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

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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.

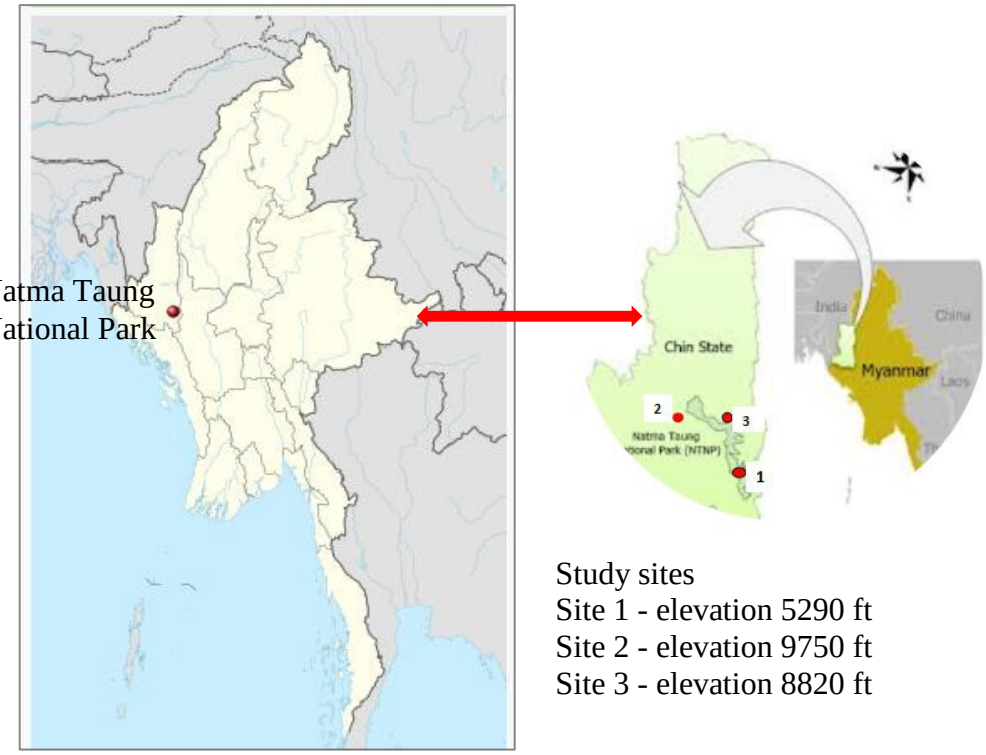


Figure 1 Map of Natma Taung National Park

Site 1 Near human habitation (Elevation 5290 ft)

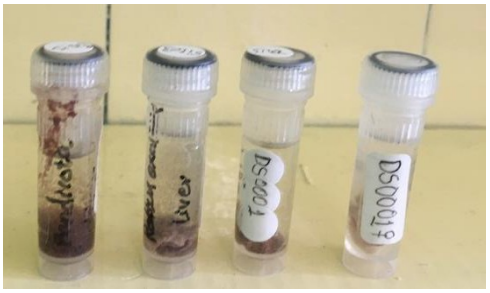
Site 2 Forest (Elevation 9750 ft) **Site 3** Near forest camp (Elevation 8820 ft)

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)

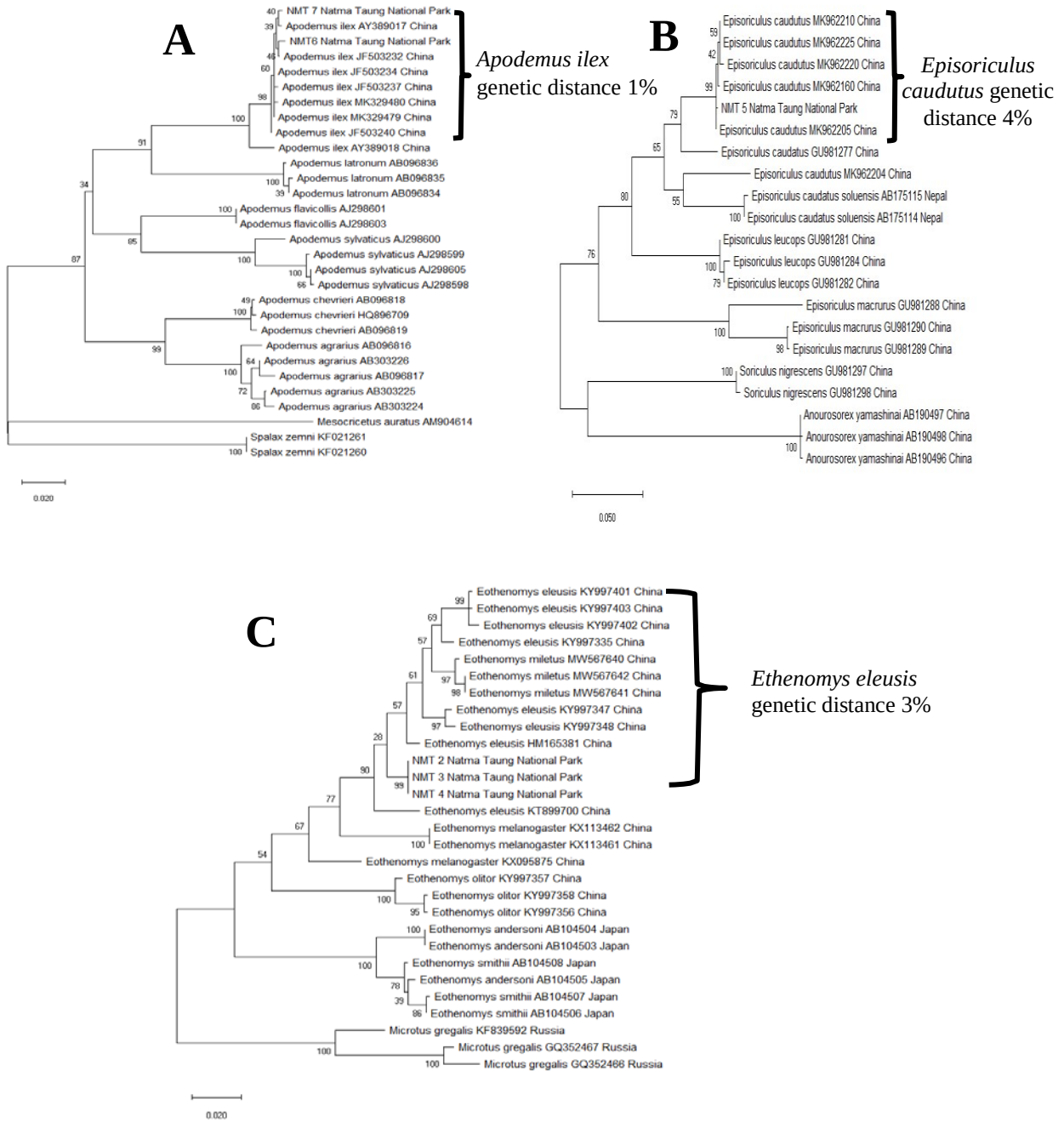


Figure 2 Maximum Likelihood (ML) tree constructed using mtDNA *Cytb* data of the (A) *Episoriculus caudutus* (B) *Ethenomys eleusis*, and (C) *Apodemus ilex* from Natma Taung National Park of Myanmar. Bootstrap values, expressed as a percentage of 1000 replications, are given at each node. The scale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