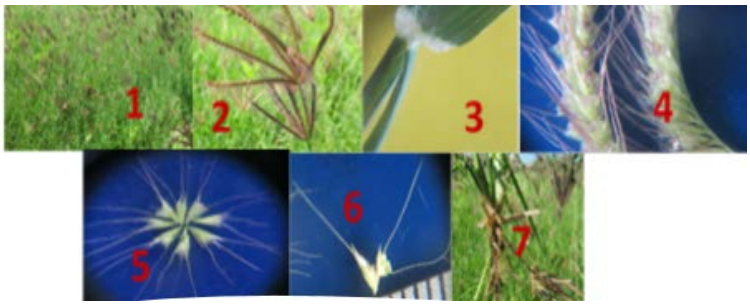


Figure (17) 1. Habit, 2. Inflorescence, 3. Ligule, 4. Spike, 5. Spikelet, 6. Floret, 7. Roots

Perennial. Culm 60.0-70.0 cm high, scabrous. Leaf sheath scabrous on the margins, ligule membranous fringe, leaf blade linear. Inflorescence spike-liked raceme, Spikelet paired or tried, 6-flowered, 3-awned. Lower glume narrowly linear. Upper glume narrowly linear, lower lemma ovate, awned, 3-nerved, awn 5.1 mm long, hair on the apex, light green color; lower palea ovate, 1.0-1.2 x 0.7-0.9 mm, membranous, acute at the apex, nerve less, upper floret ovate, awned, awn 5.1 mm long, 3-nerved; upper palea, awned, awn 4.8-5.5 mm long, ovary ovoid, 0.5 mm long, style 2, 0.5 mm long, stigma 2, 1.0 mm long, brown color, plumose.

6.Family - Poaceae (Gramineae)
Sub-family - Panicoideae (Tribe Paniceae)
Scientific Name - *Pennisetum parpareum* L.
Myanmar Name - Myet -Ya

Figure (18) 1. Habit, 2. Inflorescence, 3. Ligule, 4. Spikelet, 5. Floret

Perennial, erect, tufted. Culm 60-120 cm high, Leaf-sheath glabrous; ligule hairy ring; leaf blade linear-lanceolate, Inflorescences spike-like dense cylindrical or false panicle, peduncle and rachis scabrous. Spikelet paired; one sessile and one pedicellate, spikelet subtended by involucres bristles, spikelet falling with the involucres. Sessile spikelet linear-lanceolate, 2-flowered. Lower floret staminate and upper bisexual, awn less. Pedicellate spikelet linear-lanceolate, 2-flowered. Both florets staminate.

7.Family	-	Cyperaceae
Sub-family	-	Cyperoideae (tribe Cypereae)
Scientific Name	-	*Actinoscripus grossus* (L.F.) Goetah & D.A.Simpson
Myanmar Name	-	unknown

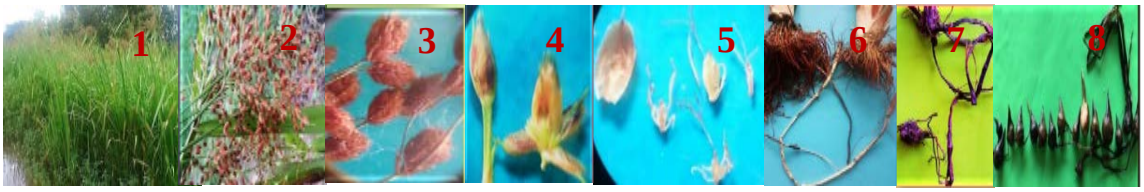


Figure (19) 1. Habit, 2. Inflorescences, 3. Spikelet, 4. Floret,. 5. Flowering glume, 6. Ovary with perianth, 7, 8. Stoloniferous with bulb

Herbs, perennial, rhizomes with stolon long and slender ending in small tubers(bulb), aquatic. Culm about 300 cm in length, compressed trigonous, erect, cespitose, scabrous, greyish green, culm base bulbous and reddish brown near to rhizome. Leaves 3 - 4; leaf sheath 3 - 5 cm long, reddish brown in color, spongy, glabrous, ligulate; leaf blade flat, margins scabrous, recurved. Involucre bracts 4 - 6, linear, flat, margin scabrous on the upper part, surfaces glabrous, yellowish green spreading. Inflorescence terminal large, corymbiform, decompound anthela; many rays; spike ovoid to globose 20 - 30 spikelet; spikelet ovoid to globose, terete, many flowered, reddish brown. Flower bisexual, acropetally deciduous, arranged in spiral. Empty glume absent. Flowering glume 1, ovate to broadly ovate, boat shaped. Perianth 3. Stamens 3; filaments slender; anthers connective present beyond anthers., apiculate, verrucose, pale yellow; style about 1.0 mm long, slender, glabrous, caducous, style and ovary flattened; obovoid to sub-ellipsoid, compressed trigonous, cuneate at the base not dilated, persistent, stigma 3.

8.Family	-	Cyperaceae
Sub-family	-	Cyperoideae (tribe Cypereae)
Scientific Name	-	*Eleocharis acutangular* (Roxb.) Schult
Myanmar Name	-	unknown

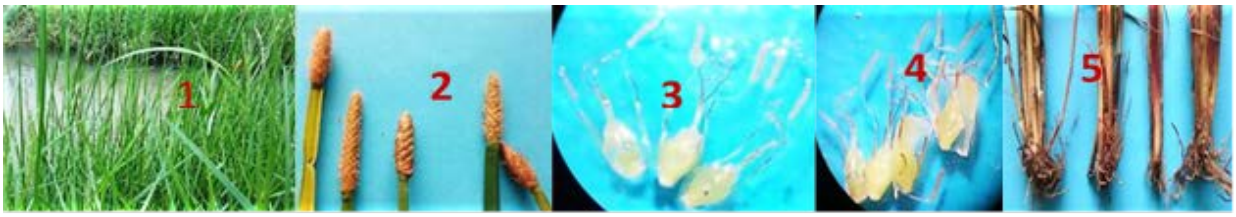


Figure (20) 1. Habit, 2. Inflorescence, 3. Ovary with perianth, 4. Flowering glumes, 5. Roots

Herbs, perennial, fibrous roots, aquatic. Culm 40 - 75 cm in length, narrowly trigonous, erect, stout, spongy, smooth, light-green. Leaves 2 - 3, leaf sheath 5.0 - 10.0 cm in length, membranous, lower portion of basal sheath brownish, leaf blade is minute, acute at the apex, membranous, pale green. Involucre bract absent. Inflorescence cylindrical terminal spike, broader than the culm, without ray or ray let; spike single globose-oblong; spikelet terete, apex acuminate, with many flowered. Flower bisexual, loosely imbricate, closely appressed to main

axis, acropetally deciduous. Perianth absent. Empty glume absent. Flowering glume broadly ovate, strongly persistent. Stamens 2, filaments later produced perianth appendage; anthers, linear, shortly apiculate, ovary, broadly obovoid, turgidly and biconvex, glabrous but shallowly pitted in longitudinal rows, pale yellow; style about 0.8 mm long, flat, style base subulate deltoids, reddish brown, persistent; stigma 3, recurved.

9.Family - Cyperaceae
Sub-family - Cyperoideae (tribe Cypereae)
Scientific Name - *Pycerus flavidus* (Rertzius) T.Koyama
Myanmar Name - unknown

Figure (21) 1. Habit, 2. Inflorescence, 3. Spikelet, 4. Floret, 5. Ovary with perianths, 6. Stoloniferous roots

Herbs, annual, stoloniferous roots. Leaves 3-5, leaf sheath about 3.0 cm long, membranous, reddish brown, glabrous; leaf blade, linear, flat, margins scabrous, surfaces glabrous, apex acute, recurved, brightly green. Involucre bract 2-4, linear, erect, rigid spreading, yellowish green, margin scabrous on the upper part, surfaces glabrous. Inflorescence simple or compound congested anthela; 7 - 13 rays; congested into a single or head like cluster of spikes; spikelet spreading, and forming a globose spike, each with 8 -14 flowered, greenish yellow. Flower bisexual, acropetally deciduous, distichous. Empty glume1.Flowering glum 1, broadly ovate. Perianth 2 – 3, slightly flat. Stamens 2; filaments minute, later produced perianth appendages; anthers about 1.0 mm long, yellow, connective present beyond anthers. Ovary about 1.0 x 1.0 mm, ob.-ovoid, biconvex, slightly compressed, minutely apiculate, cuneate at the base, distinctly verrucose; style about 1.0 mm long, slender, glabrous, style base not dilated, persistent, pale brown; stigma 2.

10. Family - Cyperaceae
Sub-family - Cyperoideae (tribe Cypereae)
Scientific Name - *Lipocarpha chinensis* Osbeck
Myanmar Name - unknown

Figure (22) 1. Habit, 2. Inflorescence, 3. Spike, 4. Spikelet, 5. Ovary, 6. Fibrous roots

Herbs, annual, fibrous roots, aquatic. Culm about 27.0 - 30.0 cm in length, angular, concave both sides, erect, slender, cespitose, smooth, greenish, glabrous. Leaves 5-7; sheath closely compressed to the culm, greenish -brown; ligule membranous, Involucre bract 6, very narrowly linear, Inflorescences compound very narrowly spike anthela, broader than the culm. Spike densely; each spike with 2-5 spikelet, ellipsoid, spikelet terete, 11- flowers. Flower bisexual, spirally arranged, empty glume 3, lanceolate, middle nerved to form awned, perianth 1, persistent. Stamen 1, flattened, ovary narrowly linear, stigma 2, strongly recurved, fimbriate.

11. Family - Cyperaceae
Sub-family - Caricoideae (tribe Sclerieae)
Scientific Name - *Scleria biflora* Roxb
Myanmar Name - unknown

Figure (23) 1. Habit, 2. Inflorescence, 3. Spike, 4. Spikelet, 5. Floret, 6. Ovary, 7. Root

Herbs, annuals, fibrous roots, terrestrial. Culm 25.0 – 60.0 cm in length, greyish green. Leaves ranked weak cauline, sheath tubular, sheaths 3 sided, loosely surrounding the culm. Involucre bracts about 15.0 x 1.0 cm, sheathing with brown pubescence, branchlets setaceous much longer than spikelet, but not over topping the total inflorescence. Inflorescence spike like raceme, with 2 - 3 brunches, narrow, elongate, peduncles slightly excreted from bract sheath; smooth peduncles. Flower mostly unisexual, male and female or male and some bisexual; 1 or 2 in a cluster narrowly ovoid; mostly unisexual; female spikelet 4 or 5 glumes and 1 female flower; male spikelet with 7 - 9 or more glumes and male flower. Empty glume absent. Perianth absent. Stamens 2 - 3; filaments slender; anthers short. Ovary spherical, surface reticulate, purplish brown, apex with a purple tip or stipitate, short gynophore or cupula, stigma 2, purplish brown.

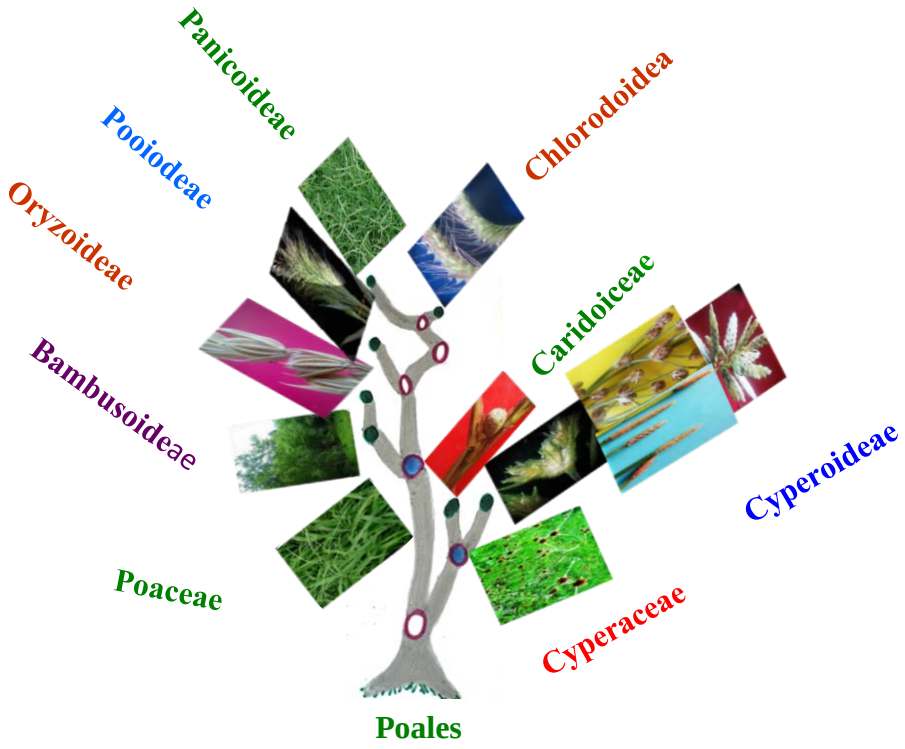


Figure (24) Evolutionary trends of Poaceae and Cyperaceae under clade Order Poales

Useful of Wild Grasses and Wild Sedge Species for Daily Household Materials

Figure (25) *Arundinella pumila* (Hochst) Steut, the whole plant is used for broom

Figure (26) *Eleocharis acutangular* (Roxb.) Schult. Culm fiber makes hat and roof materials for house

Discussion and Conclusion

According to cladistic analysis of APG IV (2016), family Cyperaceae stands more primitive evolutionary trends and it distance 6 families far from family Poaceae under clade of Order Poales by their morphological and molecular data. Poaceae is one of the largest and most so difficult morphological features among the flowering plants and the most economic important family and very widely adaptable status on various environmental conditions than other families. Cyperaceae is growing preferable in wet and swamp aquatic areas and some species is very useful for various point of humanity and economic status *(Actinoscripus*; tuber for food and *Eleocharis*; used for fiber). Poaceae is identified and classified by vegetative characters in genus *Meloncanna arundina* Darkison, tribe Bambusaceae in Sub- family Bambusoideae, this is more evolutionary advance characters than family Cyperaceae. Bamboo floret's stamens is six, Bor (1960).*Oryza sativa* L. and *Oryza grandiglumis* (Doell) Prodhl in tribe Oryzeae of Sub-

family Oryzoideae are six stamens , lemma and palea textures are strongly rigid and cover the sexual organs this is closely related evolutionary primitive features with Bambusoideae and differ from other Sub- families of Poaceae and Cyperaceae, and agree with principles evolutionary status of Baker (1965*). Arundinella pumoila* (Hochst) Steut tribe Arundinellae in Sub- family Pooideae is reed like primitive culm but broadly advance open panicle inflorescence and its advance evolutionary features, Baker (1965), Hafliger and Schole (1981). *Chloris Pilosa* Schmach in tribe Chlorideae of Sub-family Chloridoideae with rarely 3- awned structure. It is so great advance character spikelet from other species for distribution of floret characters in family Poaceae. *Pennisetum parpareum* L. in tribe Paniceae of Sub- family Panicoideae is densely bristles spikelet advance modified character and readily distributed than other recorded 10 species, this characters agree with phylogenetic principles of Baker (1965), Dassanayake et. al (1968). In family Cyperaceae, genera of *Actinoscripus grossus* (L.F.) Goetah & D.A.Simpson in tribe Cypereae of Sub-family Cyperoideae) and *Scleria biflora* Roxb in tribe Sclerieae of Sub - family Caricoideae possess tuber (bulb) advance modified underground organs and *Scleria* is very rare spherical ovary from other species of family Cyperaceae. In tribe Cypereae; *Pycerus flavidus* (Rertzius) T.Koyama possess concave ovary with 3 persistent hyaline perianths and *Eleocharis acutangular* (Roxb.) Schult is narrowly slender culm and reduce involucre bract is modified features for adaptation of evolution trends. *Lipocarpha chinensis* Osbeck is so difference characters of star shape anthela and this is own distinct primitive features for reproductive status, this is agreed with concepts of evolutionary trends of Stebbin (1965). Moreover, the simplicity to complexity of both two families; Poaceae and Cyperaceae are integration of some features but so quite differences morphological features in evolution under Order Poales in graminoids clade of monocotyledonous gramineous flowering plants. This research highlights and supports the future outcome researches for graminoids clade's evolutionary trends of systematics science record in region, Myanmar.

Acknowledgements

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MEIOTIC CHARACTERS OF *ZEA MAYS* L. CV. PAUK PAUK PYAUNG NI AND PHU SAR PYAUNG IN SHAN STATE

Su May Naung[1], Thi Thi Htun[2] & Thein Tun Oo[3]

Abstract

The two cultivars of *Zea mays* L., Pauk pauk pyaung ni and Phu sar pyaung were studied to determine the morphology and meiotic characters. This research was done in Department of Biology, Kyaing Tong Education Degree College, Shan State during June to December 2020. In morphology, the silk and kernel color of the two *Z. mays* L. cultivars were significantly different. The meiotic chromosomes behavior of all studied *Z. mays* L. cultivars were observed 2n = 20, in the diakinesis stage. The lowest univalent, trivalent and quadrivalent (ring, chain and total) chromosomes were observed in *Z. mays* L. cv. Pauk pauk pyaung ni. The maximum mean value of ring and total bivalent chromosomes were also found in *Z. mays* L. cv. Pauk pauk pyaung ni. The chiasmata frequency and normal pollens of cv. Pauk pauk pyaung ni was also significantly superior than the *Z. mays* L. cv. Phu sar pyaung. The chromosome configuration of the *Z. mays* L. cv. Pauk pauk pyaung ni was 0.05 I + 1.35 oII + 8.38 cII + 0.15 III + 0.03 oIV + 0.03 cIV and 0.38 I + 2.15 oII + 7.18 cII + 0.23 III + 0.03 oIV + 0.03 cIV in cv. Phu sar pyaung. In *Z. mays* L. cv. Pauk pauk pyaung ni was found the low laggard and bridge chromosomes in anaphase I and telophase I. Therefore, the *Z. mays* L. cultivar Pauk pauk pyaung ni should be selected for inbreed experiment for future researches.

Keywords meiotic characters, chiasmata frequency, chromosome configuration

Introduction

Zea mays L. is a grain crop belonging to the grass family Poaceae (Paliwal, 2000) and is the only cultivated species of importance in the tribe Mydeae (Salian, 2007). The center of origin for *Z. mays* L. has been established as the Mesoamerican region, now Mexico and Central America (Watson & Dallwitz, 1992). According to Harris *et. al*. (2007), maize is the third most important crop in the world, after rice and wheat. The crop is of significant economic importance worldwide as human food, animal feed and as a source for a large number of industrial products (Paliwal, 2000). In developing countries maize forms part of the staple diet (Du-Plessis, 2003). Rouanet (1992) stated that maize is an industrial raw material for a growing range and variety of food and non-food products as it is used for human consumption, animal feed and for industrial purposes.

In Myanmar, Khaing Khaing Htwe (2020) stated that, maize is the second most important cereal after rice, and is grown in the whole country except in Mon State. It is important for animal feed for domestic livestock farms, and for export. In 2017-2018, about 1,437 thousand metric tons of maize was exported to China and the Philippines. As demand for maize has increased annually since 2009, the maize growing area has expanded (Anonymous, 2018). About 50 % of Myanmar maize production is exported.

Maize is a naturally outcrossing species, which makes its genetic architecture (diversity, linkage, recombination, etc.) more similar to other outcrossing organisms such as humans rather than self-pollinating plants (Wallace *et. al*., 2013). While its genetics are similar to humans, maize retains the major strength of plant genetics: the ability to self-cross and quickly produce homozygotes or F_2 populations.

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Meiosis is a specialized mechanism by which sexually reproducing species reduce their genomes from diploid to haploid, and the underlying mechanisms are highly conserved among eukaryotes (John, 1990). In general, the process of meiosis is under genetic control and consists of commitment and initiation, homologous chromosome pairing and synapsis, interhomolog reciprocal recombination, disjunctive segregation, and haploid gamete or gametophyte formation. Interhomolog chromosome recombination between parental chromosomes during meiotic prophase I establishes physical connections for bipolar spindle attachment between the homologs, and it generates novel allelic combinations (Murphy & Bass, 2012).

Meiosis is an event of high evolutionary stability that culminates in the reduction of chromosome number in gametes. Cytological events of meiosis are controlled by a large number of genes acting from pre-meiosis to the post-meiotic mitoses (Golubovskaya, 1989). Mutations of these genes may cause anomalies that impair plant fertility (Curtis & Doyle, 1991).

In Myanmar, cytogenetic analysis was done by various researchers on many species. More comprehensive studies are needed and in particular a thorough examination of meiosis and post meiotic characters among some local cultivars. Thus, the meiotic chromosome behaviors on two local cultivars of *Zea mays* L. was carried out in this research work. The aim and objectives of this research are to study the meiosis characters of two *Z. mays* L. cultivars, to compare their morphological differences and genetic stability.

Materials and Methods

The seeds of *Zea mays* L. cultivars, Pauk pauk pyaung ni (Accession No. 011446) and Phu sar pyaung (Accession No. 011793) were used for this study. The seed samples were obtained from Seed Bank, Department of Agricultural Research, Nay Pyi Taw, Yezin, Mandalay Region. The PMC (Pollen Mother Cells) of these cultivars were used in meiosis analysis.

The two cultivars of *Zea mays* L. were sown in the Kyaing Tong Education Degree College, Kyaing Tong Township, Shan State in June - December 2020 for morphological characters and meiotic analysis. Each plot size was 3.05 meter in length and 0.91 meter in width with twenty plants per plot. Each cultivar was sown in two rows, 0.46 meter between rows and 0.31 meter between plants.

For meiotic analysis, the young floral buds were collected between 6 a.m. to 10 a.m. about nine weeks after sowing, which were fixed in Carnoy's I (1886) solution. The sixty cells for each cultivar were studied. The Pollen Mother Cell (PMC's) was studied by squash technique (Belling, 1921) using 2% acetocarmine stain. The meiotic chromosome configurations were also studied in each phase of division (metaphase, anaphase, telophase and pollen characters). The data collected for all the meiosis characters were recorded.

Results

The morphological characters and meiotic chromosome behaviors of two *Zea mays* L. cultivars were described in Table 1 to 2 and Figure 1 to 4.

Taxonomic Description

Family	-	Poaceae
Scientific Name	-	*Zea mays* L.
English Name	-	Maize
Myanmar Name	-	Pyaung

Annual erect herbs, monoecious; stems 230 – 280 cm high, solid, well-defined nodes and internodes, 13 – 18 jointed swollen nodes; internode 10 – 22 cm in length, the last node end with tassel. Leaves simple, alternate and distichous, exstipulate, sessile; blades linear-lanceolate, 37 – 102 cm long and 4.0 – 10.0 cm wide, the margin entire, hairy, the apex acuminates, scarcely strigose on both surfaces; ligule 0.5 – 1.0 cm long, auriculate. Male inflorescences or tassels terminal paniculate, 33 – 45 cm long, 22 – 33 branched. Female inflorescences or ears axillary, 2 to 3, series of paired spikelets in longitudinal rows, the rows usually even number, 12 – 16. Male spikelets paired, one sessile and other pedicellate, with paired glumes; glumes overlapped, bracteate, outer lemma 3-nerved, inner palea 2-nerved, unisexual, zygomorphic; perianth modified into 2 fleshy lodicules, opposite the lemma and alternate the stamens; stamens 3; filaments free, short; anthers versatile, dithecous, dehiscent by longitudinal slit, pale yellow; gynoecium absent. Female spikelets paired, arranged in rows on the central axis or cob, sessile, with paired glumes, thick near the base of ovary, bracteate, represented by lemma and palea, unisexual, zygomorphic; perianth usually absent, sometimes 2, scaly lodicules; androecium absent; gynoecium monocarpellary, unilocular; ovary superior, dome shaped, ovary single ovuled, basal placentation; style long, silky, filiform; stigma long, hairy; fruits or kernels caryopsis, various coloured.

Tasseling Period: Varied according to cultivars.

Outstanding characters of cv. Pauk pauk pyaung ni

Plant height 239 – 249 cm; ear height 183 – 204 cm; jointed swollen nodes 17 – 18 and internodes 10 – 22 cm long. Leaf blades 37 – 99 cm long and 5.5 – 10.0 cm wide. Male inflorescences or tassels 33 – 38 cm long with 22 to 29 branches. Female inflorescences or ears 3 per plant; silk color pale yellow; ears 28 – 30 cm long and 15 – 17 cm in diameter; female florets arranged in 14 – 16 rows per ear; cobs diameter 10 – 13 cm; kernel purple in color.

Tasseling Period: 60 – 79 days

Outstanding characters of cv. Phu sar pyaung

Plant height 230 – 280 cm; ear height 149 – 217 cm; jointed swollen nodes 13 – 15 and internodes 14 – 19 cm long. Leaf blades 37 – 102 cm long and 4.0 – 9.5 cm wide. Male inflorescences or tassels 41 – 45 cm long with 26 to 33 branches. Female inflorescences or ears 2 – 3 one per plant; silk color red; ears 29 – 30 cm long and 14 – 18 cm in diameter; female florets arranged in 12 - 14 rows per ear; cobs diameter 12 – 14 cm; kernel pale white and pale purple in color.

Tasseling Period: 55 – 67 days

Meiotic characters

The chiasmata frequencies were calculated according to synapsis of chromosome arm at metaphase I. In *Z. mays* L. cv. Pauk pauk pyaung ni was observed the highest number of frequency of chiasmata (18.43 ± 1.25) than the cv. Phu sar pyaung (16.93 ± 1.53). The formulae of chromosome constitution were 0.05I + 1.35 oII + 8.38 cII + 0.15 III + 0.03 oIV + 0.03 cIV in *Z. mays* L. cv. Pauk pauk pyaung ni and 0.38 I + 2.15 oII + 7.18 cII + 0.23 III + 0.03 oIV + 0.03 cIV in cv. Phu sar pyaung as recorded in table 1.

It was observed that the normal diakinesis stages (2n = 20) was observed in all studied cultivars of *Zea mays* L. as shown in Figure 2. The metaphase chromosome behavior of the two *Z. mays* L. cultivars were described as the mean value of univalent, bivalent (ring, rod and total), trivalent, chain and ring quadrivalent, frequency of chiasmata and meiotic configuration in table 1. Among two *Zea mays* L. cultivars, the cv. Phu sar pyaung was possessed the high mean number of univalent chromosome (0.38 ± 0.59) than the cv. Pauk pauk pyaung ni (0.05 ± 0.20).

The maximum mean number of ring (8.38 ± 1.35) and total (9.73 ± 0.73) bivalent chromosomes was found in *Z. mays* L. cv. Pauk pauk pyaung ni, while the *Z. mays* L. cv. Phu sar pyaung was possessed the highest rod bivalent chromosome (2.15 ± 1.13). The cv. Phu sar pyaung was stated that the higher trivalent chromosome than the cv. Pauk pauk pyaung ni. The ranges of quadrivalent (chain, ring and total) were slightly different to each other in the two studied *Z. mays* L. cultivars (Table 1).

The anaphase I and telophase I chromosome behaviors of the two cultivars of *Z. mays* L. were described in Table 2 and Figure 2. The maximum mean number of laggard and bridge chromosomes at anaphase I and telophase I were found in *Z. mays* L. cv. Phu sar pyaung. In *Z. mays* L. cv. Pauk pauk pyaung ni was not occurred the laggard chromosome in anaphase I and bridge chromosome in telophase I, it possessed the lowest mean number of bridge chromosome in anaphase I and laggard chromosome in telophase I (Table 2).

In meiotic division II (Metaphase II, Anaphase II and Telophase II) analysis, synchronous chromosome segregation was found in two studied *Z. mays* L. cultivars (Figure 3). The normal and abnormal spore tetrads were also observed in two studied *Z. mays* L. cultivars as seen in table 2 and figure 4. The mean number of micronuclei at tetrad and abnormal pollen character of *Z. mays* L. cv. Pauk pauk pyaung ni was lower than the cv. Phu sar pyaung.

Table 1 Comparison on metaphase I characters of two cultivars of *Zea mays* L.

	cv. Pauk pauk pyaung ni $\overline{X}$ **± S. E**	**cv. Phyu sar pyaung** $\overline{X}$ **± S. E**
No. of Chromosome	20	20
No. of Cells Studied	60	60
Univalent	0.05 ± 0.20	0.38 ± 0.59
Rod Bivalent	1.35 ± 1.12	2.15 ± 1.13
Ring Bivalent	8.38 ± 1.35	7.18 ± 1.38
Total Bivalent	9.73 ± 0.73	9.33 ± 0.93
Trivalent	0.15 ± 0.60	0.23 ± 0.06
Ring Quadrivalent	0.03 ± 0.26	0.03 ± 0.26
Rod Quadrivalent	0.03 ± 0.26	0.03 ± 0.26
Total Quadrivalent	0.07 ± 0.36	0.06 ± 0.36
Frequency of Chiasmata	18.43 ± 1.25	16.93 ± 1.53
Meiotic configuration	0.05I + 1.35 oII + 8.38 cII + 0.15 III + 0.03 oIV + 0.03 cIV	0.38 I + 2.15 oII + 7.18 cII + 0.23 III + 0.03 oIV + 0.03 cIV

$\overline{X}$ = mean
S. E = Standard Error
I = univalent, oII = open bivalent, cII = closed bivalent, III= trivalent,
oII = open quadrivalent, cIV = ring quadrivalent

Table 2 Comparison on mean number of laggard, bridge at Anaphase I and Telophase I; Tetrad and pollen characters in two cultivars of *Zea mays* L.

	cv. Pauk Pauk Pyaung ni **X ± S. E**	**cv. Phyu sar pyaung** $\overline{X}$ **± S. E**
Laggard chromosome at anaphase I	-	0.17 ± 0.38
Bridge chromosome at anaphase I	0.10 ± 0.31	0.33 ± 0.48
Laggard chromosome at telophase I	0.03 ± 0.18	0.33 ± 0.48
Bridge chromosome at telophase I	-	0.07 ± 0.25
Micronuclei at tetrad	10.00 ± 2.83	23.00 ± 2.83
Normal Pollen Character	60.80 ± 4.02	55.00 ± 3.08
Abnormal Pollen Character	39.20 ± 4.02	45.00 ± 3.08

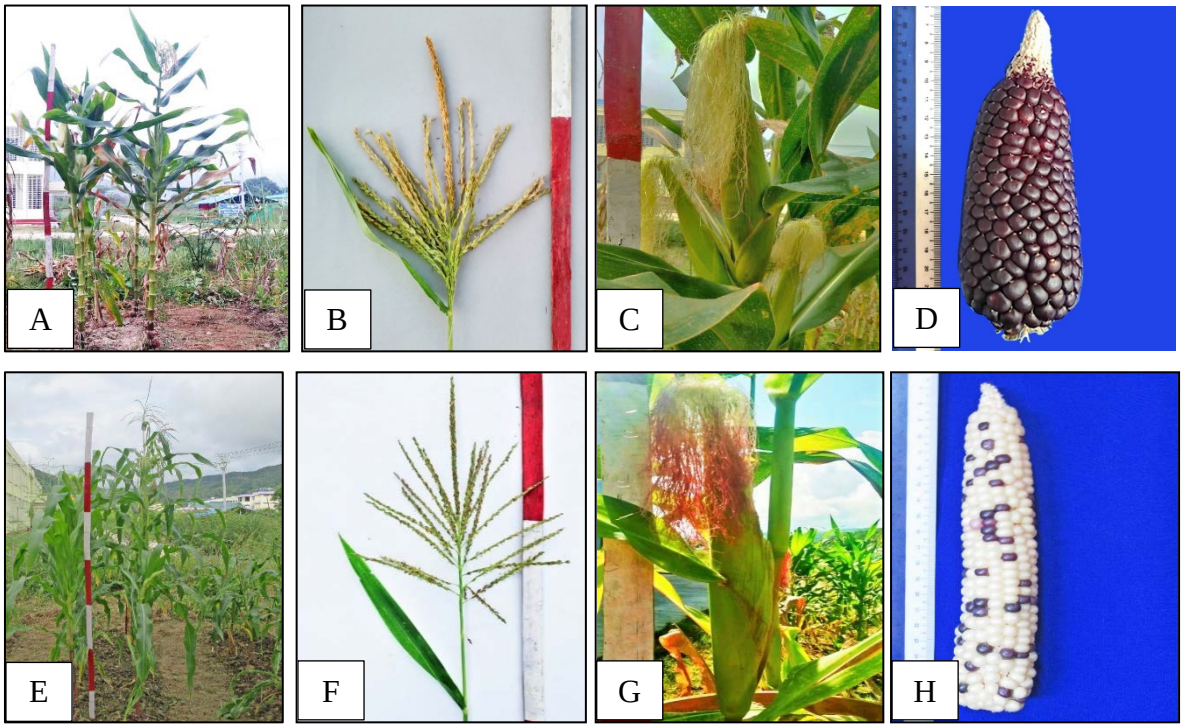

Figure 1 Morphological characters of *Zea mays* L. cv. Pauk pauk pyaung ni and Phu sar pyaung

A. Habit of cv. Pauk pauk pyaung ni
B. Tassel character of cv. Pauk pauk pyaung ni
C. Ear character of cv. Pauk pauk pyaung ni
D. Cob character of cv. Pauk pauk pyaung ni
E. Habit of cv. Phu sar pyaung
F. Tassel character of cv. Phu sar pyaung
G. Ear character of cv. Phu sar pyaung
H. Cob character of cv. Phu sar pyaung

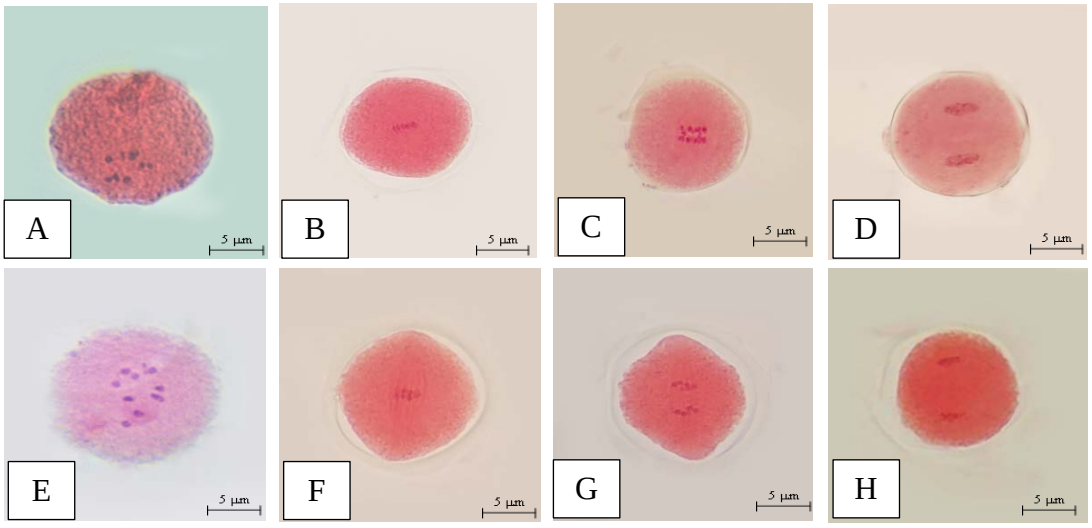

Figure 2 Diakinesis, Metaphase I, Anaphase I and Telophase I characters of *Zea mays* L. cv. Pauk pauk pyaung ni and Phu sar pyaung

A. Diakinesis character of cv. Pauk pauk pyaung ni

B. Metaphase I character of cv. Pauk pauk pyaung ni
C. Anaphase I character of cv. Pauk pauk pyaung ni
D. Telophase I character of cv. Pauk pauk pyaung ni
E. Diakinesis character of cv. Phu sar pyaung
F. Metaphase I character of cv. Phu sar pyaung
G. Telophase I character of cv. Phu sar pyaung
H. Telophase I character of cv. Phu sar pyaung

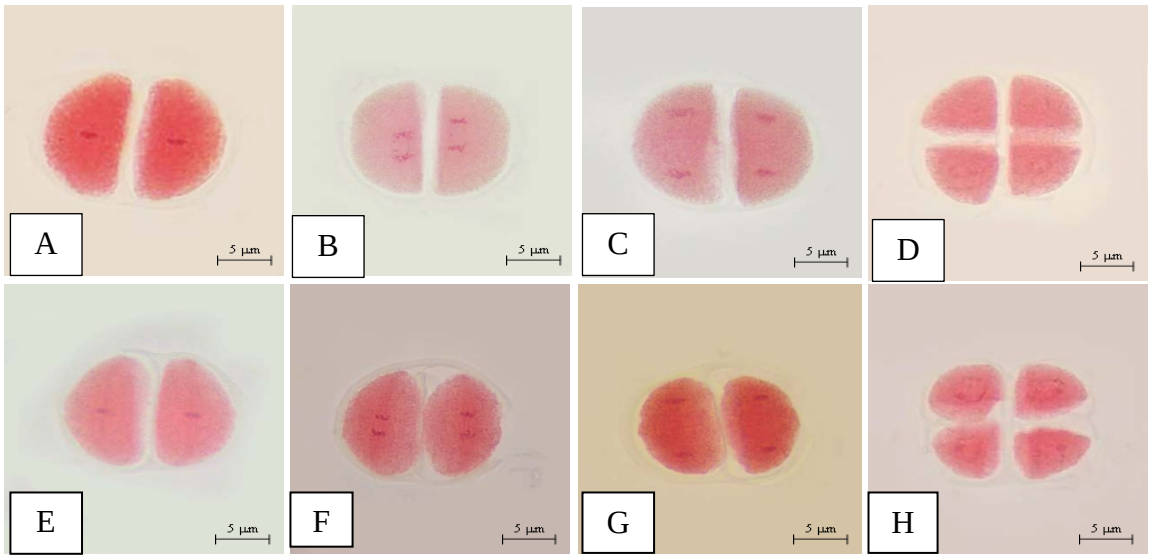

Figure 3 Metaphase II, Anaphase II, Telophase II and Tetrad characters of *Zea mays* L. cv. Pauk pauk pyaung ni and Phu sar pyaung

A. Metaphase II character of cv. Pauk pauk pyaung ni
B. Anaphase II character of cv. Pauk pauk pyaung ni
C. Telophase II character of cv. Pauk pauk pyaung ni
D. Tetrad character of cv. Pauk pauk pyaung ni
E. Metaphase II character of cv. Phu sar pyaung
F. Anaphase II character of cv. Phu sar pyaung
G. Telophase II character of cv. Phu sar pyaung
H. Tetrad character of cv. Phu sar pyaung

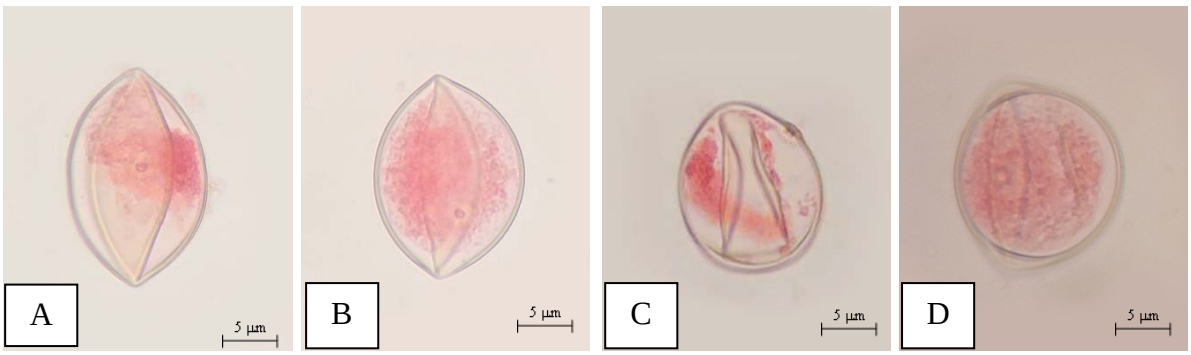

Figure 4 Normal and abnormal pollen characters of *Zea mays* L. cv. Pauk pauk pyaung ni and Phu sar pyaung

A. Normal pollen character of cv. Pauk pauk pyaung ni
B. Normal pollen character of cv. Phu sar pyaung
C. Abnormal pollen character of cv. Pauk pauk pyaung ni
D. Abnormal pollen character of cv. Phu sar pyaung

Disscusion and Conclusion

The morphology and meiotic chromosome characters of the two *Z. mays* L. cultivars were studied. The two studied *Z. mays* L. cultivars cv. Pauk pauk pyaung ni and Phu sar pyaung were slightly different in most morphological characters, while significantly different in silk and kernel color. These finding were agreement with (Rosemary, 2000), the different kernel colors yellow, red and purple are produced by the carotenoids or the anthocyanin pathway. White color, results from the lacks of pigments produced from either pathway.

In the meiotic behavior, chromosome number, meiotic stages, as well as abnormalities were studied. The chromosome number of two studied maize cultivars were 2n = 20 at diakinesis. This observation was agreement with Khah *et. al.* (2018), meiosis was found normal in the PMCs of *Zea mays* L. plants which showed regular 10 bivalents (2n = 20) at diakinesis. A varying range of various meiotic irregularities were observed during the separation and anaphase movement of the chromosome.

In univalent chromosome of two *Z. mays* L. cultivars, the *Z. mays* L. cv. Pauk pauk pyaung ni (0.05 ± 0.20) was significantly inferior to the cv. Phu sar pyaung (0.38 ± 0.59). Due to this condition of the cytological anomalies, the laggard and bridge chromosomes of the cv. Phu sar pyaung was observed in anaphase and telophase than the *Z. mays* L. cv. Pauk pauk pyaung ni. This observation was agreed with Khah & Verma (2017a), who stated that the chromosome clumping at metaphase sometimes led to their inability of bivalents to separate at anaphase, thus leading to the sticky bridges. The observed disturbed polarity might have occurred due to spindle disturbances and presence of high frequency of univalents. The chiasmata frequency of the cv. Phu sar pyaung (16.93 ± 1.53) was also lower than the cv. Pauk pauk pyaung ni (18.43 ± 1.25). This result was agreement with Sjödin (1970), who suggested that the presence of univalent chromosomes resulted in the discrease of chiasma frequency.

Although the *Z. mays* L. cv. Phu sar pyaung was possessed the high rod bivalent chromosome (2.15 ± 1.13), the cv. Pauk pauk pyaung ni was stated that the maximum ring (8.38 ± 1.35) and total bivalent chromosome (9.73 ± 0.73). Among two studied *Z. mays* L. cultivars, the mean number of normal pollen character in cv. Pauk pauk pyaung ni (60.80 ± 4.02) was higher than the cv. Phu sar pyaung (55.00 ± 3.08). This result was agreed with Sumner (2003), Khin Swe Lai (2010) and Chaw Su Lwin (2016) also stated that the cultivars were possessed the high ring bivalent and total bivalent chromosome number produced the high percentage of fertile pollen.

The meiotic abnormalities, formation of multivalents have been observed. The *Z. mays* L. cv. Phu sar pyaung was possessed the high number of trivalent chromosome (0.23 ± 0.06) than the cv. Pauk pauk pyaung ni (0.15 ± 0.60). The mean number of quadrivalent chromosome (ring, chain and total) was not significantly different between two cultivars. Risso-Pascotto *et. al.* (2005) proposed the meiotic process was typical of polyploids, with chromosomes associating in bi-, tri-, and quadrivalents. Precocious chromosome migration to the poles, laggards and micronucleus formation were found in both meiosis I and II, resulting in tetrads with micronuclei in some microspores. The frequency of abnormal pollen mitosis varied among flowers and also among inflorescences. All plants were equally affected. Sterile pollen grains resulted from this abnormal pollen development.

In *Z. mays* L. cv. Pauk pauk pyaung ni was not observed the laggard and bridge chromosome in anaphase I and telophase I. The *Z. mays* L. cv. Phu sar pyaung was possessed high mean number of the laggard and bridge chromosome in both anaphase I and telophase I. Therefore, the high mean number of micronuclei in tetrad (23.00 ± 2.83) and abnormal pollen

(45.00 ± 3.08) was also found in cv. Phu sar pyaung. This finding was agreement with Bajpai and Singh (2006) stated that the meiotic abnormalities which generally affect pollen viability have troubled sexual reproduction. In conclusion, the morphological character of the two studied maize cultivars was slightly different while in silk and kernel color were significantly different. The total bivalent, chiasmata frequency and pollen fertility of the *Z. mays* L.cv. Pauk pauk pyaung ni was superior. Therefore, the *Z. mays* L. cv. Pauk pauk pyaung ni should be selected for inbreed experiment for future researches.

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SCREENING OF PHYTOCHEMICAL AND ANTIMICROBIAL ACTIVITY OF *BORASSUS FLABELLIFER* L. ROOTS (PALMYRA PALM)

Nwe Nwe[1], Sanda Myint[2], Phyu Phyu Aung[3]

Abstract

Borassus flabellifer Linn. is one of the medicinally important plants in family Arecaceae. The study was under taken to investigate the phytochemical and antimicrobial activities of *Borassus flabellifer* L. root. The phytochemical analysis of alkaloids, α-amino acid, carbohydrate, glycosides, phenolic compounds, polyphenol, reducing sugars, saponins, starch, terpenoids, tannins, and steroids were present. Antimicrobial activity was analyzed by agar well diffusion method against six strains of microorganism, namely *Escherichia coli*, *Saccharomyces cerevisia*, *Pseudomonous fluorescens*, *Bacillus pumilus*, *Bacillus subtilis*, and *Candida albicans*. Methanol extract of root (12.95-14.68 mm) were active against all test strains. Ethanol and watery extracts (13.23-15.64 mm) against four test strains. Petroleum ether extract did not show antimicrobial activity. Among the various crude extracts, the ethyl acetate extract showed the maximum zone of inhibition was exhibited in *C. albicans* (22.26 mm) compared to tested microorganisms. Therefore, the ethyl acetate extract of the palm root has showed consistently significant inhibitory activity on different tested organisms.

Keywords *B. flabellifer* roots, Phytochemical and Antimicrobial

Introduction

Borassus flabellifer L. is one of the important horticultural crops in many countries. The palm is the family Palmae, or more recently reclassify in Arecaceae, and sub family Coryphoideae with 1200-4000 species. This plant is widely distributed, and cultivated in tropical Asian countries such as Thailand, Bangladesh, India, Myanmar, Sri Lanka, Malaysia, etc.

The different parts of the *B. flabellifer* L. are being used for medicinal properties, such as the various ailments like secondary syphilis, antiperiodic, heart burns, liver, and spleen enlargement (Sandhya *et al.* 2010).

The germinating shoot of the Palmyra palm is either as food, and used for medicinal purposes. The nutritional analysis of the roots has shown 8.54% protein, 23.53% carbohydrates, and 7.29% crude fiber. These roots are found to be high in calories. They are rich in starch, but poor in fats, and proteins (Schneider and wolfing, 2004).

Phytochemical are naturally occurring chemicals produced by plants. They are biological active, and may affect human health. The preliminary phytochemical screening tests may be useful in the detection of the bioactive principles, and subsequently may lead to the drug discovery, and development. Thus, present study was investigated to the phytochemicals, and antimicrobial activities of *Borassus flabellifer* L. roots, and in order to understand their health benefits.

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Materials and Methods

Botanical Studies

Sample collection and preparation

The root samples of *Borassus flabellifer* L. were collected from the Kyauk-pa-daung Township, Mandalay Region. It was peeled and washed with water, minced into small pieces, and dried in the shade for one week then the dried roots were made powder in electric motor grinder. The powdered samples were stored in airtight containers to prevent moisture changes, and contamination.

Chemical Studies

Phytochemical Investigation of *Borassus flabellifer* L. roots

Phytochemical tests of *Borassus flabellifer* L. roots were carried out, to investigate the presence, and absence of phyto-constituents such as alkaloids, α- amino acids, reducing sugar, carbohydrates, flavonoids, glycosides, phenolic compounds, polyphenols, saponins, starch, terpenoids, tannins, and steroids. Phytochemical tests were done according to the methods of (Trease and Evans 1980, Marini-Bettolo, *et al*., 1981, and M-Tin Wa, 1972).

Preparation of crude Extract

Extraction from dried *Borassus flabellifer* L. roots were done using aqueous, ethanol, methanol, ethyl acetate, and petroleum ether as the extraction solvents. 5 g of the powdered sample with 50 ml of solvent in a conical flask, wrapped with aluminum foil, and kept for one week. The sample was shaken twice a day, and filtered using what-man filter paper No. 32. Then, the obtained filtrates were collected, and concentrated using water-bath until a crude viscous extract was obtained.

Antimicrobial activity

Test extracts: Ethanol, Methanol, Ethyl acetate, Petroleum ether, and water.

Medium used in Antimicrobial Activity Test (Ando, 2004)

	(Assay Medium)
Glucose	0.5 g
Yeast extracts	0.2 g
Agar	1.8 g
Distilled water	100ml

Test Organisms used in Antimicrobial Activities (NITE, 2005)

Sr No.	Test Organisms	Infections
1.	*Escherichia coli*	Cholera, diarrhea and vomiting, urinary tract infections
2.	*Bacillus subtilis*	Fever
3.	*Bacillus pumilus*	Wound and burn infection, eye infection
4.	*Saccharomyces cerevisiae*	Food spoilage and fever
5.	*Pseudomonas fluorescens*	Rice disease
6.	*Candida albicans*	Candidiasis, cardiac infection, skin infection

Test for antimicrobial Activity by Agar Well Diffusion Methods (Collins, 1965)

Antimicrobial activity was carried out at the Department of Botany, University of Magway. Sterized cork borer was used to make the wells (8 mm in diameter) in the autoclaved basal antimicrobial test medium. Screening of antimicrobial activity of various crude extracts,

such as ethanol, methanol, ethyl acetate, petroleum ether, and aqueous extract were using by agar well diffusion method.

Assay medium containing glucose (0.5 g), yeast (0.2 g), agar (1.8 g), and distilled water (100 ml) were placed into the sterilized conical flask, and plugged with cotton wool, and then autoclaved at 121 °C for 15 min. After cooling down to 40 °C, 0.5 ml of suspended strain was inoculated to the assay medium with the help of a sterilized disposable pipette near the burner.

About 20 ml of medium was poured into the sterilized petri-dishes and allowed to set the medium. After solidification, the wells (8 mm diameter) were punched in the agar, and 20 µl of extracts were loaded into the wells. The plates were incubated at room temperature 37°C for 24-48 hours. After 24-48 hours of incubation, the antimicrobial activity was assessed by measuring the diameter of the zone of inhibition, and reported on the scale of millimeter.

Results

Scientific Name - *Borassus flabellifer* Linn
English Name - Palmyra palm
Myanmar Name - Htan
Common Name - Toddy palm, Sugar palm
Family - Arecaceae
Part Used - Fruits, Male Inflorescences, and Roots

Outstanding Characters of *Borassus flabellifer* L.

Palmyra palm is a tall dioeciously tree, and single-stemmed evergreen tree up to 30 m high. The plant contains its crown of leaves at the top position.

The leaves are leathery, gray green, palmately fan-shaped, 1-3 m wide, and folded along the midrib, lanceolate, 0.6 -1.2 m along, petiole edges along with hard horny spine scent serrates.

Inflorescence is a spadix. The flowers are unisexual with male spadix branching, and female spadix simple. Male flowers are minute, mixed with scaly bracteoles, sunk in cavities between large overlapping leaves. Female flowers are solitary, scattered on a sparingly branched spadix.

The coconut like fruits are three sided, rounded or more or less oval, 12-15 cm wide, and capped on the greatly enlarged perianth. When the fruit is young, this kernel is hollow, soft as jelly, and translucent like ice, sweetish, and potable. The flowering and fruiting period is December to June.

Figure-1 Habit

Figure-2 Leaves

Figure-3 Mature staminate inflorescence

Figure-4 Pistillate inflorescence

Figure-5 Fruits

Figure-6 Germinating shoots or roots

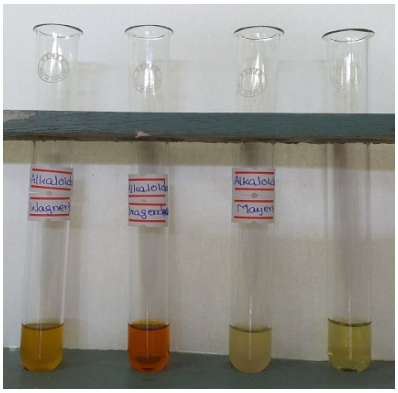
Phytochemical Investigation of Root Sample by Test Tube Methods

The preliminary phytochemical screening of *B. flabellifer* L. roots consisted of alkaloids, α amino acid, carbohydrates, glycosides, phenolic compounds, polyphenols, reducing sugar, saponin, starch, tannins, terpenoids, and steroids. However, flavonoids are not found on it. The results were shown in (Table 1).

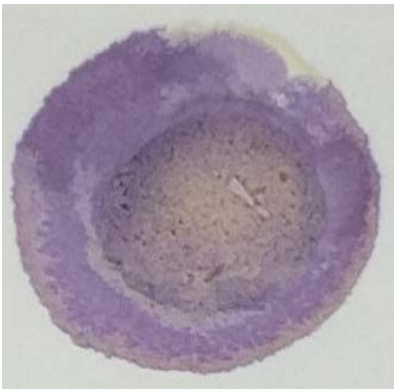
Table 1 Phytochemical constituents of *Borassus flabellifer* L. Root

No.	Tests	Solvent extraction	Test Reagents	Observation	Results
1.	Alkaloids	EtOH	Wagner's reagent	Orange ppt	+
			Dragendorff's reagent	Reddish brown ppt	+
			Mayer's reagent	White ppt	+
2.	α amino acid	H_2O	Ninhydrin reagent	Violet color	+
3.	Carbohydrates	H_2O	10% α-naphthaol & Conc H_2SO_4	Red ring	+
4.	Flavonoids	EtOH	Conc: H_2SO_4, Mg ribbon	-	-
5.	Glycosides	EtOH	10% lead acetate	White ppt	+
6.	Phenolic compounds	H_2O	10% $FeCl_3$	Green	+
7.	Polyphenol	EtOH	1%$FeCl_3$&$K_3Fe(CN)_6$	Greenish blue	+
8.	Reducing Sugar	H_2O	Benedict's solution	Green	+
9.	Saponin	H_2O	Distilled water	Frothing	+
10.	Starch	H_2O	I_2 solution	Deep blue	+
11.	Terpenoids	EtOH	Acetic anhydride, & Conc: H_2SO_4	Reddish brown	+
12.	Tannins	H_2O	10% Lead acetate	White ppt	+
13.	Steroids	H_2O	Acetic anhydride, & Conc: H_2SO_4	Black	+

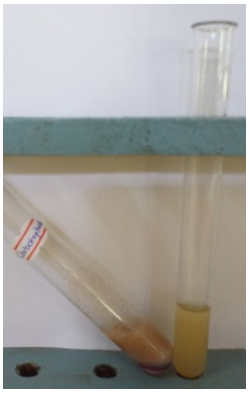
(+) = Presence (-) = Absence (ppt) = Precipitate



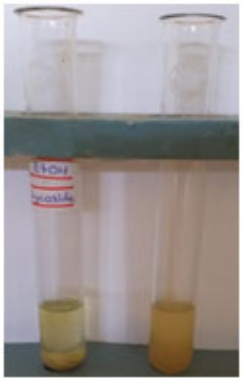
1. Alkaloids



2. α amino acid



3. Carbohydrates



4. Glycosides

5. Phenolic compound

6.Polyphenol

7. Reducing sugar

8. Saponins

9. Starch

10. Terpenoids

11. Tannins

12. Steroids

Figure 7 Phytochemical constituents of *Borassus flabellifer* L. Root

Antimicrobial Activity of Various Crude Extracts of *Borassus flabellifer* L. Root

Screening of antimicrobial activity of root extracts were carried out on different strain of microorganisms by agar well diffusion method. The measureable zone diameter of growth inhibition reflects the degree of antimicrobial activity. The results of the antimicrobial activities were presented in (Table 2).

Table 2 The Antimicrobial Activity of Roots Extract of *Borassus flabellifer* L.

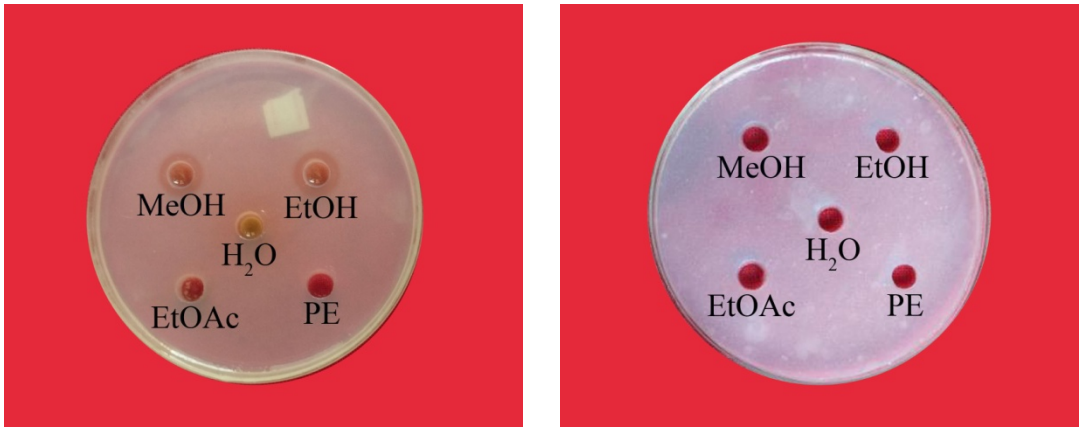
Test microorganisms	Zone of inhibition (mm diameter)				
	Methanol	**Ethanol**	**Water**	**Ethyl acetate**	**Petroleum ether**
Escherichia coli	14.68 (++)	14.75 (++)	14.48 (++)	15.28 (++)	-
Bacillus subtilis	13.12 (+)	14.33 (+)	-	-	-
Bacillus pumilus	14.33 (++)	-	13.37 (+)	-	-
Pseudomonas fluorescens	13.56 (++)	13.24 (+)	14 .28 (+)	-	-
Saccharomyces cerevisiae	12.95 (+)	-	-	-	-
Candida albicans	13.10 (+)	13.23 (+)	15.64 (++)	22 .26 (+++)	-

Agar well - 8mm

10mm - 13mm (+) low activity
14mm - 19mm (++) medium activity
20mm - above (+++) highest activity
(-) No activity

In the present study, the antimicrobial activity of different extracts of *Borassus flabellifer* L. root were tested by agar well diffusion method on six test organisms. Among them, ethyl acetate extract showed the highest activity (22.26 mm) on *Candida albicans* followed by *Escherichia coli* (15.28mm) respectively, and did not show the activity on other four test organisms. The watery extract also showed the medium activity on *Candida albican*s (15.64 mm). Except the petroleum ether extract, all extracts showed the medium activity on *Escherichia coli*. In the methanol extract showed all test organisms (13.10-15.64 mm), and petroleum ether extract did not showed all tested organisms (Table 2 & Fig- 8).

1. *Escherichia coli*



2. *Bacillus subtilis*

3. *Bacillus pumilus*

4. *Pseudomonas flurescens*

5. Sa*cchoromycae cerevisiae*

6. *Candida albicans*

Figure 8 The antimicrobial activity of roots extract of *Borassus flabellifer* L.

Discussion and Conclusion

In this research, the preliminary phytochemical screening of *Borassus flabellifer* L. roots contains alkaloids, α amino acid, carbohydrates, glycosides, phenolic compounds, polyphenols, reducing sugar, saponin, starch, tannins, terpenoids, and steroids. However, flavonoids are not found on it.

In phytochemicals test, alkaloids are one of the largest groups of phytochemicals that have led to the invention of powerful pain killer medications. Also, alkaloids are the most efficient therapeutically significant plant substance (Kam and Liew, 2002).Glycosides are used as antibiotic, anticancer, anti-diabetic, purgative treatment of congestive heart failure, and cardiac arrhythmia.

Tannins are also for antiviral, antimicrobial, and antitumor properties. Steroids are known to be an important cardio tonic activities possess antimicrobial property, and also used in herbal medicines, and cosmetics. Saponins are also important therapeutically as they are shown to have anticancer activity. It is also necessary for activity of cardiac glycosides (Doughari, 2012).

In this research, the screening of antimicrobial activity of various crude extracts such as petroleum ether, methanol, ethanol, ethyl acetate, and water were investigated by employing agar well diffusion method against six species of microorganisms such as *E.coli*, *B. subtilis*, *B. pumalis*, *P. fluroscens*, *S. cerevisia*, and *C. albicans*. The larger the diameter of clear zone the more potent the microbial activity.

In the present study, the Palmyra palm root extracts have showed different degrees of antimicrobial activity ranging from (13.12 mm to 22.26 mm) against studied microorganisms. Ethyl acetate and watery extracts exhibited maximum zone of inhibition against *C. albicans* (22.26 mm & 15.64 mm) respectively. Screening of antimicrobial for inviting us to present research paper activities of these various crude extracts, the ethyl acetate extracts of *Borassus flabellifer* L. root exhibited significant antimicrobial activity.

From this study, it was concluded that *B. flabellifer* L. roots contained many biological active phytochemical and antimicrobial activity. It is recommended that further investigation on vitamins, amino-acids, isolation of active compounds, and efficiencies of this plant are needed for novel herbal medicine.

Acknowledgements

I would like to thank all members of the Myanmar Academy of Art and Science (MAAS) for inviting us to present research paper. I would like to express their profound gratitude to the Department of Higher Education, Ministry of Education in Myanmar, for provision of opportunity to do this research. I would like to express our deepest thanks to Rector Dr. Khin Maung Oo, University of Magway, for allowing to do research paper. I am also grateful thanks to Dr. Sanda Myint, Professor and Head, University of Magway, for her kind permission to use the research facility for the research work.

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ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI FROM FLOWER OF *CATHARANTHUS ROSEUS* (L.) G. DON

Ei Pyae Myo Win[1], Yee Yee Myint[2]

Abstract

In this research, various kinds of fungi were screened from flowers of *Catharanthu sroseus* (L.) G. Don. The plant specimen of *C. roseus* was collected from Hlaing Township, Yangon Region, near the Oakkyin bus-stop, on the Insein Road. The direct inoculation method was used to isolate different strains of fungi on Potato Glucose Agar medium. Five strains of fungi were found out in this experiment. These strains of fungi were designated as F1, F2, F3, F4 and F5. The morphological and microscopically characters, colony appearances, hyphae, spore shape, appearance of conidiophores, characteristics of spore head, starch hydrolysis test and urease test were carried out at Microbiology Laboratory, Department of Botany, University of Yangon. Isolated fungi F2 from flower of *Catharanthus roseus* was assumed to be the genus *Mucor* sp. and F3 was also to be the genus *Aspergillus* sp. based on their morphological and microscopic characters. Starch hydrolysis and urease tests were also investigated F2, F3 and F5 are positive result of starch hydrolysis. Except F2, all strains were possessed urease activity. All of fungal isolates were cultured on six different agar such as Czapek dox agar (CZA), Potato dextrose agar (PDA), Potato sucrose agar (PSA), Sucrose yeast agar (SY), Yeast malt extract agar (YMA), and Sabouraud's dextrose agar (SDA) respectively and their reverse color and observed color were recorded. These isolated fungal strains did not grow well on Sabouraud agar medium. Among them potato sucrose medium is the best for growth of these isolates. These media were applied as fermented broth for antimicrobial activities. The antimicrobial activity of fermented broths from all isolated strains were examined on ten test organisms by paper disc diffusion method and fermentation were carried out for 1 day to 10 days. The fermented broths of all isolated strains F1 to F5 selectively showed good antimicrobial activity from 4 to 6 days on eight tests organisms. Secondary metabolite of activity strains were extracted with ethyl acetate (1:1Vol/Vol) and their antimicrobial activity was also investigated on ten tests organism. The crude of five strains showed excellent antimicrobial activity on eight test organisms.

Keywords *Catharanthus roseus*, fungi, media, *Mucor*, *Aspergillus*, incubate, isolate, Antimicrobial activities

Introduction

Endophytes are microorganisms that inhabit in living tissues of various plants. Endophytes are mainly colonize vegetative parts but are also found in reproductive organs. These endophytes are organisms that colonize internal plant tissues without causing apparent harm to their host. Fungi, bacteria, actinomycetes and mycoplasma are groups of microorganisms and these are reported as endophytes of plants (Arnold and Lutzoni, 2007).

Endophytes protect plants against herbivores, insect attacks or tissue invading pathogens and they are mutualistic and commensalis relationship with its host (Marcellano *et al*., 2017). Filamentous fungi are organisms that can break down organic matter, releasing phosphorus, oxygen, nitrogen and carbon into the atmosphere and the soil (Svahn, 2015). *Catharanthus roseus* (L.) G. Don is important to explore endophytic microflora in the medicinal plant.

It belongs to the family Apocynaceae. Medagascar periwinkle is also known as Thinbaw-ma-hyno-pan. Two common cultivars of *Catharanthus* which is named on the basis of their flower color that is the pink flowered "Rosea" and the white flowers "Alba" Monika and Sharma (2013).

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In this study, the effective endophytic fungi were screened from flower of *Catharanthus roseus* (L.) G. Don that was grown on Potato glucose agar medium by direct plate method (Atlas, 1993). The growth effect of these isolates were studied on six media such as Czapek dox agar (CZA), Potato dextrose agar (PDA), Potato sucrose agar (PSA), Sucrose yeast agar (SYA), Yeast malt extract agar (YMA), and Sabouraud's dextrose agar (SDA), respectively. Their potential of fermented broth extracts and ethyl acetate solvent extracts were preliminary carried out using the ten tested organisms. The Aim of this research is to isolate and identify the endophytic fungi from flower of *Catharanthus roseus* (L.) G. Don and to find out their potential of antimicrobial activities from fermented broth extracts and ethyl acetate solvent extracts against test organisms.

Materials and Methods

Samples collection and surface sterilization (Arnold *et al*., 2007)

The flower specimens were collected in early January of 2018 from Hlaing Township,Yangon Region, near the Oakkyin Bus-stop, on the Insein Road. This plant was identified as *Catharanthus roseus* according to Dassanayake and Fosberg (1981). So, this plant was identified into *Catharanthus roseus* (L.) G. Don by using available literature in the Department of Botany. Endophytic fungi were isolated from flower of *Catharanthus roseus* (corolla lobe F1, corolla lobe F2, corolla tube F3, anther F4 and calyx F5). Each 0.5 cm of sterilized segments were directly cultured on Potato glucose agar medium. Single colonies were picked up and sub cultured on this medium respectively. Morphological characters of isolated fungi were visually checked and microscopic characters of these fungi were examined under microscope.

Isolation of endophytic fungi from flower of *Catharanthus roseus* (Atlas, 1993)

Endophytic fungi were isolated from flower of *Catharanthus roseus* (corolla lobe F1, corolla lobe F2, corolla tube F3, anther F4 and calyx F5). Each 0.5 cm of sterilized segments were directly cultured on Potato glucose agar medium. Single colonies were picked up and sub cultured on this medium respectively. Morphological characters of isolated fungi were visually checked and microscopic characters of these fungi were examined under microscope. Procedure for isolation of Fungi from flower of *Catharanthus roseus* (L.) G. Don.

The effect of isolated fungi F1, F2, F3, F4 and F5 on different medium

To obtain the optimum growth and production of fungal metabolites (F1, F2, F3, F4 and F5), the culture were grown in different media namely Czapak dox agar (CZA), Potato sucrose agar (PSA), Sucrose yeast agar (SYA), and Sabouraud's dextrose agar (SDA), respectively. Their observed and reversed color and sizes of colonies were examined and recorded.

Potato Glucose Agar (Atlas, 1993)	**Composition per Liter**
Peeled potato	200 g
Glucose	20 g
Agar	20 g
Distilled water	1000 ml
pH	5.6

Potato Sucrose Agar Medium (Atlas, 1993)	
Peeled potato	200.0 g
Sucrose	20.0 g
Agar	20.0 g
pH	5.6

Biochemical Tests

Urease test (Christensen, 1946)

Starch hydrolysis test (Dubey and Maherwari, 2002)

Extraction of crude compounds from the fungal isolates F1, F2, F3, F4 and F5 (Collado and Pelaez, 1996)

Pieces of fungal mycelium (3mm) were transferred to 5 petridishes containing 30 ml of potato sucrose agar medium and were stand cultured for 7 days at room temperature. The 3mm of 7 days old culture inoculum were incubated in 250 ml flask containing 100ml of Potato sucrose broth for 21 days at room temperature. The fungal mat was separated from the fermented broth using filter paper and this broth was thoroughly mixed with ethyl acetate 1:1. The organic layer containing metabolites was separated from broth layer using funnel separating method. The separated organic layer containing the metabolites was concentrated to dryness at the temperature of 70°C to evaporate the solvent. After completing solvent evaporation, the crude extracts were weighed and recorded.

Extraction of Bioactive Metabolites from Selected Fungal Strains (Strobel and Sullivan, 1999)

Selected fungal strains F-1 to F-5 were inoculated into five conical flasks containing PSB fermentation medium. 100 ml of the fermented broths in 250 ml of the flasks were incubated for 7 days at room temperature as shown in Figure.

The fermented broth 100 ml from each of the fermented flasks was separately taken out at seven days fermentation. Each separated broth (35 ml) was extracted with ethyl acetate (35 ml) by shaking in the separating funnel, and collected the solvent layer from the aqueous phase. Then, the collected solvent layer was concentrated to obtain an organic solvent extract. Ethyl acetate (1.0 ml) was added in each organic extract, and stirred with a glass rod to get the uniform extract, then the extract (60µl) was applied for each disc. The paper disc size was 10.0 mm. The test plate size was 11.5 cm. The paper disc diffusion assay was done according to the method of (Davis and Stout, 1971).

Results

The outstanding characters of *Catharanthus roseus* (L.) G. Don

ScientificName : *Catharanthus roseus* (L.) G. Don
Family Name : Apocynaceae
Elglish Name : Cape periwinkle, Rose periwinkle, Rosy periwinkle and "Old-Maid".
Myanmar Name : Thinbaw-mahnyo

Perennial shrubs. Leaves opposite and decussate, simple, laminae oblong, margin entire, apex obtuse, base cuneate both surfaces pubescent, petiolate, exstipulate. Inflorescences terminal and axillarycymose. Flowers bracteate, ebrateolate, pedicellate bisexual complete,pink; sepal 5, aposepalous, green;petals 5, sympetalous, corolla lobe (salver shape), pink with a long corolla tube, the throat of the corolla tube hairy. Stamen 5, epipetalous, anther sagittate, dithecous, dorxifixed. Ovary, bicarpellary, stigma capitate, style long, filiform, marginal placentation, superior. Fruits follicle. Seeds small, numerous. The flowering and fruiting period throughout the years.

a. Habit b. Inflorescence

Figure 1 *Catharanthus roseus* (L.) G. Don

A total of five strains of fungi were isolated from flowers of *Catharanthus roseus* (L.) G. Don that grown on Potato glucose agar medium (PGA) during 7 days by direct inoculation method. Their culturing periods of fungal isolates were significantly different. These isolates were denoted as F1 (corolla lobe), F2 (corolla lobe), F3 (corolla tube), F4 (anther) and F5 (calyx), respectively. The colony of fungal isolates F1, F2 and F3 were grown on PGA medium during 3 days but isolate F4 and F5 were well grown during 7 days. The individual colonies of filamentous fungi were picked up and kept on PGA medium to get pure colony. Their morphological and microscopic properties of fungal isolates showed differences in colony observe and reverse color. The observe color of colonies on PGA of isolate F1 and F5 were white margin and center black, F2 was white and F3 was cottony white and F4 was margin pale yellow and center black. The reverse color of fungal isolates had F1, F3 and F5 were white, pale orange in F2 and yellow in F4 respectively. The sizes of colony on PGA medium were all a like (30x30 mm) except F3 (40x40 mm). The colony characters and microscopic characters of isolated fungi from flower of *Catharanthus roseus* shown Figures 2 to 7.

Microscopic Character of fungal isolates F1, F2, F3, F4 and F5

Fungal isolate F1

Hyphae were septate, hyaline and conidiophore were long, erect, and conidia head round. Conidia were 1- celled and round.

Fungal isolate F2

Hyphae were septate, hyaline. Sporangiophores were hyaline, short and often banched and bear terminal round spore-filled sporangia, brown. The sporangial walls were dissolved. Spores were round or oblong, numerous spores present in sporangia.

Fungal isolate F3

Hyphae were septate, hyaline and conidiophores were long, erect, globose and dark. Vesicle was globose. Phialide were biseriate, covering the entire surface of the vesicle. Conidia were 1- celled, chain, mutulae present, round, black, wall with spine.

Fungal isolate F4

Hyphae were septate and hyaline. Sporangiophores were long, erect and sporangium head globose, round and radiate. The spores were round.

Fungal isolate F5

Hyphae were septate, hyaline and conidiophore were long, erect, and conidia head round. Conidia were 1- celled and round.

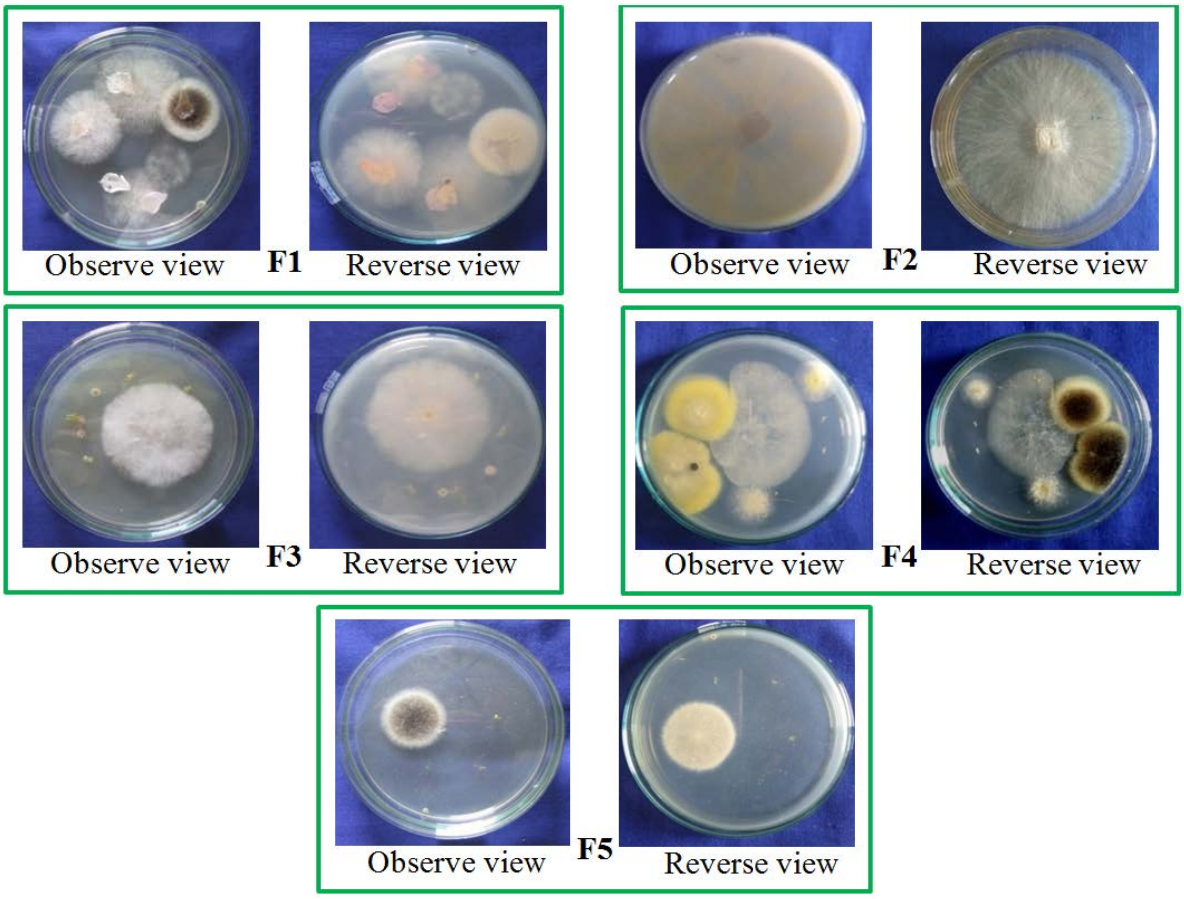

Figure 2 Isolated fungi F1, F2, F3, F4 and F5 from flower of *Catharanthus roseus* (L.) G. Don. grown on Potato Glucose Agar medium

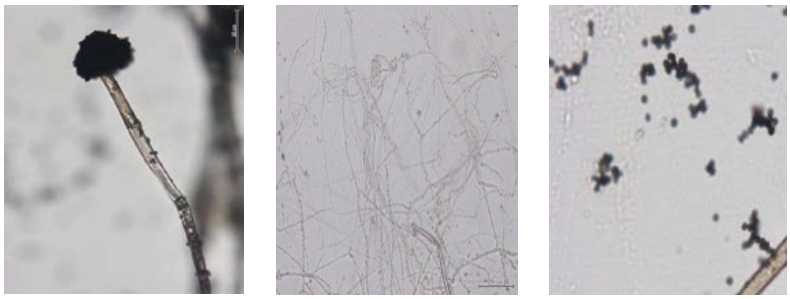

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Figure 3 Microscopic character of isolated strain F1

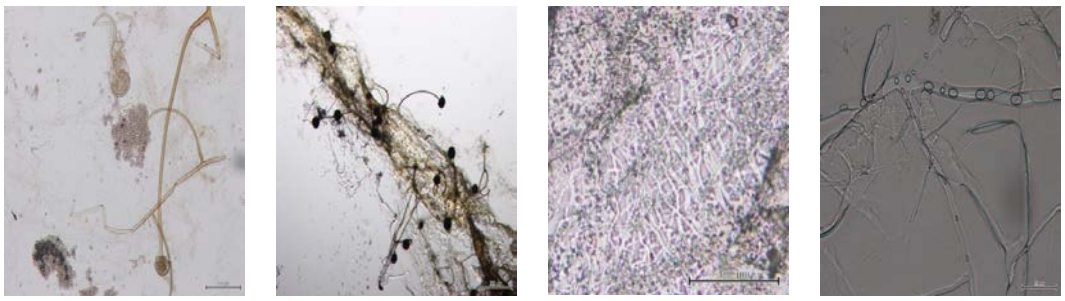

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Figure 4 Microscopic character of isolated strain F2

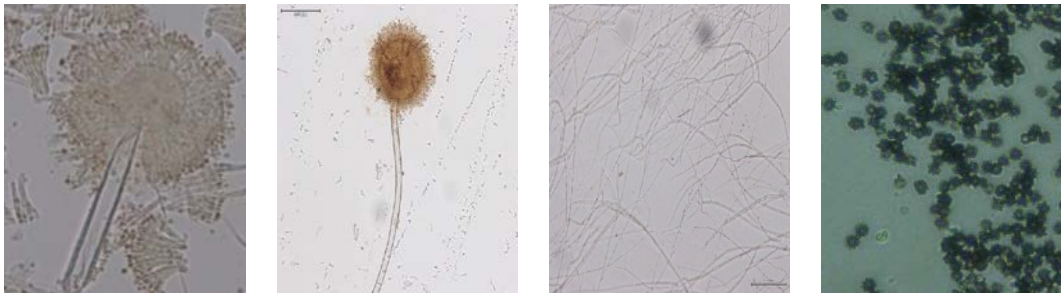

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Figure 5 Microscopic character of isolated strain F3

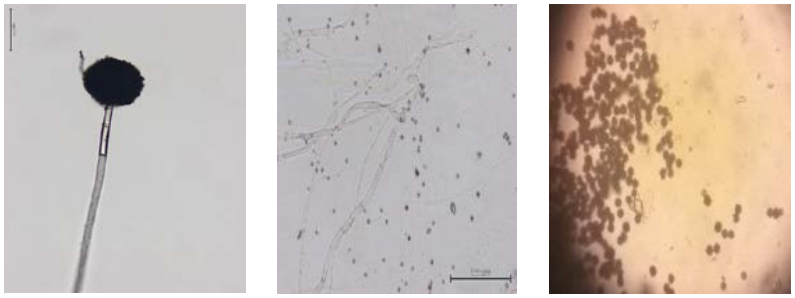

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Figure 6 Microscopic character of isolated strain F4

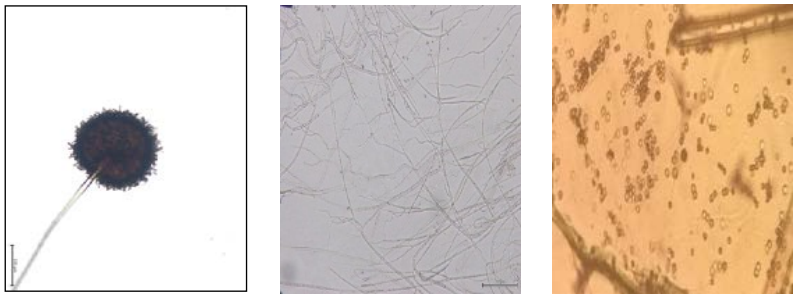

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Figure 7 Microscopic character of isolated strain F5

Effect of different media for antimicrobial metabolite production

These fungal isolates F1, F2, F3, F4 and F5 were cultured on Czapek dox agar (CZA), Potato dextrose agar (PDA), Potato sucrose agar (PSA), Sucrose yeast agar (SYA), Yeast malt extract agar (YMA) and Sabouraud's dextrose agar (SBA). Among these media, all fungal isolates were well grown on potato sucrose medium. The largest size of colony was observed in F2 on Potato sucrose agar (20×20 mm) followed by the second largest size 14×12mm in F5, 11×12 mm in F3, 18×18 mm in F1 and 16×20 mm in F4. So, potato sucrose media were also used as fermentation medium for antimicrobial activities.

Antimicrobial activities of potato sucrose fermented broth extracts from fungal isolates F1, F2, F3, F4 and F5

In Potato sucrose broth, the effective activity of antimicrobial test showed at 4-5 day. The potato sucrose broth was suitable for the production of antimicrobial metabolites from these endophytic fermented broth extracts as F3 showed maximum diameter zone of growth inhibition was observed by against *E. coli* (16.4 mm) followed by *S. aureus* (15.2 mm) and *B. Subtilis* (15.3 mm). Similarly, the maximum zone of inhibition was produced by F5 towards *B. subtilis*,

(15.5 mm) diameter zone of inhibition, *X. oryzae* (15.7mm) and *S. cerevisiae* (15.0 mm). It is therefore this medium should be further used for extraction of secondary metabolites from isolated endophytic fungi F1, F2, F3, F4 and F5 from the flower of *Catharanthus roseus* (L.) G. Don.

Table 1 Antimicrobial activities of fermented broth extract from isolate F1 in PSB

Test Organisms	measurement of inhibitory zones(mm)									
	D1/ pH6	D2/ pH5	D3/ pH4	D4/ pH3	D5/ pH2	D6/ pH2	D7/ pH2	D8/ pH2	D9/ pH1	D10/ pH1
E.coli	-	-	-	-	10.0	9.2	7.2	9.6	10.6	-
S.aureus	-	-	-	7.4	8.2	9.2	-	-	-	-
B.subtilis	-	-	-	8.4	11.2	9.4	8.0	-	-	-
X.oryzae	-	-	-	-	-	-	7.2	10.6	10.0	-
A.tumefaciens	-	-	-	7.4	11.2	12.0	7.0	7.0	7.0	-
S.typhi	-	-	-	-	7.0	8.6	9.8	9.8	9.2	-
P.aureginosa	-	-	-	-	11.6	8.2	8.0	8.2	8.0	-
S.cerevisiae	-	-	-	-	8.2	8.0	8.0	8.0	7.0	-
A.flavus	-	-	-	-		-	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-

10-12mm = weak activity,
13-18mm = high activity,
>18mm= very high activity
Size of Paper disc = 6mm/D=Day C = Control with potato glucose broth
Test organisms
Escherichia coli,
Staphylococcus aureus,
Bacillus subtilis,
Xanthomonas oryzae,
Agrobacterium tumefaciens,
Salmonella typhi,
Pseudomonas auregiosa,
*Saccharomyces cerevis*iae,
Aspergillus flavus,
Candida albican

Table 2 Antimicrobial activities of fermented broth extract from isolate F2 in PSB

Test Organisms	measurement of inhibitory zones(mm)									
	D1/ pH6	D2/ pH6	D3/ pH5	D4/ pH4	D5/ pH3	D6/ pH2	D7/ pH2	D8/ pH2	D9/ pH1	D10/ pH1
E.coli	-	-	-	7.0	9.0	10.0	9.0	12.0	7.0	7.0
S.aureus	-	-	-	7.0	8.0	7.0	8.0	-	-	-
B.subtilis	-	-	-	11.0	7.0	10.0	-	-	-	
X.oryza	-	-	-	7.0	7.0	-	-	-	-	-
A.tumefaciens	-	-	-	7.0	7.0	9.0	8.0	-	-	-
S.typhi	-	-	-	7.0	7.0	10.0	-	-	-	-
P.aureginosa	-	-	-	7.0	7.0	-	-	-	-	-
S.cerevisiae	-	-	-	8.0	7.0	9.0	-	-	-	-
A.flavus	-	-	-	-	-	-	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-

10-12mm = weak activity,
13-18mm = high activity,
>18mm= very high activity
Size of Paper disc = 6mm/D=Day C = Control with potato glucose broth
Test organisms
Escherichia coli,
Staphylococcus aureus,
Bacillus subtilis,
Xanthomonas oryzae,
Agrobacterium tumefaciens,
Salmonella typhi,
Pseudomonas auregiosa,
*Saccharomyces cerevis*iae,
Aspergillus flavus,
Candida albican

Table 3 Antimicrobial activities of fermented broth extract from isolate F3 in PSB

Test Organisms	measurement of inhibitory zones(mm)									
	D1/ pH6	D2/ pH6	D3/ pH5	D4/ pH4	D5/ pH3	D6/ pH2	D7/ pH2	D8/ pH2	D9/ pH1	D10/ pH1
E.coli	-	-	-	12.0	16.4	11.4	9.8	11.2	10.0	10.0
S.aureus	-	-	-	8.4	11.8	15.2	10.2	9.6	9.6	10.0
B.subtilis	-	-	-	13.0	15.3	10.0	9.6	9.6	10.2	10.2
X.oryza	-	-	-	7.0	11.5	11.2	15.0	12.2	9.4	9.2
A.tumefaciens	-	-	-	10.0	12.2	13.8	10.0	8.2	8.2	8.2
S.typhi	-	-	-	9.4	9.0	11.2	10.0	9.2	9.2	9.6
P.aureginosa	-	-	-	8.0	15.0	10.0	10.4	9.8	9.2	9.2
S.cerevisiae	-	-	-	9.4	9.0	9.0	8.4	8.2	7.2	7.0
A.flavus	-	-	-	-	-	8.0	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-

Table 4 Antimicrobial activities of fermented broth extract from isolate F4 in PSB

Test Organisms	measurement of inhibitory zones(mm)									
	D1/ pH6	D2/ pH6	D3/ pH5	D4/ pH4	D5/ pH3	D6/ pH2	D7/ pH2	D8/ pH2	D9/ pH1	D10/ pH1
E.coli	-	-	-	7.2	7.8	6.4	-	-	-	-
S.aureus	-	-	-	7.0	9.2	-	-	-	-	-
B.subtilis	-	-	-	9.6	9.6	8.8	-	-	-	-
X.oryza	-	-	-	-	-	-	-	7.8	7.0	7.0
A.tumefaciens	-	-	-	10.2	8.6	-	-	-	-	-
S.typhi	-	-	-	-	-	-	-	-	8.0	7.4
P.aureginosa	-	-	-	9.2	-	-	9.2	-	-	-
S.cerevisiae	-	-	-	9.0	-	-	-	-	-	-
A.flavus	-	-	-	-	-	-	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-

Table 5 Antimicrobial activities of fermented broth extract from isolate F5 in PSB

Test Organisms	measurement of inhibitory zones(mm)									
	D1/ pH6	D2/ pH 5	D3/ pH4	D/4 pH3	D5/ pH3	D6/ pH2	D7/ pH2	D8/ pH2	D9/ pH 1	D10/ pH 1
E.coli	-	-	-	7.6	10.2	11.4	10.0	10.0	10.0	-
S.aureus	-	-	-	8.2	15.0	15.0	11.2	11.0	9.8	7.0
B.subtilis	-	-	-	7.0	15.5	10.0	10.4	10.6	-	-
X.oryzae	-	-	-	8.0	15.7	11.2	9.2	9	9.0	-
A.tumefaciens	-	-	-	10.6	10.8	13.8	12.0	12.0	-	-
S.typhi	-	-	-	9.8	12.4	11.2	12.0	10.2	7.2	-
P.aureginosa	-	-	-	-	11.4	10.0	9.4	9.8	9.5	-
S.cerevisiae	-	-	-	9.4	13.2	15.0	8.2	10.0	7.0	-
A.flavus	-	-	-	-	-	-	-	-	-	-
C.albicans	-	-	-	-	-	-	-	-	-	-

10-12mm = weak activity,
13-18mm = high activity,
>18mm= very high activity
Size of Paper disc = 6mm/D=Day C = Control with potato glucose broth
Test organisms
Escherichia coli,
Staphylococcus aureus,
Bacillus subtilis,
Xanthomonas oryzae,
Agrobacterium tumefaciens,
Salmonella typhi,
Pseudomonas auregiosa,
*Saccharomyces cerevis*iae,
Aspergillus flavus,
Candida albican

Extraction of secondary metabolites from F1, F2, F3, F4 and F5 with ethyl acetate

The crude extracts of all isolated fungal strains was studied for test organisms. Among isolates F1, F2, F3, F4 and F5 showed the highest yield of crude extracts obtained from isolate F1, F3 and F4 (0.2 g) followed by F2 and F5 (0.1 g). All extracts gave brown color except F2 (yellow). The highest fresh weight was obtained from F2 (9.6 g) followed by F4 (8.4 g), F3 (8.2 g), F5 (7.2 g) and the least fresh weight was obtained from F1 (5.2 g). The maximum amount of mycelium biomass was obtained from F3 (2.2 g), followed by F4 (1.9 g). The biomass of mycelium was observed by F2 (1.7 g) and (1.5 g). The 30µl of all of these fungal secondary metabolites extract the showed effectively against tested organisms. The isolates F1 and F3 showed maximum activity against *A. tumefaciens* with a diameter of inhibition zone 46 mm followed by *S. aureus* with a diameter 42 mm. Among these fungal isolates, the F3 and F5 showed the highest activity because the zone of inhibition of test organisms ranges between 30-40 mm and 28-40 mm. The results of extraction of antimicrobial secondary metabolites from fungal isolates F1, F2, F3, F4 and F5 with ethyl acetate indicated in Table 6, Figures 8 to 12.

Table 6 Antimicrobial activities of fermented broth extract from isolated strains

Isolated strains / Test organisms	F1	F2	F3	F4	F5
Escherichia coli	35	35	35	35	30.5
Staphylococcus aureus	42	30	30	30	28
Bacillus subtilis	40	27	35	30	28
Xanthomonas oryzae	22	30	40	35	33
Agrobacterium tumefaciens	46	35	35	30	40
Salmonella typhi	35	42	40	30	30
Pseudomonas aeruginosa	40	38	38	30	32
Saccharomyces cerevisiae	40	30	36	35	30.5
Aspergillus flavus	35	22	35	27	40
Candida albicans	30	37	35	35	*40*

Size of paper disc = 10mm, Control = 95% ethyl acetate

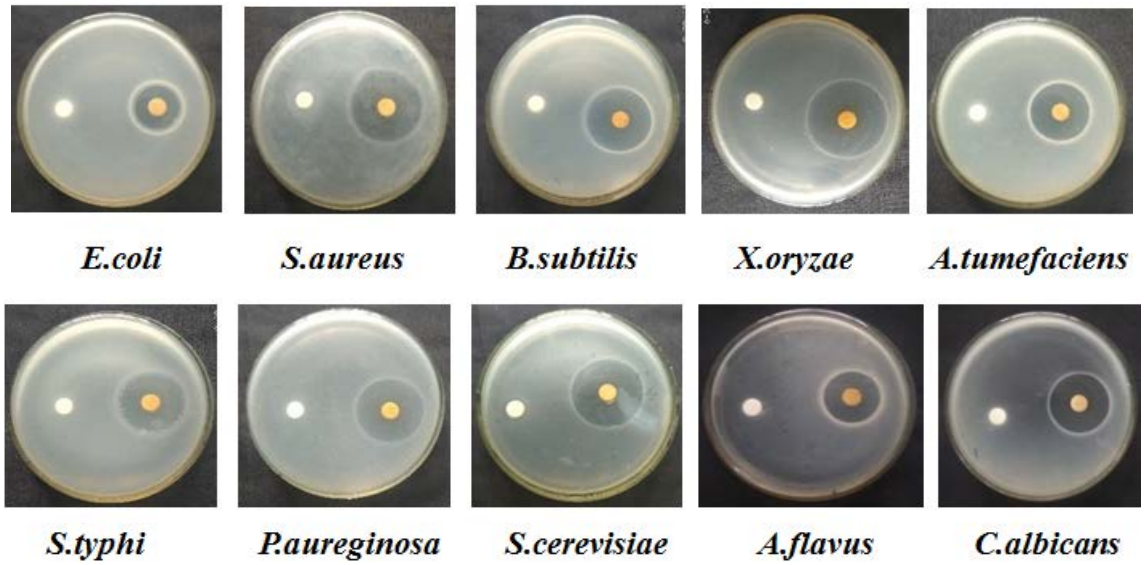

Figure 8 Inhibitory zones of Isolated strain F1 from crude extract

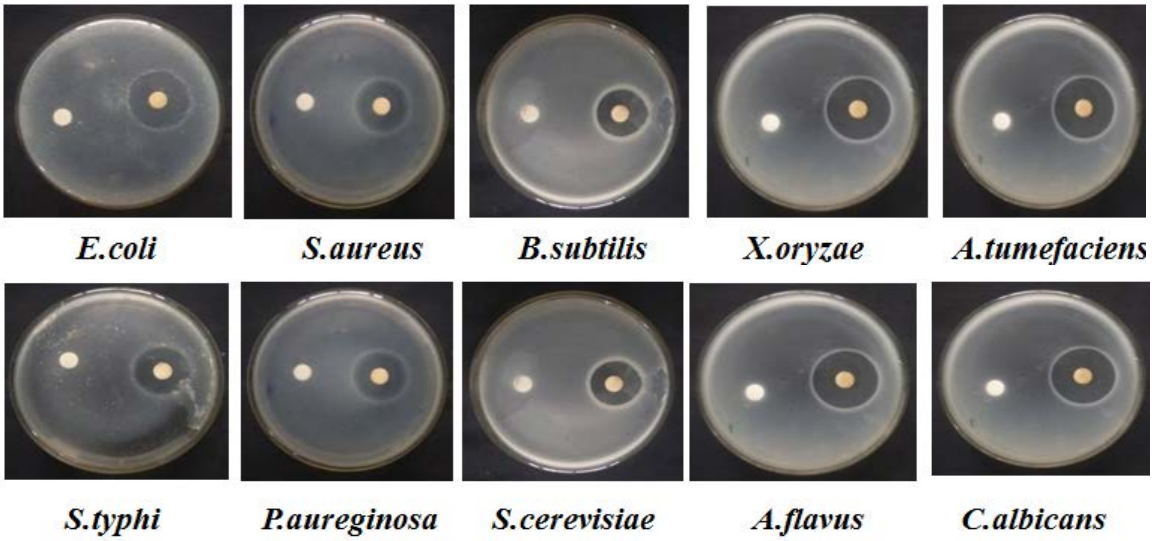

Figure 9 Inhibitory zones of Isolated strain F2 from crude extract

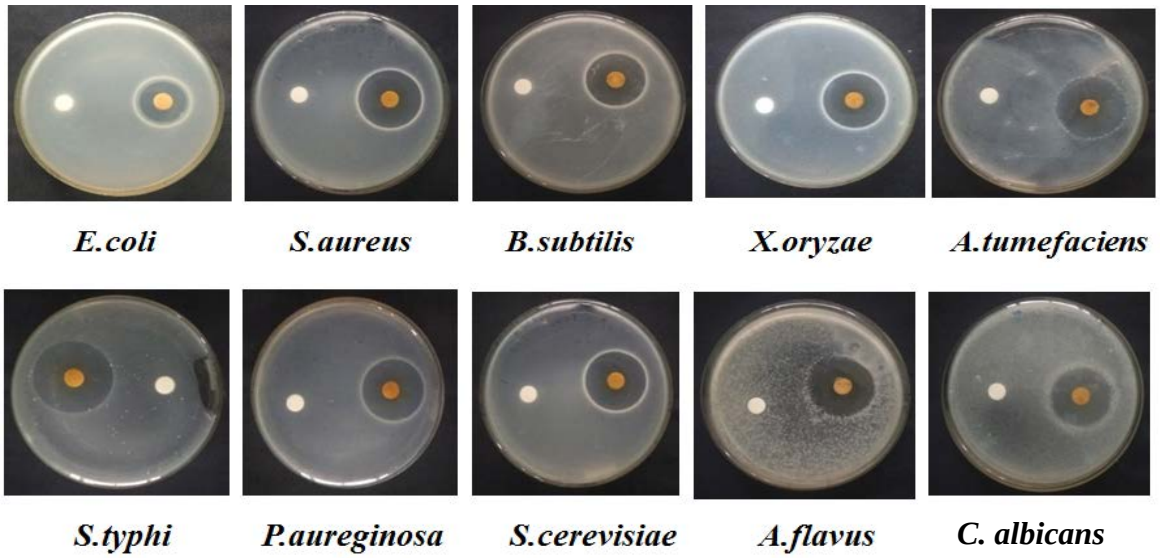

Figure 10 Inhibitory zones of Isolated strain F3 from crude extract

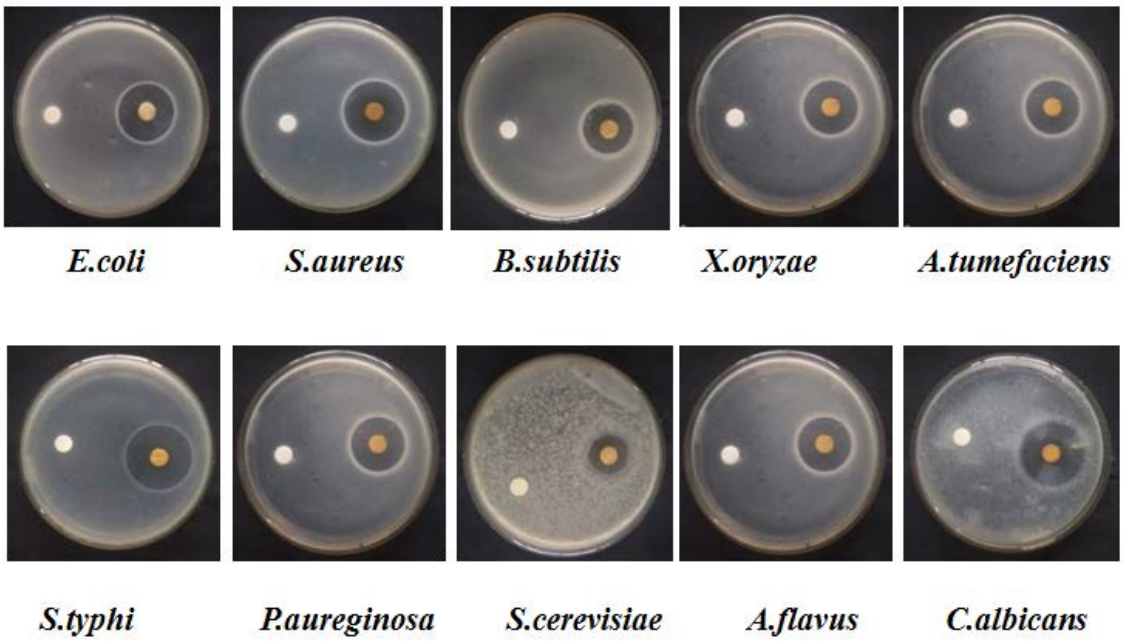

Figure 11 Inhibitory zones of Isolated strain F4 from crude extract

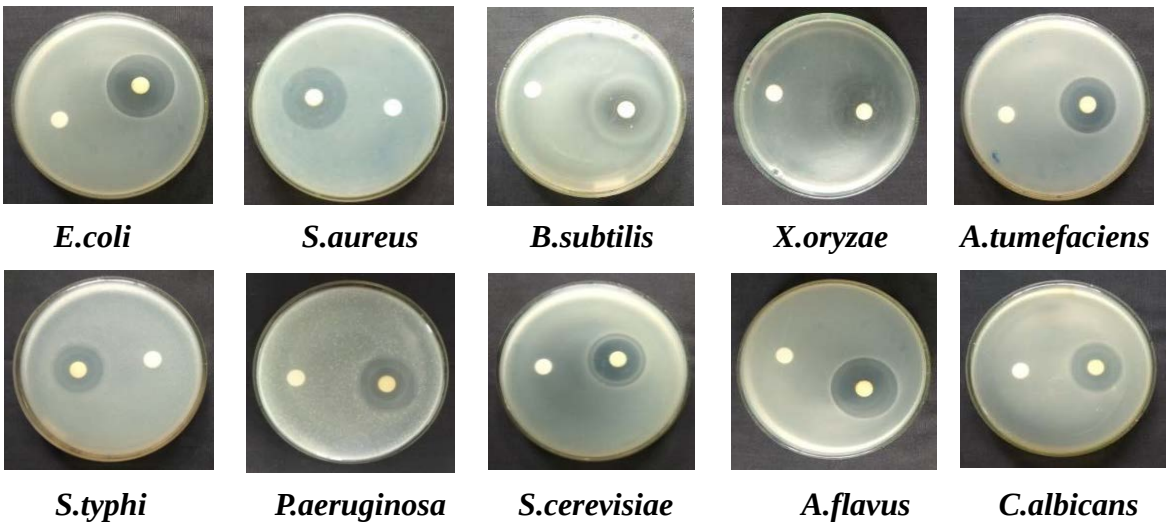

Figure 12 Inhibitory zones of Isolated strain F5 from crude extract

*E.coli = Escherichia coli, S.aureus = Staphylococcus aureus, B.subtilis = Bacillus subtilis, X.oryzae = Xanthomonas oryzae, A.tumefaciens = Agrobacterium tumefaciens, S.tyhi = Salmonella typhi, P.aeruginosa = Pseudomonas aeruginosa, S.cerevisiae = Saccharomyces cerevis*iae, *A.flavus =Aspergillus flavus, C.albicans = Candida albican*

Discussion and Conclusion

The flower specimens of *Catharanthus roseus* (L.) G. Don was collected from Hlaing, Township,Yangon Region. It belongs to the family Aypocynaceae. Their morphological character of this plant was confirmed the scientific name with the literature of Dassanayake and Fosberg (1981) and Soe Myint Aye (2002).The five strains of fungi were isolated from flower of *Catharanthus roseus* (L.) G. Don. that grown on Potato glucose agar medium. These isolated

fungi were denoted as F1, F2, F3, F4 and F5. Based on their morphological and microscopic character, these isolated fungi F2 and F3 were identified into Genus Level

(*Mucor* and *Aspergillu*s).

Ayob and Simarani, (2016) observed two strains of filamentous fungi from violet color of *Catharanthus roseus* showed hyphae septate and spore rounded shaped. Sreekanth *et al*., (2017) found that twenty-five endophytic fungi were isolated from different tissue of Catharanthus roseus. The 48% from leaves, 44% from stems and 4% from each of the roots and flowers were investigated.

Kidd *et al*., (2016) said that colony of *Mucor were* very fast growing white to yellow with the development of sporangia, sporangiophore hyaline, erect, simple, globose to spherical, multispore present in sporangia. As characters of fungal isolate F2 are in accordance with Kidd *et al*., (2016).

During the starch hydrolysis and urease test, the isolates F2, F3 and F5 were positive effect on starch hydrolysis test as well as only F2 did not produce urease enzyme that bioactive compound were explored from endophytic fungi *Aspergillus fumigatus* strain KARVSO4 from Piper crocatum Ruiz and Pav. More components were observed in ethyl acetate extracts indicating its potential exploration for other bioactive substances having pharmaceutical values. *Aspergillus niger* and *A. flavus, Eurotium amsteldom* and *Fusarium* sp. showed positive effect on starch hydrolysis and urease tests but urease activity was only found in *Aspergillus niger* (Shivanni *et al.,* 2015). The amylase, lipase, pectinase and protease activity was observed in several species of *Muco*r. Alves *et al.,* (2006). The amylase and protease could be produced by *Aspergillus niger* and *Aspergillus flavus* (Ayanda, 2013).

So the characters of isolated fungi F1, F2, F3, F4 and F5 were agreed in Alves *et al*., (2006), Ayanda (2013) and Shivanni *et al.,* (2015).

Barnett and Hunter (1979) observed that the conidiophore of *Aspergillus* sp. were clevate swelling bearing phialides at the apex or rading from the entire surface and conidia are one celled, globose often various color and mass and dry basipetal chains.

Pumphrey and Christran (1996) observed that glucose is the most readily metabolized sugar but most fungi could use sucrose and they require ammonia, nitrate and nitrite for nitrogen sources. Zhang *et al*., (2012) discovered that the 11 strains of endophytic fungi were separated from the healthy stems of *Artemisa annua. Aspergillus* sp. isolated from *Artemisa annua* showed actively against *Escherichia coli and Staphylococcus aureus, Tricoderma rubrum* but *Mucor sp.* indicated against *Rhizotonia cerealis.*

Potato dextrose broth and ethyl acetate solvent was suitable for secondary metabolites extraction from *Aspergillus* sp. and *Mucor* sp. isolated from *Artemisa annua.* Astuti (2017) observed that bioactive compounds were explored from endophytic fungi *Aspergillus fumigatus* strain KARVSO4 from *Piper crocatum* Ruiz & Pav.

The best bioactive compound producing medium was Potato dextrose broth at 29ºC and pH 5 supplemented with starch or fructose as carbon sources and nitrogen sources. More components were observed in ethyl acetate extracts indicating its potential exploration for other bioactive substances having pharmaceutical values. The result of finding in antimicrobial activity of isolated F1, F2, F3, F4 and F5 were in accordance with previous finding Pumpharey and Christran (1996) and Zhang *et al*., (2012).

To sum up, all isolated fungal secondary metabolite broth extracts exhibited antimicrobial activities in all strains of test organisms. The results showed that among them, Potato sucrose broth was found to be the best for extraction of secondary metabolites and only ethyl acetate

solvent was used to extract secondary metabolites from these fungal strains F1, F2, F3, F4 and F5.

In this experiment, the F1 extract showed highly against *Agrobacterium tumefaciens* with a diameter 46 mm and *Staphylococcus aureus* with a diameter 42 mm but F3 and F5 was the zone of inhibition of tested organisms range between 30-40 mm and 28-40 mm. Isolated F3 and F5 broth extracts showed broad spectrum activity against *Escherichia coli* and *Xanthomonas oryzae* and ethyl acetate extracts showed good activity in antimicrobial tests. These isolated fungal metabolites could be affected on human respiratory tract problems, skin disease, dysentery and phytopathogens. The result of finding showed that ethyl acetate extracts secondary metabolites isolated from flower of *Catharanthus roseus* should be further studied for purification of compounds for medicine and pharmaceutical uses.

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GONAD DEVELOPMENT AND CONDITION INDEX OF GREEN MUSSEL, *PERNA VIRIDIS* (LINNAEUS, 1758) FROM YE ESTUARY, MON STATE

Win Win Nwe[1]

Abstract

The study on the gonad development and condition index of green mussel, *Perna viridis* from Ye Estuary was conducted from January to December 2017. Sample collection was made by the help of local mussel collector during low tide. Ripe gonads were in good numbers from January and March and peak spawning ones were observed from April to July.The occurrence of spawning and partially spent specimens of both sexes recorded from April to October indicated that spawning took place during a prolonged period. *P. viridis* exhibited year-round gametogenesis and monsoon spawning. Surface water temperature at Ye estuary varied between 27.60°C in August and 32.00°C in March. CI_{shwt} and $CI_{commercial}$ values were high in June and low in July and December. High CI values in June may be due to ripe gonad states and food available from the river runoff. The low CI value in July coincides with the peak spawning stages of male and female mussels. The study indicated that there is obvious seasonal variation in the mussel condition and gonad maturation.

Keywords Condition index, Gonad Development, Mon State, *Perna viridis*, Ye estuary

Introduction

Green mussel, *Perna viridis* is a commercially important mussel species that distributes widely in the Indo-Pacific regions. It can grow in the optimum temperature ranging from 26 ºC to 32ºC and salinity ranging from 27‰ to 33 ‰ (Power *et al*., 2004). Sexes of this species are separate and gonad tissue of a sexually matured male shows creamy-white in colour, while that of the female appears reddish (Al-Barwani *et al*., 2013). *P. viridis* in tropical countries has been shown to spawn all year-round with two peaks which coincide with monsoon seasons (Soon & Ransangan, 2014).

Condition index (CI) is generally regarded as an indicator of the health status of mussels and the commercial quality of bivalve population (Crosby and Gale, 1990; Al-Barwani *et al*., 2011). The biotic and abiotic conditions of the environment such as food availability, temperature and salinity control the condition of mussel (Seed and Suchanek, 1992). Poor conditions in bivalves can be resulted by the limitations in food availability that is caused by unfavourable changes in environmental conditions (Bayne, 1976). Through the study of the condition index of *P. viridis* in Ye estuary, the spawning time of this mussel can also be estimated. The data observed from the mussels in Ye Estuary can also serve as baseline information for assessing the impacts of any future changes in the ecosystem.

Materials and Methods

Study area

Sample collection was conducted at Sitaw, Ye estuary (Lat. 15º 11' N, Long. 97º 48' E). Sitaw in Ye estuary is an intertidal rock shore area and rich mussel beds are located in subtidal rocky stretches up to the river (Fig. 1). This area is known for a daily level fishery of green mussel and oysters, locally called Be Won and Ka Mar. Mussels are removed from the natural beds, mainly for local consumption as food and for local fishermen's income. The mean values of salinity and temperature were monthly recorded by using a water monitor and refractometer.

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There were marked fluctuations in the salinity during monsoon seasons. The average salinity of the surface water varied from 6.38‰ in June to 30.5‰ in March. Surface water temperature ranged from 27.60°C in August to 32.00°C in March. Seasonal variations in the environmental parameters of Ye Estuary are primarily influenced by the prevailing monsoon regime. During the study period, the southwest monsoon commenced by the last week of May and the highest rainfall of 1628.14 mm was recorded in July 2017.

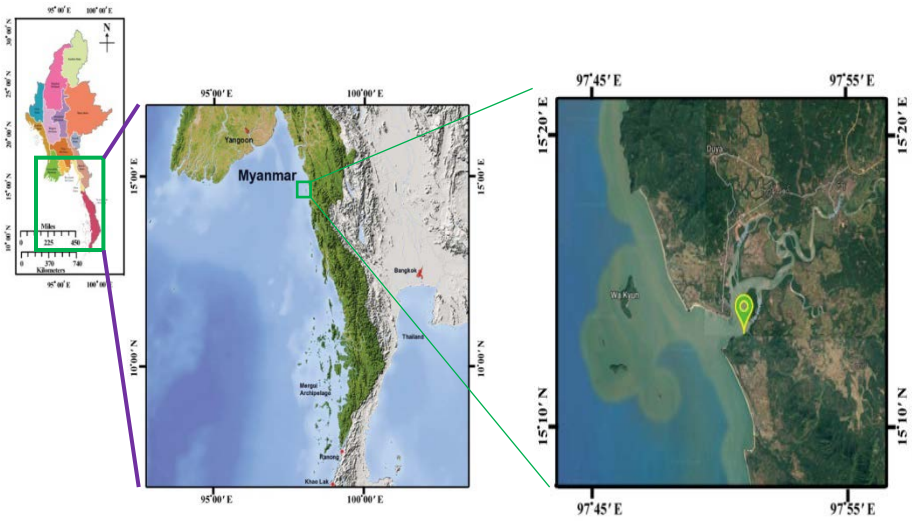

Figure 1 Map showing the sample collection site in Sitaw, Ye Estuary (Source: Google Maps).

Sample Collection

Samples were randomly collected from the subtidal rocky natural mussel beds under about 10 meters in Ye estuary on a monthly basis by the local mussel collector. The collection was made during low tide and samples were collected by using iron implements like chisels and kept in fishing net bags tied around their waist. The collected mussels were cleaned and extraneous water was wiped and transported to the laboratory using the insulated box.

Histological analysis of seasonal gonad development

For the study of gonad development, tissue sections were cut and processed according to standard histological techniques (Howard *et al.*, 2004). Sections of mantle, male and female gonads were immersed in Davidson's fixative (Shaw and Battle, 1957) for one week. The fixed tissues were washed in running tap water overnight and then dehydrated by the usual procedure in ascending grades of alcoholic series as 50%, 70% and 90% alcohol for 1 h, and 100% alcohol for 30 min. The tissues were first soaked in Alcohol-Acetone solution (1:1) for 30 min and then in xylem for 30 min to remove alcohol. After that tissues were infiltered in 2-3 changes of molten paraffin of melting point 58-62°C, and then embedded in wax at 58-60°C, made into blocks which were labeled. Paraffin blocks were trimmed to a suitable size and sections of tissue were cut using a microtome at 10 µm thickness. The resulting ribbons containing tissue sections were fixed on the glass slides using Mayer's egg albumin glycerol (1.1v/v) as an adhesive. Slides were placed in xylene to deparaffinise and were given brief dips in grades of alcohol (100%, 90%, 70% - 20 minutes each) to dehydrate. Hydrated slides were stained in Herris' hematoxylin (2-5 min). Sections were de-stained in acid alcohol, and counter stained in Eosin (1min). Slides were further dehydrated in alcohol and then transferred to xylene (15 min). Slides were mounted in DPX and a cover slip was applied and labeled. Thin layer of albumin was used as an adhesive. Sections were observed and photographed under a light microscope. For the interpretion of tissue structures and the stages of male and female gonads were followed

by the works of Narasimham (1980), Vasanthi *et al*., (2004), Hagger *et al*., (2008), Soumady (2012) and Mcfarland (2016).

Condition index

For the study of condition index (CI), the total weights of 50 mussels were first determined up to 0.01 g by using an electronic digital compact scale (SF-400A). Mussels were then opened; identified sex; tissue was removed from the shell and blotted to remove excess water to ensure accuracy before weighing the tissue. The individual weight of tissue and shell were then determined. CI_{shwt} recommended by Lucas and Beninger (1985) was used for calculating mussel condition index (CI).

$$CI_{shwt} = \frac{\text{Dry soft tissue weight (g)}}{\text{Dry shell weight (g)}} \times 100\,\%$$

According to the recommendation of Hickman and Illingworth (1980), $CI_{commercial}$ (meat yield) was used for studying the variations in wet meat percentage or the percentage edibility (% edibility).

$$CI_{commercial}\,(\text{Meat yield}) = \frac{\text{Wet tissue weight (g)}}{\text{Whole (live) weight (g)}} \times 100\,\%$$

To evaluate the variation of monthly CI values with reference to the mean CI during the study period, a CI ratio was calculated as:

$$\text{CI ratio} = \bar{x}\ CI_{month} / (\bar{x}\ CI_{all\ month}).$$

Based on the values obtained, the monthly CI which exceeded its annual mean was classified as "high (CI ratio >1)" and the remaining as "low (CI ratio <1)" (Hickman, 1991).

Result and Discussion

Gonad Development

The gonadal tissue of a sexually matured male mussel appears creamy-white in color, while that of the female is bright orange in color (Fig. 2 A & B). From the gonad examinations, it was observed that 50% of the mussel of both sexes were matured by about 40 mm. However, Sreenivasan *et al*., (1989) observed that the sexes were matured by about 20 mm and Power *et al*., (2004) reported that sexual maturity occurs at 15-30 mm shell length.

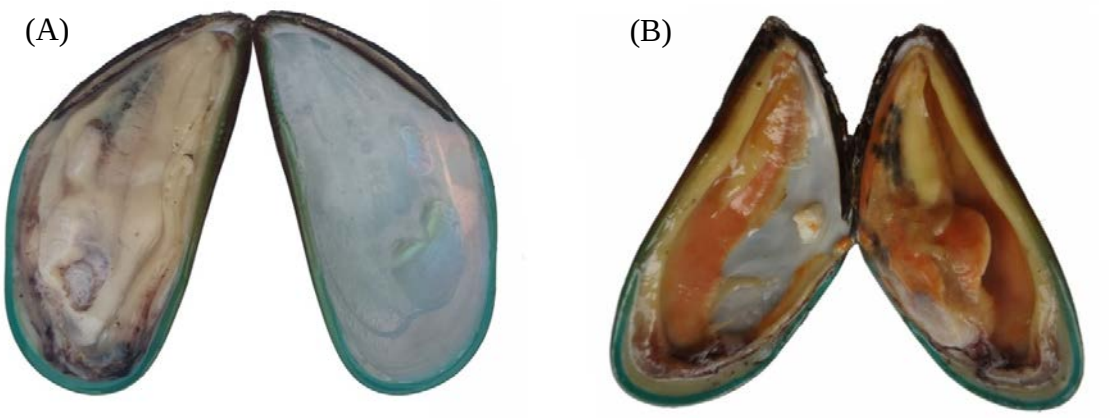

Figure 2 The internal morphology of male and female mussels, *Perna viridis* (A) ♂ Male with milky color, (B) ♀ Female with bright orange color.

Four main stages were distinguished in the reproductive cycle of *Perna viridis* from Ye estuary (Fig. 3 & 4). The percentage composition of different stages of maturity of males and females showed that developing gonads (Stage I) were encountered in November and December in male mussels and October to December in female ones. Ripe gonas (Stage II) were in good numbers from January and March, while partially spawned mussels (Stage III) were found in good numbers from April to July in both sexes. Spent and spent resorbtive individuals (Stage IV) were predominant from September to October in male mussles and August to September in female ones. However partially spawned mussels were observed in almost all months (Fig. 5 A & B).

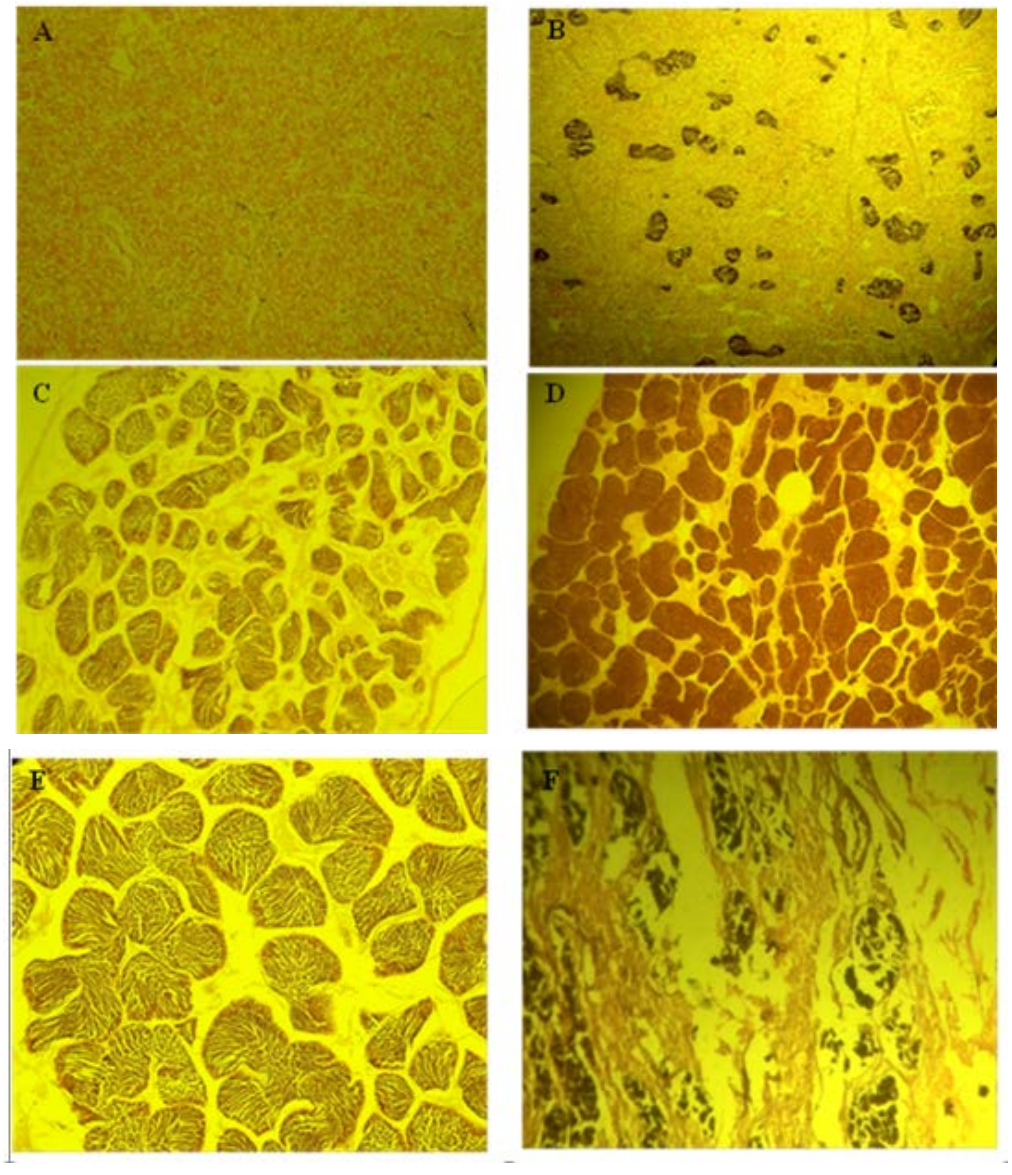

Figure 3 Photomicrographs of male gonads at different stages in sexual cycle of *Perna viridis* (10 x).

(A-C) developing/re-developing stages; (D) ripe gonads; (E) spawning in progress; (F) spent/resting gonads.

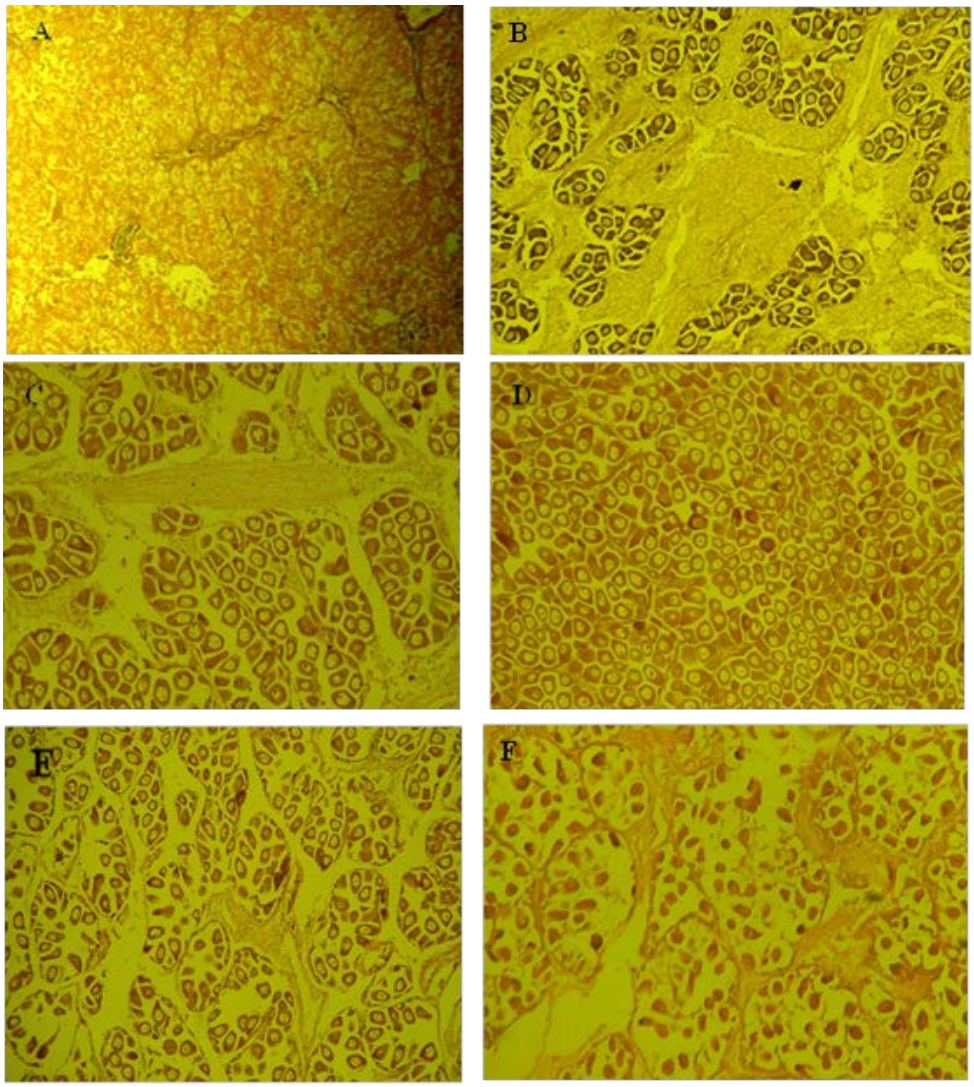

Figure 4 Photomicrographs of female gonads at different stages in sexual cycle of *Perna viridis* (10 x).(A-C) developing/re-developing stages; (D) ripe gonads; (E) spawning in progress; (F) spent/resting gonads.

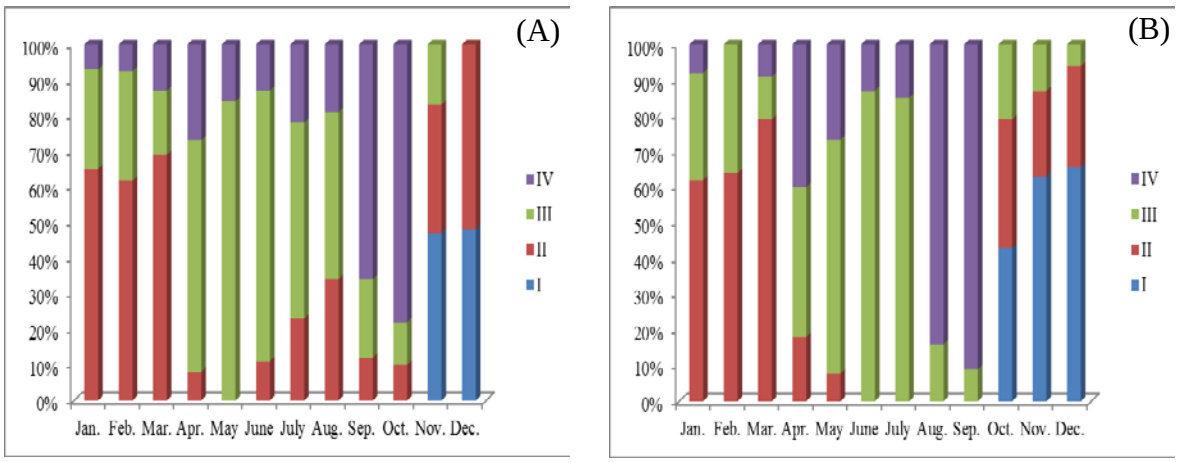

Figure 5 Stages of maturity among males and females *Perna viridis*.: A) Male; B) Female (I: developing/re-developing stage; II: ripe stage; III: spawning stage; IV: spent/resting stage)

The occurrence of spawning and partially spent specimens of both the sexes recorded from April to October indicates that spawning took place during a prolonged period. Though there was a drop in salinity and temperature during the monsoon season (May to October), ripe and spawning individuals continued to be present. This indicates that *Perna viridis* could spawn in salinities less than 30‰ also. Ripe individuals of both sexes were recorded with more numbers from January to March and peak spawning ones were observed from April to July. This indicates that there was peak spawning among green mussels during these months. Narasimham (1980) reported two main spawning periods for *P. viridis* from the east coast of India and stated that the spawning periods were seemingly associated with the seasonal distribution of temperature. Lee (1985) reported a single breeding period that extended from June to September for *P. viridis* population in the Victoria Harbour, Hong Kong. However, Cheung (1993) reported two breeding periods (July-September & November-March) per year for the population dynamics of *P. viridis* in the Tolo Harbour, Hong Kong. Yoshiyasu *et al*., (2004) reported that *P. viridis* in the Sagami Bay, Japan, reproduces successfully and spawns from summer to early autumn.

During the study period, the peak reproductive activity in April for male mussels and in May for female ones coincided with after rising in temperature in March (32.20˚C). Rajagopal (1998 a, b) stated that the temperature regulates the onset of reproductive activity of the mussels along the southeast coast of India. Chen *et al*., (1998) also reported that the spawning period, May-September, was observed when the temperature is 23-26°C. Rajagopal *et al*., (1998a) indicated that peak reproductive activity of *P. viridis* coincided with rising water temperature along the east coast of India. Temperature and food availability have been demonstrated as important decisive factors for somatic growth and gonadal development in bivalves (Seed and Suchanek, 1992; Ceballos *et al*., 2000). The annual reproductive cycle of *P. viridis* has a direct bearing on the degrees of fatness or condition, which has been related to environmental factors such as temperature, salinity and food availability (Rao *et al*., 1975; Nagabhushanam and Mane, 1975; Qasim *et al*., 1977; Ajithakumar, 1984; Parulekar *et al*, 1982; Rivonker *et al*., 1993 and Rajagopal *et al*., 1998a).

Condition Index (CI)

Variations in the value of condition index (CI) were studied by two methods; percentage of meat weight in shell weight of the mussel (CI_{shwt}) and meat weight in total weight of the mussel ($CI_{commercial}$). The highest CI_{shwt} (74.46 %) was recorded in June and the lowest (41.86 %) in December 2017 (Fig. 6A). Wet meat percentage or percentage edibility ($CI_{commercial}$) also followed similar trends as CI, with the highest meat percentage in March and June before spawning, which declined sharply after spawning in July. $CI_{commercial}$ values ranged from 25.61% in July to 37.32 % in June.The range of wet meat percentage observed in the study was comparable with the observations of Narasimham (1980) from natural beds and with the observations of Rivonker *et al*., (1993) and Rajagopal (1998b) from the suspended culture of *P. viridis* along the Indian coast. The comparison of CI values in their absolute terms between different periods was found irrelevant due to the differences in the methods of determination. Many workers applied different methods using wet tissue weight or volume in relation to shell cavity volume or weight.

Distinct peaks in CI with the values of 1.24 in June and 1.20 in October; a decrease was observed in May (0.88) and in July (0.82) which improved later in August. CI (CI_{shwt}) also displayed a continuous decline from March to May which later improved in August. Meat yield or percentage edibility ($CI_{commercial}$) also displayed similar patterns as CI_{shwt} in mussels from Ye estuary. Generally, the percentage edibility of mussels was low in December and July and high in June and August to September months (Fig. 6B). The index values were low when

spawning and spent resorbtive specimens occurred in considerable numbers in the population in July, too (Fig. 5). Mussels from Ye estuary generally showed low condition when compared to Surathkal and Someshwara mussel beds that yielded mussels with high condition (Sasikumar, 2007).

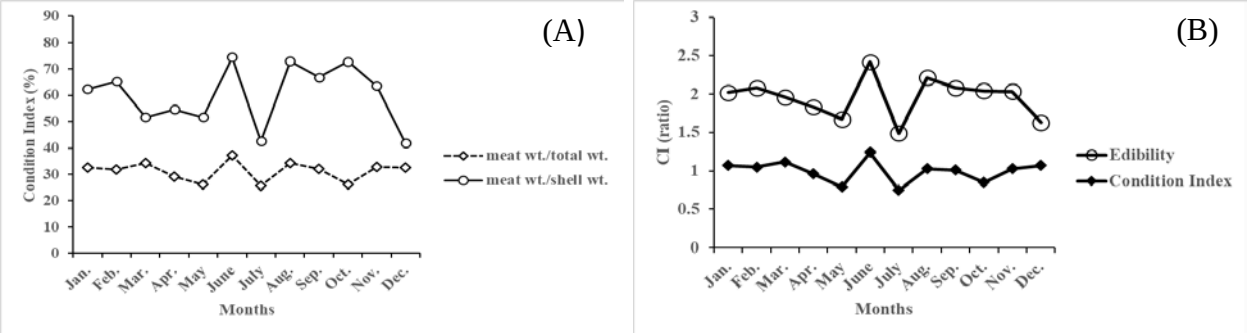

Figure 6 Condition Index of *Perna viridis* during the study period: A) Percentage of condition index; B) CI ratio = $\bar{x}$ CI_{month} / ($\bar{x}$ $CI_{all\ month}$).

The highest CI was observed in the monsoon season, followed by post moonsoon season and premonsoon seasons (Table 1). In the present study, relatively higher water temperature prevailed during March-May, June and December in the mussel bed with a difference of about 5°C between the maximum (32.00˚C) and minimum (27.60˚C) values. It was observed that the CI as well as the percentage edibility corresponded with these seasonal patterns in temperature, with poor mussel condition in pre monsoon and post monsoon. Temperature is a principal factor in controlling the broader aspects of the annual cycle of mussels (Seed, 1976). Chatterji *et al*., (1984) stated the changes in temperature can affect the growth rate of adult mussels in the tropical waters though they are exposed to thermally stable. Nair and Appukuttan (2003) also reported that the temperater changes can affect the development, growth, survival and settlement in spat. The optimum temperature for normal growth in green mussels is between 26 and 32°C (Sivalingam, 1977). The temperature (27.60˚C - 32.00˚C) recorded from the mussel beds in Ye estuary may be within the tolerable limits for this species.

Table 1 Seasonal variation in the condition index and edibility of green mussels from Sitaw

Season	Condition Index	Edibility
Premonsoon (February – May)	0.9800	0.945
Monsoon (June – September)	1.0225	1.028
Post monsoon (October – January)	0.9875	0.990

Conclusion

The study area covers the mussel bed off Sitaw, Ye estuary where green mussel, *Perna viridis* contributes to a significant fishery of commercial importance. Monthly monitoring of established *P. viridis* populations revealed year-round reproductive activity The observed year-round reproduction was fueled by an ability to maintain adequate energy reserves with minimal seasonal variation, which allowed for continuous gametogenesis. Peak spawning activity from April to July may be due to the trigger of rise in temperature during March and April. High CI values in June may be due to ripe gonad states and high food availability from the river and followed by low CI values in July may be due to peak spawning stages of male and female mussels.

Acknowledgements

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MARINE BENTHIC ALGAE OF SITTWE COASTAL AREAS, RAKHINE STATE

Win Htet San[1], Mya Kyawt Wai[2]

Abstract

A total of 19 species of marine benthic algae belonging to 13 families of 11 orders collected from the Sittwe Coastal Areas (lat. 20º 09´ N, Long. 92º 54´ E), Rakhine Coastal Region from December 2021 to August 2022 were identified. The identification of species was carried out based on the external and internal morphologies, herbarium, and living plants in the field. In the present study, 10 species, 9 genera of Rhodophyta, 5 species, 2 genera of Chlorophyta and 4 species, 4 genera of Phaeophyta had been identified. Among these algae, *Bangia atropurpurea*, *Catenella impudica*, *Polysiphonia subtilissima*, *Ulva flexuosa*, *Ulva clathrata* and *Colpomenia sinuosa* were newly recorded for Sittwe Coastal Areas. C*ompsonema serpens* and *Chondracanthus intermedius* were first recorded for Myanmar. The descriptive key emphasized to the species of these algae was provided. A brief note on the potential uses of each alga was described. Some ecological notes of these species were briefly described.

Keywords Ecological notes, Herbarium, Identification, Marine Benthic Algae, Morphology, Potential uses.

Introduction

Seaweeds are part of a complex ecosystem essential to marine life; the delicate filamentous species are important food sources for different animals (snails, sea urchins, crabs, fishes, turtles, etc.). Agal stocks provide dissolved organic matter, an important food source for bacteria, fungi, and protozoa. And finally, they provide habitat and nourishment for the higher links in the food chain; fishes, seabirds, and marine mammalian species. Juveniles get support, protection and coverage from predators in dense agal stands.

Since ancient times, seaweeds are a direct food source for humans. In China and Japan, more than 70 species of marine algae are consumed, and in both countries, there are marine farms which are hundreds of square kilometers in extent (aquaculture systems), where specific species (*Porphyra*, *Laminaria*, *Undaria*) are cultivated for human consumption. There are an almost infinite variety of healthcare products available commercially; lotions, bath gels, shampoos and soaps for skin protection and as a source of vitamins and minerals (Braune and Guiry (2011).

In Myanmar, floristic studies on some marine benthic algae of various localities such as Mazin coastal areas by Mya Kyawt Wai and Soe Htun (2009), Gwa coastal areas by Soe Pa Pa Kyaw and Soe Htun (2012), and Soe-Htun (2009a, b, c), Kyaikkhame coastal areas by Sein Moh Moh Khine (2012), Campani and Maungmagan coastal areas by Myo Min Tun (2013), Sit Thu Aung (2012) and Kalagauk coastal areas by Thet Htwe Aung (2013) had been undertaken.

As many as 122 genera and 307 species of seaweeds grow along the coastal areas of Myanmar (Kyi-Win 1972, Kyaw Soe and Kyi Win 1977, Soe-Htun 1998). A total of 229 marine benthic algae with 61 taxa, belonging to 22 genera of Chlorophyta, 44 taxa, belonging to 17 genera of Phaeophyta and belonging to 79 genera of Rhodophyta grow along the three coastal regions (Soe-Htun 2010). A total of 261 species of marine benthic algae under 121 genera, comprising 72 taxa belonging to 26 genera of Chlorophyta, 45 taxa belonging to 18 genera of

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Phaeophyta and 144 taxa belonging to 77 genera of Rhodophyta growing along the three coastal regions of Myanmar were recorded by Soe-Htun *et al.* 2021.

According to previously records, *Padina antillarum, Gelidium pusillum, Peyssonnelia rubra, Gracilaria canaliculata, Gracilaria foliifera, Ceratodictyon repens* and *Ulva rigida* from ittwe coastal area were reported by Kyaw Soe and Kyi Win (1977) and Soe-Htun *et al* (2009 a, b, c).

The objectives of this study are - (1) to know the morphology of marine benthic algae based on the external and internal structures and ecological features, (2) to show the distribution of marine benthic algae within the Sittwe Coastal Areas and (3) to understand the potential utilization of these algae for the benefits to the local people living in Sittwe township.

Materials and Methods

The marine benthic algae were collected in the intertidal and shallow subtidal zones of the Sittwe coastal areas (Lat. 20º 09´ N, Long. 92º 54´ E) from December 2021 to August 2022. In the field, the algae were observed the form of live materials growing in the natural beds and noted in the description of the site location. To remove the adhering materials, the plants were washed with seawater in the field. And then the plants were dried in a sheltered place for about 12 hrs and preserved in plastic bags. In the Phycological Research Laboratory of the Department of Marine Science in Sittway University, the healthy plants were thoroughly washed with the help of a painting brush in the sterile seawater to remove the adhering materials such as sand particles and other debris as well as epiphytes. And then the plants were identified under the binocular and compound microscopes. After that specimens were fixed and preserved in 5% formaldehyde in seawater and also mounted on the herbarium sheets. Herbarium, slides, liquid-preserved and living specimens were used for detailed investigations emphasized vegetative (external and internal) structures.

For the experiments on the cross sections of the thalli were made by hand cutting using double-edged razor blades. Sizes of vegetative organs were studied under the compound microscope using an ocular meter. Microscopic measurements were recorded in micrometer (µm) using the ocular meter. All materials prepared for observations are deposited in the Herbarium of the Department of Marine Science, Sittway University.

The useful and important characteristics, vegetative (external and internal) and reproductive structures were photographed under the light microscope with a digital camera and the observed results from digital photographs are processed by Adobe Photoshop CS5. This study has basically followed the classification system used by Lee (2008) and Guiry and Guiry (2022). Potential uses of these algae were recorded from the literature available.

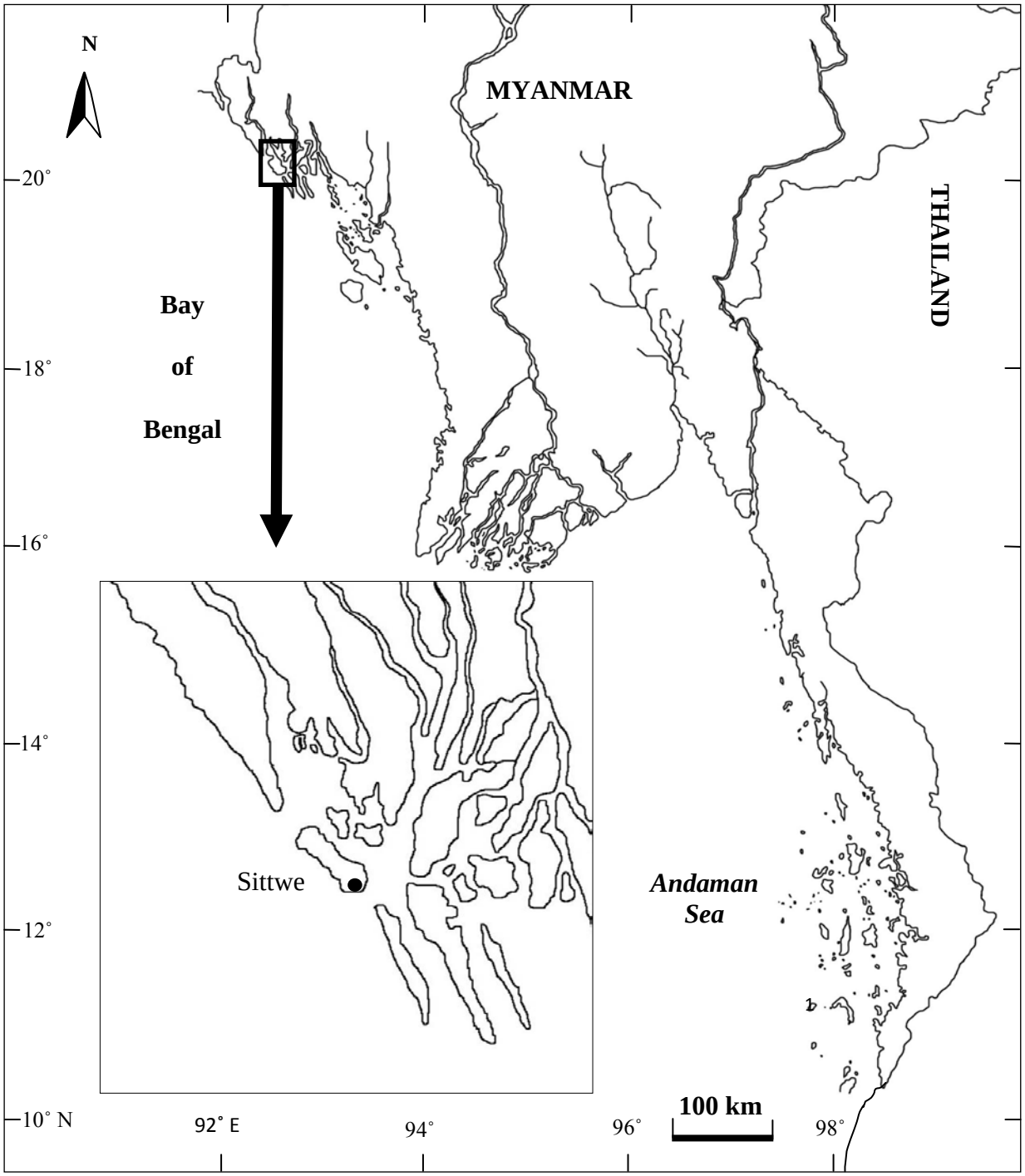


Figure 1 Map showing the collection sites of the marine benthic algae in Sittwe coastal areas.

Results

Table 1 Classification, Distribution and Potential Uses of Red Algae found and identified from Sittwe Coastal Areas

Phylum	**Rhodophyta**		**Distribution**			**Potential Uses**
Class	**Bangiophyceae**		**R**	**A**	**T**	
Order	I	Bangiales				
Family		Bangiaceae				
Genus		*Bangia* Lyngbye, 1819				
Species	1.	*B.atropurpurea* (Mertens ex Roth C.Agardh, 1824	+	-	-	fodder, fish meal, manure and organic fertilizers
Class	**Florideophyceae**					
Order	II	Gelidiales				
Family		Bangiaceae				
Genus		*Gelidium* J.V. Lamouroux 1813				
Species	2.	*G. pusillum* (Stackhouse) Le Jolis, 1863	+	-	-	fodder, fish meal and manure
Order	III	Peyssonneliales				
Family		Peyssonneliaceae				
Genus		*Peyssonnelia* Decaisne, 1841				
Species	3.	*P. rubra (Greville)* J.Agardh, 1851	+	+	+	fodder and manure
Order	IV	Gigartinales				
Family		Gigartinaceae				
Genus		*Chondracanthus* Kutzing, 1843				
Species	4.	*C. intermedius* (Suringar) Hommersand,1993	+	-	-	raw material for carrageenan production
Family		Caulacanthaceae				
Genus		*Catenella* Greville, 1830				
Species	5.	*C. impudica* (Montagne) J. Agardh, 1852	+	+	-	carrageenan, fodder, fish meal, human foods, manure and salad
Order	V	Gracilariales				
Family		Gracilariaceae				
Genus		*Gracilaria* Greville, 1830				
Species	6.	*G. canaliculata* Sonder, 1871	+	+	+	agar, drugs, fodder, fish meal and human foods
	7.	*G. foliifera* (Forsskal) Boergesen , 1932	+	-	+	agar, drugs, fodder, fish meal and human foods
Order	VI	Rhodymeniales				
Family		Lomentariaceae				
Genus		*Ceratodictyon* Zanardini, 1878				
Species	8.	*C. repens* (Kutzing) R.E.Norris,1987	+	+	-	fodder and manure
Family		Rhodomelaceae				
Genus		*Laurencia* F.Schmitz, 1889				
Species	9.	*Laurencia sp.*	+	+	-	drugs fodder and fish meals
Order	VII	Ceramiales				
Genus		*Polysiphonia* Greville, 1823				
Species	10.	*P. subtilissima* Montagne, 1840	+	+	+	fodder, drugs and agar

* (R= Rakhine Coastal Area, A= Ayeyawaddy Delta and Gulf of Moattama Area, T= Taninthayi Coastal Area)

1. *Bangia atropurpurea* (Mertens ex Roth) C.Agardh, 1824 (Fig.2)

Womersley 1922: 34-36, figs. 3.D-H; Chihara 1970: 57, Pl. 29, Fig. 2; Soe-Htun *et al*. 2009b: 120, fig. 6; Guiry and Guiry 2022.

Description. - Filaments are initially erect, unbranched with single series of cells 10-13 µm broad, becoming more than one cell broad in the upper portion. Each young thalli attached by the lowermost cells; thalli are rose to dark red in colour.

Ecological notes. - Young plants occur in subtidal zones on rhizoids and blades of *Padina antillarum* with *Compsonema serpens.*

2. *Gelidium pusillum* (Stackhouse) Le Jolis, 1863 (Fig.3-4)

Womersley 1922: 133-136, figs. 39. E-K; Taylor 1967: 354-355, Pl. 45, fig. 4; Durairatnam 1961: 50, Pl. 13, figs. 1-5; Smith 1969: 195, Pl. 44, fig. 1; Kyaw Soe and Kyi Win 1977: 103, fig. 179; Soe-Htun *et al*. 2009b: 127, fig. 22; Guiry and Guiry 2022.

Description. - Plants are creeping, small, dense, 13 mm tall, brownish red in colour, forming short loosely tufts. Erect branches are freely branched and compressed to flattened at upper; differentiated into horizontal thread-like, cylindrical, 11mm in diameter and attached to substratum by small attaching pads, branched opposite.

Ecological notes. - Plants are found on rocks and coralline tones in the upper intertidal zones.

3. *Peyssonnelia rubra* (Greville) J.Agardh, 1851 (Fig.5-6)

Dawson 1954: 424, fig. 36c; Abbott and Hollenberg 1976: 371, fig. 310; Kyaw Soe and Kyi Win 1977: 123, fig.216; Soe-Htun *et al*. 2009c: 147, fig.3; Guiry and Guiry 2022.

Description. - Plants are membranaceous, somewhat calcified on the lower side, rose red to deep red; loosely attached by the entire lower face directly or by numerous short rhizoids and with distinct radial and faint concentric lines.

Ecological notes. - Plants found common in most subtidal habitats on dead corals, rocks and other hard surfaces.

4. *Chondracanthus intermedius* (Suringar) Hommersand, 1993 (Fig.7-8)

Yang and Kim 2016: 520-523, figs. 3.a-h; Guiry and Guiry 2022.

Description. – Thalli are cartilaginous and flexible, purplish red to black with bluish iridescence in colour, attached firmly to the substratum by a small discoid holdfast forming a low-growing tuft. Axes are cylindrical at the base and compressed, irregularly branched, strongly recurved, and adhering to each other by a secondary attachment.

Ecological notes. - Plants grow in intertidal zone adhered to exposed rocks.

5. *Catenella impudica* (Montagne) J.Agardh, 1852 (Fig.9-10)

Kyi Win 1972: 7; Kyaw Soe and Kyi Win 1977: 130, fig. 231; Sein Moh Moh Khaing 2012: 87-88, figs. 4.4.3. A-F; Jha *et al*. 2009: 138, figs. a-c; Guiry and Guiry 2022.

Description. - Plants are creeping, complanate-expanded, 1-2 cm high, blackish violet in colour. Branches have 1-3 segments, articulate with irregularly di - or tri-chotomously branched and outsends from the joint; the younger segments slender, with the terminal ones more terete and acute; the older segments broader and more flattened. The segments are elliptical- oblong and strongly constricted at the nodes.

Ecological notes. - Plants grow in intertidal zone adhered to exposed rocks.

Key to the species of *Gracilaria* Grevielle

1a. Branches club- shaped; fronds rounded.....*Gracilaria canaliculata*

1b. Branches fin- shaped; fronds flattened.....*Gracilaria foliifera*

6. *Gracilaria canaliculata* Sonder, 1871 (Fig.11-12)

Hla Hla Cho 1975: 48-59, figs. 40-42; Kyaw Soe and Kyi Win 1977: 134, fig. 239, A.1-2; Soe-Htun *et al*. 2009c: 150-151, figs. 9-10; Guiry and Guiry 2022.

Description. – Fronds are short cylindrical attached by means of a discoid holdfast, constricted below when old, purplish red or dark greenish in colour. The young plants are twisted, creeping, branching and attached to the rocks or other hard substrates; branches up to 20 mm in length and 1-2 mm broad. The mature branches conspicuously articulated with oblong and ovoid segments.

Ecological notes. - Plants grow in exposed places at the littoral zone.

7. *Gracilaria foliifera* (Forsskal) Boergesen, 1932 (Fig.13-14)

Durairatnam 1961: 63, Pl. 31, fig. 2; Taylor 1967: 446, Pl. 55, fig. 1; Kyaw Soe and Kyi Win 1977: 135, fig. 241; Jha *et al*. 2009: 121, figs. a-b; Soe-Htun *et al*. 2009c: 151-152, fig. 11; Guiry and Guiry 2022.

Description. - Plants are compressed, bushy firmly attached to the substratum with a discoid holdfast, brownish to yellowish red or faded in colour. Plants twisted, creeping, branching and attached to the rocks or other hard substrates, branches up to 2 cm in length and 0.1 cm broad. The fronds are conspicuously compressed or flattened throught out the thallus, branched proliferations along the margins or at the upper parts of the branches.

Ecological notes. - Plants occur common on rocks and shells in quiet in the subtidal zones associated with *Laurencia sp., G. canaliculata and G. pusillum.*

8. *Ceratodictyon repens* (Kutzing) R.E.Norris, 1987 (Fig.15-17)

Jha *et al.* 2009: 163, Fig.a; Soe-Htun *et al.* 2009c: 153, fig. 13; Guiry and Guiry 2022.

Description. - Plants are erect and filiform, light red in colour, 4 cm in height, forming extremely dense and dichotomously alternately; branches spread out in subflagellate manner, apex of the segments often narrow, upper segment slender, very well attached to rocks, creeping shoots.

Ecological notes. - Plants occur on the rocks at the subtidal zone associated with *Laurencia sp.* and *G. canaliculata*.

9. *Laurencia sp.* (Fig.18-19)

Description. - Thalli are solitary tufts from a common disc-shaped base, 2 cm tall, dark red to brown in colour. The erect shoots are cylindrical or markedly flattened, 4 mm in height, branching pinnate or radial. The branch tips are blunt and terminating in a small depression containing a single apical cell.

Ecological notes. - Plants occur on solid substrate in the subtidal zone, especially in wave - exposed locations associated with *C. sinuosa.*

10. *Polysiphonia subtilissima* Montagne, 1840 (Fig.20-22)

Soe-Htun *et al.* 2009c: 161, figs. 32-33; Jar San and Soe-Htun 2014: 2-5, figs. 2-18; Guiry and Guiry 2022.

Description. - Young plants are composed of siphons, reddish brown in colour, 2750 µm high; soft, arising from a creeping base; erect filaments divided into two branches below, alternately branched above; attached by scattered unicellular rhizoids.

Ecological notes. - Plants are found on rocks in subtidal zone attached in rocks.

Table 2 Classification, Distribution and Potential Uses of Green Algae found and identified from Sittwe Coastal Areas

Phylum	**Chlorophyta**		**Distribution**			**Potential Uses**
Class	**Ulvophyceae**		**R**	**A**	**T**	
Order	I	Ulvales				
Family		Ulvaceae				
Genus		*Ulva* Linnaeus, 1753				
Species	1.	*U. rigida* C.Agardh, 1823	+	-	-	human foods, drugs and fish meal
	2.	*U. compressa* Linnaeus, 1753	+	+	+	
	3.	*U. flexuosa* Wulfen, 1803	+	+	-	
	4.	*U. clathrata* (Roth) C.Agardh,1811	+	+	+	
Order	II	Cladophorales				
Family		Cladophoracceae				
Genus		*Chaetomorpha* Kutzing, 1845				
Species	5.	*C. linum* (O.F.Muller) Kutzing,1845	+	+	+	fodder, fish meal, human foods and organic fertilizers

* (R= Rakhine Coastal Area, A= Ayeyawaddy Delta and Gulf of Moattama Area, T= Taninthayi Coastal Area)

Key to the species of *Ulva* Linnaeus

1a. Thallus flat lamina, margins of thallus usually slenderly dentate.....*Ulva rigida*
1b. Thallus cylindrical filaments.....2
2a. Thalli are erect, tube-shaped hollow, narrow sheet with ruffled edges…..*U. compressa*
2b. Thallus usually sparsely branched at the base, few small proliferations,
constricted at intervals with tubular blades……..…….……*U. flexuosa*
2c. Thallus usually densely branched from main branches, more proliferations with thin cylindrical blades.....*U. clathrata*

1.*Ulva rigida* C.Agardh, 1823 (Fig.23-24)

Durairatnam 1961: 18, Pl. 1, fig. 1; Kyaw Soe and Kyi Win 1977: 42, fig. 42; Wormersley 1984: 142-144, figs. 44D. 45 G-J; Norris 2010: 39-40, fig. 18; Guiry and Guiry 2022.

Description. - Blades of thalli are dark green in colour, a flat lamina, deeply divides, 1-3 cm height, with folded margins, forming small rounded to orbicular slightly wavy blades with short and thick stipe, surface somewhat crinkled, usually with microscopic teeth along the blade margins, epiphytic on other algae.

Ecological notes**. -** Plants grow on rocks, or other algae in the intertidal pools.

2.*Ulva compressa* Linnaeus, 1753 (Fig.25-26)

Durairatnam 1961: 18, Pl. 1, fig. 7; Taylor 1969: 64, Pl 3, fig. 3; Smith 1969: 52, Pl 5, fig. 7; Guiry and Guiry 2022.

Descriptions. - Thalli are erect, tube-shaped hollow, 4 cm length and 0.2 cm width, narrow sheet with ruffled edges and light to medium green in colour. Thalli are commonly becoming compressed in upper blade, usually much branched near the base.

Ecological notes. - Plants grow on pneumatophores of mangrove trees and mud flat.

3. *Ulva flexuosa* Wulfen, 1803 (Fig.27-28)

Abbott and Hollenberg 1976: 76, fig. 30; Abbott and Huisman 2004: 48-49, figs.6. A-C; Jha *et al*. 2009: 9, figs. a- b; Guiry and Guiry 2022.

Description. - Young thalli are yellowish green in colour, usually unbranched, 3 mm height, simple or occasionally branched at the base, rarely with secondary branches; branches have cylindrical, becoming inflated bent or flexuous, sometimes constricted at intervals, epiphytic on other algae. Thallus is erect and attached to substratum by a small round basal disk.

Ecological notes. - Plants grow as epiphytes on the *P. antillarum* and *G. pusillum* in the upper and middle intertidal zone.

4. *Ulva clathrata* (Roth) C.Agardh, 1811 (Fig.29-31)

Abbott and Hollenberg 1976: 73-74, figs 27-29; Myo Min Tun 2013: 27-29, figs. 4.2.1. A-B; Guiry and Guiry 2022.

Description. - Thalli are light to yellowish-green in colour, profusely branched, 4 mm height, taperingly branched at the base; branches are cylindrical to slightly compressed and repeatedly branched, becoming dense floor, many thin branchlets produced almost throughout. In surface view, cells are in more or less longitudinal series; in narrow portions and either similarly ordered or more arranged in broader older portions.

Ecological notes. - Plants are found in the upper intertidal zones as a densely mat.

5. *Chaetomorpha linum* (O.F.Muller) Kutzing, 1845 (Fig.32-34)

Taylor 1967: 71, Pl 2, fig. 8; Abbot and Hollenberg 1976: 101, fig. 60; Kyaw Soe and Kyi Win 1977: 57, fig. 76; Wormersley 1984: 176, Pl 13, fig. 2; Guiry and Guiry 2022.

Descriptions. - Thalli are rigid, slightly curved, composed of loosely entangled and uniseriate filaments. Surface view of cells are yellowish green in colour, cylindrical in shape, 32-45 µm wide.

Ecological notes. - Plants grow in shallow, sheltered water and mangrove tree trunk in the intertidal zone.

Table 3 Classification, Distribution and Potential Uses of Brown Algae found and identified from Sittwe Coastal Areas

Phylum	**Phaeophyta (Ochrophyta)**		**Distribution**			**Potential Uses**
Class	**Phaeophyceae**		**R**	**A**	**T**	
Order	I	Ectocarpales				
Family		Chordariaceae				
Genus		*Compsonema* Kuckuck, 1899				
Species	1.	*C. serpens* Setchell & N.L.Gardner, 1922	+	-	-	Unknown
Family		Scytosiphonaceae				
Genus		*Colpomenia* (Endlicher) Derbes & Solier, 1851				
Species	2.	*C. sinuosa* (Mertens ex Roth) Derbès & Solier, 1851	+	+	-	human foods, drugs, fodder, fish meal and manure
Order	II	Dictyotales				
Family		Dictyotaceae				
Genus		*Dictyota* J.V.Lamouroux, 1809				
Species	3.	*D. adnata* Zanardini, 1878	+	+	-	alginate, drugs, fodder, fish meal, human food and manure
Genus		*Padina* Adanson, 1763				
Species	4.	*P. antillarum* (Kutzing) Piccone, 1886	+	-	+	the production of a jelly-like sweetmeat, fertilizer, human food

* (R= Rakhine Coastal Area, A= Ayeyawaddy Delta and Gulf of Moattama Area, T= Taninthayi Coastal Area)

1. *Compsonema serpens* Setchell & N.L.Gardner, 1922 (Fig.35-36)

Smith 1969: 110- 111, Pl. 3, fig. 3; Abbott and Hollenberg 1976: 160- 162, fig. 128; Norris 2010: 194- 195, fig. 93; Guiry and Guiry 2022.

Description. - Plants are minute, microscopic size, epiphytic, brownish in colour, lower portion of irregularly branched prostrate filaments with a few endophytic rhizoids attached to host; above erect, unbranched filaments, up to 175 µm tall.

Ecological notes. - Algae are found in intertidal zone as epiphytic and endophytic on *P. antillarum* with *U. flexuosa* and *B. atropurpurea.*

2. *Colpomenia sinuosa* (Mertens ex Roth) Derbes & Solier, 1851 (Fig.37-38)

Arasaki 1871: 34, fig. 103; Setchell 1931: 49, fig.2; Abbott and Hollenberg 1976: 204, fig. 168; Jha *et al*. 2009: 80, figs. a-b; Guiry and Guiry 2022.

Description. - Thallus is hollow, yellowish brown in colour, 4 cm in diameter and attached by a broad basal disc. Thallus surface is smooth and twisted in some portion, filled with seawater when young but broke down later. Young plants are approximately round, becoming flattened, expanded and irregularly convoluted.

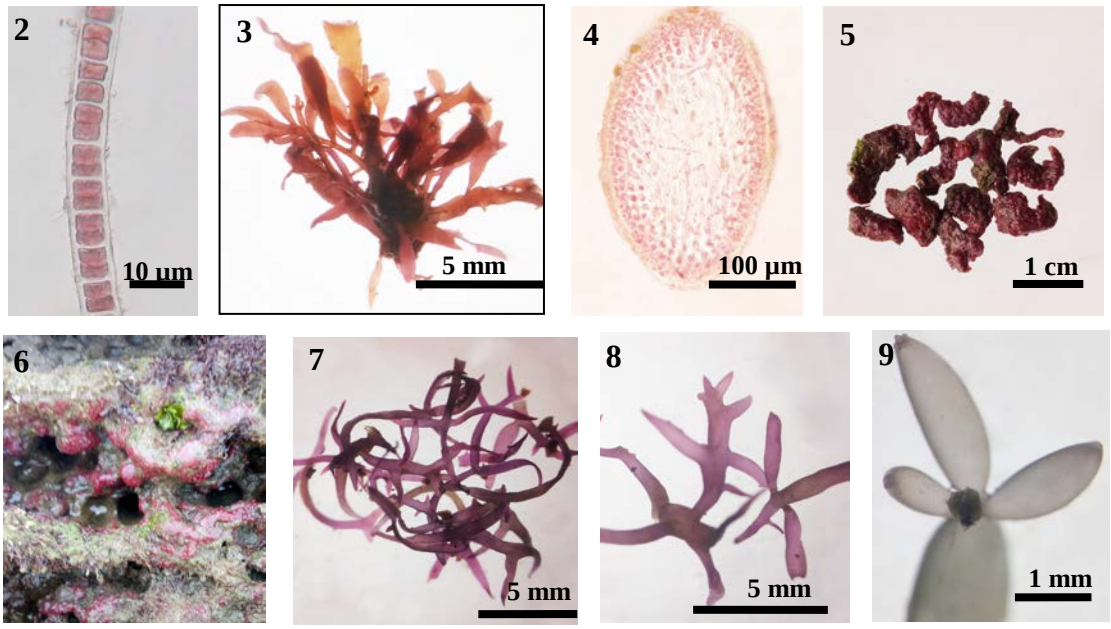
Ecological notes. - Plants are found in intertidal rocks and epiphytic on other large algae such as *Laurencia sp.*

3. *Dictyota adnata* Zanardini, 1878 (Fig.39-40)

De Clerck 2003: 32, fig.7; Soe Pa Pa Kyaw 2008: 19-22, figs. 1-8; Soe Pa Pa Kyaw and Soe Htun 2012: 269-282, figs. 2-9; Guiry and Guiry 2022.

Description. - Branches are 3 cm long, obtriangular, apices obtuse or sometimes acute, non-twisted, margins entire with poliferations and fuscicules of rhizoids arising from the underside margins of thallus. Thalli are dichotomously branches.

Ecological notes. – Plants attached to the pneumatophores of mangrove trees and other substrate in upper intertidal zone.



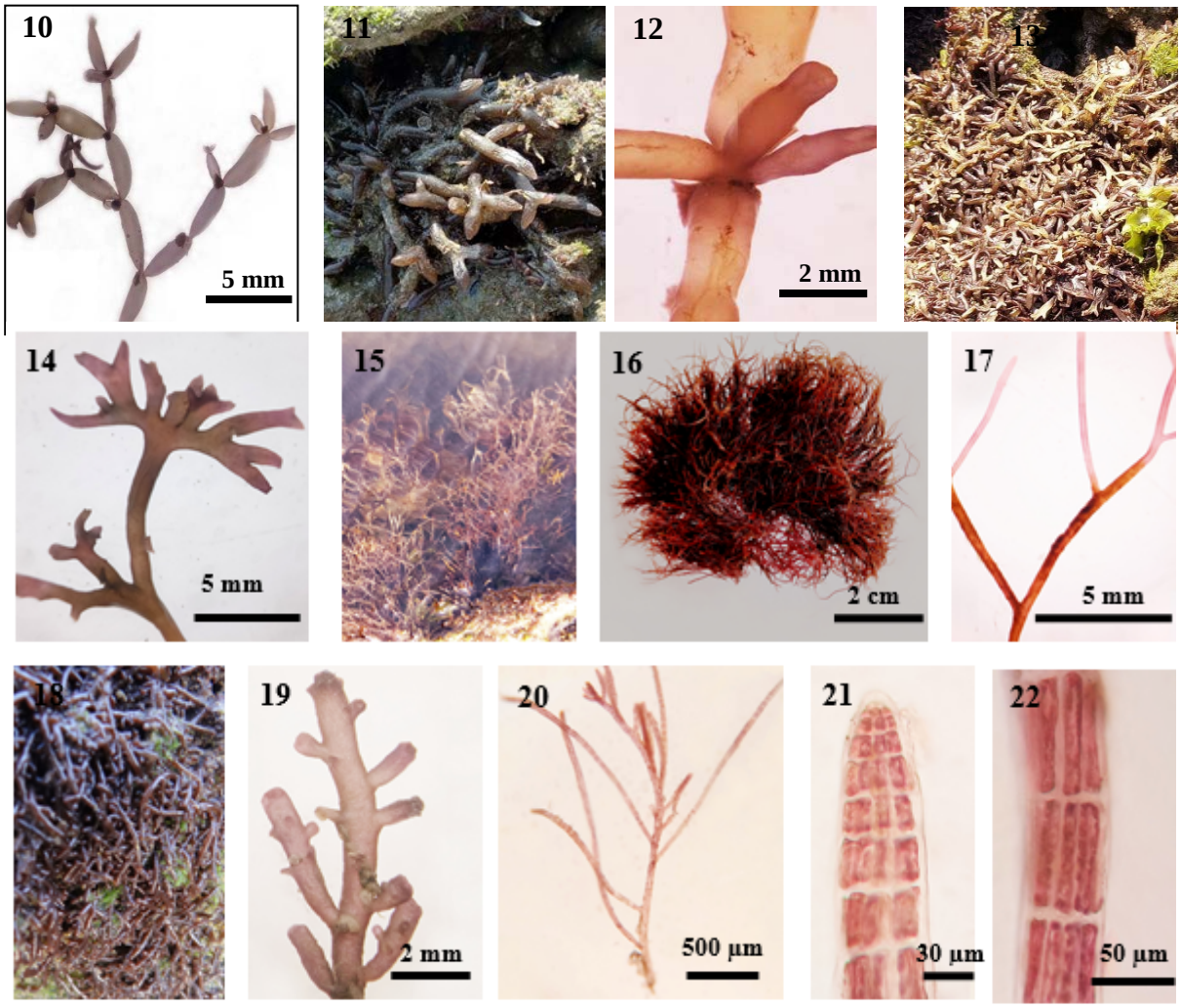


Figure 2-22. The morphological structures of red algae. (2) Erect filament of *Bangia atropurpurea* showing single series; (3) Habit of *Gelidium pusillum*; (4) Cross section showing several axes; (5)-(6) Habit of *Peyssonnelia rubra*; (7)-(8) Habit of *Chondracanthus intermedius*; (9) Branch system showing 3 segments of *Catenella impudica*; (10) Habit; (11) Habit of *Gracilaria canaliculata*; (12) Branch system; (13) Habit of *G. foliifera* (14) Branch system showing flattened thallus; (15)-(16) Habit of *Ceratodictyon repens*; (17) Branch system; (18) Habit of *Laurencia sp.*; (19) Blunt tip of branch; (20) Habit of *Polysiphonia subtilissima*; (21) Tip of thallus; (22)

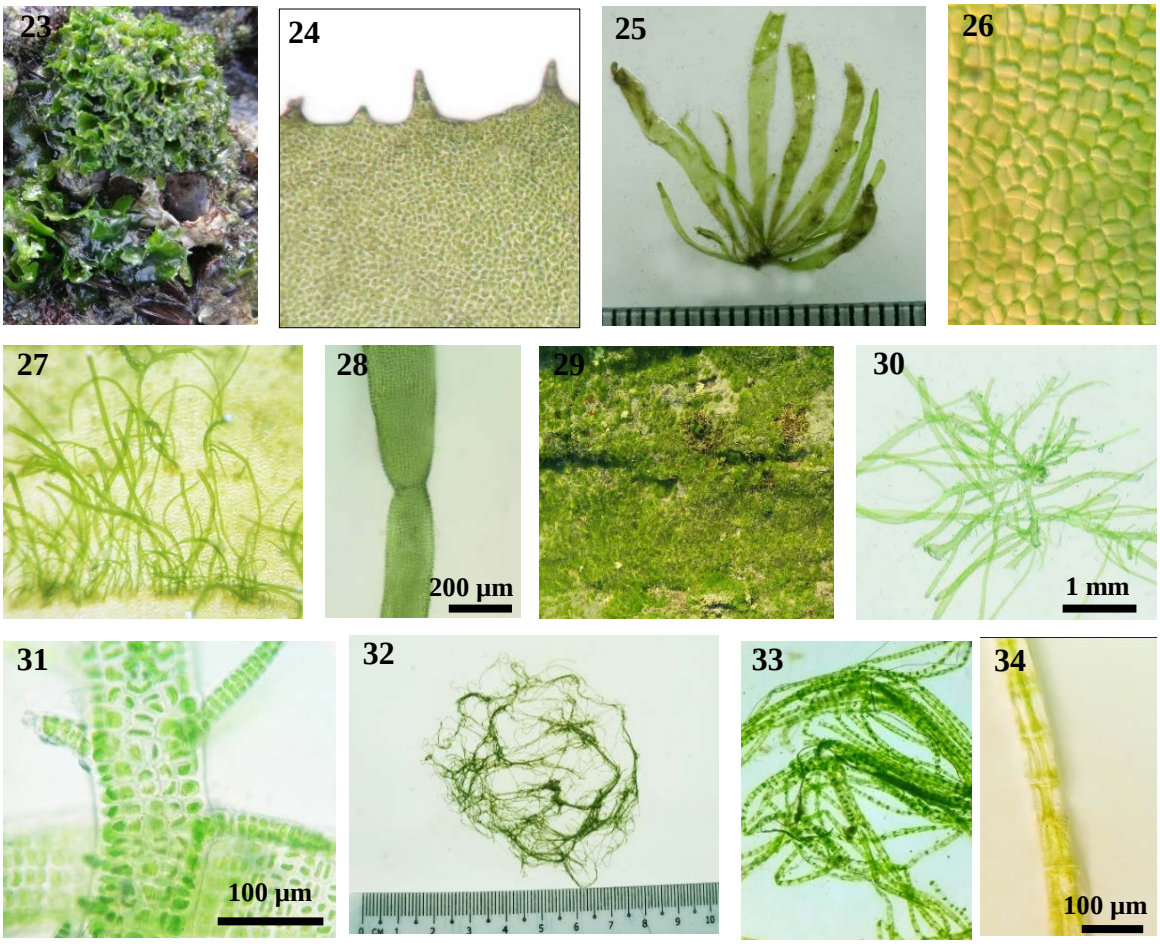


Figure 23-34. The morphological structures of green algae. (23) Habit of *Ulva rigida* (24) Blade margin showing microscopic teeth; (25) Habit of *U. Compressa*; (26) Surface cell; (27) Habit of *U. flexuosa*; (28) Thallus showing constricted at interval; (29) Habit of *U. Clathrata*; (30) Habit showing dense floor; (31) Branch system showing thin branchlets; (32) Habit of *Chaetomorpha linum*; (33)-(34) Surface view of the filaments.

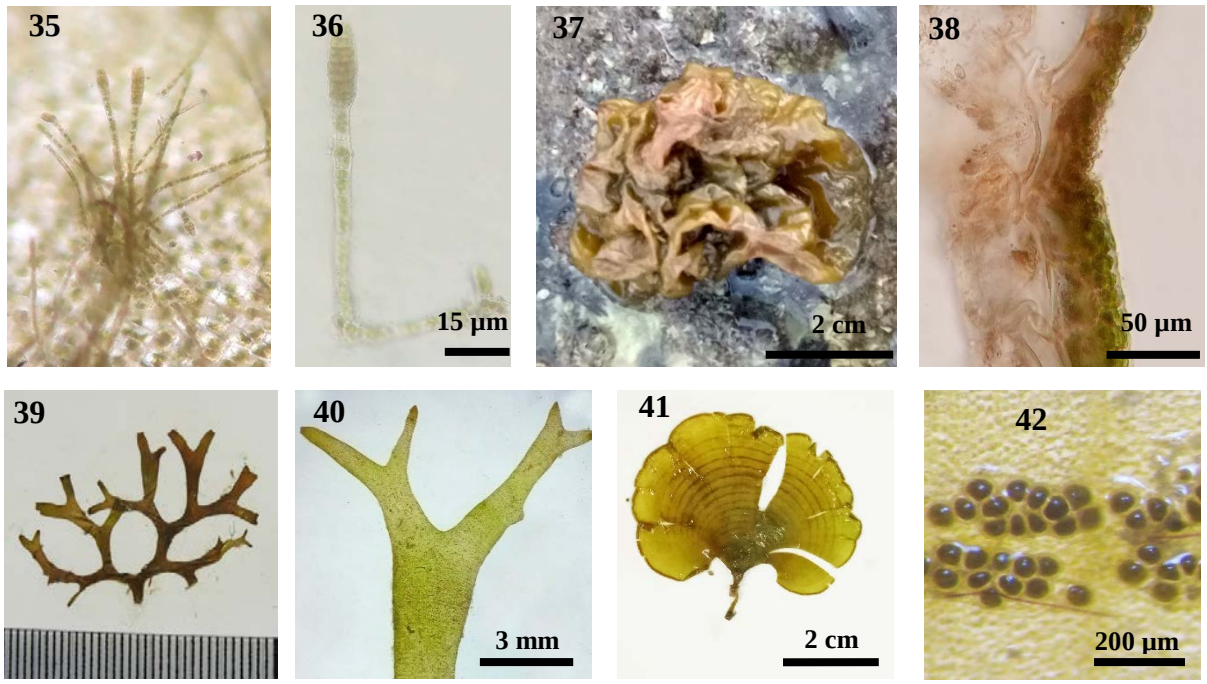


Figure 35-42. The morphological structures of brown algae. (35) Habit of *Compsonema serpens*; (36) Erect filament with terminal plurilocular reproductive structures; (37) Habit of *Colpomenia sinuosa* showing hollow form; (38) Cross section showing 1-2 layers of cortex cells; (39) Habit of *Dictyota adnate*; (40) Dichotomus branches; (41) Habit of *Padina antillarum*; (42) Surface view showing tetrasporangial sori.

4. *Padina antillarum* (Kutzing) Piccone, 1886 (Fig.41-42)

Misra 1966: 158-159, fig. 84; Kyaw Soe and Kyi Win 1977: 80, fig. 130; Wynne and De Clerck 1999: 286-295, figs. 1-10; Soe-Htun *et al.* 2009a: 95-96, fig. 9; Guiry and Guiry 2022.

Description. - Plants are pale yellow to reddish brown in colour, 4-7 cm tall and 4.5- 6.5 cm in breadth developing from rhizomatous disc; fan-shaped lobes. Thallus has two cell-layered in upper portion, three cell-layered in middle portion and four cell-layered or more near the base in mature plant. Margin of the thallus is entire or irregularly separated and deeply split into several lobes with involute margin at the apex. Reproductive organs possess on both sides of each hair line, forming fertile rows, 0.6-1 mm in breadth.

Ecological notes. - Plants are found in the intertidal and subtidal zone with *G. foliifera*.

Discussion

In the present study, the study area was with a salinity range 33-34 ‰, at water temperature 30-32° C. The diversity of a total of 19 species of marine benthic algae belonging to 15 genera, 13 families of 11 orders was recorded from Sittwe coastal areas and identified as *Bangia atropurpurea* (Mertens ex Roth) C.Agardh, *Gelidium pusillum* (Stackhouse) Le Jolis, *Peyssonnelia rubra* (Greville) J. Agardh, *Chondracanthus intermedius* (Suringar) Hommersand, *Catenella impudica* (Montagne) J. Agardh, *Gracilaria canaliculata* Sonder, *G. foliifera* (Forsskal) Boergesen, *Ceratodictyon repens* (Kützing) R.E.Norris, *Laurencia sp*., *Polysiphonia subtilissima* Montagne, *Ulva rigida* C. Agardh, *U. compressa* Linnaeus, *U. flexuosa* (Wulfen), *U. clathrata* (Roth) C. Agardh, Linnaeus, *Chaetomorpha linum* (O.F.Muller) Kutzing, *Compsonema serpens* Setchell & N.L.Gardner, *Colpomenia sinuosa* (Mertens ex Roth) Derbès & Solier, *Dictyota adnata* Zanardini and *Padina antillarum* (Kutzing) Piccone.

Firstly, the distinct characters of *B. atropurpurea* are the presence of erect filaments and unbranched with single series of cells. In 1922, Womersley previously identified *B. atropurpurea* which has a dense mass of flaccid and is basally attached by rhizoids from several suprabasal cells. In the present study, it had not been found densely mass because it was so young. *G. pusillum* are abundantly distributed during the study period. In this study, external morphology, branched systems and internal cell structures of *G. pusillum* are closely similar to those of this plant described by Smith (1969). Kyaw Soe and Kyi Win (1977) recorded *G. pusillum* from Myanmar. Moreover, *P. rubra* can be seen as loosely attached plants to rocks by the entire lower face or by numerous short rhizoids. Thalli of *C. intermedius* are cartilaginous and flexible forms. The characters of *C. intermedius* are closely similar to the observations on this plant by Yang and Kim (2016) especially in presence of recurving axes. The younger segments of *C. impudica* are slender in shape and the older segments are broader and more flattened. The two species of *Gracilaria* such as *G. canaliculata* and *G. foliifera* can be recorded in the present study. Fronds of the former species are short and cylindrical and attached by means of a discoid holdfast. Plants of the later species are twisting, creeping and branching, and attached to the rocks or other hard substrates. In *Laurencia sp*., the tips of branch are blunt and terminate in a small depression containing a single apical cell. The prominent features of *P. subtilissima* are the presence of soft and erect filaments divided into two branches. In surface view, thallus has usually one per segment with reddish brown in colour and square to elongate in shape.

In Chlorophyta, the wavy blades of *U. rigida* usually have microscopic teeth along the blade margins and are epiphytes on other algae. The presence of microscopic teeth along the blade margins is an identical factor in the description of Norris (2010).

Thalli *of U. compressa* are commonly becoming compressed in upper blade, usually much branched near the base. Thalli of *U. flexuosa* are becoming inflated, are bent or flexuous forms, sometimes constricted at intervals in the present study. The information from the field is identically similar to the observations of Jha *et al.* (2009). Thalli of *U. clathrata* are taperingly branched at the base and cylindrical to slightly compressed in shape. The records are closely similar to observations of young plants of these plants by Abbott and Hollenberg (1976). Thalli of *C. linium* is rigid, slightly curved, composed of loosely entangled and uniseriate filaments.

In Phaeophyta, plants of *C. serpens* are minute and microscopic size. The growth forms and physical features mentioned above are identically alike to the observations by Abbott and Hollenberg (1976), and Norris (2010). They described the presence of terminal plurilocular reproductive structures of that plant. Plants of *C. sinuosa* are filled with air and irregularly lobe vesicles with corrugated surface. The morphology of inner cells and shape of cells is closely related to the descriptions of Abbott and Hollenberg (1976), Kyaw Soe and Kyi Win (1977) and Abbott and Huisman (2004). Branches of *D. adnata* are obtriangular, apices obtuse or sometimes acute, non-twisted, margins entire with poliferations and fuscicules of rhizoids. Plants of *P. antillarum* are pale yellow to reddish brown in colour; fan-shaped lobes. Reproductive organs possess on both sides of each hair line, forming fertile rows, 0.6-1 mm in breadth. The reproductive patterns of present study agree well with the key to species by Wynne and De Clerck (1999) and Misra (1966). In Myanmar, Mya Kyawt Wai (2008) also studied the vegetative and reproductive structures of *P. antillarum*.

Conclusion

Sittwe is the ordinary plentiful seaweeds comparatively other places. Seaweeds were richly growing along the Sittwe Point because of abundance of rock reefs. Conspicuously *C. serpens* can be seen as epiphytes in Sittwe Point. The most overflowingly seaweeds of Sittwe coastal area are *G. pusillum, G. canaliculata, G. foliifera, C. repens, Laurencia sp., U. clathrata, U. rigida, C. sinuosa* and *P. antillarum*. And then *B. atropurpurea, P. subtilissima, U. flexuosa* and *C. serpens* could be searched as epiphytes or parasites on other large algae. Moreover, *P. rubra* was found by firmly attaching to rocks in the field. Moreover, *U. compressa*, *C. linum* and *D. adnata* were found in the pneumatophores of mangroves in this study. They are also important food sources for different animals such as snails, sea urchins, crabs, fish, sea turtles and so on. *Catenella spp*., locally known as "Kyaukpwint" has been utilized as traditional sea vegetable in Myanmar (Soe-Htun *et al*. 1999). Finally, seaweeds play an essential role around the world and are saving the life of animals.

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SPECIES COMPOSITION AND ABUNDANCE OF AMPHIPODS FROM SEAWEEDS AND SEAGRASSES OF THE SOUTHERN RAKHINE COAST

Aung Aung Htaik[1]

Abstract

A total of 25 species of amphipod were recorded from the southern Rakhine Coast. *Elasmopus palu*, *E. pectenicrus*, *Hyale crassicornis* and *H. galateae distorta* were by far the most numerous and widespread species at all stations. The composition of amphipod fauna was higher in seaweed habitats than the others. *Maera quadrimana* and *M. serrata* were recorded only on *Halimeda opuntia* and *Gracilaria canaliculata*. *Melita zeylanica* showed the common occurrence on all habitats. *Parelasmopus suensis* was recorded only on *Gracilaria canaliculata*. The highest abundance of amphipod at all stations was observed from November to March and the lowest from June to September. The abundance and species composition of amphipod were related to the algal blooming periods.

Keywords Amphipod, abundance, composition, algal blooming period

Introduction

Amphipods are peracarid crustaceans that are divided into four suborders, Gammeridea, Hyperiidea, Caprellidea and Ingolfiellidea. The arrangement of the thoracic legs into forward and backward direction is one of the unique features of amphipods. A second unique characteristic is the biramous swimming legs (pleopods) and the thrusting legs (uropods).

The gammaridean amphipods are the dominant group of pericaridan crustaceans. Gammarid amphipods are small in size (1-8 mm), shrimp like crustaceans. The gammarideans, which are mostly free-living, occupy a wide variety of habitats; in rocky crevices, on coral rubble, on algae and seagrasses, burrowing in sediment, living in fixed or mobile tubes and living in invertebrate hots. Gammarid amphipods are important food items for fish and seabirds. They constitute a significant part of the demersal plankton, and inhabit every available substrate. A number of species are associated with seaweeds. Many species are found among the various crevices of live coral and coral rubbles in reef complexes. Amphipods are distributed according to the habitats, availability of food supplies and feeding methods. They are important in marine food web as secondary consumers.

This study is firstly record concerned with marine amphipod for these areas. Moreover, the research papers studied on marine amphipods from Myanmar coastal areas are very rare. The objective of this study was to know the seasonal composition and abundance of amphipod fauna of the seaweeds and seagrasses from the southern Rakhine coast.

Materials and Methods

The study areas, Poelaung Gyaing (Lat. 17° 12' N and Long. 94° 31' E), Wetthay (Lat. 17° 10' N and Long. 94° 28' E), Magyi (Lat. 17° 5' N and Long. 94° 27' E) Chaungtha (Lat. 16 º 57' N, Long. 94º 26' E) and Ngwesaung (Lat. 16° 52' N and Long. 94° 22' E), were situated in the Ayeyarwady Region, southern part of Rakhine coast. Sampling location from the study areas are shown in Fig. 1. Amphipod samples were collected from June, 2013 to May, 2015.

Seaweed and seagrass samples were collected by placing a 1 m x 1 m quadrat within 10 m interval along 100 m transect line and removing all the samples by scraping. The samples were quickly transferred to the 120 µm net. Plants are rinsed thoroughly with water and shaken

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to dislodge the fauna from them. After sieving, the specimens were preserved in 5% formalin and rose-bengol solutions for further analysis and identifications. The amphipods in these substrates tended to move very fast so the samples of these substrates were collected by hand and quickly transferred to a plastic bag. A small amount of 5% formalin was added and the plastic bag was tied and transported to the laboratory. Amphipod samples were analyzed under compound and stereomicroscope to species level as possible. Species identification was made by using Barnard and Karaman 1991, Myers 1981 and 1985.

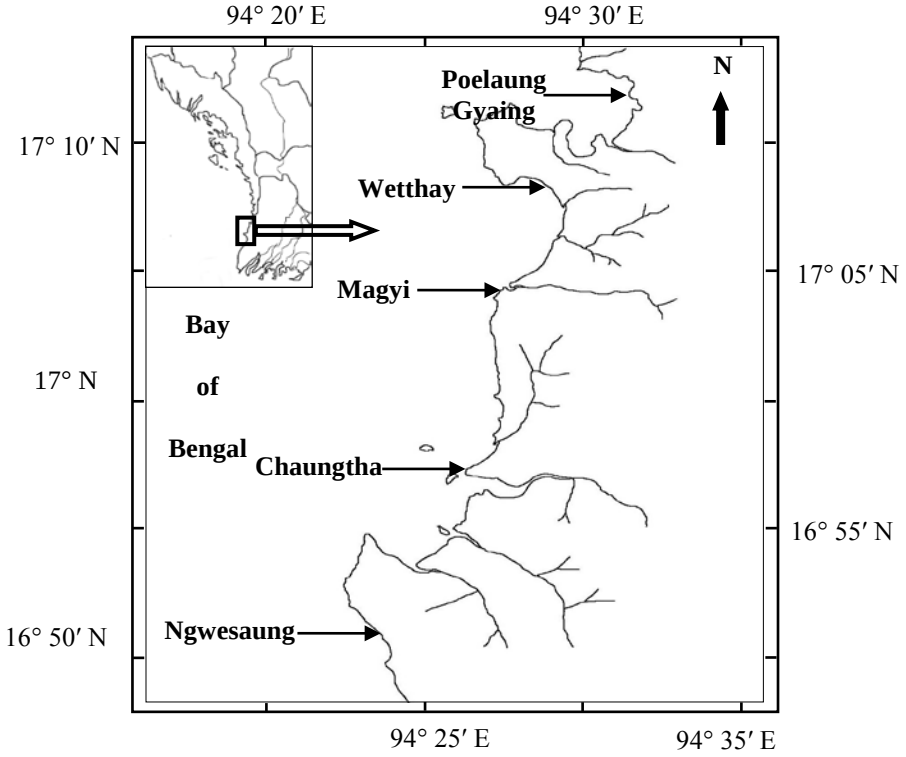

Figure 1 Map showing the sample collection sites of the study areas.

Environmental parameters

The seasonal variations of sea surface temperature and salinity of the study areas was shown in (Fig. 2). The seasonal range in temperature varied from 27°C to 36.5°C in Ngwesaung, 26.9°C to 32.1°C in Chaungtha, 26.9°C to 31.5°C in Magyi, 26.9°C to 31.5°C in Wetthay and 26.5°C to 32.1°C in Poelaung Gyaing. The salinity range was 23.3 ‰ to 33 ‰ in Ngwesaung, 23.3 ‰ to 34 ‰ in Chaungtha, 23.3 ‰ to 32 ‰ in Magyi, 24.3 ‰ to 33.6 ‰ in Wetthay and 25.5 ‰ to 33.1 ‰ in Poelaung Gyaing. (Fig. 2).

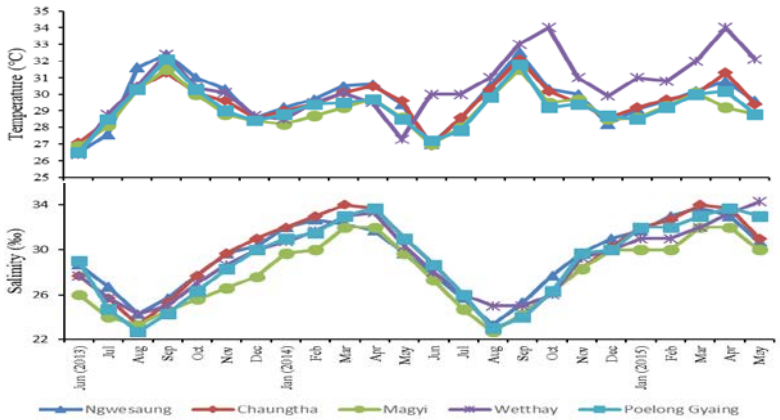

Figure 2 Seasonal changes of sea surface temperature and salinity at sampling stations.

Results and Discussion

Species compositions and abundance

A total of 25 species of amphipod belonging to 19 genera of 12 families were identified from all studied areas of the southern Rakhine coast. Family Gammaroidae was represented by 6 species, followed by Hyalidae with 4 species, Aoridae with 3 species, families Ampithoidae, Isaeidae, Leucothoidae with 2 species each and families Dexaminidae, Eophliantidae, Ischyroceridae, Lysianassidae, Phliantidae, Pleustidae with 1 species respectively. The distribution of amphipod species from the studied areas was shown in Table. 1.

Table 1 Distribution of amphipod species from the studied areas

No.	Name	Ngwesaung	Chaungtha	Magyi	Wetthay	Poelaung Gyaing
1.	*Ampithoe ramondi*	+	+	+	+	+
2.	*Cymadusa imbroglio*	-	+	+	+	+
3.	*Globosolembos ovatus*	+	+	-	-	-
4.	*Bemlos unicornis*	+	+	+	+	+
5.	*B. aequimanus*	-	+	-	+	-
6.	*Paradexamine rewa*	-	+	+	+	+
7.	*Bircenna dronga*	+	+	+	+	+
8.	*Hyale crassicornis*	+	+	+	+	+
9.	*H. galateae distorta*	+	+	+	+	+
10.	*H. rubra*	+	+	+	+	-
11.	*Parhyale hawaiensis*	+	+	+	+	-
12.	*Gammaropsis atlantica*	+	+	+	+	+
13.	*G. digitata*	+	+	-	+	-
14.	*Erichthonius brasiliensis*	-	+	-	+	-
15.	*Leucothoe diemenensis*	-	-	+	+	-
16.	*Leucothoella bannwarthi*	-	-	-	+	-
17.	*Parambasia nui*	+	-	-	-	-
18.	*Melita zeylanica*	+	+	+	+	+
19.	*Elasmopus palu*	+	+	+	+	+
20.	*E. pectenicrus*	+	+	+	+	+
21.	*Maera quadrimana*	+	+	+	+	+
22.	*M.serrata*	+	-	+	+	-
23.	*Perelasmopus* sp.	-	-	+	+	-
24.	*Pereionotus alaniphlias*	-	-	-	+	+
25.	*Parapleustes pulchellus*	+	+	+	+	+
	Total	17	19	18	23	14

The highest numbers of species were found in Wetthay station. The lowest was in Poelaung Gyaing station. *Ampithoe ramondi*, *Bemlos unicornis*, *Bircenna dronga*, *Hyale crassicornis*, *H. galateae distorta*, *Gammaropsis atlantica*, *Melita zeylanica*, *Elasmopus palu*, *E. pectenicrus*, *Maera quadrimana* and *Parapleustes pulchellus* were common at all stations. *Leucothoella bannwarthi* was only found in Wetthay station and *Parambasia nui* was recorded only in Ngwesaung station. Joseph (1978) recorded that 26 species of amphipods from 19 species of algal habitats. Seven species of six families of gammarid amphipod were recorded from seagrass beds of Libong Island, Trang Province, Thailand. Mondal *et al.* (2010) were identified 29 species of amphipods from all the nine habitats of southeast coast of India.

Ampithoe ramondi, *Bemlos unicornis*, *Bircenna dronga*, *Hyale crassicornis*, *H. galateae distorta*, *Gammaropsis atlantica*, *Melita zeylanica*, *Elasmopus palu*, *E. pectenicrus*, *Maera quadrimana* and *Parapleustes pulchellus* were dominated at all stations. The genera *Ampithoe* and *Cymadusa* were abundant in most of the algae and seagrass environments (Edgar 1983). *Ampithoe ramondi*, *Gammaropsis atlantica*, *Elasmopus pectenicrus* and *Erichthonius brasiliensis* were known to be circumtropical species (Appadoo 1997).

In the mean abundance of amphipod, its range from 50-150 ind/m^2 at Ngwesaung, 84-184 ind/m^2 at Chaungtha, 57-120 ind/m^2 at Magyi, 54-137 ind/m^2 at Wetthay and 24-72 ind/m^2 at Poelong Gyaing respectively (Fig. 3 and 4).

In the present study, the composition of amphipod fauna was higher numbers in seaweed habitats than the others (Table. 2). The melitids of the genus *Elasmopus* was commonly distributed on all seaweed. Appadoo (1997) stated that *Elasmopus pectenicrus* and *E. brasiliensis* have a high percentage frequency of occurrence only on algae. Moreover, the species occurring mainly on plant substrates (seaweed and seagrass) were *Maera quadrimana*, *Melita zeylanica*, *Gammaropsis atlantica*. *Ampithoe ramondi* was also found on coral rubble. Rao (1975) indicated that *Maera* and *Hyale* species were commonly distributed in the intertidal zone of the Indian coast.

It can be seen that amphipod species were also unevenly distributed in different seaweeds and seagrasses. The Hyalidae, *Hyale crassicornis*, *H. galateae distorta*, *H. rubra* and *Parhyale hawaiensis* were recorded on almost all the algae except for *Ulva* species. Their occurrence was coincided with the branched and complex type of seaweeds. The melitids of the genus *Maera* found only on a few species of algae. *Maera quadrimana* and *M. serrata* were recorded only on *Halimeda opuntia* and *Gracilaria canaliculata*. *Melita zeylanica* showed the common occurrence on all habitats. *Parelasmopus suensis* was recorded only on *Gracilaria canaliculata* among the others.

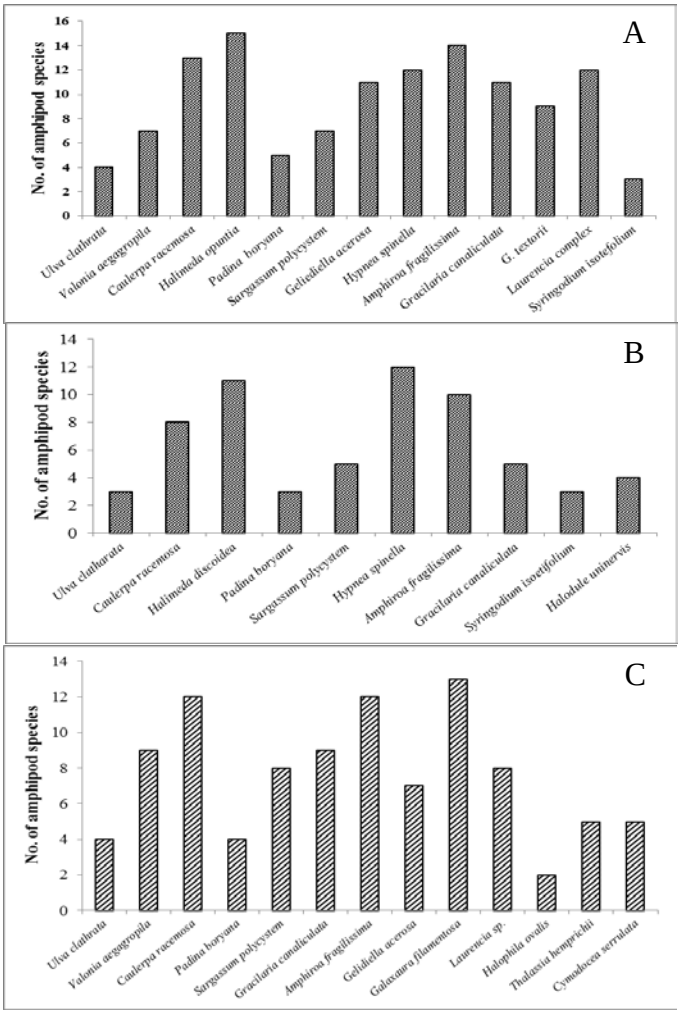

Figure 3 Distribution of ampipods on different seaweeds and seagrasses from (A) Ngwesaung, (B) Chaungtha, (C) Magyi.

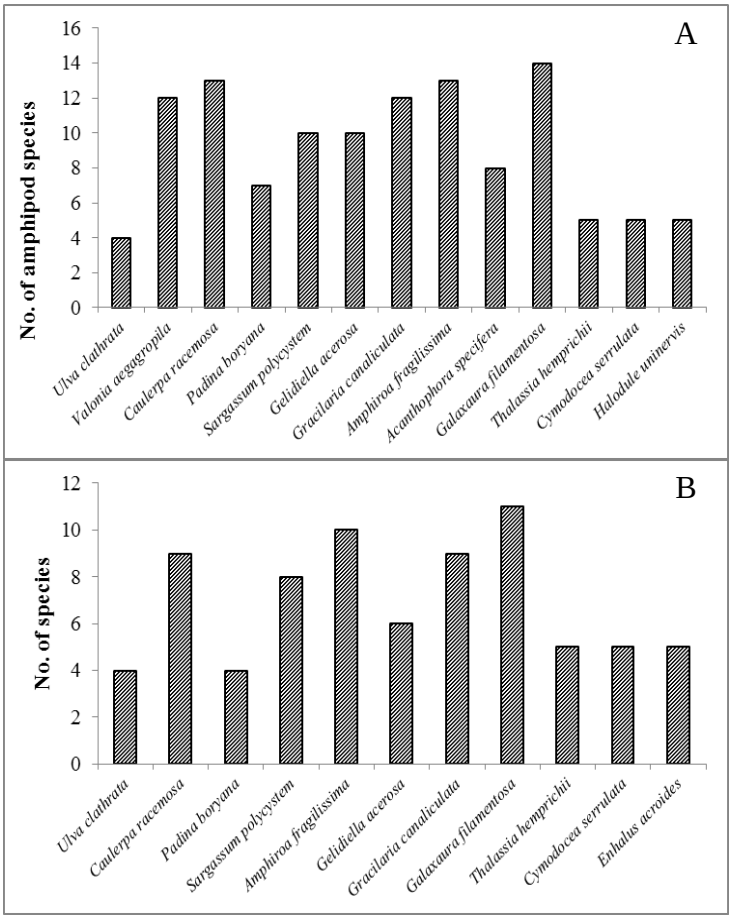

Figure 4 Distribution of ampipods on different seaweeds and seagrasses from (A) Wetthay, (B) Phoelaung Gyaing.

Ampithoe ramondi was commonly found on all algae whereas *Cymadusa imbroglio* only on a few algal categories. Ampithoids are the dominant groups that commonly herbivorous and prefer vegetative ecosystem such as seagrass bed and algae bed (Barnard 1970 and Myers 1985). Among the isaeid, *Gammaropsis atlantica* and *G. digitata* were observed only on *Caulerpa racemosa*, *Amphiroa fragilissima* and *Gelidiella acerosa*. Viejo (1999) observed that the gammarid amphipods were the most abundant group on both *Cystoseira* and *Sargassum*. The composition and density of the phytal fauna is influenced by the structure, texture, colour and the physical and developmental stage of alga. (Sarma and Ganapati 1972). In the present study, *Hyale* sp. was the abundant group of the algal habitats. Buschmann (1990) stated that the amphipods inhabit the algal resources as a refuge.

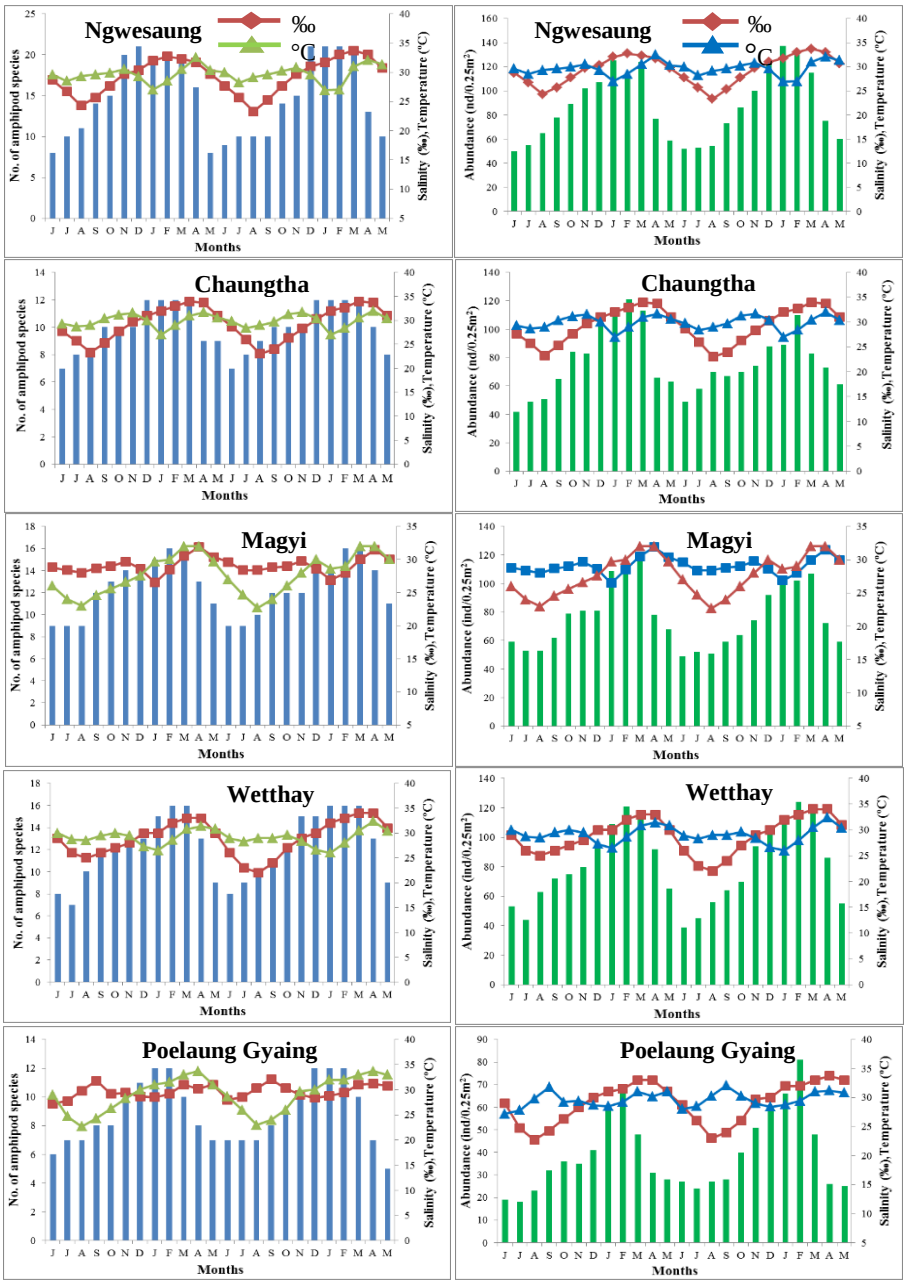

Figure 5 Comparison between species composition and abundance of amphipods in different salinity and temperature.

The Aoridae, *Bemlos unicornis* and *B. aequimanus* distributed only on some algal species. *B. aequimanus* was observed only in *Caulerpa racemosa* and *Amphiroa fragilissima*.

Seasonal variation of species composition and abundance of amphipod are shown in Fig. 5. It can be seen that both the composition and abundance of amphipod are higher in November to March but lower in June to September. This result is also coincident with the period of algal bloom. Soliman et. al., (2005) indicated that the abundance of macroalgae was the most important factor influencing amphipod distributions. Several studies have demonstrated that the assemblage structure of epibenthic marine fauna was influenced by the habitat heterogeneity and complexity (Edgar 1983).

As seen in Figure 5., the species composition and abundance of amphipods was correlated with the increasing salinity in all stations. In general, the increased number of species was coincided with the gradually increased of salinity at all stations. Moreover, the low salinity periods were also noticed that the species composition of amphipod was decreased. But the effect of temperature was not influenced markedly on species composition and abundance of amphipods. In all stations, the abundance of ampipod was also related to the salinity regime of the study sites. The high salinity period from December to March was resulted in an increased in abundance of amphipod.

Conclusion

A total of 25 species of amphipod were recorded from the studied areas. *Elasmopus palu*, *E. pectenicrus*, *Hyale crassicornis* and *H. galateae distorta* were by far the most numerous and widespread amphipods at all stations, very widespread were also *Ampithoe ramondi*, *Melita zeylanica*, *Maera quadrimana* and *Parapleustes pulchellus*. The composition of amphipod fauna was higher numbers in seaweed habitats than seagrasses and unvegetated habitats. *Elasmopus* sp. was the most abundant group at all stations. The abundance and species composition of amphipod were related to the algal blooming periods. The result of this study indicated that the seaweed and seagrass habitats and their complexity are important for the amphipod fauna. Amphipod and other associated epifauna provide tropic links between primary producers and predatory fishes. Consequently, understanding the role of seaweed and seagrass dominated habitats is necessary and these plants should not be destroyed through human activities like harvesting of seaweeds and seagrasses from their natural habitats, coastal developments, recreational activities and beach cleaning activities that can deplete valuable seaweeds and seagrasses from the coast.

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FINE-SCALE SPATIAL AND TEMPORAL HETEROGENEITY OF PHYTOPLANKTON IN THE INNER TOKYO BAY, JAPAN

Khin Khin Gyi [1]

Abstract

The spatial and temporal variations of phytoplankton were governed by the physicochemical properties of the water column such as water stratification, vertical mixing, temperature and salinity gradients, and nutrient supply. In this study, the salinity gradient was pronounced in the upper layer, and a strong stratification developed in the water column which in turn influenced large variability in the vertical distributions and cell concentrations of phytoplankton. In terms of cell concentration, nine species such as *Lauderia annulata, Skeletonema* sp., *Thalassiosira* spp., *Chaetoceros* sp., *Thalassionema frauenfeldii*, *T. nitzschioides*, *Pseudo-nitzschia* sp., and *Heterosigma akashiwo* dominated the phytoplankton community, and the percentage of their abundance comprised 89.5% of the total phytoplankton concentration. Among these species, the cells of *Pseudo-nitzschia* sp. and *H. akashiwo* increased at night, approximately 4 to 8 times higher than in the daytime, due to the changes in the water mass. At the most depth range, all species had higher cell concentrations in the upper layers above the pycnocline where Chl-*a* showed the maximum. However, a significant decrease in cell concentration was noted below the pycnocline where the hypoxic water mass was observed.

Keywords hypoxic, phytoplankton, pycnocline, salinity, stratification

Introduction

The phytoplankton community in the vertical water column is highly heterogeneous and related to the small-scale physical hydrodynamic gradients such as water mass stability (Mellard et al., 2011), mixing (Maznah et al., 2016), and light availability (Tilzer and Goldman, 1978). Abiotic water conditions varied naturally at different timescales in a day (Gast et al., 2014), related to the periodic oscillations of the tidal currents, which consecutively influence short-term changes in the phytoplankton community (Blauw et al., 2012; Khin Khin Gyi, 2020).

The inner region of Tokyo Bay is a eutrophic semi-closed coastal embayment due to urban runoff, river discharge, and industrial wastes from metropolitan areas (Furukawa and Okada, 2006). As a result of freshwater discharge from rivers in Tokyo Bay, a sharp horizontal and vertical salinity gradient was prominent. Moreover, the formation of anoxic or hypoxic water mass during stratification and the vertical distribution of nutrients may result in great fluctuations of phytoplankton abundance, composition, and the vertical patchiness of algal blooming in the inner Tokyo Bay. Therefore, to understand the controlling factors of the phytoplankton community, many previous studies from various aspects of the ecological approaches had already been conducted in Tokyo Bay (e.g., Han, 1988; Han and Furuya, 2000; Koibuchi and Isobe, 2007; Nakane et al., 2008; Bouman et al., 2010). However, their works mainly focused on seasonal and monthly variations, and a few studies have achieved a sampling frequency of nearly weekly intervals. It is remaining to explore how the phytoplankton fluctuate at a short timescale of the day. Here in this paper, a fine-scale vertical structure of phytoplankton was observed at 1 m depth intervals, dealing with the environmental properties of the water column between day and night hours. The objectives of the present study were (1) to clarify the fine-scale spatiotemporal heterogeneity of phytoplankton distributions in species-level, and (2) to observe the species-specific distribution patterns within the communities during different hours of the study period.

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Materials and Methods

Sampling site and sample collection

Phytoplankton samples were collected using a vortex submersible pump to sample simultaneously throughout the vertical water column from 0 to 20 m at every 1 m interval at a fixed station of 23 m deep in the inner Tokyo Bay (35°30′30″ N, 139°50′00″ E), Japan (Fig. 1). Day and night samplings were conducted at the same point of the above-mentioned fixed station from a training and research vessel "Hiyodori" of Tokyo University of Marine Science and Technology at a local time 10:00 and 20:45 (JST) on 21 September 2017. A total of 42 samples were collected from 21 distinct vertical water layers within 0-20 m depth to understand the fine-scale distribution of phytoplankton.

Physical and chemical analyses

Hydrographic profiles of water temperature, salinity, sigma-t, and dissolved oxygen were observed using a CTD (AAQ-PRO 2, JFE Advantech, Tokyo, Japan) equipped with sensors for in vivo chlorophyll-*a* fluorescence (Chl-*a*) and turbidity. For inorganic nutrient analyses (NO_3+NO_2, PO_4, SiO_4, and NH_4), 15 mL of water sampled at each depth was filtered through a 0.20 µm pore size syringe filter (DISMIC-25CS, Advantec, Tokyo, Japan) on the boat deck and frozen at -20°C until analysis. Nutrient concentrations were determined using an automated nutrient analyzer, QuAAtro (SEAL Analytical, Hampshire, UK) in the laboratory.

Phytoplankton analysis

Water samples of 500 mL were taken for phytoplankton analysis during each CTD cast by attaching a pump intake hose with a CTD and immediately fixed with formalin (final concentration 1%). For the abundance, phytoplankton samples were analyzed with a FlowCAM (Fluid Imaging Technologies, Inc., ME, USA) in triplicate under the autoimage mode.

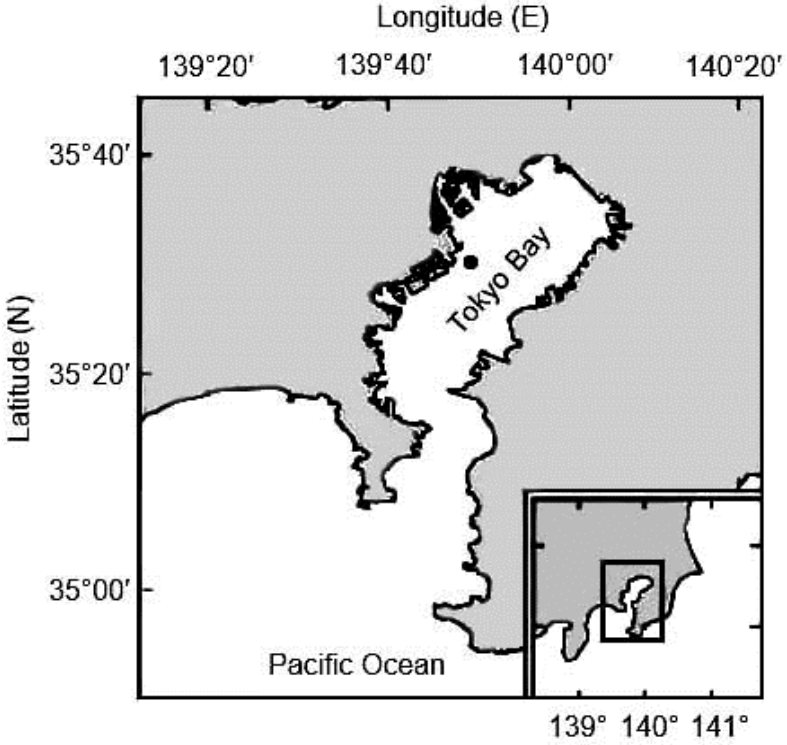

Figure 1 Sampling site in the inner Tokyo Bay, Japan.

Results and Discussion

Hydrological conditions

In the daytime (10:00), the upper layer (top 6 m) was marked with a strong pycnocline, being recognized by the drastic changes in the water salinity (27.95-30.96). At the same time, the fluctuations in the water temperature (22.83-23.13°C) and dissolved oxygen concentration (4.54-5.85 mg L^{-1}) were noted, especially in the lower limit of the pycnocline (4-6 m). The concentration of Chl-*a* showed a maximum value (> 5 µg L^{-1}) in the upper layer (3-4 m) but decreased sharply in the lower limit of the pycnocline, then gradually decreased to the minimum value (1.5 µg L^{-1}) in the 14-20 m depth. Below the pycnocline, a well-mixed water layer was observed at 7-11 m, but a weakly stratified layer (3 m thick) existed underneath, which separated the intermediate (7-11 m) and the bottom mixed layer depth (14-20 m). Simultaneously, the bottom water became hypoxic (≤ 2.5 mg L^{-1}) at 14-20 m depth (Fig. 2. a).

At night-time (20:45), a multi-stepped structure in the water column was eroded, while the pycnocline deepened to 5-10 m with the vertical salinity gradients (28.47-32.45) which separated the surface and the bottom layers. The maximum values of water temperature (23.45°C), dissolved oxygen (8.96 mg L^{-1}), and Chl-*a* concentration (11.77 µg L^{-1}) were observed in the surface layer coincided with the upper limit of the pycnocline but decreased rapidly in the pycnocline till its lower limit, then gradually decreasing towards the bottom. Below the pycnocline, the water mass became nearly hypoxic (≤ 2.5 mg L^{-1}), and the thickness increased to 10 m. Based on the profiles, salinity variations resemble density variations, indicating that salinity is more important than water temperature in determining the density field in our observation area. Turbidity and dissolved oxygen concentration showed a negative trend in both observation periods (Fig. 2. b).

Dissolved inorganic nutrients

The vertical distribution of inorganic nutrient concentrations during the day and night times (Figs. 3. a, b) showed that nitrate and nitrite, phosphate, silicate, and ammonium concentrations varied between 8.1 and 39.2 µM L^{-1}, between 1.1 and 3.5 µM L^{-1}, between 22 and 56.8 µM L^{-1}, and between 1.6 and 14.7 µM L^{-1}, respectively during the study period. The concentration of each inorganic nutrient showed significant peaks in the surface layer above the pycnocline but decreased below it.

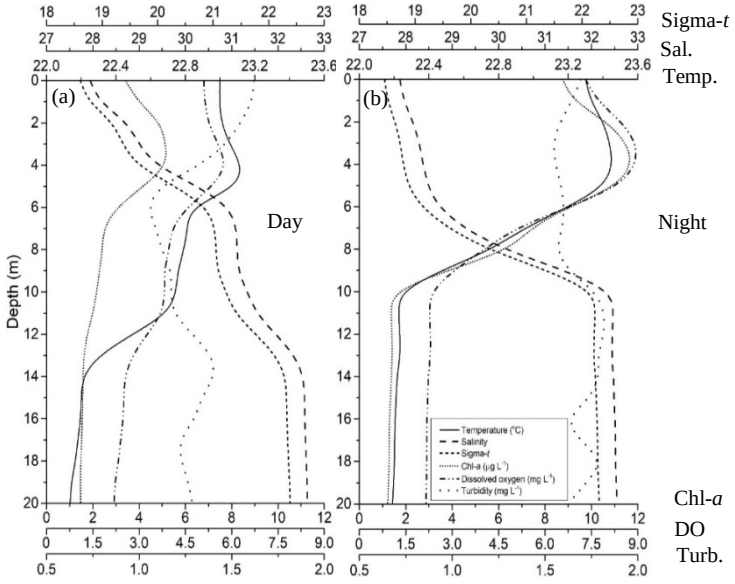

Figure 2 Day and night vertical distributions of water temperature (°C), salinity, sigma-*t*, Chl-*a* (µg L^{-1}), dissolved oxygen (mg L^{-1}), and turbidity (mg L^{-1}).

Spatial and temporal distributions of total phytoplankton

The vertical distribution of the total phytoplankton community showed the same pattern between day and night (Figs. 4. a, b). The phytoplankton concentration was higher in the surface layer where Chl-*a* showed the maximum but decreased sharply in the pycnocline till its lower limit, then little variation was found below the pycnocline towards the bottom. During the observation, the pycnocline was deepening at night which increases the surface layer of phytoplankton abundance. Moreover, the phytoplankton concentration and Chl-*a* values increased double at night due to a significant increase in the cell concentrations of *Pseudo-nitzschia* sp. and the Raphidophycean flagellate, *Heterosigma akashiwo* occurred in the upper 0-5 m and 5-8 m depths, respectively.

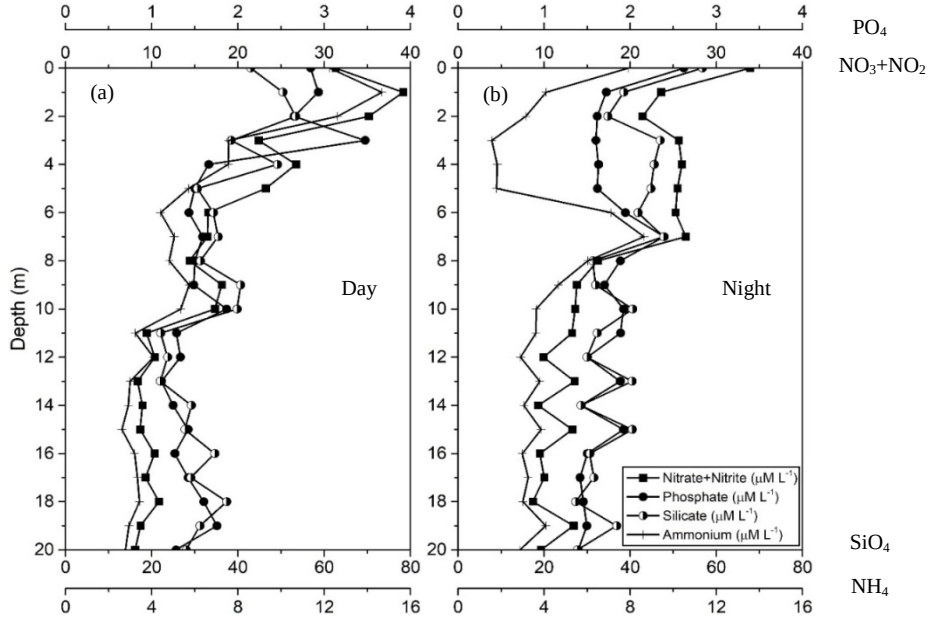

Figure 3 Day and night vertical distributions of nitrate and nitrite (μM L^{-1}), phosphate (μM L^{-1}), silicate (μM L^{-1}), and ammonium concentrations (μM L^{-1}).

Phytoplankton assemblage composition

During the study period, a total of 26 phytoplankton taxa, including 11 diatoms (Bacillariophyceae), 13 dinoflagellates (Dinophyceae), and 1 species of Raphidophyceae and Dictyochophyceae were identified. In the phytoplankton community, diatoms were the most abundant group, accounting on average for 77% of the total cell concentration of phytoplankton. Besides, dinoflagellates comprised only 8% of the community, and other phytoplankton groups (such as Raphidophyceae, Dictyochophyceae, and the unidentified phytoplankton), altogether accounted for 15% of the total cell concentration of phytoplankton (Table 1).

In terms of percentage composition, nine phytoplankton species such as *Pseudo-nitzschia* sp. (4,411 cells mL^{-1}, 20.6%), *Heterosigma akashiwo* (3,026 cells mL^{-1}, 14.2%), *Thalassiosira* sp. 2 (3,013 cells mL^{-1}, 14.1%), *Thalassionema frauenfeldii* (2,226 cells mL^{-1}, 10.4%), *T. nitzschioides* (2,158 cells mL^{-1}, 10.1%), *Skeletonema* sp. (1,316 cells mL^{-1}, 6.2%), *Lauderia annulata* (1,235 cells mL^{-1}, 5.8%), *Thalassiosira* sp. 1 (882 cells mL^{-1}, 4.1%) and *Chaetoceros* sp. (855 cells mL^{-1}, 4.0%), were noted as the dominant species and the percentage of their abundance comprised 89.5% of the total cell concentration of phytoplankton (Table 1).

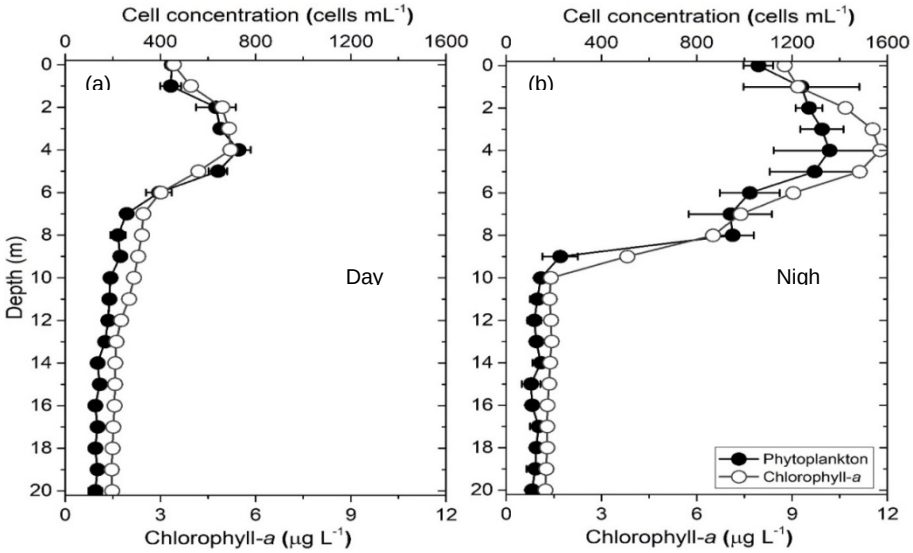

Figure 4 Day and night vertical distributions of total phytoplankton and Chl-*a* concentration.

Table 1 A list of phytoplankton in Tokyo Bay with total abundance (cells mL^{-1}) and percentage composition (%).

Phytoplankton	Total abundance	
	cells mL^{-1}	%
Bacillariophyceae		
Lauderia annulata	1,235	5.8
Skeletonema sp.	1,316	6.2
Thalassiosira sp. 1	882	4.1
Thalassiosira sp. 2	3,013	14.1
Coscinodiscus sp.	55	0.3
Chaetoceros sp.	855	4.0
Thalassionema frauenfeldii	2,226	10.4
Thalassionema nitzschioides	2,158	10.1
Meuniera membranacea	54	0.3
Pleurosigma sp.	162	0.8
Pseudo-nitzschia sp.	4,410	20.6
Dinophyceae		
Prorocentrum minimum	272	1.3
P. micans	108	0.5
Dinophysis sp.	135	0.6
Gyrodinium sp.	109	0.5
Ceratium furca	502	2.3
C. fusus	68	0.3
C. tripos	14	0.1
Gymnodinium mikimotoi	14	0.1
Oxyphysis oxytoxoides	54	0.3
Pyrophacus sp.	27	0.1
Protoperidinium oblongum	41	0.2
P. quinquecorne	95	0.4
Scrippsiella trochoidea	366	1.7
Raphidophyceae		
Heterosigma akashiwo	3,027	14.2
Dictyochophyceae		
Dictyocha speculum	41	0.2
Unidentified phytoplankton	136	0.6

Species-specific distributions of dominant phytoplankton

Fig. 5 showed species-specific patterns in the vertical distributions of nine dominant phytoplankton species during day-and-night times. In general, the samples of these dominant phytoplankton species showed higher cell concentrations during the night than day. Among these species, the cell concentrations of *Pseudo-nitzschia* sp. and *H. akashiwo* increased at night, approximately 4 to 8 times higher than in the daytime. At the most depth range, all species had higher cell concentrations in the upper layer (0-8 m) coinciding with the Chl-*a* maximum. Below it, their abundance significantly decreased and was quite steady at the greater depths, suggesting these species reveals distinct depth habitats. However, the variability does not markedly affect the pattern of diel vertical migration because most dominant phytoplankton species showed almost similar patterns in the vertical distributions between day and night. Out of these, some species such as *Thalassionema frauenfeldii*, *T. nitzschioides*, and *Skeletonema* sp. exhibited distinct vertical distribution patterns between day and night. In the daytime, *Thalassionema frauenfeldii* had a fairly even vertical distribution in the water column, but they concentrated in the upper layer (0-5 m) during the night. *T. nitzschioides* were found throughout the water column during the day; however, they were mainly distributed in the upper layer (0-5 m) during the night and were not observed below 10 m depth. *Skeletonema* sp. distributed almost at all sampling depths during the day, but they concentrated in the upper 0-5 m during the night and did not occur below 6 m depth.

Discussion

The spatial and temporal variations of the phytoplankton community in the coastal waters are governed by the physicochemical properties of the water column such as water stratification, vertical mixing, temperature and salinity gradients, and nutrient supply (Pennock, 1985). In the present study, the salinity gradient was pronounced at the upper layer (10 m) due to the influence of freshwater discharge from rivers (Horie et al., 2010). Dealing with this characteristic of water properties, a strong stratification developed in the water column, which in turn influenced the vertical distribution pattern of phytoplankton. Sprintall and Cronin (2001) reported water mass stability or formation of pycnocline which may act as a barrier in the water column, where the sedimentation of particles may be prevented by its marked density gradients. Therefore, in the present study, the vertical distribution of phytoplankton was significantly different above and below the pycnocline (see Figs. 4. a, b).

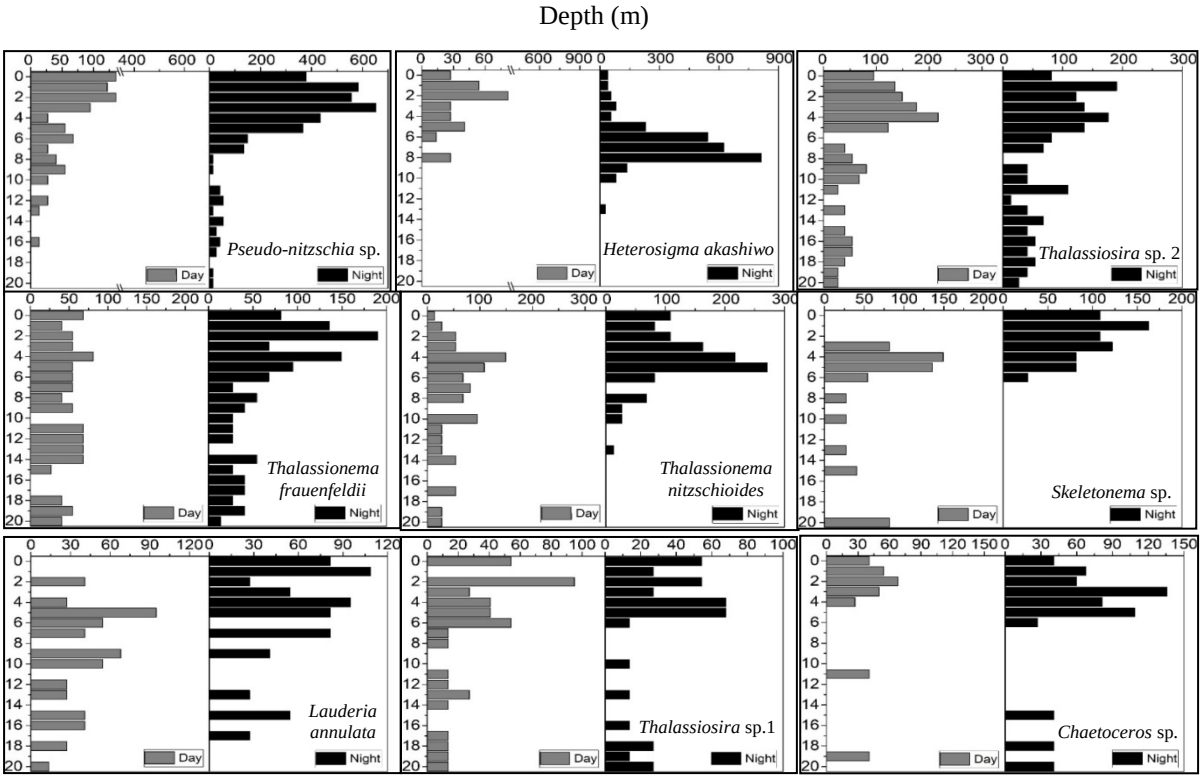

Figure 5 Species-specific vertical distribution of dominant phytoplankton (cells mL^{-1}) during day and night.

During the study period, diatoms were found to be the most dominant group in the phytoplankton community, accounting for 77% of the total cell concentration of phytoplankton. This result was consistent with the previous finding by Nakane et al. (2008) who reported the diatoms encompassed a major component (84.4%) in the phytoplankton community in inner Tokyo Bay. It was suggested that diatoms can grow rapidly in eutrophic conditions (Tada et al., 2009; Guo et al., 2014) because Tokyo Bay (especially the inner section) is one of the most eutrophic embayments in Japan (Furukawa, 2015). In terms of cell concentration, *Lauderia annulata*, *Skeletonema* sp., *Thalassiosira* sp. 1 and 2, *Chaetoceros* sp., *Thalassionema frauenfeldii, T. nitzschioides*, *Pseudo-nitzschia* sp. and *Heterosigma akashiwo* were noted as the dominant species in the community. All these species had higher cell concentrations in the upper layer (10 m) but significantly decreased below 10 m depth (Fig. 5). These distinct depth habitats may deal with the presence of pycnocline which can restrict the phytoplankton distribution. It was further considered that the nutrient concentration was higher in the upper layer due to river discharge which could give favorable conditions for their abundance.

T. frauenfeldii, *T. nitzschioides*, and *Skeletonema* sp. showed distinct patterns in their vertical distributions between day and night. They were found throughout the water column during the day but were not observed below 10 m depth during the night with exception of *T. frauenfeldii*, which was detected below 10 m depth at night (but with low concentration) (see Fig. 5). This may due to the increase of the bottom hypoxic water layer (10 m thick) at night, which can limit the distribution of phytoplankton (Rabalais *et al*., 2010). Moreover, a significant increase in the cell concentrations of *Pseudo-nitzschia* sp. and *H. akashiwo* were observed in the upper layer at night. This may probably be related to the changes in the water mass.

Conclusion

The fine-scale spatial and temporal distributions of phytoplankton were observed in the inner Tokyo Bay. The vertical profiles of the most dominant phytoplankton species did not show day and night differences in their distribution patterns; however, each species occupied well-defined depth habitats. Large variability in the vertical distribution and concentration of phytoplankton was resulting from the presence of pycnocline, which acts as a barrier in the water column, and the nutrient loads from freshwater discharge from rivers in the upper layers that might increase the cell concentration of phytoplankton. On the other hand, the water mass tends to become hypoxic below the pycnocline which might limit the distribution of phytoplankton. Therefore, it was noted that changes in the vertical distribution of phytoplankton were strongly influenced by the strength and thickness of the pycnocline and the associated hydrodynamic properties.

Acknowledgments

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LENGTH FREQUENCY DISTRIBUTION, LENGTH-WEIGHT RELATIONSHIPS AND THE CONDITION FACTOR OF THE SPADENOSE SHARK *SCOLIODON LATICAUDUS* MULLER & HENLE, 1838 IN MON COASTAL WATER

Myo Min Tun[1]

Abstract

Population parameters of the spadenose shark *Scoliodon laticaudus* were estimated for a total of 180 specimens in each station from the fish landing sites were also studied periods. The estimated length-weight relationship of *S. laticaudus* showed a negative allometric growth pattern (b<3) in Zeephyuthaung and Setse and an isometric growth pattern (b=3) in Kyaikkhami. According to length frequency distribution, the juveniles of shark were observed throughout the study period and (10-12 cm) size group was recruited to the fishery from October to December in Setse. The value of K was the highest in February at Setse.

Keywords condition factor, length frequency distribution, length-weight relationships, Mon coastal water, *Scoliodon laticaudus*, spadenose shark,

Introduction

Establishment of a relationship between length and weight is necessary for the calculation of fish condition and biomass of a fish population. Length-weight relationships (LWRs) are also useful for life history and morphological comparisons of populations from different locations and these relationships allow conversion of a growth equation in length to a growth equation in weight. Basic biological data needed for stock assessment are lacking for many of these sharks and rays, including size values and size relationships/ conversions. These data are essential for understanding growth rate, age structure and other aspects of population dynamics. Size conversions have a practical value in fisheries.

The study of distribution of the species and the stocks from which the fishery is supported forms valuable information in assessing the fishery potential. In addition, it is also important to know the estimation of population parameters. The windows version of FiSAT II is a program package consisting of methodologies for use with computers, enabling users to formulate some management options for fisheries, especially in data-sparse, tropical contexts. It was developed mainly for the analysis of length-frequency data but also enables related analyses, size-at-age, catch-at-age, selection and others.

The objective of the present study is to estimate the length frequency distribution, length-weight relationships and the condition factor of the spadenose shark *Scoliodon laticaudus* along the Mon coastal areas including Ahlayt, Sebalar, Kyaikkhami, Setse and Zeephyuthaung.

Materials and Methods

The monthly data on the length frequency of specimens collected from the study areas during the study periods were examined. A total of 180 fish samples of shark from each station were arranged with the class interval of 2 cm. And then, the mean value, mode and standard deviation were computed by excel program. The total length (from the tip of the snout to the extended tip of the caudal fin) of each sample was measured to the nearest centimeter by measuring board and the fish body weight was measured to the nearest gram by Balance (1 Kg) and. LWRs were estimated by fitting an exponential curve, $W = aL^b$ (Le Cren, 1951).

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Parameters *a* and *b* of the exponential curve were estimated by linear regression analysis over log-transformed data log W = Log *a* + *b* LogL, where W is the total weight (g), L is the total length (cm), *a* is the intercept and *b* is the slope. The degree of association between the variables was computed by the determination coefficient, r^2. The condition factor (K) of fish was determined by using the following formula:

$K= 100*W/L^3$ (Pauly 1983 as cited in Fafioye and Oluajo 2005)
Where, K = condition factor, W= weight of fish in gram and L= total length of fish in cm.

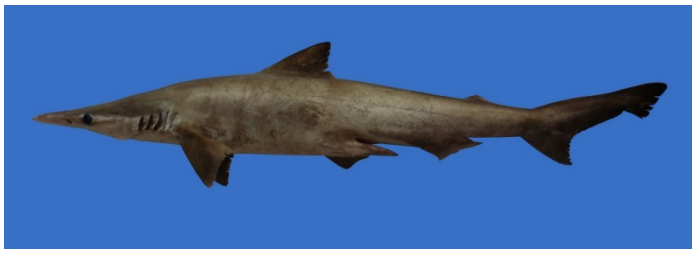

Figure 1 Selected species the spadenose shark *Scoliodon laticaudus*

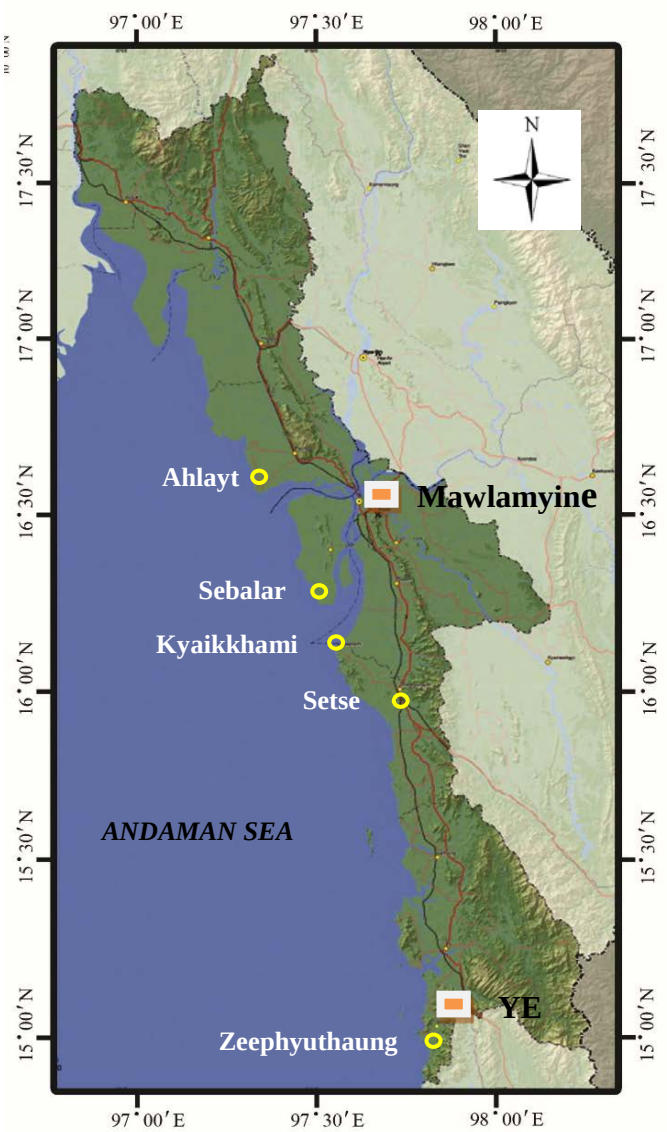

Figure 2 Map showing the sample collection sites of the study areas.

Results and Discussion

Length frequency distribution of the shark *Scoliodon laticaudus*

The length frequency data of *Scoliodon laticaudus* ranging from the size group of total length 10-12 cm to 48-50 cm were collected from October 2015 to September 2016 in Zeephyuthaung, Setse, Kyaikkhami, Sebalar and Ahlayt. In Zeephyuthaung, the size group of 30-32 cm was the most dominant in the catch in May. In October and November, the size groups of 36-38 cm and 38-40 cm occurred abundantly. In May, August and December, the size group of 32-34 cm occurred abundantly. The size group of 22-24 cm occurred the highest abundant in January. In February, May and July, the size group of 30-32 cm occurred abundantly. The size group of 26-28 cm occurred the higest abundant in March. A large number of 40-42 size groups were recorded in April and June. The size group of 34-36 cm was mostly dominant in the catch in Zeephyuthaung. The size group of 34-36 cm, 36-38 cm and 38-40 cm were the most abundant at Zeephyuthaung landing site.

In Setse, the size group of 16-18 cm was abundant in the catch in October. In November and December, the size group of 14-16 cm was dominant. The most abundant size group of 30-32 cm was observed in January, April, May and July. The size group of 12-14 cm was abundant in February. In March, the size group of 40-42 cm was abundant. In June and August, the size group of 38-40 cm was observed abundantly. The size group of 34-36 cm was abundant in September. The size groups of 30-32 cm, 32-34 cm and 34-36 cm were the most abundant at Setse landing site.

In the landing site of Kyaikkhami, the size group of 20-22 cm was found abundantly in March and May. The most abundant size group of 30-32 cm was observed from August to January. In April, the size group of 40-42 cm was abundantly observed. The size group of 34-36 cm was abundant in January and February. In June and September, the size group of 36-38 cm was abundant. In July the size group of 38-40 cm was abundant and 30-32 cm show as a peak in October. The size group of 30-32 cm, 32-34 cm and 34-36 cm were the most abundant at Kyaikkhami.

In Sebalar, the size group of 30-32 cm was abundant in October, January, February and May. In November, December and March, the size of 32-34 cm was abundantly found. The size group of 32-34 cm, 38-40 cm and 40-42 cm were abundant in March. In April, the size group of 40-42 cm and 42-44 cm were abundant. The size group of 36-38 cm was dominant in June, July and August. In September, the size group os 28-30 cm occurred abundantly. The size group of 30-32 cm, 36-38 cm and 32-34 cm were the most abundant at Sebalar and 30-32 cm shows a peak in October.

In Ahlayt fish landing site, the size group of 30-32 cm was mostly found in September, October, December and May. In March, August, September and November the size group of 32-34 cm occurred abundantly. The large group of 34-36 cm was observed abundantly in August, November and January. In February, the size group of 38-40 cm and 40-42 cm were abundant. In February and April, the size group of 38-40 cm was dominant. The size of 36-38 cm was dominant in June and November. In July, the size group of 46-48 cm was abundant. The size groups of 30-32 cm, 32-34 cm, 36-38 cm, 34-36 cm and 38-40 cm were the most abundant at Ahlayt landing site and 32-34 cm shows a peak in March. The highest mean lengths are B 36.57 ± 5.560 cm in Ahlayt and the lowest mean length, 29.03 ± 9.678 cm in Setse.

Length-weight relationships of the shark *Scoliodon laticaudus*

A total of 180 fishes were measured in each station throughout the study period. In Zeephyuthaung, the size group from 22 cm to 48 cm (TL) with mean length of 34.95 ± 4.828 cm of *Scoliodon laticaudus* were used to estimate their length-weight relationships and the mean

weight of 161.61 ± 69.797 g were observed in this study. In Setse fish landing site, the size group of this species from 10 cm to 48 cm (TL) with mean length of 29.03 ± 9.678 cm were used to estimate their length-weight relationships and the mean weight of 124.12 ± 93.116 g was found.

The size group of *S. laticaudus* from 18 cm to 46 cm (TL) with mean length of 33.62 ± 5.961 cm were studied to estimate their length-weight relationships and the mean weight of 148.73 ± 86.708 g was observed in the fish landing site of Kyaikkhami. In Sebalar, the fish ranging in size from 12 cm to 46 cm (TL) with mean length of 33.8 ± 6.436 cm were used to estimate their length-weight relationships and the mean weight of 153.75 ± 94.841g was studied. In the fish landing site of Ahlayt, the size group of this species from 20 cm to 50 cm (TL) with mean length of 36.57 ± 5.560 cm were used to estimate their length-weight relationships and the mean weight of 196.38 ± 99.158 g was observed in this study. The relationship was obtained by using the formula Log W = Log a + b Log L for males and females separately. The values of parameters "a" and "b" are expected from the linear regression equation of logarithmic conversion values of length and weight. The linear regression equation: Y = a+bX.

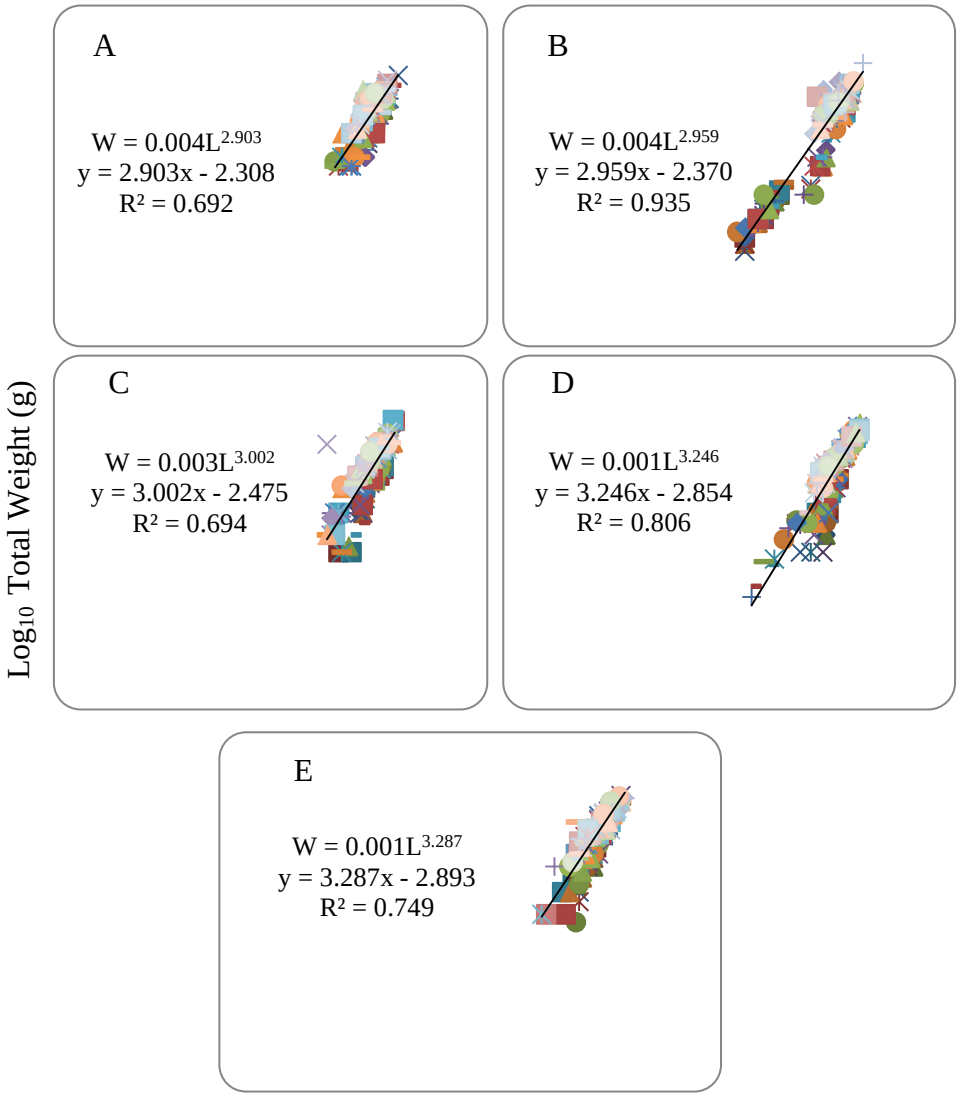

Figure 3 Length-weight relationship of *Scoliodon laticaudus* in (A. Zeephyuthaung, B. Setse, C. Kyaikkhami, D. Sebalar, E. Ahlayt) from October 2015 to September 2016.

The value of "a" need to transform antilogarithm value to express, $W = \alpha L^b$ where "α" is the antilogarithm value of "a". The exponent (b) value of *S. laticaudus* was 2.903 in Zeephyuthaung, 2.959 in Setse, 3.002 in Kyaikkhami, 3.246 in Sebalar and 3.287 in Ahlayt respectively. Their length-weight relationships were $W = 0.004L^{2.903}$, r = 0.8318 in Zeephyuthaung, $W = 0.004L^{2.959}$, r = 0.9669 in Setse, $W = 0.003L^{3.002}$, r = 0.8331 in Kyaikkhami, $W = 0.001L^{3.246}$, r = 0.8978 in Sebalar and $W = 0.001L^{3.287}$, r = 0.8654 in Ahlayt stations respectively. (Fig 3. A-E)

Condition Factor (K) of the shark *Scoliodon laticaudus*

The monthly condition factor (k) of *Scoliodon laticaudus* in dominant group (ranging from 10 to 50 cm total length) was calculated during the study period. The condition factor was estimated for different months as shown in Table 6.1. The mean values of condition factor (k) in Zeephyuthaung varied from 0.278096 to 0.491526 and the maximum condition factor values occurred in July while the minimum value was in November. The condition factor indicated increasing variation in February. In Setse, the condition factor varied from 0.223334 to 0.627087 and the highest k values was recorded in February and the lowest k values were in January. In Kyaikkhami, the k values ranged from 0.242627 to 0.463537. May was the highest k values and the lowest was in December. The condition factor at the station Sebalar varied from 0.206446 to 0.438170. The maximum condition factor values occurred in May while the minimum value was in October. In Ahlayt, the k values ranged from 0.229543 to 0.466968 and the May has the highest which is compared with the other months during the study period. The highest k values of May were found in Kyaikkhami, Sebalar and Ahlayt stations. The lowest condition factor occurred in the months of October to January. During the study period, the highest k value (0.627087) was found in May at the station Kyaikkhami. The lowest condition factor (0.206446) occurred in October at the station Sebalar. The condition factor of *S. laticaudus* for different study areas is represented in Figure 4.

Population parameters of the shark *Scoliodon laticaudus* were estimated by using the length frequency data collected from October 2015 to September 2016. The length-weight relationships of these species were estimated by using the formula '$W = aL^b$'. The resultant 'b' value can determine the growth of fish. Fish can attain isometric or allometric growth. When the 'b' value equals 3, growth of fish is isometric which means that no change of body shape as a fish grows. If the 'b' value is larger or smaller than 3, growth of fish is allometric: positive allometric if $b > 3$ (fish becomes relatively stouter or deeper-bodied as it increases in weight) or negative allometric if $b < 3$ (fish becomes more slender as it increases in weight). Hile (1936) and Martin (1949) proposed that the value of exponent (b) in fish usually ranges between 2.5 and 4.0 (Chakravarty *et al*. 2012)

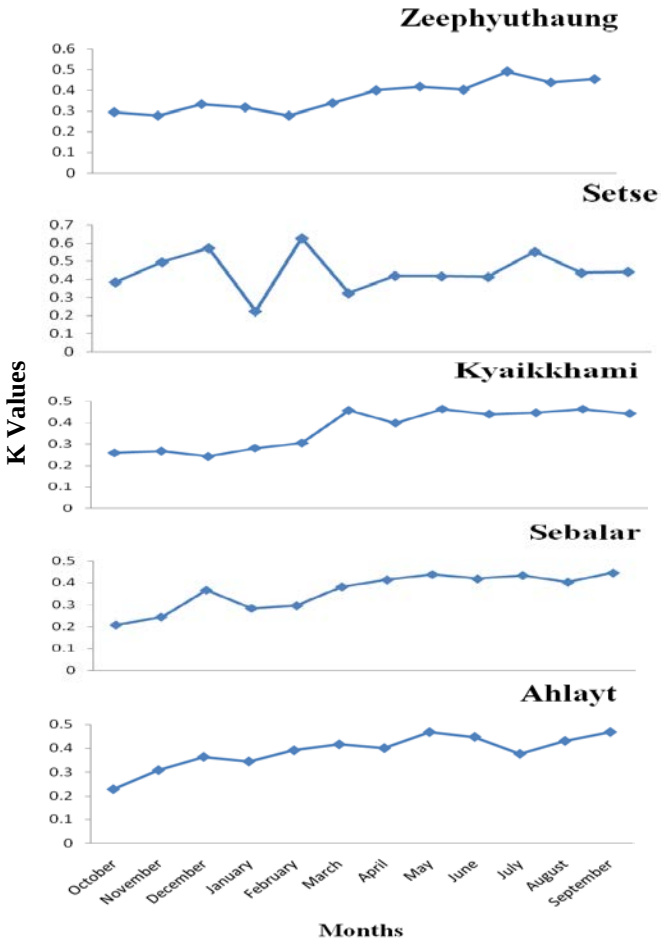

Figure 4 Monthly condition factor of the shark *Scoliodon latcaudus* in different studied areas from October 2015 to September 2016.

Length-weight relationships have a most important role in fisheries studies. In the present study, the least value of "b" for *Scoliodon laticaudus* is 2.903 in Zeephyuthaung landing site and the most value of "b" is 3.287 in Ahlayt site. Due to the fact, the length of fish used for estimation ranged from 34-40 cm in Zeephyuthaung, 30-36 cm in Setse and Kyaikkhami, 30-38 cm in Sebalar and 30-40 cm in Ahlayt landing sites. The combined relationships of males and females with indeterminate in five study areas are 2.903, 2.959, 3.002, 3.246 and 3.287, indicating that the rate of increase in body length is nearly proportional to the rate of increase in the body weight. Among them, the higher "b" value for the combined population of *S. laticaudus* from Ahlayt (3.287) was observed. The growth of *S. laticaudus* is isometric because the "b" value is almost equal to 3. The mean value was 3.002 in Kyaikkhami and *S. laticaudus* showed isometric (b= 3) growth pattern in the present study. The present estimation on 'b' value of *S. laticaudus* also shows seasonal variation. This variation is possibly due to factors related to ecosystem and biological phenomena like maturity stages, feeding behavior, competition for food, etc (Gosh *et al.*, 2009).

The value of coefficient of correlation (r) determined whether the relationship between length and weight was significant or not. Its value lies between -1 and + 1. The monthly 'r' values of the shark *S. laticaudus* in the present study were higher than 0.5. So the relation between the length and weight of the shark *S.laticaudus* was positively correlated and highly significant. When the results from 5 stations were compared in the present study, Zeephyuthaung and Setse show a negative allometric growth pattern (b<3) which mean that a fish becomes more

selender as it increases in length. The shark *S. laticaudus* in Kyaikkhami show an isometric growth pattern (b=3) which indicated that fish length increases in equal proportion with body weight and body forming maintain a constant proportion to length.

The spadenose shark (*Scoliodon laticaudus*) was available throughout the study period but the peak month slightly varied from month to month. The smallest size of *S. laticaudus* occurred in the catch in Setse and Sebalar during the study period. The largest size of this species was found abundantly in Ahlayt landing site. The length frequency distribution showed that the young ones (10-12 cm) are recruited to the fishery from October to December in Setse.

The catching rate of fish was slightly varied monthly in the study areas. The highest catch rate occurred in the premonsoon season in March to August in Zeephyuthaung; in June to November in Setse; in April, June and September in Kyaikkhami; in March and April in Sebalar and in April, July and September in Ahlayt. Among them, April has the highest catching rate month in throughout the study areas. The size group of spadenose shark (30-38 cm) was observed in all months.

The condition factor (K) of the shark *S. laticaudus* in five study areas indicated slight variation in different months. The K values in Sebalar gradually increase from January to May and showed a decline in June. The peak value occurred in September from Sebalar and Ahlayt and indicated a rapid decline in October. The condition factor was the same in almost all stations except Setse. In Setse, the k value increase from March to July and decline in August. The highest condition factor (K) (0.627087) was found in February from Setse and the lowest K value (0.206446) occurred in October from Sebalar. Condition factor have been used as an index of growth and feeding intensity. Condition factor decrease in length and also influences the reproductive cycle in fish (Abowei, 2009). Other authors showed that the values of the condition factor vary according to the season and are influenced by environmental conditions. The K value depends on physiological factors like maturity and spawning as well as food availability.

Conclusion

The length-weight relationships of the shark, *Scoliodon laticaudus* showed that the growth was isometric and there were no significant and significant variations between male and female. All allometric coefficients (b) of *S. laticaudus* estimated in these study areas varied between 2.903 and 3.287. From the estimation of length frequency distribution, *S. laticaudus* breeds throughout the study period. Each female found in study period has brood of 15 larvae in average. The length-weight relationships data can be used in fishery or biomass assessment and trophic studies in this study area of Mon coastal waters.

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FAUNAL COMMUNITY OF AHLYAT INTERTIDAL MUDFLAT UNDER ANTHROPOGENIC STRESS

Naung Naung Oo[1]

Abstract

Anthropogenic stress of Ahlyat intertidal mudflat was studied on exploited and reserved area at central Mon coastal area from January 2016 to December 2018. During the study period, species composition, diversity, evenness, and richness of mudflat fauna communities were not commonly disturbed but the species abundance were relatively common. Based on correlation index, regression coefficient, habitat loads and total biotic-abiotic factors of different habitats were negatively correlated. The prey species and predators were balanced at higher dominance level. Faunal structures of study area showed top-down scheme and affected the diversity of community negatively.

Keywords diversity indices, faunal community, mudflat, stress, structure.

Introduction

Ahlyat is one of the mudflat ecosystem structures in central Mon coastal area which covers mangrove fringe, saltmash grasses, sand dune beach, inlet creeks and huge mydflat shore. Species community structures were exploited by coastal population who live in intertidal zones. Nowadays, production of fishery sectors and over utilization of natural ecosystems have related for climate impact and pollution damage on coastal resources (de Boer *et.al*., 2001). The community structure of tropical waters provided various functions, stresses and loads on mudflat habitats and diversity.

In nature, anthropogenic activities and estuarine ecosystems have increased the impact on diversity of mudflat fisheries, vertebrates and invertebrates' fauna. The problematic correlation of natural and human impacts was altered the community structures of functional areas. Soft bottom communities like mudflat ecosystems and bare areas were supported trophic activity for avian fauna and many aquatic epi and infauna (Clarke and Warwick, 1994). Some correlation of functional procresses and human impacts of coastal systems are affected on hypotheses of anthropogenic structure of mudflats (Cyr *et.al*., 1997 a, b).

Soft bottom habitats have always been considered to be not only food sources but also especially ecosystems strengthening and to support the coastal qualities. In study areas, a vast assemblage of different forms lived in or on mudflat while the tidal period. Others preferred sandy mauflat where they burrowed into the sandflat during ebb tide. Many liked nothing better than a muddy bottom, and blacker and stickier the better. Some lived only in the fringe of mangrove, tolerating no environmental stress at all; whereas others seemed to like it better where the water was brackish and bare area, often thriving best in partially polluted areas.

The objectives of this study are: 1) to know the spcies community and diversity of Ahlyat intertidal mudflat relationship with anthropogenic stress loads, 2) to test the comparison of species composition-abundance, density-size-weight relationship of regression analysis of mudflat community structure, 3) to analyze the null hypothesis between the ecosystem data and statistical components, and 4) to know the species dominance and relative value of mudflat community in study areas.

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Materials and Methods

Study area

The mudflat of Ahlyat intertidal area (Lat. 16˚30′ N, Long. 97˚21′ E) is located in the central Mon coast (Fig. 1). Annual rainfall is 203.5 in (5.17 m) and mean air temperature is 27˚C. The water temperature ranged from 21˚- 33˚C. This study was divided into two different sites such as Site I and II faced to Gulf water of Martaban. The Site I is close to coastal population consumed by finfish and shellfish daily but the Site II is not directly accessed by local people because natural barriers like tidal creeks. Both areas have dense mangrove fringe and saltmarsh grass beds, extensive mudflats, sandflat and channels, respectively.

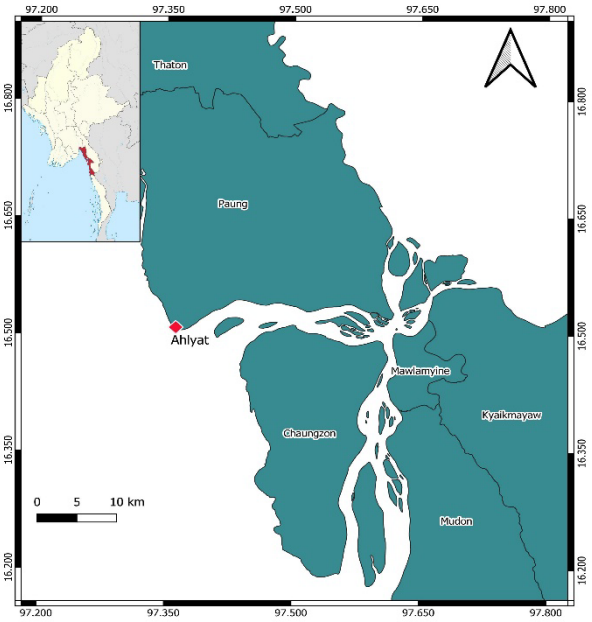

Figure 1 Map showing the collection site of Ahlyat coastal area

Sampling

The abundance and biomass of the different species were recorded from January 2016 to December 2018. In this study, quantitative analysis was used by the quadrat (50 cm × 50 cm) which was divided into a (10 cm × 10 cm) grid made of aluminium for light and durability. For each site, at least 5 transects of 125 m in length are lined perpendicularly to the shore at the interval of 50 m for each site (Fig. 2).

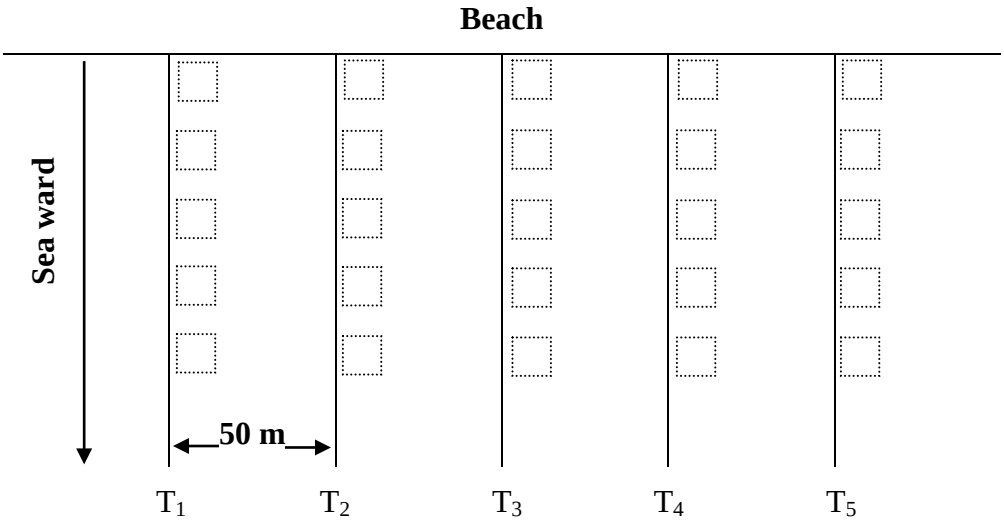

Figure 2 Systematic sampling in Ahlyat coastal area

In Ahlyat mudflat area, stress factors were analyzed by Best Professional Judgement (BPJ) as nominal values (0: no stress, 1: intermediate stress, 2: high stress) (Hyman and Leibowitz, 2001)

Statistical analysis

The species diversity, evenness, and richness indices were measured by The Shannon-Wiener species richness ($R' = S$-1/ln N), and diversity ($H' = -\sum(Pi \ln Pi)$), Simpson's Dominance Index of Diversity ($D = 1-(\sum n_i(n_i-1)/N(N-1))$) and Pielou's evenness ($J' = H'/\ln S$). According to Clarke and Warwick (1994), the multiple dimensional scaling (MDS) was calculated by the data analysis tools with non-parametric test. Species density, biomass and similarity of all organisms were calculated by average density per sampling month and Bray-Curtis similarity coefficients. The relative abundance, dominance and combination of all taxonomic groups were tested by regression analysis with an ABC graph (Abundance/Biomass Comparison) (Seys *et.al*., 1994). The W-test was analyzed the relative biomass to the disturbance system ranged of -1 to +1, being used for over exploited or unexploited species within the study areas.

Results

Composition

The MDS plot of five different sampling types in two study sites showed the difference in species composition (Fig. 3). Both study sites, the total stress factor was lower than 0.09 and composition of one-way similarity showed ($R = 0.504$, $p < 0.02$, $n = 10$). For benthic invertebrates, 50% dissimilarity was decreased 29% dissimilarity especially small bivalves and gastropods (*Dosinia angulosa* (Philippi, 1847), *Tellina palatum* (Iredale, 1929), *Bulla ampulla* Linnaeus, 1758 *B. striata* Bruguière, 1792 and *B. vernicosa* Gould, 1859).

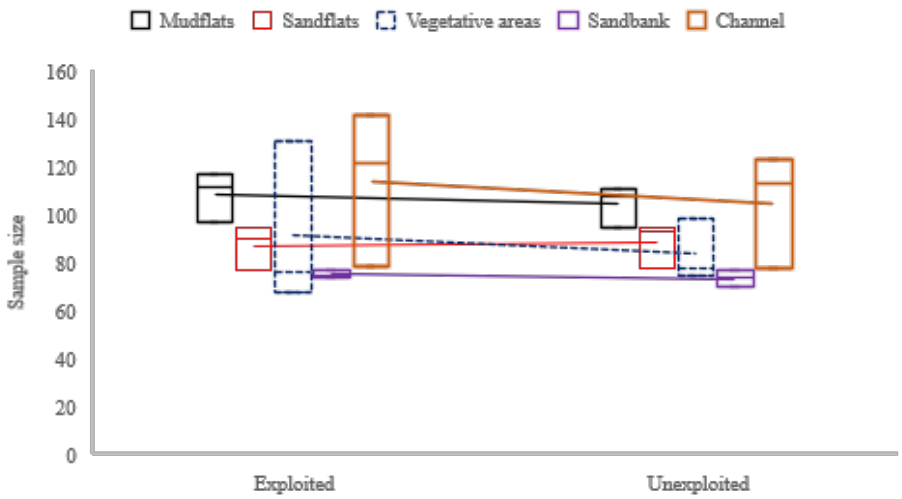

Figure 3 The multiple-dimensional scaling plot of different substrates

The total number of species per taxonomic group as shown in (Table 1). The species components of benthic substrate were highest number of sample size in Site I. The low mean diversity (*H´* of 1.7 ± 0.5 against 2.0 ± 0.3) was found in Site II (Table 2) but this result was not affective (Mann-Whitney U = 8; n = 10, p > 0.10). The highest species diversity was found in the mangrove fringe of Site I (*H´* = 2.35) and the mudflats of Site II (*H´*= 2.49). In Site I and II, the evenness and dominance factors were typically sharp at sandbank areas. The significant diversity changed positive relationship between richness with evenness and negative with dominance appeared (Spearman rank > 0.78, n = 10, p < 0.01).

The size-abundance relationship

The size-abundance relative plots did not show the differences between the two areas (Fig. 4). Regression data showed a clearly significant negative relationship between body size and abundance (for Site I: F = 501, p < 0.0001 adjusted-R^2 = 0.711, n = 203 and for Site II: F = 372 and p < 0.0001, adjusted-R^2 = 0.672, n = 181). The 95% confidence intervals for the coefficient were respectively 1.9 ± 0.2 and 2.1 ± 0.3 for Site I and Site II and for the intercept -1.4 ± 0.1 and -1.3 ± 0.2.

Table 1 The total number of species per taxonomic group

Substrates	Quantitative sample								
	Birds	Fish	Bivalves	Gastropods	Crustaceans	Saltmarsh grass	Plankton	Others	Species
Site I Exploited									
Mudflats	31	33	14	4	11	0	12	7	112
Sandflats	30	16	17	5	5	0	13	4	90
Mangrove fringe	20	20	7	2	11	0	12	4	76
Sandbank	26	18	8	3	4	0	13	2	74
Channel	32	49	13	9	10	2	14	13	142
Qualitative sample	43	59	33	15	22	2	15	14	
Mean	27.8	27.2	11.8	4.6	8.2	0.4	12.8	6	98.8
S.D	4.9	13	4.2	2.7	3.4	0.8	0.8	4.3	28.5

Site II Unexploited									
Mudflats	15	28	21	6	6	0	12	9	97
Sandflats	21	13	10	7	6	0	15	5	77
Saltmarsh grass bed	21	30	24	11	16	1	16	12	131
Sandbank	15	14	6	5	10	0	20	5	75
Channel	8	15	13	9	7	0	17	10	79
Qualitative sample	24	45	33	16	21	1	22	19	
Mean	16	20	14.8	7.6	9	0.2	16	8.2	91.8
S.D	5.3	8.2	7.5	2.4	4.2	0.4	2.9	3.1	23.6

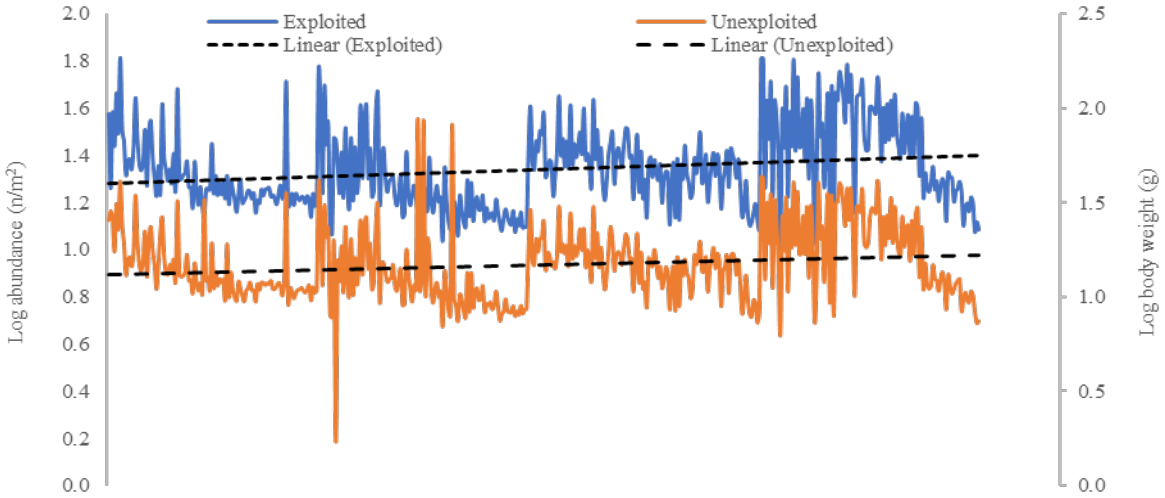

Figure 4 The log body weight (g) against the log abundance of each individual's species (n/m^2) in the study area

Table 2 Species diversity, evenness, dominance, and richness in study sites

Substrates	Diversity *H′*	Evenness *J′*	Dominance D	Richness *R′*		
				2016	2017	2018
Site I Exploited						
Mudflats	2.17	0.46	0.25	3.95	2.14	0.08
Sandflats	1.19	0.26	0.53	1.9	1.42	0.04
Mangrove fringe	2.35	0.54	0.17	5.92	3.31	0.14
Sandbank	1.33	0.31	0.39	2.58	1.8	0.05
Channel	1.49	0.3	0.5	2.01	1.44	0.03
Mean	1.71	0.37	0.37	3.27	2.02	0.07
S.D	0.52	0.12	0.16	1.69	0.78	0.04
Site II Unexploited						
Mudflats	2.49	0.54	0.21	4.82	2.34	0.12
Sandflats	1.88	0.43	0.31	3.24	1.92	0.08
Saltmarsh grass bed	2	0.41	0.24	4.2	2.9	0.06
Sandbank	1.59	0.37	0.39	2.59	1.71	0.06
Channel	1.99	0.45	0.34	2.9	1.74	0.09
Mean	1.99	0.44	0.30	3.55	2.12	0.08
S.D	0.32	0.06	0.07	0.93	0.50	0.02

Abundance-biomass comparison

Sandflats or tidal channels in Site I and mudflats or saltmarsh grass beds in Site II were showed the disturbed and undisturbed sediments abundance-biomass pattern by the W-statistic varying from - 0.052 to + 0.078 and – 0.033 to + 0.028 for the ten substrate types (Fig. 5). Benthic substratum is subjected to coastal exploitation pressures and other stress loads.

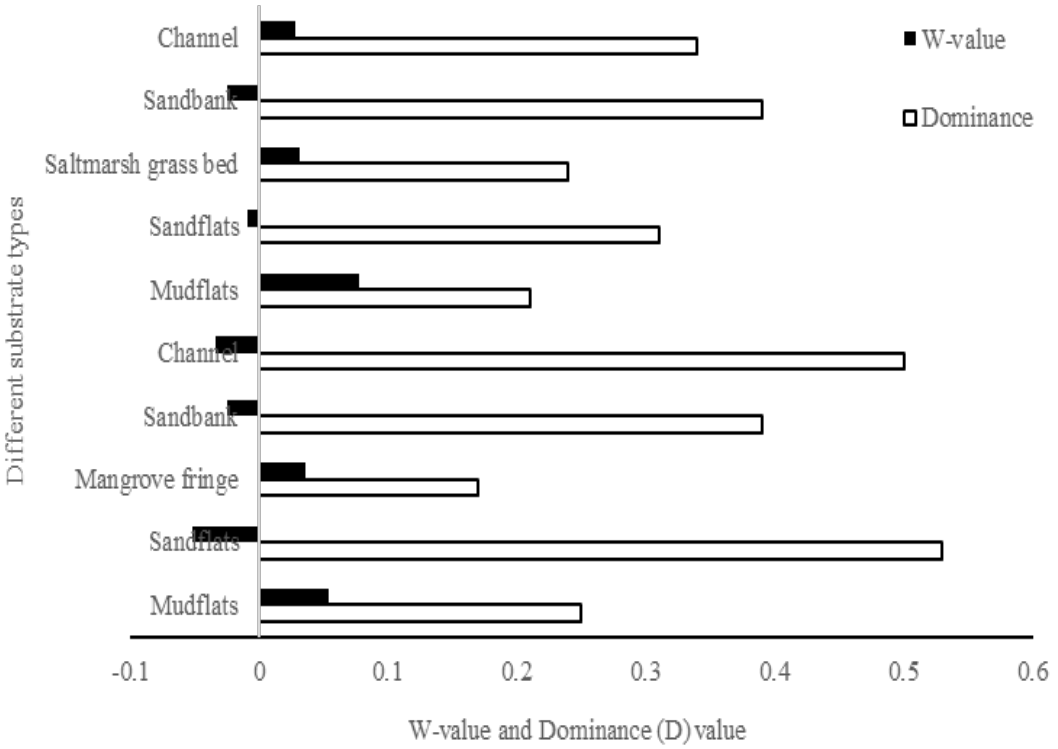

Figure 5 The Abundance-Biomass Comparison (ABC) graph of different substrate types

Table 3 The relative value of different stress factors in Site I

Stress factors	Exploited				
	Mudflat	**Sandflat**	**Mangrove**	**Sandbank**	**Channel**
Human exploitation	1	1	2	1	2
Human trampling	1	2	1	2	2
Human fisheries	0	0	0	0	1
Habitat heterogeneity	0	1	0	2	0
Desiccation	0	1	0	2	0
Grain size suitability	0	1	0	2	0
Excessive exposure	0	0	1	1	0
Storm damage	0	0	0	0	0
Hypersalinity	0	0	1	1	0
Low salinity	2	2	2	2	2
Irregular exposure	2	1	0	0	2
High water currents	0	0	0	2	2
Sedimentation	1	0	2	1	1
Low OM sedimentation	0	1	0	2	1
Shear stress	0	0	0	2	0
Substrate unpenetrability (debris etc.)	0	1	1	0	2
Low water table	0	0	1	2	2
Oxygen stress	2	2	2	0	1
OM-supply nutrient rich bay water	2	2	2	2	2
Saltmarsh grass nutrient shortage	1	1	1	1	0
Mangroves nutrient shortage	0	0	0	0	0
W-value	0.054	-0.052	0.036	-0.025	-0.033

Symbols: 0 (no stress), 1 (intermediate stress), 2 (high stress)

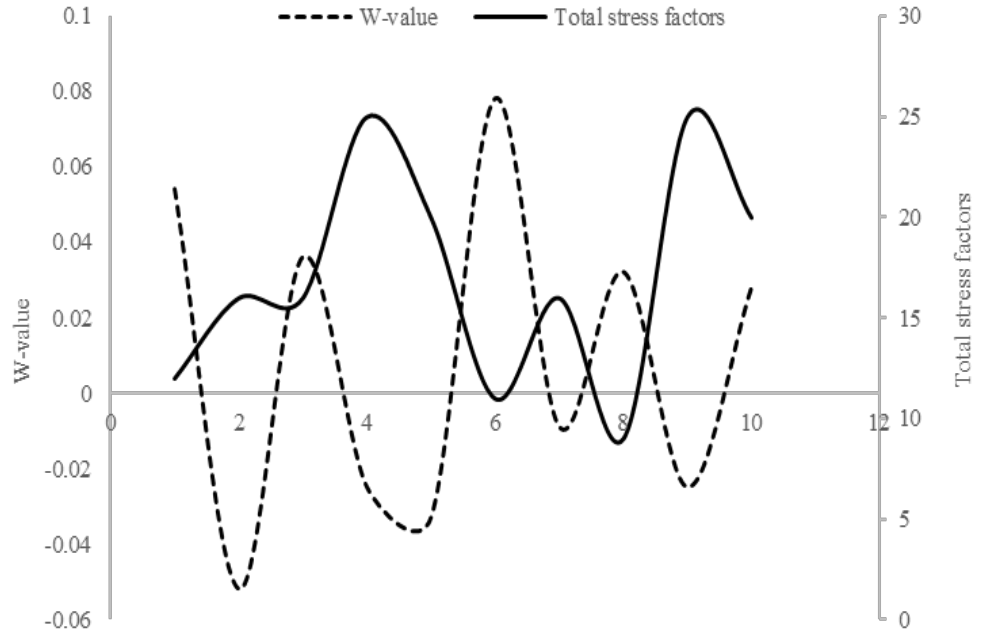

Figure 6 The total stress load compared with the W-statistics

Table 4 The relative value of different stress factors in Site II

Stress factors	Unexploited				
	Mudflat	**Sandflat**	**Saltmarsh grass**	**Sandbank**	**Channel**
Human exploitation	0	0	0	0	0
Human trampling	0	0	0	0	0
Human fisheries	2	2	2	2	2
Habitat heterogeneity	0	1	0	2	1
Desiccation	0	1	0	2	0
Grain size suitability	0	1	0	2	1
Excessive exposure	0	0	1	1	0
Storm damage	1	1	1	1	1
Hypersalinity	0	0	0	1	0
Low salinity	0	0	0	0	0
Irregular exposure	2	1	0	0	2
High water currents	1	1	1	2	2
Sedimentation	1	1	1	2	2
Low OM sedimentation	0	0	0	1	1
Shear stress	0	1	0	2	0
Substrate unpenetrability (debris etc.)	1	2	1	2	2
Low water table	0	1	0	2	2
Oxygen stress	2	2	1	0	1
OM-supply nutrient-rich gulf water	0	0	0	0	0
Saltmarsh grass nutrient shortage	0	0	0	1	1
Mangroves nutrient shortage	1	1	1	2	2
W-value	0.078	-0.009	0.032	-0.025	0.028

Symbols: 0 (no stress), 1 (intermediate stress), 2 (high stress)

In tables 3 and 4 show a half-quantitative summary of the stress factors for the ten substrate types. These tables indicated that the negative or positive W-value similar with ABC graphs of total stress factors identical disturbance levels. The comparison of human stress on Site I and II characterized by habitat community structures, the total stress load per substrate type was

compared with their W-statistic (Fig. 6). As expected, the larger the total stress load, the lower their W-value ($F = 5.867$, $p < 0.05$, $n = 10$, $R^2 = 0.42$).

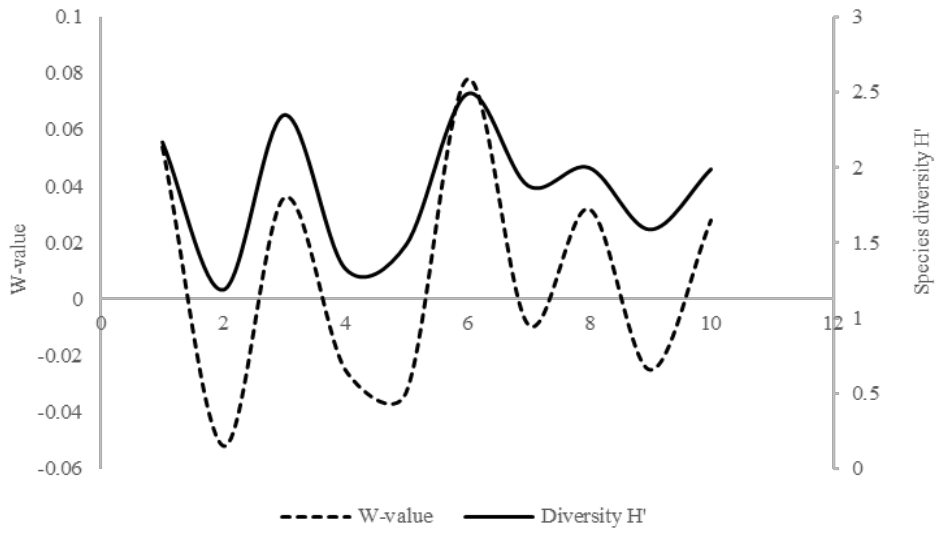

Figure 7 The correlation between the species diversity and W-statistics

Species diversity was significantly and positively related to the W-value (Fig. 7: Spearman rank = 0.967, $p < 0.0001$, $n = 10$), indicating that the highest species diversity was obtained at the highest W-value; or lowest levels of disturbance. The similar substrates (mudflats, sandflats, sandbanks, and tidal channels in both areas) were included in a Wilcoxon matched-pair test on pooled data for each substrate in each area, a significant decrease in species diversity, a decrease in evenness, and an increase in dominance in the exploited area were confirmed ($z > 2.52$, $n = 8$, $p < 0.02$).

Discussion

The settling or changing of species composition factor in the multi-factorial analysis was not easy to assess the entire study areas. The significant negative correlation between the abundance and the weight of the species as shown in (Fig. 4). The correlation of pronounced community level apparent in the taxonomic group being exploited. The relationship between the log body weight-abundance of benthic structure was negatively significant for the Site II ($F = 4.83$, d.f. = 1 and 87, $p < 0.05$), and not significant for the Site I ($F = 3.16$, d.f. = 1 and 82, $p < 0.10$).

When comparing the different benthic components, no change in species level could be statistically confirmed with different ABC plot and homogeneous equitability indices. At both sites, the composition of species assemblages was similar under exploitation to the MDS graph analysis (Fig. 3 and 7). Biomass and standing stock of shellfish covered in exploited areas due to mean annual offtake was < 5%. This mean that the exploitation rate was probably sustainable.

Minimun dissimilarity and maximum similarity indices in study areas mainly attributed to small size of gastropods and bivalves. Further study needed to check the increase or decrease of opportunistic species is being caused by the sediment disturbance which people create by merely walking or digging. The total stress load of the substrate in each area is reflected in the W-value (Fig. 6). The total stress load was increased and significantly correlated with decreasing species diversity, increasing dominance, and decreasing evenness. The W-value is probably a good indicator of stress load and offers new opportunities as an independent variable in impact studies.

Conclusion

Faunistic structure of Ahlyat intertidal mudflat is strongly related with anthropogenic stress and abiotic stress factors. Benthic components and human exploitation were balanced under the structuring of top-down process in study areas. The biological indicators and ecological systems of Ahlyat mudflat were stated that intermediate disturbance hypothesis seem to hold in communities comprising mobile aquatic invertebrates.

Acknowledgements

I would like to express my gratitude to Dr. Aung Myat Kyaw Sein, Rector of Mawlamyine University; Dr. Khine Khine San, Dr. Myint Thida, and Dr. Mar Lar Aung, Pro-rectors of Mawlamyine University for their permission to undertake this research work. I would like to express my sincere thanks to Dr. Nyo Nyo Tun, Professor, and Head of the Department of Marine Science at Mawlamyine University, for her advice and needful assistance. My final thank goes to local people from my study areas, for their assistance in the sample collections and Daw Lwin Lwin who has put in back-breaking hours studying seashells but more importantly has kept me focused.

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COMPOSITION AND DIVERSITY OF PHYTOPLANKTON SPECIES FROM ANDIN COASTAL WATER, YE TOWNSHIP, MON STATE

May Sabai Htay[1], Thida Nyunt[2]

Abstract

Phytoplankton species were studied from Andin coastal water from December 2019 to August 2020. A total of 74 species of phytoplankton were identified and comprised 36 species of centric diatoms, 29 species of pennate diatoms and 9 species of dinoflagellates. The percentage composition of centric diatoms ranges from 63% to 68%, pennate diatoms range from 30% to 35% and dinoflagellates with 2% were observed. Species composition and abundance were found to be highest at Station III (2597 no/m³) and lowest at Station II (2191 no/m³). The index values encountered from Andin coastal water ranged between 3.73 and 3.76 for the species diversity index (*H'*), between 0.85 and 0.87 for evenness index (*E'*), and between 9.28 and 9.49 for species richness index (*D'*). The maximum salinity value of 30‰ was found in February and the minimum value of 24‰ was found in August. The highest water temperature of 31°C was found in February and the lowest water temperature value of 28°C and was found in August. This paper represents part of the ongoing effects to revise and document the phytoplankton of Andin coastal water.

Keywords Phytoplankton, Andin coastal water, composition, diversity, diatoms, dinoflagellates.

Introduction

Phytoplankton is mostly microscopic drifting organisms, solitary or colonial and unicellular algae. They live near the surface of the sea because, like all plants, they require light for photosynthesis. They are single-celled organisms, primary producers that serve as the base of the marine food chain. Diatoms are the most important group of phytoplankton. Dinoflagellates are the second most abundant phytoplankton.

Kyi Win (1972) identified 341 species of phytoplankton along the Myanmar coastal region. Zar Ni Ko Ko (2014) reported 60 species of diatoms and 21 species of dinoflagellates widely distributed in the Elphinstone Island waters, Myeik Archipelago. Nyunt Sandar Aung (2011) studied the littoral diatoms and dinoflagellates of Setse coastal area. Zaw Moe Aung (2011) studied the primary productivity of marine phytoplankton in Setse waters. Zin Mar Phyo (2012) examined the phytoplankton of Setse and Kyaikkhami coastal waters. In addition, Thida Nyunt (2013) classified the 112 species of phytoplankton collected Mon coastal waters. Aung Kyaw Lwin (2018) studied 48 species of phytoplankton from Ahlyat coastal water. Wai Yan Tun (2019) observed 50 species belonging to 20 families and 27 genera of diatoms and dinoflagellates from Ankhae coastal water. Recently, Thu Thu Myat Noe Kyaw (2021) observed 66 species belonging to 23 orders, 30 families and 37 genera of diatoms and dinoflagellates from Hnyigarok tidal creek, Ye Township, Mon State.

The purposes of this study are to know the diversity of phytoplankton and to realize the species composition and abundance of phytoplankton from Andin coastal water.

Materials and Methods

Phytoplankton samples were collected monthly from Station I (Lat 15°19'16.78"N and Long 97°42'16.77"E), Station II (Lat 15°18'7.07"N and Long 97°43'29.37"E), and Station III (Lat 15°16'27.69"N and Long 97°42'33.15"E) in Andin coastal water from December 2019 to August 2020 (Fig. 1). The samples were collected from the surface water by towing the

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phytoplankton net horizontally, during the neap tide in the day time. Phytoplankton net (length of 50 cm, mouth diameter with 24 cm, mesh size 28 µm was towed for 5 minutes one time in the study area. All samples were retained in polythene bottles and preserved in 2% formaldehyde buffer with seawater immediately. Then, the specimens were carried to the Department of Marine Science, Mawlamyine University. The water temperature was determined by an ordinary mercury thermometer and the salinity of seawater was measured by using a refractometer. The specimens were identified by light microscope Olympus (H20BIMF 200) and are measured by using an ocular micrometer. This study has followed by the classification system used by Allen and Cupp (1930), Davis (1955), Newell and Newell (1963), Hendey (1964), Shirota (1966), Weber (1966), Sourina (1968), Yamaji (1962, 1969, 1971), Guiry (2016), Al-Kandari *et al.* (2009) and Al-Yamani and Saburova (2019).

The diversity indices of phytoplankton for each sample, species diversity (*H´*), evenness (*E´*) and richness (*D´*) were calculated, using the formula of Shannon and Wiener (1963) and Pielou (1975).

$$H' = -\sum Pi * \mathrm{Log}_e Pi$$
$$E' = H'/\ln S$$
$$D' = S\text{-}1/\ln N$$

Where, *H'* is the index of species diversity,
E' = the index of species evenness
S is the total number of species,
D' is the index of species richness,
Pi is the proportional abundance of i^{th} species (ni/N),
ni is the total number of individuals of i^{th} species and
N is the total number of individuals in a station and the total number of species.

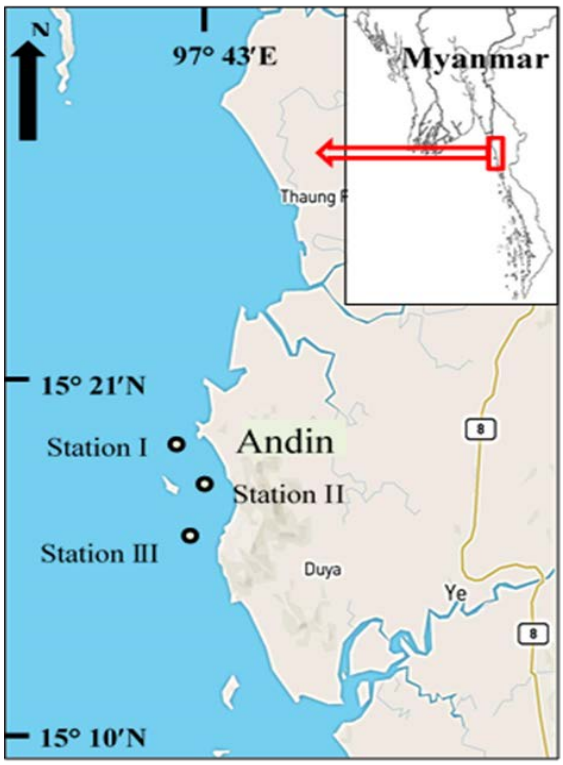

Figure 1 Collection site of phytoplankton from Andin coastal water, Ye Township, Mon State.

Results

Composition

A total of 74 species comprising 36 families and 44 genera were recorded and identified. Among them, the phytoplankton community of Andin coastal water was composed of 65 diatoms species representing 29 families and 9 species of dinoflagellates representing 7 families. Distribution of diatom species at Andin coastal water as shown in Table 1. Distribution of dinoflagellate species at Andin coastal water as shown in Table 2. Percentage composition of phytoplankton group as in Figure 2.

Table 1 Distribution of diatom species at Andin coastal water

Family	Species Name	St I	St II	St III
Diatom				
Hyalodiscaceae	1. *Hylodiscus subtilis*	+	+	+
Paraliaceae	2. *Paralia sulcata*	+	+	+
Coscinodiscusceae	3. *Coscinodiscus asteromphalus*	+	+	+
	4. *C. excentricus*	+	+	+
	5. *C. gigas*	+	+	+
	6. *C. nitidus*	+	+	+
	7. *C. marginatus*	+	+	+
	8. *C. radiatus*	+	+	+
Rhizosoleniaceae	9. *Rhizosolania alata*	+	+	+
	10. *R. bergonii*	+	+	+
	11. *R. calcar-avis*	+	+	+
	12. *R. imbricata*	+	+	+
	13. *R. setigera*	+	+	+
	14. *R. styliformis*	+	+	+
	15. *R. stolterfothii*	-	+	-
	16. *Guinardia striata*	-	+	+
Asterolampraceae	17. *Asteromphalus cleveanus*	+	+	+
Triceratiaceae	18. *Triceratium reticulum*	+	+	+
Lauderiaceae	19. *Lauderia annulata*	+	+	+
Skeletonemataceae	20. *Skeletonelma costatum*	+	+	+
Thalassiosiraceae	21. *Thalassiosira decipiens*	+	+	+
	22. *Planktoniella sol*	-	+	+
Stephanodiscaceae	23. *Cyclotella striata*	+	+	+
Hemianulaceae	24. *Hemiauulus hauckii*	+	+	+
	25. *H. sinensis*	+	+	+
Eupodiscaceae	26. *Odontella sinensis*	+	+	+
	27. *O. mobilliensis*	+	+	+
Lithodesmiaceae	28. *Lithodesmium undulatum*	+	+	+
	29. *Ditylum sol*	+	+	+
Bellerocheaceae	30. *Bellerochea horologicalis*	+	+	+
Streptothecacecae	31. *Streptotheca indica*	+	+	+
	32. *S. temesis*	+	+	-
Chaetocerotaceae	33. *Chaetoceros curvisetus*	+	-	-
	34. *C. lorenzianus*	+	+	+
	35. *C. peruvianus*	+	+	+
Leptocylindraceae	36. *Leptocylindrus danicus*	+	+	+
Surirellaceae	37. *Surirella ovalis*	+	+	+
Entomoneidaceae	38. *Entomoneis alata*	+	+	+
Thalassionemataceae	39. *Thalassionema nitzshoides*	+	+	+

	40. *Thalassionema frauenfeldii*	+	+	+
	41. *Thalassiothrix longissima*	+	+	+
Tabellariaceae	42. *Asterionella japonica*	+	+	+
Naviculaceae	43. *Navicula distans*	+	+	+
	44. *N. elegan*	-	-	+
	45. *N. pusilla*	-	+	+
	46. *Gyrosigma tenuissimum*	+	+	+
Diploneidaceae	47. *Diploneis chersonensis*	+	+	+
Pleurosigmataceae	48. *Pleurosigma angulatum*	+	+	+
	49. *P. elongatum*	+	+	+
	50. *P. normanii*	+	-	+
	51. *P. marium*	+	+	+
Catenulaceae	52. *Amphora spectibilis*	-	+	-
Bacillariaceae	53. *Vibrio paxillifer*	+	+	+
	54. *Nitzschia angularis*	+	+	+
	55. *N. clausii*	+	+	+
	56. *N. seriata*	+	+	+
	57. *N. lanceolata*	+	+	+
	58. *N. linearis*	+	+	+
	59. *N. longissima*	+	+	+
Plagiotropidaceae	60. *Plagioltropis tayrecta*	+	+	+
Achnanthidaceae	61. *Planothidium delicatulum*	+	+	+
Fragilariaceae	62. *Fragilaria crotonensis*	+	+	-
	63. *F. islandica*	+	+	+
	64. *Synedra ulna*	+	+	+
	65. *Centranella reicheltii*	+	+	-

Table 2 Distribution of dinoflagellate species at Andin coastal water

Dinoflagellate				
Prorocentraceae	66. *Prorocentrum mican*	+	-	-
Dinophysaceae	67. *Dinophysis caudata*	+	-	-
Gonyaulaceae	68. *Gonyaulax grindleyi*	+	+	+
Diplopsalis	69. *Diplopsalis lenticula*	+	+	-
Protoperidiniaceae	70. *Protoperidinium pentagonum*	+	+	+
Podolampadaceae	71. *Podolampas palmipes*	-	+	-
Ceratiaceae	72. *Tripos furca*	+	+	+
	73. *T. muelleri*	+	+	+
	74. *T. fusus*	+	+	+
	Total of species	67	69	64

Symbol: + = present.

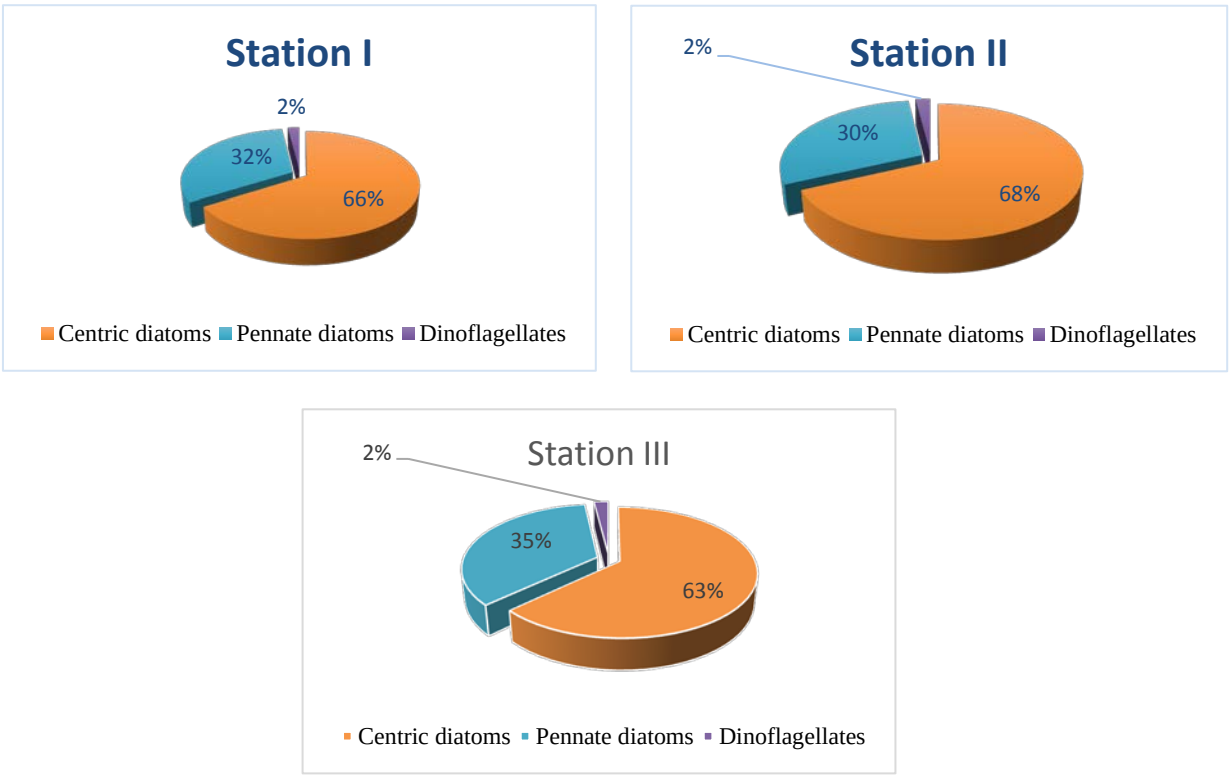

Figure 2 Percentage composition of phytoplankton group at Andin coastal water

Diversity and Density

Phytoplankton species diversity was calculated by the formula of Shannon and Wiener (1963) and Pielou (1975), and the result indicated that the diversity index (*H'*) was in Station I (3.73), followed by Station II (3.76), Station III (3.74). The evenness (*E'*) values of phytoplankton species were found in Station I (0.85) followed by Station II (0.87), Station III (0.86). Species richness (*D'*) values were found in Station I (9.43), Station II (9.49), Station III (9.28). The highest Shannon index was found in Station II and the lowest index was found in Station I. The high density of phytoplankton was found in Station III and phytoplankton density was found to be low in Station II. Phytoplankton species diversity indices and density were presented in Figures (3, 4). The highest temperature 31˚C was measured in February and the lowest temperature 28˚C was found in August during the study period. During the study period, the maximum salinity value of 30‰ was found in February and the minimum value of 24‰ was found in August.

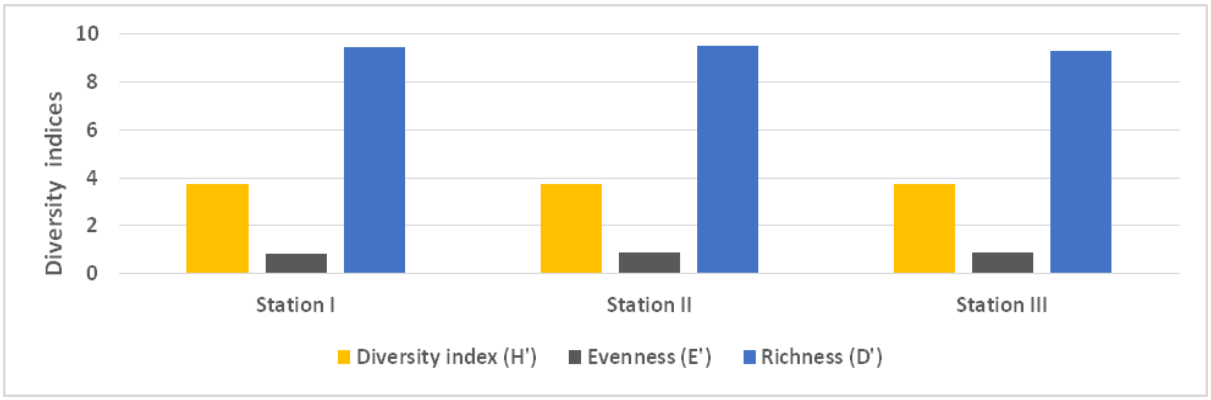

Figure 3 Phytoplankton species diversity indices at Andin coastal waters

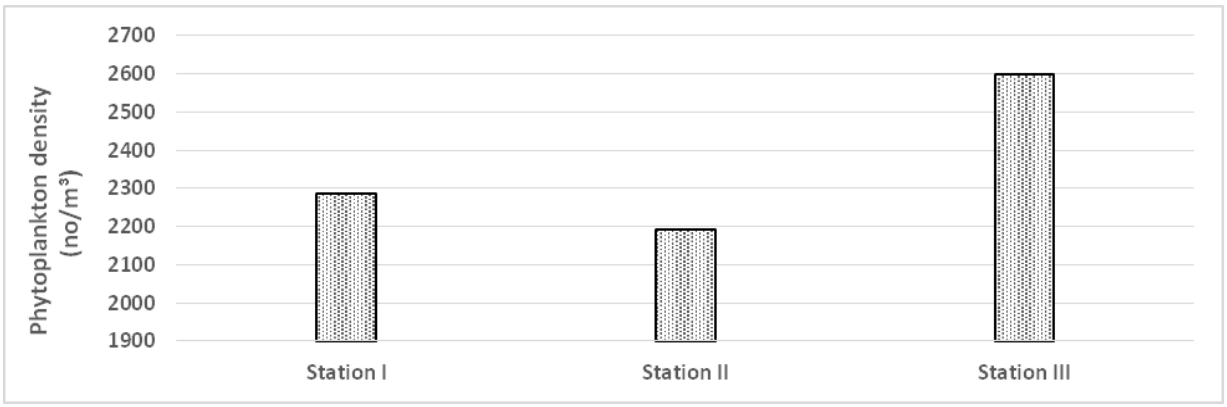

Figure 4 Phytoplankton density at Andin coastal water (no/m³)

Discussion

In the present study, a total of 74 species were identified. 65 species belonged to diatoms, 9 species to dinoflagellates. Among them, diatoms species were most abundannt as compared to dinoflagellates. The environmental parameters such as salinity and temperature are very important for growth and dispersal of phytoplankton on which zooplankton and some higher consumers depend for their existence. Thu Myat Noe Kyaw (2021) described, maximum temperature was observed in February (2021) 31°C and minimum temperature was observed in August (2021) 26°C. In the present study the highest water temperature (31°C) was observed in February and the lowest water temperature (28°C) was observed in August. Thu Myat Noe Kyaw (2021) described, the higher salinity values in dry months (February-April) are related to the high rate of evaporation from water bodies; however, salinity values were very low in the rainy season. In the present study salinity values were gradually increased to the highest value of 30‰ in February but the salinity was lowered to 24‰ in August because of heavy raining.

Phytoplankton community structure was reported by three different terms diversity index, Shannon's species diversity index (*H'*), evenness index (*E'*) and species richness index (*D'*). During study period the highest diversity value (*H'*) 3.76 whereas the lowest one, 3.73 was found in the Andin coastal water. Thida Nyunt (2013) mentioned that the diversity index values 2.05-3.2 was recorded from Mon coastal waters.

In the present study the highest value of evenness index (*E'*) 0.87 whereas the lowest 0.85 was found in the Andin coastal water. Thida Nyunt (2013) described the highest values of evenness index (*E'*) as 0.95 and the lowest 0.54 was found from Mon coastal waters.

The maximum richness index value (*D'*) 9.49 however the minimum 9.28 was found in the Andin coastal water. Thida Nyunt (2013) described the maximum richness index value as 5.17 and the minimum value 2 was found from Mon coastal waters.

Phytoplankton species composition richness, population density, and primary productivity will vary from coast to coast and sea to sea depending upon the varying hydrobiological features. The abundance of phytoplankton can directly or indirectly contribute to the development of fishery in a certain area, such as Andin coastal water.

Conclusion

Bacillariophyceae is the most diverse and high abundance class because it possesses a wide range of the environmental variables. The most abundant species of *Coscinodiscus asteromaphalus, C. gigas, C. nitidus, C. excentricus, C. marginatus, Rhizosolenia calcar-avis, Asteromphalus cleveanus, Hemiaulus hauckii, Lithodesmium undulatum, Lauderia annulata* and *Diploneis chersonensis* were observed in the Andin coastal water. Density during this period indicated that Station III had the richness of phytoplankton species when compared to the two stations. In the present study was observed increase of water temperature and salinity cause increasing of phytoplankton abundance and diversity. The diversity and density of phytoplankton communities are essentially reflecting the resource supply in the ecosystem. Phytoplankton of Andin coastal water was composed of seventy-four phytoplankton species in which diatoms numerically dominated in the community. Regarding species richness, density, and diversity of phytoplankton, it could be concluded Andin coastal water is a protective water area. This study on the status of phytoplankton will provide information for resource conservation and fisheries management on local and regional scales. Moreover, a sea without phytoplankton could not support the zooplankton and the other larger animals. Further studies of the distribution and abundance of phytoplankton in the Andin coastal water should be continued long term.

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GENOME-WIDE IDENTIFICATION AND CHARACTERIZATION OF CATALASE (*CAT*) GENE IN ZHIKONG SCALLOP REVEALS GENE EXPANSIONS AFTER EXPOSURE TO THE PST-PRODUCING DINOFLAGELLATE, *ALEXANDRIUM MINUTUM*

Sein Moh Moh Hlaing[1]

Abstract

Bivalves can accumulate high concentrations of paralytic shellfish toxins (PSTs) produced by toxic algae that may induce oxidative stress. Catalase (CAT) is one of the important antioxidant enzymes involved in scavenging the high level of ROS and plays a significant role in the protection of aerobic organisms against oxidative stress by degrading hydrogen peroxide. In the present study, a total of two *CAT*s were identified in the *Chlamys farreri* genome. Sequence characterization revealed *CfCAT* protein sequences contained proximal heme-ligand signature sequence (^{354}RLFSYSDTH^{362}), two N-glycosylation sites (^{440}NFS^{442} and ^{482}NFT^{484} domains), the proximal catalase active site signature (^{64}FNRERIPERVVHAKGGGA^{81}), the peroxisome-targeting signal in the C-terminus ^{495}QKL^{497} and 12 amino acids (H^{89}, N^{171}, F^{154}, S^{122}, R^{112}, N^{142}, Y^{325}, K^{169}, I^{311}, W^{277}, Q^{331}, and Y^{260}), which were identified as the putative residues involved in NADPH binding. Eight amino acids: R^{320}, H^{364}, R^{365}, N^{369}, F^{409}, R^{432}, Y^{404}, and R^{457} were identified as the heme-binding site residues. Three conserved catalytic amino acids (H^{166}, N^{148}, and Y^{137}) and catalase signature sequences were essential for the structure and function of *CfCAT*s. The homology of deduced amino acid sequences revealed that *CfCAT*s had high identity with catalases from other mollusks. RNA-Seq data analysis revealed there were no regulation patterns of scallop *CAT*s were significantly induced in the kidney after exposed to *Alexandrium minutum* (AM-1). Gene expression analysis in the scallop revealed all *CAT*s being predominantly expressed in the mantle, gill, muscle, and hepatopancreas after feeding the scallop with PST-producing dinoflagellates. All these results indicate that CAT involves an important role in counteracting oxidative stress in *C. farreri* by PST accumulation. The tissue-, species-, and toxin-dependent expression pattern of scallop *CAT*s might be involved in their functional diversity in response to toxin exposure.

Keywords *Chlamys farreri*; catalase; gene expansion; paralytic shellfish toxins (PSTs); *A. minutum*; expression profiling

Introduction

Reactive oxygen species (ROS) are generated as a by-product of aerobic metabolism where the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are formed when molecular oxygen chemically oxidizes electron carriers (Chaitanya, Shashank et al. 2016). Through the per-oxidizing of cellular proteins, nucleic acids, lipids, enzyme deactivation, down-regulation of redox-sensitive processes, and signaling pathways, the excessive production of ROS in cells can harm a variety of cellular components and immune dysfunction. Antioxidant enzymes are crucial in protecting organisms from the potentially harmful consequences of oxidative stress caused by various environmental stresses (Arockiaraj, Easwvaran et al. 2012). Oxidative stress is caused by the over-production of ROS and damage to various organs has occurred as a critical factor for organisms responding to environmental challenges. (Li, Wang et al. 2022). All aerobic species have developed enzymatic defense mechanisms against ROS including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and peroxiredoxins (Prx) to prevent oxidative stress. Among these systems, catalase (EC 1.11.1.6) is a major enzyme of the enzymatic antioxidant system that catalyzes the breakdown of hydrogen peroxide (H_2O_2) to water and oxygen and is essential to life. This enzyme is ubiquitous among aerobic

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organisms and plays an important role in protecting cells from oxidative damage by ROS (Ken, Lin et al. 2000).

Additionally, the expression level of CAT in cells can also affect many other biological processes (Wu, Li et al. 2016). Studies on the structure and regulation of catalase genes and proteins have been reported in plants (McClung 1997), bacteria (Storz and Tartaglia 1992), and mammals (Park, Kim et al. 2004). To date, more studies about catalase have focused on aquatic organisms including fish, crustaceans, and mollusks (Gao, Ishizaki et al. 2016). Bivalve mollusks, like other aquatic invertebrates, are continually exposed to possibly harmful xenobiotics and a wide range of toxic stressors in their environment. In mollusks, cloning and characterization of CAT gene sequences have been reported for the scallop *Chlamys farreri* (Li, Ni et al. 2008), abalone *Haliotis discus discus* (Ekanayake, De Zoysa et al. 2008), oyster *Crassotrea hongkongensis* (Zhang, Fu et al. 2011), pearl oyster *Pinctada fucata* (Guo, Zhang et al. 2011), mussel *Cristaria plicata* (Xilan, Gang et al. 2011), and clam *Meretrix meretrix* (Wang, Yue et al. 2013). Previous studies on the effects of heavy metal exposure, bacterial challenge, and PST exposure on antioxidant enzyme responses, particularly CAT responses, have already been conducted in various bivalve species (Gao, Ishizaki et al. 2016). But few studies have documented how toxins or exposure to harmful algae species alters the gene expression in bivalves.

Filter-feeding bivalves can accumulate paralytic shellfish toxins (PSTs) produced by marine dinoflagellates through the consumption of algae. The genus Alexandrium spp which is strongly associated with harmful algal blooms (HABs) and are the most potent marine biotoxins (Li, Sun et al. 2017). Saxitoxin (STX) and its variants which can reversibly bind the voltage-gated Na+ channels (NaV) of excitable cell membranes and block the conduction of nerve signals are the main components of the class of neurotoxins known as paralytic shellfish toxins. This stress on the body's metabolism and paralysis of the neuromuscular system is caused by this blockage of nerve signals. (Bricelj, Connell et al. 2005, Li, Sun et al. 2017, Wang, Liu et al. 2021).

Scallops, as filter feeders may consume toxic dinoflagellates that can accumulate high levels of neurotoxins in their tissues, tolerate a higher concentration of PSTs, and retain these toxins for a more extended period than other bivalve species (Tan and Ransangan 2015). Based on the genomic resources, the present study performed systematic identification of *CAT* genes in the commercially important bivalve species known as the Zhikong scallop, *Chlamys farreri* (Jones et Preston, 1904, also known as Chinese scallop) which is farmed as aquaculture in China (Li, Sun et al. 2017). After being challenged by the PST-producing dinoflagellate, *Alexandrium minutum*, the expansion of scallop *CAT* genes and their tissue-specific expression were revealed and examined using RNA-seq datasets. These results will help understand the molecular mechanisms in *C. farreri* after exposure to *A. minutum*, and could be used to protect this commercially important species from oxidative stress and PST-induced antioxidant responses in bivalves.

Materials and Methods

Sample Collection

The Zhikong scallop, *C. farreri* was collected from Xunshan Group Co., Ltd. (Rongcheng, Shandong Province, China) and Zhangzidao Group Co., Ltd. (Dalian, Liaoning Province, China), respectively. To analyze the effects of PST-producing dinoflagellate, two-years-old adult scallops of *C. farreri* were acclimated in filtered and aerated seawater at 12–13°C and depurated for three weeks by feeding non-toxic algae, *Isochrysis galbana* (7.5×10^5 cells/mL) (Romero - Geraldo and Hernández - Saavedra 2014) as the control group then maintained

separately with aeration during the exposure experiments (Escobedo-Lozano, Estrada et al. 2012).

Genome-Wide Identification and Sequence Analysis of *CAT* Genes in *C. farreri*

The transcriptome and whole-genome sequence databases (Li, Sun et al. 2017, Hu, Li et al. 2019) of *C. farreri* were searched to identify the *CAT* genes using all available *CAT* protein sequences of invertebrates and vertebrates, including *Homo sapiens, Mus musculus, Xenopus tropicalis, Bos taurus, Rattus norvegicus, Danio rerio, Gallus gallus* and some mollusks species: *Pinctada fucata, Unio tumidus, Haliotis discus hannai, Crassostrea gigas, C. hongkongensis, Argopecten irradians, Mimachlamys nobilis, Hyriopsis cumingii, Meretrix meretrix, Sus scrofa,* and *Cristaria plicata, Azumapecten farreri* queries from NCBI, Ensembl, and OysterBase online databases to obtain candidate *CAT* sequences. ORF finder was used to translate the candidate *CAT* sequences and the predicted *CAT* proteins were aligned to public online databases including UniProt. The predicted amino acid sequence was conducted by BLASTP against the NCBI non-redundant (Nr) protein sequence database (e-value set: 1E-05). All candidate *CAT*s with a significant BLAST hit were obtained and the presence of *CAT* domain was further verified by Conserved Domains Database and SMART tools. The compute pI/MW tool was used to predict the pI value and MW (kDa). "Multiple EM for Motif Elicitation" (MEME) version 5.4.1 was used to find conserved motifs in *CfCAT* proteins. The physicochemical properties of *CAT* proteins of scallops were performed using the ProtParam tool. The subcellular localization of *CAT* proteins was analyzed through ProtParam, CELLO (Yu, Chen et al. 2006), EukmPLoc 2.0 (Chou and Shen 2010), and WoLF PSORT II (Horton, Park et al. 2006). The percentages of similarity and identity of full-length amino acid sequences between *CfCAT* and *CAT* proteins from other organisms were calculated using the Sequence Identity and Similarity (SIAS) tool. The exon/intron structure analysis of *CAT* was carried out from the scallop genome GFF3 gene annotation file using the Gene Structure Display Server. The secondary structure of *CAT* proteins was predicted by JPred 4 server program (Drozdetskiy, Cole et al. 2015) and the SOPMA program (Geourjon and Deleage 1995). The tertiary structure of scallop *CAT* proteins was predicted using the Phyer2 tool.

Multiple Sequence Alignment and Phylogenetic Analysis of *CAT* Gene Family

Multiple sequence alignments of *CAT* proteins were performed using Clustal W (Larkin, Blackshields et al. 2007), then edited by Genedoc software (version 2.7.0) (Nicholas 1997). The *CAT* protein sequences from scallops and other representative species were employed for phylogenetic analysis. The phylogenetic tree of *C. farreri CAT*s was constructed with MEGA 7.0 by using the maximum likelihood (ML) method with bootstrap values as 1000 replicates were applied as the best-fit model LG + G + I model (LG model and Gamma distribution with Invariant sites)(Kumar, Stecher et al. 2016).

Expression analysis of *CAT* genes in scallops exposed to PST-producing dinoflagellate, *A. minutum*

The strains of *A. minutum* were cultivated independently in F/2 medium with a light-dark cycle of 12:12 h, then harvested when the cell density approached 5×10^4 cells/mL in the exponential growth phase by centrifugation of 2500 g/10 min (Hwang and Lu 2000, Navarro, Munoz et al. 2006, Garcia-Lagunas, Romero-Geraldo et al. 2013). Then, each scallop was fed once a day with 3 L volume with a final cell density of 2.5×10^3 cells/mL during the feeding experiments. There were 6 groups with 3 individuals of scallops collected and fed with the PST-producing dinoflagellate, *A. minutum* (AM-1 strain) on days 0, 1, 3 (acute response), 5, 10, and 15 (chronic response) exposure. The mantles, gills, muscles, kidneys, and hepatopancreas of the scallop were dissected, washed with sterile seawater, and frozen at -80°C for subsequent RNA

extraction. Total RNA was extracted from these tissues using the conventional guanidinium isothiocyanate method (Hu, Bao et al. 2006). RNA-Seq libraries were constructed using the NEB Next mRNA Library Prep Kit following the manufacturer's instructions and were subjected to PE125 sequencing on the Illumina HiSeq 2000 platform. The RNA-seq reads were mapped to the *C. farreri* genome using Tophat (ver 2.0.9) (Trapnell, Pachter et al. 2009), and the expression of all *CAT* genes was normalized and represented in the form of RPKM. The fold changes in *CAT* expression for each test point were calculated as $(RPKM_{test}\text{-}RPKM_{control})/RPKM_{control}$ (Cheng, Xun et al. 2016). Significant differences between the experimental and control groups were determined using an independent-samples T-Test ($p < 0.05$, $p < 0.01$, and $p < 0.001$, $n = 3$). Statistical analyses were carried out with GraphPad Prism 5.0 software (Mavrevski, Traykov et al. 2018).

Results

A total of two *CAT* genes were identified in *C. farreri* genomes including *CfCAT*1 and *CfCAT*2. Basic information regarding their genome position, encoding protein length, intron number, protein characterization, and sub-cellular localization prediction were analyzed. According to sequence analysis, the coding sequences of *CfCAT*s ranged from 1494 to 1563 bp in length and encoded proteins from 497 to 520 amino acids (aa). The predicted molecular weight (MW) of *CAT* proteins ranged from 56.26 kDa to 59.12 kDa with average theoretical pIs ranging from 7.22 to 8.66 in *C. farreri*, respectively. The instability index of *CAT* proteins was predicted to be stable (instability index ≥40). According to the variable of isoelectric point (pI) values, two *CfCAT*s were alkaline amino acids in character. The aliphatic index of *CfCAT* conserved proteins ranged from 58.87 to 61.01. The values of the grand average of hydropathicity (GRAVY) showed all *CAT* proteins in scallops were found to be hydrophilic proteins based on a grand average of hydropathicity (GRAVY) analysis where the hydrophobic protein: $0 < GRAVY < 2$; hydrophilic protein: $-2 < GRAVY < 0$. The sub-cellular predictions showed that the *CfCAT*1 and *CfCAT*2 proteins were mainly localized in peroxisomal. As a result of various exon/intron organization patterns, a total of 11 introns were identified in both *CfCAT* genes. About 44.42% of *CfCAT*1 and 43.66% of *CfCAT*2 were composed of regular secondary structural elements, which accounted 28.46% and 26.76% for α-helices, 15.96% and 16.90%, for β-sheet, as well as 49.62% and 50.91% for the random coil. To understand the active site structure-function relationships, the predicted tertiary structure of *CfCAT*s proteins was shown in Fig. 1 (a and b). The four antiparallel β-strands dominated by α-helices are found in *CfCAT*1 and *CfCAT*2, respectively.

Conserved Structures of *CAT* Genes in *C. farreri*

Multiple sequence alignment of scallop *CAT* proteins with their homologs in other selected species revealed the presence of conserved structural domains (Fig. 2). Two CAT family signature sequences were found in the deduced amino acid sequence of *CfCAT*s that contained the catalase proximal heme-ligand signature motif, ^{354}RLFSYSDTH3^{62} was completely conserved as both amino acid composition and location in both *CfCAT*s. A highly conserved catalase active site signature motif, ^{64}FNRERIPERVVHAKGGGA^{81}, was also found in both *CfCAT*s with two synonymous amino acid substitutions (D-65 to N and A-79 to G). Two putative N-glycosylation sites, ^{440}NFS^{442} and ^{482}NFT^{484} domains, and the peroxisome-targeting signal in the C-terminus ^{495}QKL^{497} were predicted in *CfCAT*s. The C-terminal tri-peptide, Gln (Q), Lys (K), and Leu (L) were identified as the putative internal peroxisomal targeting signal PTS1 in *CfCAT* proteins. Twelve amino acids: H^{89}, N^{171}, F^{154}, S^{122}, R^{112}, N^{142}, Y^{325}, K^{169}, I^{311}, W^{277}, Q^{331}, and Y^{260} were identified as the putative residues involved in NADPH binding and three conserved catalytic amino acid residues, H^{166}, N^{148}, and Y^{137} were close to α5″, in β7′, and β6 in

all species. Eight amino acids: R^{320}, H^{364}, R^{365}, N^{369}, F^{409}, R^{432}, Y^{404}, and R^{457} were identified as the heme-binding site residues.

Phylogenetic Relationship of *CAT*s between Bivalves and Other Organisms

BLAST analysis showed that the deduced amino acid sequences of *CfCAT*1 and *CfCAT*2 exhibited a high identity with those of other bivalve mollusks CATs with *A. irradians* (78.31%, 68.82%), *M. nobilis* (66.6%, 91.16%), *P. fucata* (80.23%, 66.06%), *M. meretrix* (66.79%, 62.44%), *C. hongkongensis* (64.87%, 62.85%), *C. plicata* (78.69%, 66.86%), *H. discus discus* (72.74%, 62.04%), *C. gigas* (65.06%, 62.04%), and *A. farreri* (96.16%, 70.68%). The phylogenetic analysis of *CAT* amino acid sequences from 17 selected species was conducted (Fig. 3). Each member of *CAT* in scallops clustered into well-supported separate clades with its orthologues of other species. The phylogenetic analysis showed that a clear clade division of *CAT* proteins was found between vertebrate *CAT* members, and their corresponding *CAT* orthologous in bivalve mollusks. The scallop *CAT* members can be classified into a major clades. *CfCAT1* and *CfCAT2* were firstly clustered to the origin of the main bivalve branch and closely positioned to the bivalve scallop (*A. farreri*, *A. irradians*, and *M. nobilis*) *CAT* sub-clusters. Bivalve mollusks, oysters, and fish catalase sub-clusters were observed within the main bivalve cluster. The most relationship of the evolution of *CfCAT*s was that of marine bivalves, the next was freshwater bivalves, oysters, and fish, then the last was vertebrates.

Expression Regulation of Scallop *CAT*s after Toxic Dinoflagellate Exposure

To understand the antioxidant defensive mechanism of scallop *CAT*s in response to PST-producing algae challenge, RNA-Seq analysis was performed to determine the expression pattern of *CAT* genes in the mantle, gill, muscle, kidney, and hepatopancreas of *C. farreri* after exposed to the PST-producing dinoflagellate, *A. minutum* (AM-1 strain). *CfCATs* were significantly differently expressed ($P< 0.05$) according to their RPKM values. The two *CfCAT* genes were regulated in all tissues but they exhibited different expression patterns with fold changes higher than 0.5 at least one test point. The relative mRNA expression of *CfCAT*1 showed both up and down-regulation at different time points (days 1, 3, and 10) in the mantle with an increase in fold changes (1.8, -0.4, and 0.3, ($P<0.05$)) compared with the control group, while being acutely up-regulated on day 1 in muscle with 4.34-fold, $P<0.01$ during exposure experiment (Fig. 4 (a)). Similarly, the inductive expressions of *CfCAT*2 were observed after AM-1 exposure in these organs at days 3 and 5 (fold changes: 2.21 and 0.83, $P<0.05$). A chronic induction of *CfCAT*2 was detected at day 15 in the gill and hepatopancreas (fold changes: 0.83 and 0.52, $P<0.05$) after AM-1 challenge (Fig. 4 (b)). Whereas, the expression profiles of *CfCAT*s showed little change in expression levels in the kidney between control and exposure groups after being exposed to toxic algae throughout the whole experiment. They were likely to be constitutively expressed and were assumed to be not toxin-inducible.

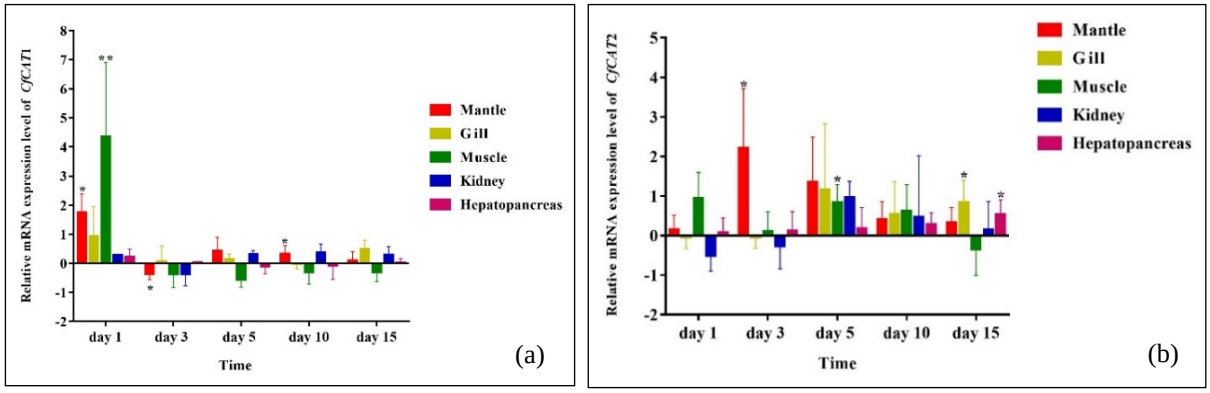

Figure 4 The expressions of (a) *CfCAT*1 and (b) *CfCAT*2 in the mantle, gill, muscle, kidney, and hepatopancreas were measured by RNA-Seq datasets after AM-1 challenge. Bars represented means ± SE; n = 3 (each group/each time point). *P < 0.05 vs control group at the same time. Different asterisks indicate significant differences between groups ($P < 0.05$, $P < 0.01$, and $P < 0.001$).

Discussion

The catalase (CAT) gene family members of *C. farreri* were identified and characterized at the genomic level. The whole-genome identification of the *CAT* gene was conducted in a scallop, then two copies of *CAT* gene were observed. The varied number of introns contributes to the variation in the length of *CAT* genes in scallops which were organized into 11 introns. Multiple introns of *CfCAT*s showed that these genes have a strong relationship between the evolution of gene structures and the sub-functionalization of proteins encoded by the genes (Lynch and Conery 2000). For understanding the protein charge stability, the theoretical pI is the pH at which a particular molecule carries no net electrical charge (Gasteiger, Hoogland et al. 2005). The *CfCAT*s were found to be alkaline/basic in characters showing the proteins which separate based on their relative content of acidic and basic residues, whose value is represented by the isoelectric point (pI). All *CAT* proteins in scallops were predicted to be stable where the instability index is ≤40), and the aliphatic index of a protein is regarded as a positive factor for the increase in the thermo-stability of globular proteins (Enany 2014). The high aliphatic index of *CfCAT*s indicated that these proteins are thermo-stable over a wide range of temperatures. Most of the amino acid residues have stronger hydrophilicity, whereas the N-end and C-end regions have hydrophobicity (Xu, Yu et al. 2017). The values of GRAVY in all *CAT*s in scallops are between -2 and 0, classified as hydrophilia proteins. The functional analysis features of proteins determined by subcellular localization which provides information about the biological and cellular functions (Ebersbach, Galli et al. 2008). The subcellular localization prediction indicated that all *CfCAT* proteins were predicted in peroxisomal locations. The catalase of *C. maenas* and *M. galloprovincialis* also were localized in the peroxisomes stated by (Orbea, Dariush Fahimi et al. 2000). The tertiary structure is primarily due to interactions between R groups of amino acids that make up the protein (Lumry and Eyring 1954). The three-dimensional predicted protein structure of *CfCAT*s showed their active site position.

The similarity and identity of *CfCAT*s amino acid sequences among marine bivalves are quite high ranging from 95.06% and 96.16% to 10.62% and 5.75% (*A. farreri* and *H. cumingii*), freshwater bivalve, *C. plicata* (83.11% and 78.69%) in *CfCAT*1 while the ranging from 89.37% and 91.16% to 12.14% and 7.09% (*M. nobilis* and *H. cumingii*), *C. plicata* (67.93% and 66.86%) in *CfCAT2* reflecting the high conservation of *CAT*s in bivalve mollusks. While *CAT*s in vertebrates are much less conserved with the identity ranging from 10.81% and 4.41% in *CfCAT1* where to 9.48% and 4.21% with *X. tropicalis* in *CfCAT2*. This result assumed that the

molecular evolution of *CAT*s was consistent with the species taxonomy. The proximal heme-ligand signature sequence, the proximal active site signature, and the signal sequences were identified in *CfCAT*s and *CAT*s of bivalves By multiple sequence alignments (Li, Ni et al. 2008, Gao, Ishizaki et al. 2016, Xia, Huang et al. 2016). It was supposed that the *CAT* proteins of *C. farreri* encode a putative peroxisomal catalase via the internal peroxisomal targeting signal (PTS1), QKL which was revealed to direct the interaction between catalase protein and PTS1 receptor PEX5 in the cytosol (Abu-Romman 2016). Twelve conserved amino acid residues were identified in the structure of *CfCAT*s which belong to the NADPH-binding catalase family (Putnam, Arvai et al. 2000). There were 15 amino acid residues in *MyCAT*, ten amino acids in *AwCAT* were identified in the sequence as NADPH binding sites and nine of them were completely conserved with human *CAT* and *CfCAT*s (Li, Ni et al. 2008). The *CfCAT*s first clustered with bivalve *CAT*s indicating that scallop *CAT*s are widely distributed with their corresponding *CAT*s orthologous from other species and evolutionarily conserved and may be derived from a common ancestor.

Up- and down-regulated gene expressions of *CfCAT*s in the tissues of scallops were observed. The up-regulation of *CfCAT*s expression displayed a time-dependent pattern in response to *A. minutum* exposure. For the inducible *CfCAT* copies, an immediate up-regulation was observed at 1-3 days, which was the scallops' acute response in the presence of the toxic dinoflagellate, and then a decrease of expression was observed at 5 days. However, sub-chronic exposure (10-15 days) may reflect the accumulating effect of prolonged PST exposure. We observed the significant inductive expression of *CfCAT*s in mantle and gill after *A. minutum* exposure, as these two organs are the first organs of directly large contact with the toxic dinoflagellate cells during the filtration process (García-Lagunas, de Jesus Romero-Geraldo et al. 2016). Furthermore, muscle is the most important tissue of protein deposition for the metabolism of nutrients, immune responses, and energy storage in response to Alexandrium cells during filtration (Sun, Xuan et al. 2015) where the significant expression up-regulation of *CfCAT*s was observed after toxic dinoflagellate exposure. It was suggested that the hepatopancreas is the major organ involved in the bioaccumulation and detoxification response to PST challenge occur (Huang, Peng et al. 2018), so the transcriptional up-regulation of *CfCAT*2 was detected in hepatopancreas after oxidative stress induced by AM-1 exposure. If the generation of ROS is under a low level, the antioxidant enzyme expressions should be up-regulated that contribute to enduring the oxidative stresses whereas the down-regulation of *CfCAT*1 in the mantle is associated with increased ROS production and oxidative damage by toxic exposure (Radak, Suzuki et al. 2016). The importance of catalase induction at the transcriptional level as an adaptive antioxidant defense system response in bivalves under algal toxin exposure. Sub-chronic exposure might be caused by strong oxidative stress in tissues that are frequently in contact with PST-producing dinoflagellate. The regulation of *CAT* genes revealed that these genes play a protective role in the defense against oxidative stress and the adaptative responses of scallops caused by AM-1 challenge. The functional variety of scallop *CAT*s is involved in the tissue-specific regulation of *CAT*s that might contribute to adaptation response to the harmful effects of PST-producing algae and could be as tissue-responsive indicator genes with PSTs challenge.

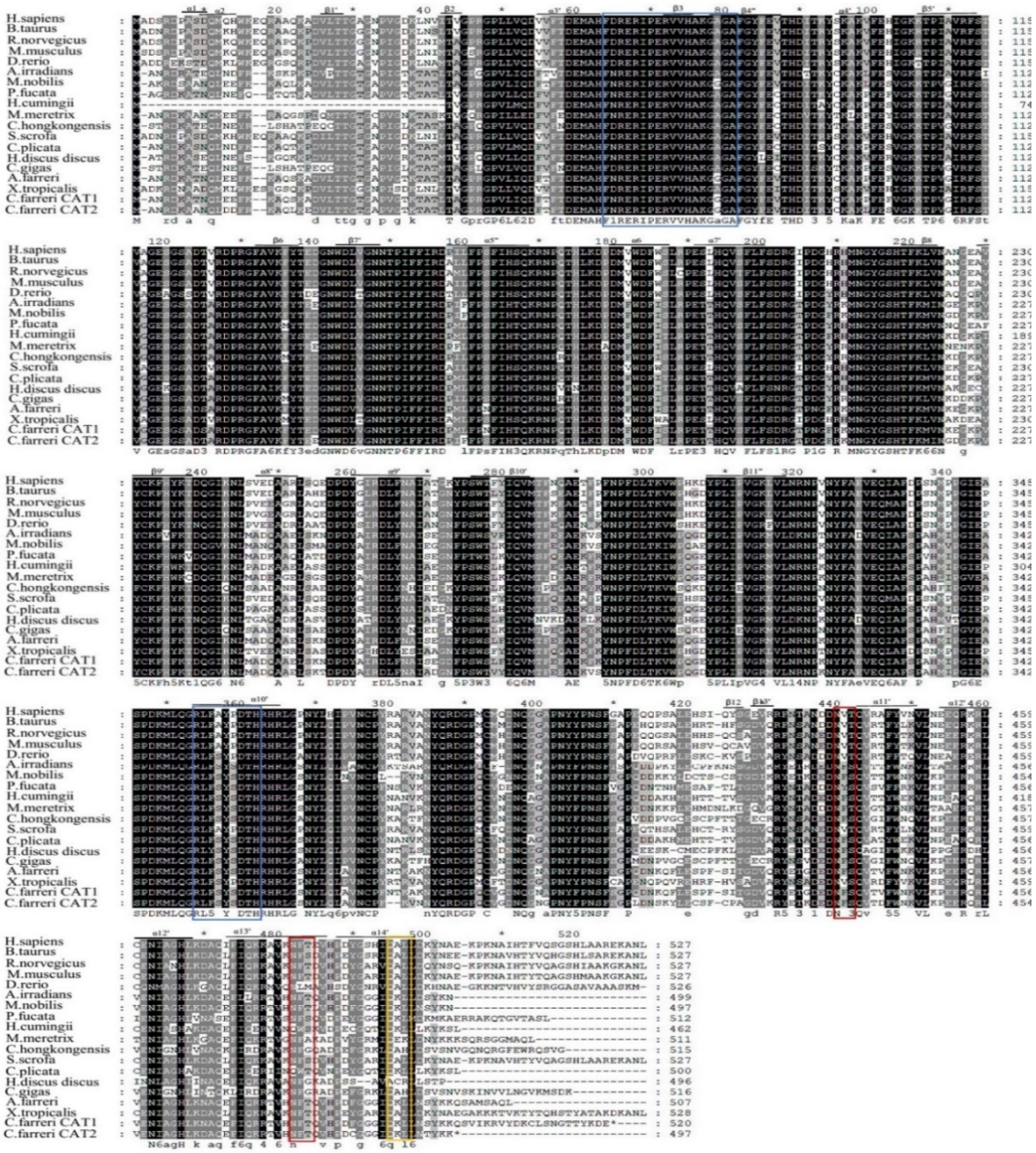

Figure 2 Multiple sequence alignment of *CfCAT*s in scallops with other homologous vertebrates and mollusks catalase amino acid sequences. The proximal active site signature and the proximal heme-ligand signature are boxed in blue color. The conserved catalytic amino acids (H^{71}, N^{144}, and Y^{354}) are shown in the down arrow. Peroxisome targeting signal (QKL) is boxed in yellow color. The predicted secondary structure is indicated by α or β (α is α-helices and β is β-sheets).

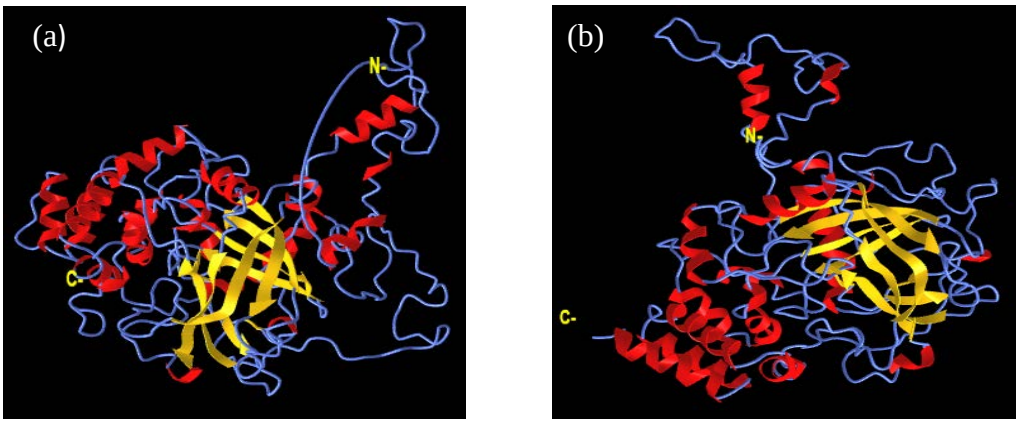

Figure 1 Three-dimensional predicted protein structures of (a) *CfCAT*1 and (b) *CfCAT*2. Models were visualized by color from N to C terminus and organized in order as gene sequences. Different sub-family proteins have similar protein models. Red ribbons represent α-helices and yellow arrows indicate β-sheets.

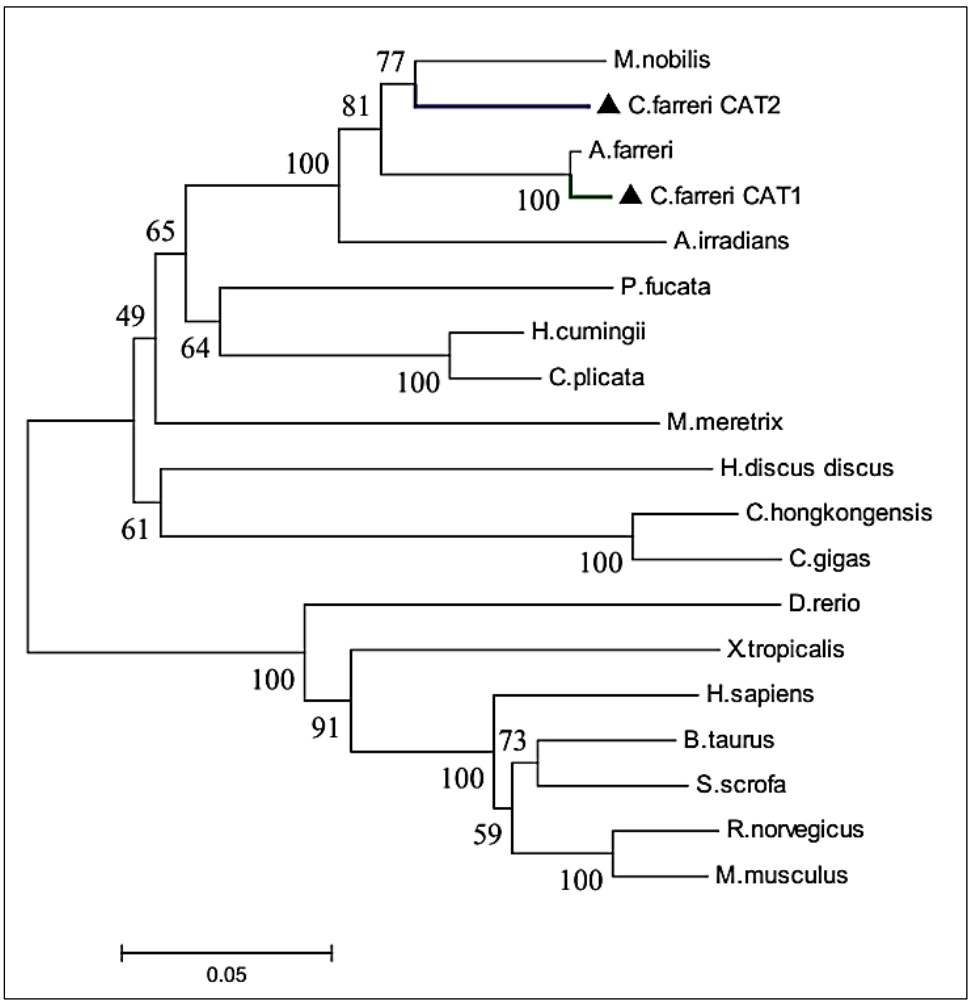

Figure 3 Phylogenetic tree of *CAT* proteins from *C. farreri* and other selected organisms. The tree was constructed using the maximum-likelihood (ML) method with LG + G + I module. Numbers at the branch point of the node represent the value resulting from 1000 replications. Branches of *CfCAT*1 and *CfCAT*2 proteins are marked with black triangles.

Conclusion

Two *CfCAT* genes were scanned and systematically characterized from the *C. farreri* genome that belongs to the NADPH-binding catalase family. After ingesting PST-producing algae, no regulated expression of *CfCAT*s was observed in the kidney of scallop, but inducible *CfCAT*1 and *CfCAT*2 were either up-or down-regulated in the mantle, gill, muscle, and hepatopancreas with acute and chronic regulation after *A. minutum* exposure. These results indicated that the response of scallop *CAT*s to PST-producing dinoflagellate was dependent on scallop species and tissues. We found diversified responsive profiles of scallop *CAT* genes after the toxic algae challenge, suggesting the catalase implied in the protective role of oxidative stress which provides a better understanding of mollusks' defensive mechanisms against the harmful effects of PST accumulation which might contribute to the adaptive evolution of scallop.

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POPULATION PARAMETERS OF WHITE SARDINELLA, *SARDINELLA ALBELLA* (VALENCIENNES, 1847) FROM NGA YOKE KAUNG COASTAL AREA

Soe Thaw Thaw Tun[1]

Abstract

This study was conducted to estimate population parameters of white sardinella, *Sardinella albella* collected from Nga Yoke Kaung coastal area from June 2019 to May 2020. A total of 853 specimens of *S. albella* ranging the length 11.3 cm to 20.1 cm were used to analyze with FiSAT II. In present findings, length-weight relationship equation of this species was $W = 0.0712\ L^{2.2431}$ ($r = 0.91$) which showed a negative allometric growth pattern. The estimated mean condition factor indicated that they were in good condition. The asymptotic length (L_∞) and growth rate (K) were 21.53 cm (TL) and 0.98 year^{-1} respectively. The theoretical age at birth (t_0) and growth performance index (ϕ') were -0.18 yr^{-1} and 2.657 respectively. Natural mortality, fishing mortality, and total mortality for *S. albella* were estimated as 1.9, 1.99, and 3.89 respectively. The exploitation rate of *S. albella* from the present study was 0.51 which indicated that they were not overexploited.

Keywords growth parameters, length-weight relationship, mortality parameters, Nga-Yoke-Kaung coastal area, *Sardinella albella*, white sardinella.

Introduction

Sardinella albella (white sardinella) is a small, coastal, and pelagic species under Family Clupeidae which are widespread in tropical and subtropical oceans' warmer regions. When compared to other clupeids fish, by a combination of characteristics, including the second supramaxilla's symmetrical paddle shape, the last two anal fin rays being considerably larger, and the hind border of the gill opening having two distinct fleshy outgrowths (Whitehead, 1985). The French ichthyologist Achilles Valenciennes initially described and published this species in the literature in 1847 under the name *Kowala albella*. The local name of Sardinella was well-known as ‘Nga Kone Nyo’ in the Rakhine Coastal Region and ‘Baung Kyae’ in Myeik Archipelago (Nyo Nyo Tun, 2013).

Estimating fish landings requires knowledge of the length-weight relationship (LWR). It provides information about the population at that time and location. The cube of the fish's length often determines how much it weighs. The hypothesis that larger fish of a particular length are in better condition is supported by the length-weight relationship, which also gives a way to determine the condition factor, which shows the "Well-being of the fish" (Bagenal and Tesch, 1978).

The von Bertalanffy growth model, which is used to estimate, is appropriate for the observed growth of the majority of fish species. According to this idea, length is a function of the animal's age. To determine the rate of population decay, mortality rates were estimated. Evaluating mortality rates is essential for estimating fish population abundance. The more the fishing effort and demand for fishmeal, the production can be greater and it may lead to the overexploitation of stock.

Several studies on the population dynamics of *Sardinella* were carried out along the coast of India. However, there is relatively little information available in Myanmar regarding the *Sardinella* population parameters. Therefore, an attempt was made to investigate the length-

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weight relationship, growth, mortality, and exploitation rate of *S. albella* from the Nga-Yoke-Kaung coastal area.

Materials and Methods

Sample Collection

This study was carried out at two stations: Sin Ma (Lat 16º 43′N and Long 94º 22′ E) and Nga Yoke Kaung (Lat 16º 31′ N and Long 94º 17′ E) (Fig.1) from June 2019 to May 2020. Fresh fish samples were collected monthly by random sampling from drift gillnet and purse seine net in fish landing centers in Nga Yoke Kaung. A total of 853 *S. albella* ranging the length from 11.3 cm to 20.1 cm collected monthly were used for these estimations. The total length of fish was measured from the tip of the snout to the tip of the tail to the nearest mm and the length measurements were converted into length frequencies with constant class intervals of 1 cm. Fish were weighed nearest to 0.1 grams by using a digital weighing balance.

Length-weight Relationship Analysis

The length and weight relationship of fish was calculated by the equation of Pauly, 1983;

$$W= aL^b$$

Where, W= weight of fish in grams, L= total length of fish in cm, a= constant, and b=exponent. The values for 'a' and 'b' coefficients were calculated and linear regression equation was obtained by using natural logarithmic transformations. The correlation (r=R) which is a degree of association between the length and weight was obtained from the graph.

Condition Factor (k)

The condition factor (k) of fish was estimated by using the formula

$$k= 100*W/L^3 \text{ (Pauly, 1983)}$$

Where, k= condition factor, W= weight of fish in gram, and L= total length of fish in cm.

Estimation of Growth Parameters

The Electronic Length Frequency Analysis (ELEFAN) I program in the FiSAT software package was used to analyze the length data and estimate the von Bertalanffy growth parameters L_∞ and K. The length data were sorted into 1 cm size class intervals. Pauly's empirical equation (Pauly, 1979) was used to estimate the value of theoretical age at length zero (t_0):

$$\text{Log } (-t_0) = -0.392 - 0.275\text{Log } L_\infty - 1.038\text{LogK}$$

The resultant values of growth parameters (L_∞, K, t_0) were substituted in the von Bertalanffy growth equation;

$$L_t= L_\infty (1- e^{-K (t-t_0)})$$

Where L_t is the length at age t, L_∞ is the asymptotic length that is the mean length of fish would reach if they were to grow indefinitely, K is the growth coefficient or the rate at which L_∞ is approached and t_0 is the age of the fish at zero length.

The growth performance index (ø′) was calculated from the resultant values of asymptotic length (L_∞) and growth coefficient (K) using the equation:

$$ø'= 2\text{Log } L_\infty + \text{Log K (Pauly and Munro, 1984)}$$

The longevity of this species was estimated using the equation:

$$T_{max}= 3/K+t_0 \text{ (Pauly, 1983)}$$

Where T_{max} is the approximate maximum age fish would reach.

Estimation of Mortality Parameters

Using the length-converted catch curve provided by the FISAT II tool, the total mortality coefficient (Z) was calculated. The empirical equation of Pauly (1980) was used to calculate the natural mortality rate (M) using a mean surface temperature (T) of 27°C:

$$\text{Log } M = -0.0066 - 0.279 \text{ Log } L_{\infty} + 0.6543 \text{ Log } K + 0.4634 \text{ Log } T$$

Where M is the natural mortality and K refers to the growth rate of the VBGF. Fishing mortality (F) was estimated by the following relationship

$$F = Z - M \text{ (Gulland, 1969)}$$

Where, F= fishing mortality, Z= total mortality, and M= natural mortality. The exploitation rate (E) was estimated from the equation: E= F/Z (Gulland, 1969).

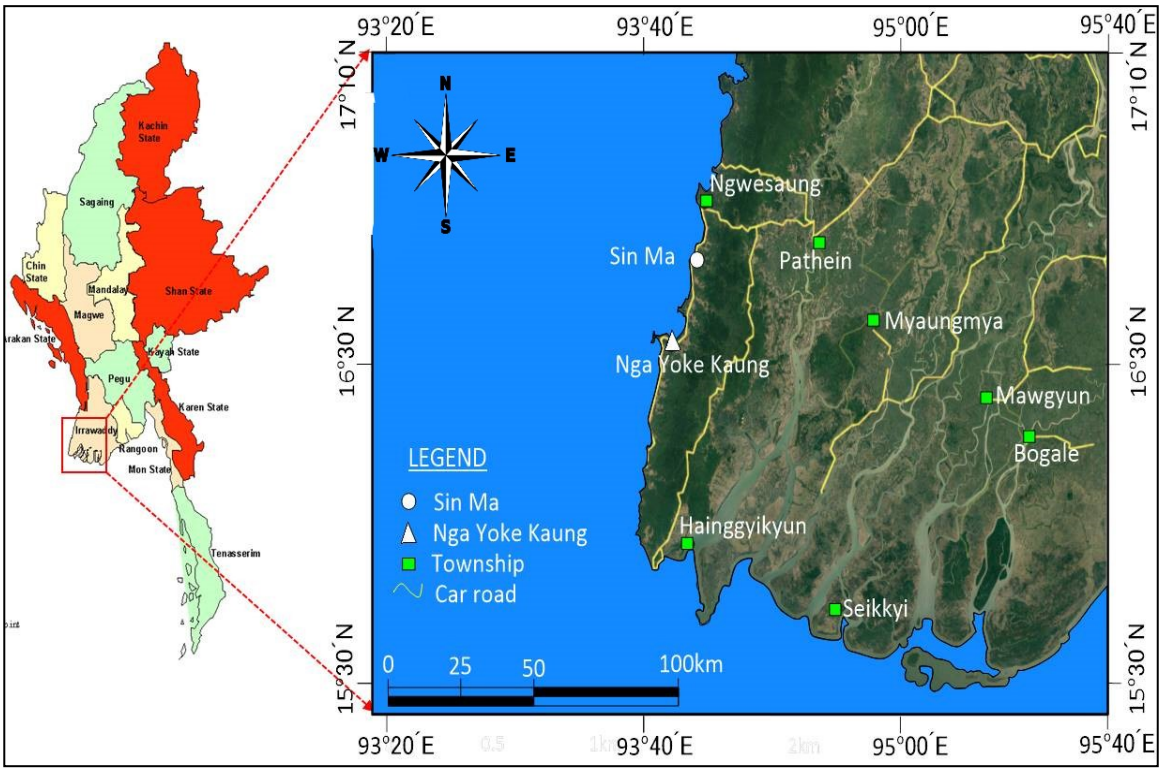

Figure 1 Map showing the locations of sample collecting area

Results

Length-weight Relationship Analysis

The linear regression of the length-weight relationship of *Sardinella albella* and their logarithmic transformations are mentioned in Figure 3.

The equation thus derived in respect of the length-weight relationship of *S. albella* was as follows:

$$W = 0.0712\ L^{2.2431},\ r = 0.91$$

The corresponding logarithmic regression equation can be represented as follows:

$$\text{Log } W = -2.6423 + 2.2431 \text{Log } L$$

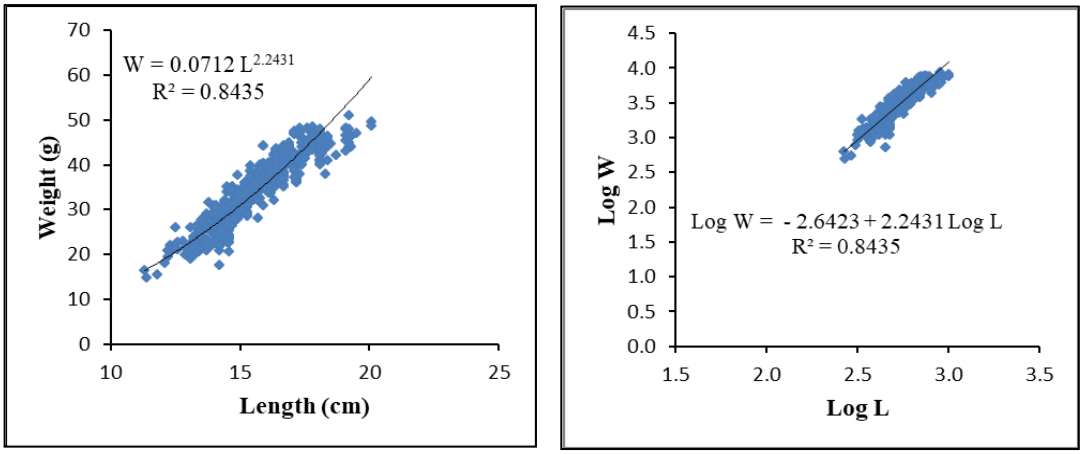

Figure 3 Length-weight relationship of *Sardinella albella* (June 2019 – May 2020) and their logarithmic transformations.

Condition Factor

The condition factor (k) which is the ratio between total length and weight was calculated for *S. albella* represented in Figure 4. The condition factor (k) for *S. albella* was 0.82-0.96 and the mean value was 0.89 ± 0.04. Nearly fifty- four percent of *S. albella* was higher than the mean value. The highest condition factor value was recorded in September while the lowest value was in May.

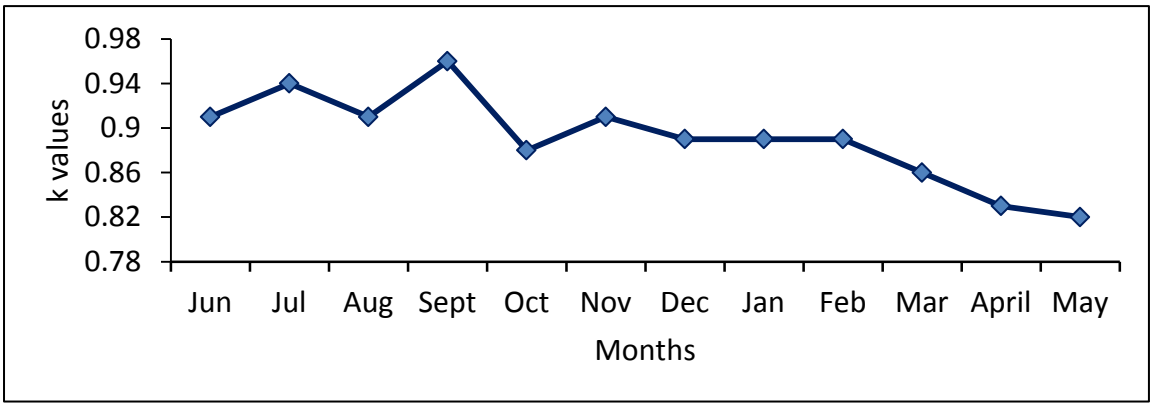

Figure 4 Monthly condition factors for *S. albella* (June 2019 – May 2020).

Growth Parameters

From the analysis of length-frequency data by the ELEFAN I, the estimates of growth parameters obtained were; asymptotic length (L_∞) =21.53 cm and growth coefficient (K) = 0.98 per year (Figs. 5. A and 5. B). The growth performance index ø´ was 2.657 and the estimated t_0 value was -0.18. Thus, the von Bertalanffy growth equation of *S. albella* can be expressed as $L_t = 21.53\ (1\text{-}e^{-0.98\ (t+0.18)})$. Accordingly, the total length attained by *S. albella* is 10.47 cm, 14.76 cm, 17.38 cm, 18.99 cm, 19.97 cm, and 20.58 cm at the end of 0.5, 1, 1.5, 2,2.5, and 3 years in its life respectively. Longevity (T_{max}) of *S. albella* calculated from Pauly's equation is 2.8 years.

Mortality Parameters

The natural mortality (M) of *S. albella* obtained from Pauly's empirical formula at 27˚C annual average sea surface temperature was 1.9 $year^{-1}$. The estimation of Z calculated by the length-converted catch curve method was 3.89 $year^{-1}$(Fig. 5. C). The computed fishing mortality (F) was 1.99 $year^{-1}$. Smaller sizes (yellow dots) had to be excluded, and large size fish had only a

few samples therefore had to be excluded from mortality calculation, so only the black dots were included to determine the total mortality. The exploitation rate (E) of this species was 0.51.

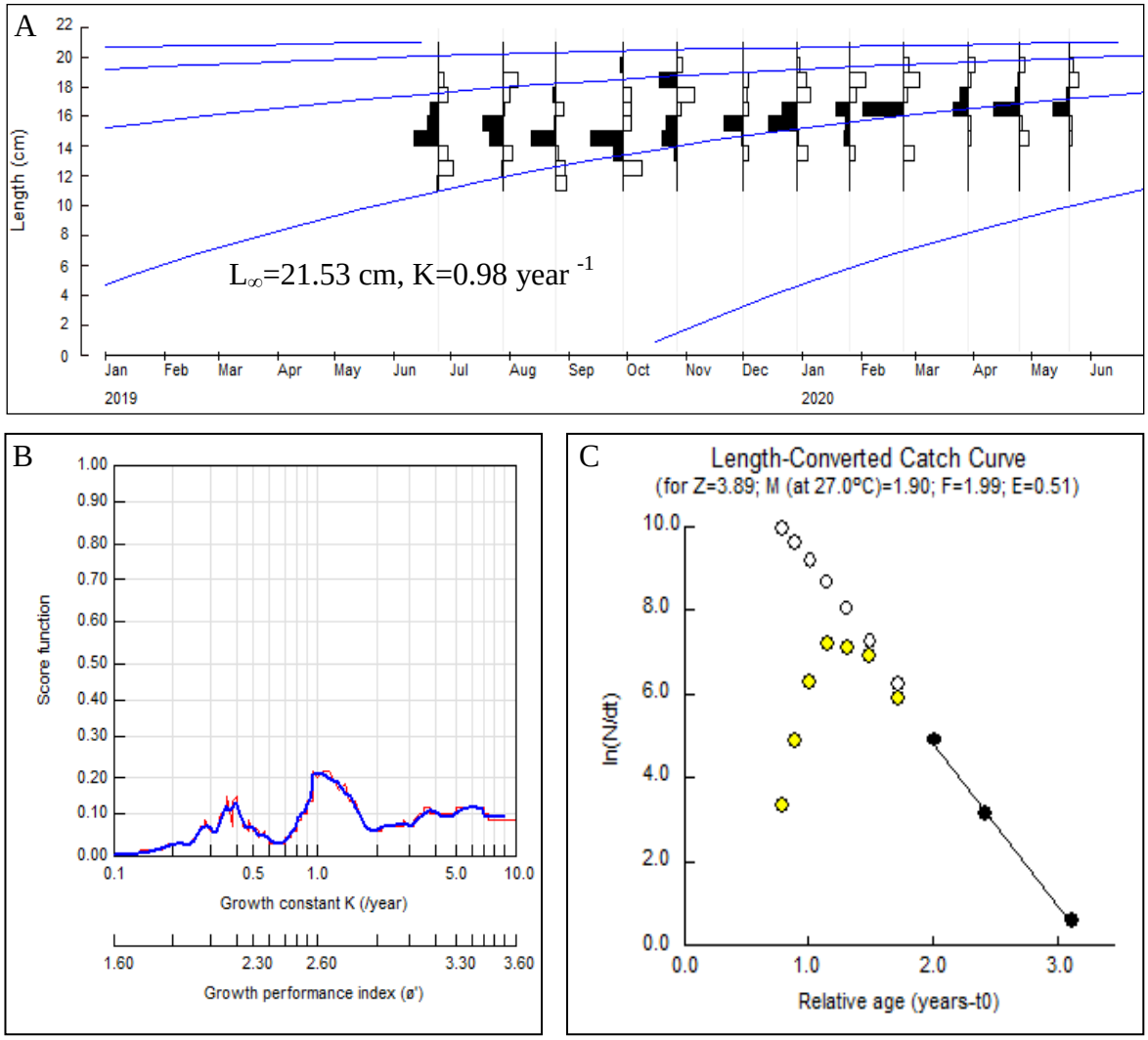

Figure 5 A-C Estimated population parameters of *S. albella*: A). von Bertalanffy growth curves of *S. albella* superimposed on the restructured length-frequency histograms; B) Estimation of K; C) Length-converted catch.

Discussion

The formula "W= aL^b" was used to approximate the length-weight relationship. Fish growth can be determined by the result "b" value. Fish can grow allometrically or isometrically. The growth of fish is isometric when the "b" number is 3, meaning that there is no change in the fish's body form as it develops. Fish grow allometrically if the "b" value is greater or less than 3. If b>3, the fish becomes relatively stouter or deeper-bodied as it grows in length; if b<3, the growth pattern is negative, this mean that the fish becomes slenderer as it grows in weight (Khin May Chit Maung, 2016). In the present study, the 'b' value obtained from the regression line of the graph for *Sardinella albella* was 2.2431, indicating a negative allometric in growth. Thus present result matches the result of Le Cren, 1951. He suggested that the value of exponent ‘b’ is to be equal to ‘three’ or usually lies between ‘two’ and ‘four’.

A correlation coefficient (r) measures the strength of the relationship between the two variables, length and weight. The value of the coefficient of correlation (r) lies between -1 and

+1 (Banerjee 2004). The present result of the r value was 0.91, indicating that the length-weight relationship was positively associated as shown by an r-value above 0.5 (Omogoriola, 2011). As a result, there was a high correlation between the length and weight of *S. albella*.

A quantitative indicator of the fish's health state is the condition factor. Fish are deemed to be in good condition if their condition factor value is more than 0.56. (Bennet, 1970). The fact that these species' condition factors were higher than their average values in the study indicated that they were in good condition. Similar to this, Nyo Nyo Tun (2013) reported that the condition factors of *Sardinella* species from Myeik water were close to 1.1 and came to the conclusion that these species' populations were in good health.

For the fishery to be managed sustainably, estimation of population parameters is also crucial. The estimated asymptotic length (L_∞) of *Sardinella albella* in the present study was 21.53 cm. This value was higher than the values estimated at 13.3 cm by Sekaran (1955), 17cm by Nair (1960), 13 cm by Bennet (1961), and 16.8 cm by Makwaia and Nhwani (1992). Aripin and Showers (2000) described the L_∞ value for this species as 20.2 cm from the Philippines. Nyo Nyo Tun (2013) stated that the growth parameters, L_∞ = 17.85 cm and K = 0.54 year $^{-1}$ observed for *S. albella* from Myeik coastal waters. Abdussamad *et al*. (2010) estimated the L_∞ value of this species from the Gulf of Mannar coast was 20.8 cm (Table.1).

The growth curvature (K) observed was 0.98 year^{-1}. As shown in Table. 1, the K value from the present study was lower than the findings from Bennet (1961), Makwaia and Nhwani (1992), Aripin and Showers (2000), and Abdussamad *et al.* (2010).The growth performance index (ϕ') from the present study was higher than the value reported by Nyo Nyo Tun (2013) and less than the result described by Aripin and Showers (2000).

Qasim (1973) stated that a high K value indicated a strong metabolic rate, and such fish matured at an early age that was greater than L_∞. The current finding indicates that *Sardinella* species have short lifespans due to the high K value and longevity estimation (T_{max}). The length increases per unit of time, which is known as the growth rate. In the present study, there was a significant increase in length during the initial year of life, following which the rate of growth progressively decreased with increasing age. Fish grow longer as they age, but their growth rates gradually slow (Sparre and Venema, 1992).

Table 1 Comparison of population parameters estimates for *S.albella* from different localities.

$L\infty$ TL, cm	K yr^{-1}	ϕ'	F yr^{-1}	M yr^{-1}	Z yr^{-1}	E	localities	References
13.3	1.44	2.41					Mandapam area, India	Sekharan, 1955
17	1.1	2.5					Palk Bay, Gulf of Manaar	Nair, 1960
13	1.65	2.45					Gulf of Manaar, India	Bennet, 1961
16.8	1.15	2.51	1.9	1.8	3.7	0.51	Dares Salaam, Tanzania	Makwaia and Nhwani, 1992
20.2	1.6	2.82	3.48	2.62	6.1	0.57	Tawi-Tawi, Philippines	Aripin and Showers , 2000
20.8	1.1		3.06	2.19	5.25	0.58	Gulf of Mannar	Abdussamad *et al.*, 2010
17.85	0.54	2.236	1.49	1.36	2.85	0.52	Myeik, Myanmar	Nyo Nyo Tun , 2013
21.53	0.98	2.657	1.99	1.9	3.89	0.51	Nga Yoke Kaung, Myanmar	Present study

Estimated mortality rates were used to determine the rate of population death. The mortality rates from the present study were lower when compared to the rates (Z = 6.1, M = 2.62, F = 3.48) reported by Aripin and Showers (2000) from the Philippines, (Z = 3.7, M = 1.8, F = 1.89) by Makwaia and Nhwani (1992) from Tanzania, (Z = 2.85, M = 1.36, F = 1.49) by Nyo Nyo Tun (2013) from Myeik waters as mentioned in Table 1. Natural mortality refers to death brought on by predators such as cannibalism, disease, stressful circumstances during spawning, malnutrition, and age. The death rates of the same species might vary based on the predators and competitors, which are also impacted by fishing activity. The death rate from fishing in the current research was considerably greater than the mortality rate from natural causes.

The rate of exploitation is the rate at which fish resources are used up by fishing activities. The optimal exploitation rate (E_{opt}) for any exploited stock is 0.5 (Gulland, 1971).The rate of exploitation obtained in this study was 0.51 which indicated that the resource of *S. albella* in Nga Yoke Kaung coastal water was not overexploited.

Conclusion

Using length weight frequency data, population parameters such as growth parameters, mortality parameters, and exploitation rate of *Sardinella albella* were estimated and discussed. According to the present study, it could be concluded that *S. albella* from Nga Yoke Kaung coastal area was in good condition. The present estimate of the exploitation rate indicated that the population of this species in the study area is not overexploited. The present study will provide information about fishable stocks and also will contribute additional information to the existing knowledge. This information is required for consideration of the management of measures of the species in the future. This will be the primary contribution to the biological study of these species, as well as information for fishery managers to better manage them.

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ISOLATION AND FERMENTATION CONDITIONS OF SOIL FUNGUS NFO-9 FROM MANGROVE SOIL MAGYI COASTAL AREA

Nan Htwe Htwe Maung[1], Moe Moe Aye[2]

Abstract

In the course of isolation of soil fungi, thirteen different kinds of fungi were isolated from five mangrove soil samples. These soil samples were collected at Magyi Channel. The isolation was undertaken by serial dilution method and using Low Carbon Agar medium (LCA medium). Potato Glucose Agar Medium (PGA medium) was utilized as transfer medium to get pure culture. Antimicrobial activities of these fungi were evaluated by agar well diffusion assay with five test organisms. Among them, eight fungi showed antimicrobial activity. Especially, NFO-9 gave the best antibacterial activity on *Pseudomonas fluorescens*. Therefore,different fermentation parameters of NFO-9 were studied by the fermentation period, age and size of inoculums, pH and fermentation medium on *Pseudomonas fluorescens*.

Keywords antibacterial activity, fermentation, soil fungi**.**

Introduction

Mangroves are coastal wetland forests established at the intertidal zones of estuaries, backwaters, deltas, creeks, lagoons, marshes and mudflats of tropical and subtropical latitudes. Mangrove forests are referred to as mangrove swamps, tidal forests, tidal swamp forests or mangals and are considered as transition zone between terrestrial and marine habitats. Approximately 25% of the world's coastline is dominated by mangroves distributed in 112 countries and territories encompassing an area of 181,000 sq km worldwide. Mangrove stands are located in sheltered places and the mouths of valleys in most cases. The mangrove floor varies from sandy to muddy.

Fungi from mangrove soil play an important role in the nutritive cycle and support the mangrove ecosystem. They commonly occur as saprophytes on decomposing organic matter such as wood, stem, leaf etc, and as symbiosis of plants and animals as parasites of plants in mangrove ecosystem. Fungi being universal organisms occur in all types of habitats and are the most adaptable organisms.

Marine fungi are found in all divisions of fungi and most probably evolved the association independently on many occasions. Mangrove derived microbes especially fungi have long been recognized as a potential source of novel and biologically potent metabolites (Suja *et al,* 2013; (Chioma *et al,* 2016; Sandhu *et al,* 2014).In recent years, the isolation of marine fungi and screening of antimicrobial activity has gained more attention (Styrobel, 2003).

The aims of this research were to isolate soil fungi from mangrove soil to study the isolation method and to investigate the effect of fermentation and pH of selected fungus on *Pseudomonas fluorescens*.

Materials and Methods

Material: The soil samples were collected from mangrove swamp soil of Magyi tidal creek, Shwe Thaung Yan sub-Township, Ayeyarwaddy Region. The isolation of fungi were carried out by serial dilution method (NITE, 2004).

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Method: Serial dilution method

Soil was air-dried at room temperature and then grounded and sieved. One gram of soil sample was suspended in 10 ml of distilled water and was shaken for 15 minutes. 0.1 ml of this dilution water was added to 5 ml of distilled water and shaken for 5 minutes. 0.5 ml of this dilute water (5 ml + 0.1 ml) was added to 4.5 ml of distilled water and shaken for 5 minutes. Then, 1.0 ml of dilute water (0.5 ml + 4.5 ml) was added to 4.0 ml of distilled water and shaken for 5 minutes. 0.1 ml of this solution was added to sterile agar medium for 3 days. After 3 days, the microorganisms were picked and purified by re-culture in glass plate containing PGA medium.

Preliminary study for antimicrobial activity

The isolated fungi were grown on PGA medium for 3 days. The isolated fungi were inoculated into 25 ml seed medium and incubated at room temperature for 3days. After 3 days, 20 ml seed culture was transferred into the 80 ml of fermentation medium and incubated at room temperature. Fermentation was carried t for 3-10 days (Ando, 2004).

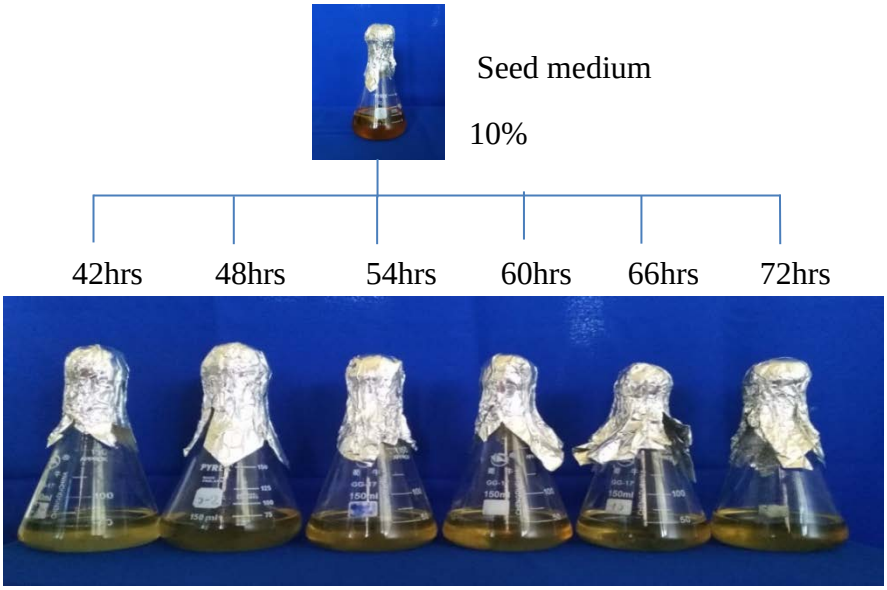

Figure 1 Procedure for the study on the effects of ages of seed culture

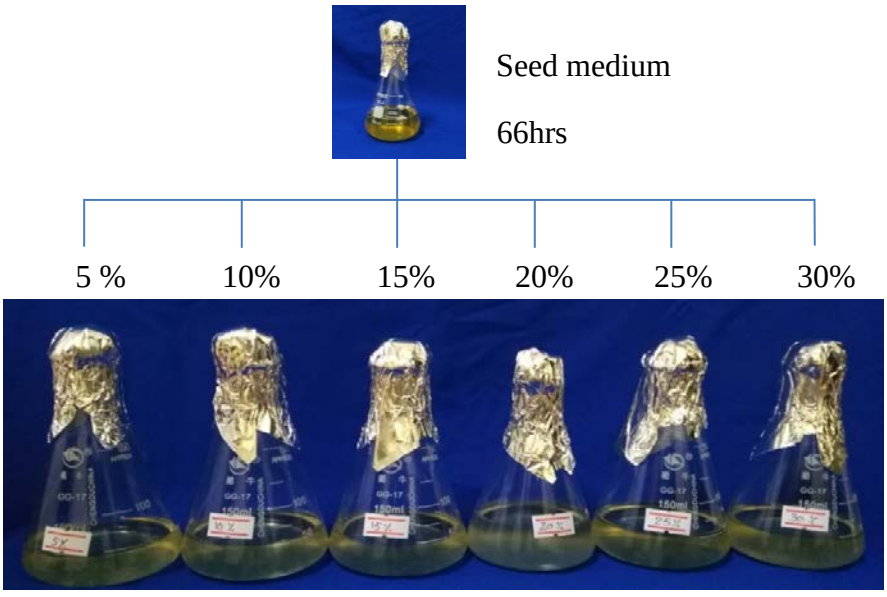

Figure 2 Procedure for the study on the effects of size of seed culture

The effect of pH on fermentation

Effects of different pH were used for antibacterial activity of pH 4, 5, 6 and 7. This different pH were adjusted by NaOH and HCL.

Study on the fermentation media of NFO-9

Fermentation was undertaken with suitable conditions of 25% sizes and 66 hrs ages of inoculum with five different media. Fermentation was carried out for 7 days and antibacterial activity test was carried out every 24 hrs.

A Medium Used for the isolation of soil fungi NITE (2004)

LCA Medium		**PGA Medium**	
Glucose	1.5 g	Potato	20g
Glycerol	1.0 mL	Glucose	1.5g
Yeast extract	0.8 g	Agar	1.8g
Polypeptone	0.4 g	DW	100 ml
K_2HPO_4	0.001g	pH	6.5
$MgSO_4.7H_2O$	0.001g		
Agar	1.8g		
DW	100 mL		
pH	6.5		
Seed Medium		Fermentation Medium	
Glucose	1.5 g	Potato Dextrose Broth	3.9g
Glycerol	1.0 mL	Glycerol	1.2 ml
Yeast extract	0.8 g	Peptone	0.6 g
Polypeptone	0.4 g	$NaNO_3$	0.8g
K_2HPO_4	0.001g	$MgSO_47H_2O$	0.001g
$MgSO_4.7H_2O$	0.001g	K_2HPO_4	0.001g
DW	100 mL	DW	100 ml
pH	6.5	pH	6.5

(After autoclaving chloramphenicol was added to the medium.)

Result

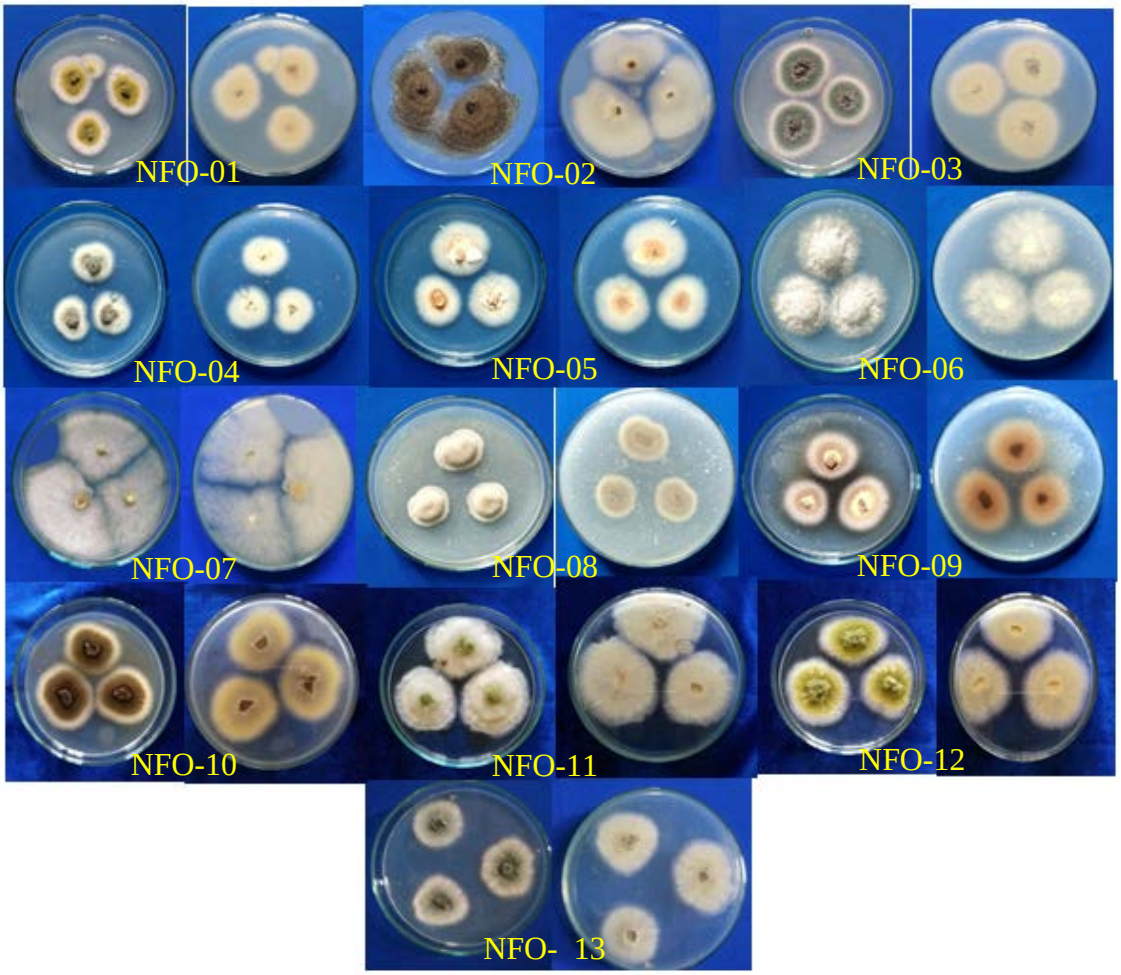

Figure 3 Morphology of fungus NFO-01 to NFO-13 (7 days old culture on PGA medium)

Table 1 Morphology of Isolated fungi NFO-1 to NFO-13

Sr No.	Isolated Fungi	Front view colour	Reverse view colour	Spore colour
1.	NFO-1	Greenish yellow	Pale yellow	Greenish yellow
2.	NFO-2	Black	Gray	Black
3.	NFO-3	Green	Pale yellow	Green
4.	NFO-4	White	White	White
5.	NFO-5	Brown	Brown	Brown
6.	NFO-6	White	White	White
7.	NFO-7	White	White	White
8.	NFO-8	Gray	Gray	Gray
9.	NFO-9	Pale brown	Pale brown	Pale brown
10.	NFO-10	Black	Gray	Black
11.	NFO-11	White	White	White
12.	NFO-12	Greenish yellow	Pale yellow	Greenish yellow
13.	NFO-13	Black	Gray	Black

Isolated Fungi and their Antibacterial Activity

In this study, eight fungi strains were tested with *Pseudomona fluorescens* by agar well diffusion method. NFO-9 gave the best activity on *Pseudomonas fluorescens.*

Table 2 Antibacterial activity of ten days fermentation of NFO-9 on *Pseudomonas fluorescens*

Fermentation period	Clear zone (mm)
1-3 days	No activity
4 day	18.21
5 day	20.94
6 day	24.31
7day	29.57
8 day	25.17
9-10 days	No activity

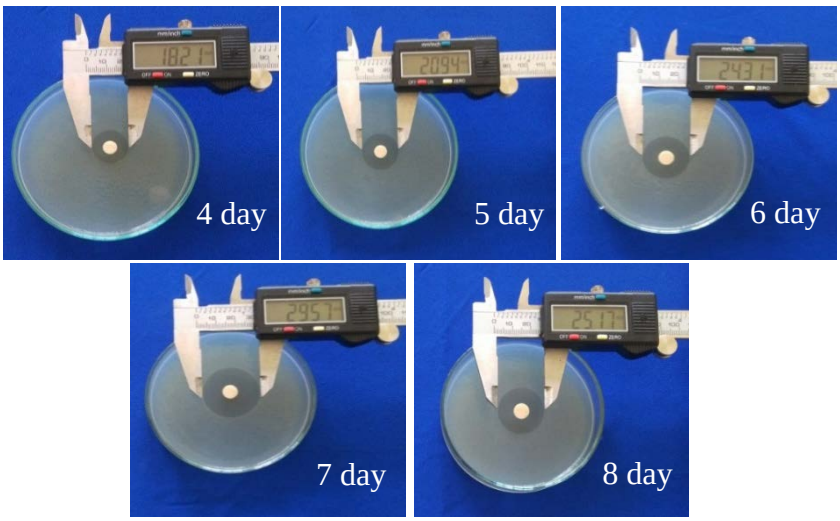

Figure 4 Antibacterial activity of ten days fermentation of NFO-9 on ***Pseudomonas fluorescens*.**

The effect of ages of inoculum on the fermentation

In the effect of age of inoculum, NFO-9 was investigated by using 42, 48, 54, 60, 66 and 72 hrs old age of inoculums. The result showed that 66hrs age of inoculum gave the highest activity (29.35mm).

Table 3 The Effects of Ages of inoculums on the Fermentation for NFO-9

Hour (hrs)	Size (mm)
2	12.55
48	6.33
54	17.90
60	26.07
66	29.35
72	26.47

The effects of sizes of inoculums on the fermentation for NFO-9

In this research work, the effect of size of inoculum was studied by using 5%, 10%, 15%, 20%, 25% and 30% inoculum. Using 25% inoculums showed significantly the highest activity (35.20 mm).

Table 4 The Effects of Size of inoculums on the Fermentation for NFO-9

Sizes %	Size (mm)
5%	24.93
10%	20.40
15%	30.84
20%	34.45
25%	**35.20**
30%	33.81

The effect of pH on the fermentation conditions of NFO-9

In this study, the highest antibacterial activity was obtained at pH-6 (37.62mm) against *Pseudomonas fluorescens*

Table 5 The Effects of pH of inoculums on the Fermentation for NFO-9

Ser.No.	pH	Antibacterial Activity (mm)
1.	4.0	24.83
2.	5.0	28.36
3.	**6.0**	**37.62**
4.	7.0	27.92

Antibacterial activity of NFO-9 on Fermentation media

In the fermentation medium (FM), the best antifungal activity was obtained by using sucrose and peptone in FM-2 (36.47 mm) followed by 27.51 mm, FM-1 (Glycerol and peptone).

Table 6 Antibacterial activity of NFO-9 on various fermentation medium

Fermentation media	Inhibitory zone (mm)
FM-1	27.51
FM-2	36.47
FM-3	23.65
FM-4	20.94

Discussion and Conclusion

Mangrove stands are located in sheltered places and in the mouths of valleys in most cases. The mangrove floor varies from sandy to muddy. Mangrove soils are of marine transported as sediment and deposited by rivers and the sea. In this isolation of marine fungi from mangrove soil samples were collected at Magyi, Shwe Thaung Yan Sub-Township, Ayeyarwaddy Region. Marine fungi were isolated by soil dilution method. In this study, 13 marine fungi were isolated. Three fungi were isolated from soil sample No.1, three from soil sample No. 2, two from soil sample No.3, two from soil sample No.4 and three from soil samples No.5. These soil fungi NFO- 06, 07, 08 and 11 have white color. NFO- 02 and 10 black colour.

Other fungi have different colour. Among them, the selected fungus NFO-9 showed potent antibacterial activity against *Pseudomonas fluorescens*.

Therefore, NFO-9 was selected for the study of the optimum fermentation condition. To study the optimization of inoculum age, the highest antibacterial activity was found at 66 hrs (29.35 mm). In the proper size of inoculum, 25% was the most suitable and the maximum activities of NFO-9 reached up (35.20 mm) followed by 20% and 30% respectively. The effect of pH was studied by varying from pH 4, 5, 6 and7. The best antibacterial activity was found at pH-6 (37.62 mm). The change in pH is also important for the enzyme activity of microorganisms, for the intermediate products, their dissociation and solubility (Rizk *et al*., 2007). Fermentation media (FM) were studied and FM-2 gave the highest activity (36.47 mm). The choice of a good fermentation medium is virtually as important to the success of an industrial fermentation as is the selection of an organism to carry out the fermentation (E1-Tayeb*et al*., 2004). Thus, the results of the optimum fermentation tests indicated that antimicrobial metabolites obtained from NFO-9 may be produced optimally in the presence of 7^{th} days fermentation period, 66 hrs age of inocuiums and 25% inoculums size,pH- 6 and FM-2. It was concluded that the present study revealed to observe the fermentation period of isoloated fungi and to investigated the optimization perameters of fermentation condition on NFO-9 against *Pseudomonas fluorescens*.

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