IDENTIFICATION OF COMMERCIALLY IMPORTANT GREEN MUSSEL FROM KYAUKPHYU, RAKHINE STATE USING MORPHOLOGICAL AND MOLECULAR APPROACH

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Abstract

The Asian green mussel is widely distributed along the Indo-pacific region, spanning from Japan to New Guinea and from Persian Gulf to South Pacific Islands. The objective of this research is to identify the green mussel species through a comprehensive approach utilizing both morphological and molecular analyses, specifically targeting the mitochondrial cytochrome "c" oxidase subunit I (COI) gene sequences. The relation between shell length (mm), wet tissue weight (g), dry tissue weight (g) and sex of the green mussels were measured. Regarding sexual differentiation, high numbers of male green mussels were abundant than female green mussels. Genomic DNA was extracted using the pet NAD Nucleic Acid Co-Prep Kit, resulting in a sequence length of 660 base pairs (bp). The phylogenetic analysis of the specimens involved a comparison of their sequences with others deposited in GenBank, revealing a close clustering with *Perna viridis* species, supported by a robust bootstrap value of 100%. This discovery holds significance for taxonomic identification and contributes to the advancement of mariculture development.

Keywords: morphological, phylogenetic, *Perna viridis*, green mussel

Introduction

The Asian green mussel (*Perna* sp.) is a type of marine bivalve mollusk under the family Mytilidae. The native habitat of the Asian green mussel is in the Indo-Pacific region, which encompasses regions between Japan to New Guinea and from Persian Gulf to South Pacific Islands (FAO, 2013). Asian green mussel Locally known as Kha-Yu-Nyo generally inhabits marine intertidal, subtidal and estuarine environments, which have high salinity and receive more nutrients from land run-off (Rajagopal *et al*., 1998). Asian green mussel is able to tolerate a wide range of salinities and temperatures (Sivalingam, 1977).

Green mussel locally known as Kha-yu-Nyo) was harvested commercially as human food in Myanmar due to their dense, fast-growing, and inexpensive source of marine protein. That makes green mussel meat as an important fishery commodity. The marine mussel genera *Perna* encompass both green and brown mussels, distinguished morphologically by variations in shell color and shape. The taxonomic classification of *Perna* recognizes three species: the green mussel (*Perna viridis*, Linnaeus 1758), the brown mussel (*Perna perna*, Linnaeus 1758), and the green-lipped mussel (*Perna canaliculus*, Gmelin 1791) (Siddall, 1980; Vakily, 1989). Identifying bivalve species based on morphology proves challenging due to the extensive diversity in shell forms and sizes. Habitat-specific traits contribute to significant variations in shell shapes, complicating species identification, even within the same species (Comesana *et al*., 2001).

To address challenges posed by morphological characteristics, recent years have witnessed the adoption of molecular biological techniques. These methods utilize various genetic markers to enhance accuracy in species identification. In this study, the suitability of mitochondrial DNA sequencing was assessed as a fundamental requirement to advance research on the green mussel. The study aimed to explore the morphology, DNA sequencing identification and the phylogeny of the green mussel species *Perna* in Kyaukphyu, Rakhine State, Myanmar.

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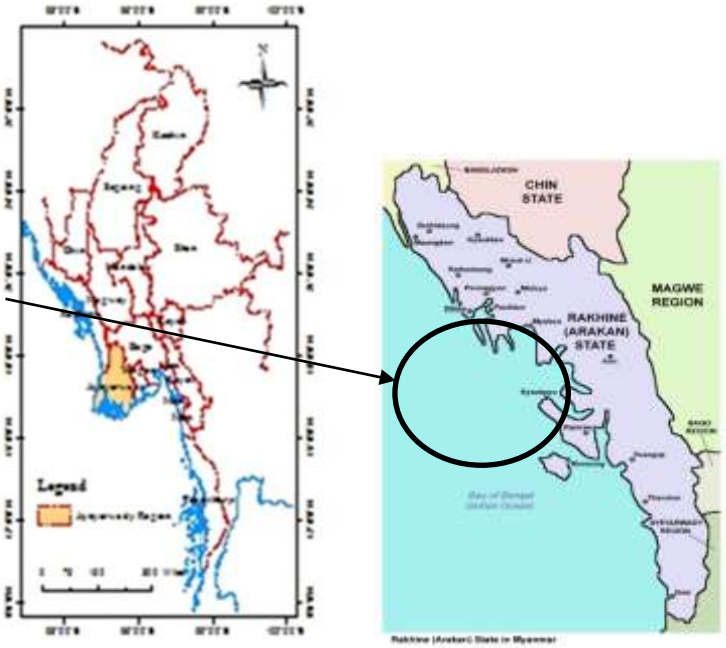
Materials and Methods

Sampling site and the study period

Green mussel samples were collected from the subtidal zone in Kyaukpyu Township, Rakhine State, Myanmar. The study site is positioned on the northwestern corner of Yanbye Island, bordering Combermere Bay, with coordinates at Latitude 19° 12' 58" North and Longitude 93° 43' 56" East (Fig. 1). The research was conducted from October 2022 to September 2023.



Myanmar Rakhine State, Kyaukphyu Township

Figure 1 Map showing location of Kyaukpyu Township of Rakhine State, Myanmar (www.googlemap.com)

Sample collection

The samples were collected during the low tide period, as it is the optimal time for harvesting green mussels inhabiting the subtidal zone (Plate 1). Subsequently, all collected samples were promptly transported to the Laboratory of Aquatic Bioscience at the University of Yangon following harvest. The gill tissue of ten specimens was kept at room temperature for DNA extraction.

(A) (B)

Plate 1 (A) *Perna* spp. (B) Internal organs of the studied green mussel

Morphological identification

Throughout the study period, a total of 50 green mussels were collected. In the laboratory, each mussel was measured its shell length using a vernier caliper, recorded to the nearest

millimeter. Subsequently, the mussels were opened, and the wet tissue weight was determined after removing excess water with absorbent tissue paper. Gender identification was conducted under a light microscope. The mussels were further characterized by examining morphometric features such as shell color, pallial line, posterior adductor muscle, posterior pedal retractor muscle, and shell length based on the criteria outlined by Rajagopal *et al*. (1998).

DNA extraction of the tissue samples for molecular identification

A total of ten green mussels were utilized for DNA extraction and subsequent gene sequencing. Prior to DNA extraction, the mussels were preserved in 70% ethanol, as illustrated in Plate (2A). The Pet NAD Nucleic Acids Co-Prep Kit was employed for the extraction of DNA from the gill tissue of the mussels, as depicted in Plate (2B). The DNA extraction procedure followed the guidelines provided by the Pet NAD Nucleic Acids Co-Prep Kit.

(A) (B)

Plate 2 (A) Fixation of *Perna* spp. (B) Pet NAD Nucleic Acid Co-Prep Kit

Polymerase Chain Reaction (PCR) amplification

To amplify the mitochondrial cytochrome gene, we designed universal primers targeting the bivalve COI region, namely 28S - LCO 1490 Forward (5'-ggtcaacaaatcataaagatattgg-3') and 28S - HCO 2198 Reverse (5'-taaacttcagggtgaccaaaaaatca-3') (Table 1). The PCR reaction consisted of mixing 5 µL of extracted DNA with 75.5 µL of water, 3.0 µL of each primer (25 µM), 0.5 µL of HS Taq DNA polymerase, 10 µL of 10× PCR buffer, and 8.0 µL of dNTPs (WizPure, Seongnam, South Korea). The PCR conditions involved 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, with a final extension at 72° C for 7 min. Subsequently, PCR products (around 700 bp) were separated and visualized via electrophoresis on a 1.5% agarose gel containing SYBR Safe DNA gel stain (WizPure, Seongnam, South Korea).

For DNA sequencing, DNA was then extracted and purified from distinct bands using the FastGene® Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Bunkyo, Tokyo). Briefly, to isolate DNA, first excised the fragment from an agarose gel using a clean scalpel. It was transferred up to 300 mg of the gel slice into a microcentrifuge tube, added 500 µl of binding buffer GP1, vortexed, and incubated at 55°C for 10-15 minutes, inverted the tube every 2-3 minutes. Next, it was applied up to 800 µl of the sample mixture from previous step into the FastGene® GP Column and centrifuged at 13,000 rpm for 30 seconds. Then, it was added 600 µl Wash Buffer GP2, and centrifuged at 13,000 rpm for 30 seconds. Wastes from the collection tube was discarded the flow-through, returned the column to the tube, and centrifuged again for 2 minutes to dry the column. Finally, 20-50 µl of elution buffer GP3 was added to the column and centrifuged at 13,000 rpm for 2 minutes to elute the purified DNA.

Table 1. Primer details for the PCR detection of the studied green mussel (Meyer, 2003)

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

Tm = melting temperature ; LCO = (Universal Primer (forward),
HCO = (Universal Primer (backward)

DNA Data Sequence Analysis and constructing of phylogenetic tree

Purified DNA products were quantified and directly used for DNA sequencing. Sequencing reactions were performed using the DNA Engine Tetrad 2 Peltier Thermal cycler (BIO-RAD) and the ABI BigDye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequences are then aligned, edited and deleted whether there are stop codons or not by using software MEGA 11 (Tamura *et al*., 2013). The mussel's gene sequence was compared with that of other bivalve species using the BLAST search available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nih.gov).

Molecular identification was conducted using mitochondrial DNA sequences within the cytochrome "c" oxidase subunit I (COI) region. The obtained sequence was then compared to mitochondrial COI data sequences from the GenBank database, specifically those associated with the Mytilidae family. The analysis confirmed the species belonging to the genus *Perna*, including *Perna perna*, *Perna viridis*, and *Perna canaliculus*. Additionally, out-group species, *Ruditapes philippinarum*, was included in the study according to Wood *et al*. (2007).

A phylogenetic analysis of the mitochondrial cytochrome c oxidase subunit 1 gene (mtDNA COI) was performed by bootstraps 1000 replicates using the Kimura-2-parameter model to create a Neighbour-Joining tree (Kimura, 1980). The genetic result of the *P. viridis* species is compared with various species of *Perna* with reference Gen Bank samples.

Results

Sex ratio, shell length and soft tissue weight of *Perna* sp. in a natural population

The soft tissue weight of male was 12.6 ± 51 g while that of female was 15.2 ± 1.78 g. The mean length of male and female were 5.6 ± 0.9 cm and 5.8 ± 1.7 cm, respectively. The soft tissue weight and the length were not significantly different in male and female green mussels (t test, $P<0.05$).

The number of females and males *Perna* sp. collected from Kyaukphyu was shown in (Table 3). Samples contained a relatively larger number of male mussels than females throughout the study period. Significant differences in the sex ratio between male and female were not found in this study.

Table 3 The sex ratio, mean shell length, soft tissue weight of studied green mussels

Gender	Mean Length	Mean Soft Weight	Number	Sex Ratio
Male	5.6 ± 0.9 cm	12.6 ± 1.51 g	32	0.56:1
Female	5.8 ± 1.7 cm	15.2 ± 1.78 g	18	

Morphological description

The mussel had two thick, smooth elongated shells with a curved shape and posterior adductor scars extended beyond the pallial line, leaving a muscle scar in a wavier or S-shaped mark. Also, it was observed a smooth mantle edges, visible concentric growth rings, the beak

curved down (i.e., where the two valves hinge together) and a pair of hinge teeth on the left valve that interlock with a single hinge tooth on the right valve.

Posterior pedal retractor muscle and PRM pesterion 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 labial palp was unridged and CTE- ctenidium was large. Inner labial palp was ridged where foot was elongated. The body of the mussel was surrounded by a mantle. Rectum and style sac were present in this mussel. Stomach was small. According to morphological characters, the mussel was tentatively identified as belonging to the species *Perna perna* or *Perna viridis* or *Perna canaliculus.*

DNA sequencing and constructing of phylogenetic tree

The PCR analysis performed on the green mussel specimen successfully amplifies a mitochondrial DNA of the COI region approximately 700 base pairs (bp) (Figure 3). Figure 4 illustrates DNA sequences of a 660 bp fragment were obtained from the green mussel. The results of the BLAST analysis revealed that our sample is 100.00% similar to *Perna viridis* with the accession numbers KP892919, MN119648 and KY081305 (Table 2). These accession numbers with sequence submissions originated in China and India. The findings revealed that the collected sample is a *Perna viridis* species that clustered together with members of the Mytilidae family. Moreover, 99.85 % identities with the species *Perna viridis* (MH664002) from (India) and 99.70 % to *Perna viridis* (DQ343576) from (China).

Identities with the species *Perna viridis* are much lower than to 89.74 % to *Perna picta* (DQ917602), 88.17 % to *Perna canaliculus* (MG766134), 87.58 % to *Perna perna* (NC026288) and 86.77 % to *Mytilus californianus* (KF931643) respectively. The phylogenetic tree was constructed with the 9 similar sequences obtained from the GenBank database; the accession numbers are shown in Fig (5). The graphic blast visualization is shown in Figure 5. The evolutionary divergence was minimal (3.8%) among the sequences of India (Accession numbers: MH664002, JF520794 and MN119648) and Pakistan whereas the considerable (4.4%) divergence was denoted (Figure 5) between the sequence from Pakistan and Australia (Accession number DQ343576).

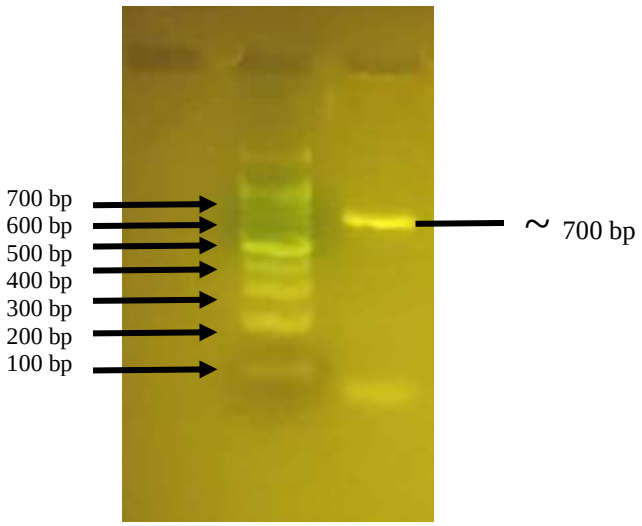


Figure 3 PCR amplification of mitochondrial DNA of the COI region of green mussels after examination with agarose gel exhibits a fragment of around 700 bp

AGCTGGGTTAATGGGGAGAAGGCTTAGGTTAATTATTCGAATTCAGCTTT……50
CTCATCCTGGGGGTAATTTTTTGAAAAATGAAAGGTTATATAATGTTGTA……100
GTAACAACTCATGCATTAGTAATAATTTTTTTTGCTGTAATGCCTTTACT……150

TATTGGTGCTTTTGGGAATTGATTACTTCCATTATGTATTGGTGGTGTTG……200
ATTTAATTTTTCCTCGTTTAAATAATTTGAGATTTTGGTTGGCACCTAAT……250
GCTTTGTACTTACTTATTTTGTCTTTTATAACGGAGAAAGGAGCTGGGAC……300
AGGTTGAACTATTTATCCACCTTTATCTTCTGGGTTGTACCATACTGGGC……350
CTGCTGTTGATATTTTGATTACGTCTTTACATTTAATTGGATTGAGTTCT……400
TTATTAGGTTCGATTAATTTTGTGAGGACTAATAAGAATATACCTACAAT……450
AAAAATAAAGGGTGAGAAATCTGAGTTGTATTTGTGGAGGATTACTGTAA……500
CCGGTGTTCTTTTAATCATTTCTGTGCCAGTTCTGGCCGGTGGGATTACT……550
ATATTGTTGTTTGATCGAAATTTCAATACTAGGTTTTTTGATCCTATTGG……600
AGGGGGAGATCCTGTTTTATTTCAGCATGTATTTTGATTTTTTGGTCACC……650
TGGAAGGTTT……660

Figure 4 Partial sequences of mitochondrial cytochrome gene of the studied mussel

Table 2 Mitochondrial cytochrome gene partial sequence identities of the studied mussel to other species in the Gene Bank

Species	Gene Bank Codes	%
Perna viridis (Linnaeus, 1758)	KP892919	100.00 %
Perna viridis (Linnaeus, 1758)	MN119648	100.00 %
Perna viridis (Linnaeus, 1758)	KY081305	100.00 %
Perna viridis (Linnaeus, 1758)	MH664002	99.85 %
Perna viridis (Linnaeus, 1758)	DQ343576	99.70 %
Perna picta (Born, 1778)	DQ917602	89.74 %
Perna canaliculus (Gmelin, 1791)	MG766134	88.17 %
Perna perna (Linnaeus, 1758)	NC026288	87.58 %
Mytilus californianus (Conrad 1837)	KF931643	86.77 %

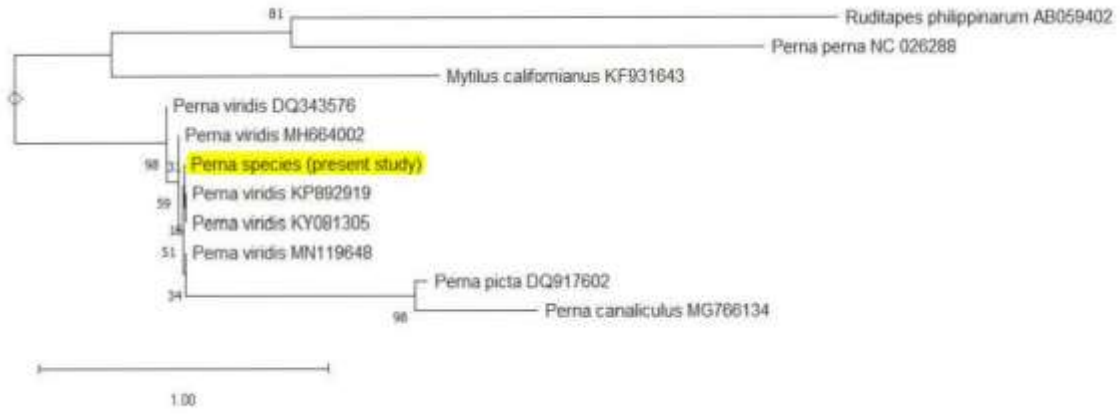


Figure 5 Phylogenetic trees are reconstructed using the Mitochondrial DNA of the COI region. *Ruditapes philippinarum* is used as out group on phylogenetic analysis

Discussion

In the present study, morphological identification along with molecular identification using the mitochondrial cytochrome gene sequencing for the identification of green mussel was conducted. Based on morphological inspection, the specimen closely resembles the Asian green mussel (*Perna* sp.) as described by Rajagopal *et al*. (2006).

Morphological characteristics, primarily focusing on shell color and shape, were examined, as outlined by Siddall (1980). *Perna* species exhibits an external shell color that is whitish under a bright periostracum, transitioning from dark brownish green anteriorly to olive-green to bright green posteriorly (Poutiers, 1998). These mussels typically feature two hinged shells connected by a posterior adductor muscle, and a robust ligament binds the two valves

together at the hinge, resulting in an equivalve shell (equally convex) with a byssal gape (Pouters, 1998). According to Siddall (1980), early larvae stages attach through proteinaceous byssal threads.

To confirm the species, DNA analysis was conducted. It was revealed that sequence identities to studied mussel species is *Perna viridis* with 100% similar to several Genebank samples (KP892919) under accession numbers MN119648 and KY081305. These sequence submissions originated in Kerala (India), Kadiapatinam (India), Zhejiang (China) and Kerala (India). The findings revealed that it is a *Perna viridis* species that clustered together with members of the Mytilidae family. The findings revealed that it is a *Perna viridis* species that clustered together with members of the Mytilidae family. Identities with the species *Perna viridis* are much lower than to 89.74 % to *Perna picta* (DQ917602), 88.17 % to *Perna canaliculus* (MG766134) and 87.58 % to *Perna perna* (NC026288). Therefore, it is highly possible that the studied mussel could not belong to species *Perna perna*. The green mussel is therefore identity as *Perna viridis*. (Linnaeus, 1758).

Bivalves commonly display an approximate balance in the distribution of males and females within populations (Gosling, 2015). Noor *et al*., (2019) conducted a study on green mussels from Pasaran Island, Indonesia, and reported morphological characteristics. At six months of age, male and female mussels exhibited shell lengths of 55.7 mm and 57.3 mm, respectively, with corresponding weights of 10.38 g for males and 9.82 g for females. In the present investigation, the mean length of males and females was 5.6 and 5.8 cm, respectively, and their soft tissue weights were 12.6 and 15.2 g, respectively. Interestingly, there were no significant differences in length and weight between *Perna viridis* populations in Myanmar and Indonesia.

Gender formation in various bivalve mollusk species is influenced by both genetic and environmental factors (Kenchington *et al*., 2002; Lee, 2015). Contradictory genetic mechanisms explanations have been proposed (Zouros *et al*., 1992; Kenchington *et al*., 2002; Yusa *et al*., 2013). Environmental factors affecting mollusk sex ratios include unfavorable habitat conditions, anthropogenic impact, food availability, temperature, and salinity (Yusa, 2007; Stenyakina *et al*., 2010; Shurova, 2013; Chelyadina, 2014). Pollutant influence may lead to masculinization in mollusk populations due to the suppression of specific genes (Ivanov, 1989). In the present study, bivalve species were collected from natural populations experiencing temperature stress and potentially reduced food availability (due to short periods of inundation during high tide). These environmental stressors may account for the observed higher male ratio in the bivalve species.

Conclusion

The identification of green mussel species in Kyaukphyu, Rakhine State, was carried out through both morphological and molecular methods. The analysis revealed that the specimens examined belonged to the species *Perna viridis*, as indicated by the sequence KY081305. The data presented here in offers valuable insights to the Department of Fisheries (DoF) regarding the taxonomy status of the species. This information is likely to be instrumental in the effective management, conservation, and sustainable utilization of green mussel resources in the future.

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