GENETIC CHARACTERIZATION OF FISHING CAT, *PRIONAILURUS VIVERRINUS* (BENNETT, 1833) BASED ON MITOCHONDRIAL CYTOCHROME B GENE AT YANGON ZOOLOGICAL GARDEN, MYANMAR

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Abstract

The fishing cat (*Prionailurus viverrinus*), categorized as a vulnerable species by the International Union for Conservation of Nature (IUCN), is increasingly threatened across its range. The fishing cat (*Prionailurus viverrinus*) is currently listed as "Vulnerable" on the IUCN Red List, with a population decline of approximately 30% over the past three generations (2010–2015) due to habitat loss and human-wildlife conflict. This study aimed to evaluate the genetic diversity of *P. viverrinus* individuals in Myanmar, particularly those housed at Yangon Zoological Garden, and to assess their genetic status in comparison with reported populations from other countries. In December 2023, fecal samples from three captive fishing cats (N=3) were collected for genetic analysis. DNA was extracted and PCR (Polymerase Chain Reaction) was used to amplify the mitochondrial cytochrome b (cyt b) gene from fecal DNA samples of fishing cats. Of the three samples, three were successfully amplified and subsequently sequenced using Big Dye terminator sequencing. Results from the NCBI database indicated that the Myanmar samples showed a 99% genetic similarity to fishing cat populations from India. Phylogenetic analysis revealed that the two Myanmar cats comprised a distinct cluster with identical haplotypes, suggesting minimal genetic divergence within this population. Comparative analyses also showed the closest genetic distances to fishing cats from Thailand and Indochina and sundaland. This study underscores the genetic relatedness of fishing cat population from Yangon Zoological Garden with other regional populations, providing valuable insights into the species' distribution and aiding conservation efforts across their range.

Keywords: Fishing cat, *Prionailurus viverrinus*, Cytochrome b, Genetic characterization, Phylogenetic analysis.

Introduction

The Fishing cat (*Prionailurus viverrinus*) is a type of wild cat under the family Felidae. Among wild cats, the family Felidae is reported to include the genus *Prionailurus* consisting of the two subspecies of *Prionailurus viverrinus* (Bennett, 1833) and *Prionailurus rhizophoreus*. The species is primarily distributed across South and Southeast Asia, inhabiting coastal mangroves, swamps, marshlands, and riverine ecosystems in countries such as India, Nepal, Sri Lanka, Bangladesh, Myanmar, and Thailand, with possible populations in Cambodia, Malaysia, and Indonesia. In Myanmar, it is recorded in the Ayeyarwady Region Meinmahla Kyun Wildlife Sanctuary; and possibly distributed in Indawgyi Wetland Wildlife Sanctuary. It is known by the local name of kyaung ta-nga (Lin and platt,2019).

The fishing cat (*Prionailurus viverrinus*), a medium-sized wild cat adapted to wetland environments, is categorized as "Vulnerable" on the IUCN Red List due to substantial habitat loss and fragmentation (Mukherjee et al., 2016).The fishing cats faced with all threats, hunting for the illegal trade has the greatest potential to do the maximum harm in minimal time (Nowell

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and Jackson, 1996) although this is likely true for some species more than others and the situation in Myanmar needs further study. It is listed as endangered in the National Red Lists of Bangladesh and Nepal (WWF, 2020).

Most species of wild cats are threatened due to a combination of habitat loss, conflict with humans, poaching and illegal wildlife trade (MacDonald et al. 2010). Wild feline species are important predators with various body types, diverse food habits, and great adaptability to the surrounding environment. These species play key roles in the natural ecosystem. Most feline species are secretive and highly vigilant, which makes them a very difficult target for field research and conservation. With the rapid development of non-invasive sampling among wild animals, fecal samples are becoming informative objects in research on wild cats.

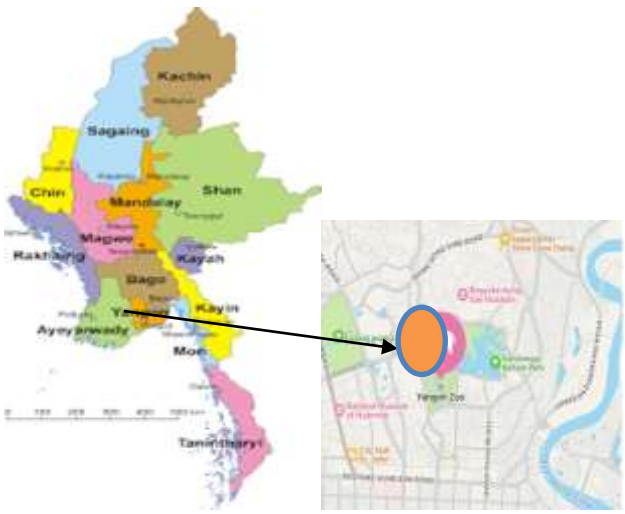
The cyt b gene is used to assess genetic diversity, species identification, evolutionary relationships, and phylogenetic studies in felid species, including fishing cats (Mukherjee et al.2016). It is frequently used in phylogenetic studies due to its stable mutation rate, making it useful for analyzing species relationships (Irwin et al.1991). It is located in mitochondrial DNA, which is maternally inherited and plays a crucial role in cellular respiration. 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 fishing cat.

The study aimed to explore DNA sequencing identification and the phylogeny of the fishing cats from Yangon Zoological Garden, Myanmar. The objective was to examine molecular techniques for precise species identification, contributing to a deeper understanding of the fishing cat population in the specified region.

Materials and Methods

Sampling site and the study period

The three fishing cats sampled in this study were captive-born at the Yangon Zoological Garden, located in Mingalar Taung Nyunt Township, Myanmar. The study site is situated north of downtown Yangon, near Kandawgyi Lake, at coordinates 16° 47′ 30″ N, 96° 09′ 34″ E (Fig. 1). The research period spanned from December 2023 to September 2024.



Myanmar Yangon Zoological Garden

Figure 1 Map showing location of Yangon Zoological Garden, Myanmar(www.googlemap.com)

Sample collection

To adhere the research ethics by avoiding invasive tissue sampling, a more sustainable method of DNA extraction was used for *P. viverrinus*. Therefore, non-invasive fecal samples were collected instead of tissue or blood samples. A total of three fishing cats from the study site were assessed (Plate 1A). Fresh fecal samples were obtained from one male and two female fishing cats at the Yangon Zoological Garden with the assistance of cat keepers. Collection took place early in the morning, prior to the enclosure cleaning, ensuring fresh samples. Each individual and its fecal sample were assigned a code—'FC' for Yangon Zoological Garden—followed by a serial number (FC1, FC2, and FC3).

(a) FC1 (female) (b) FC2 (female) (c) FC3 (male)

Plate 1 Three fishing cats (*Prionailurus viverrinus*) from study site

DNA extraction of the fecal samples for molecular identification

A total of three fishing cats were utilized for DNA extraction and subsequent gene sequencing. The feces were preserved in the sterile plastic container containing 75% ethanol, as illustrated in Plate (2A) and kept in an icebox for transport to the Molecular Biology Laboratory at the Department of Zoology. Prior to DNA extraction, samples were stored at 4°C. The Silica Extraction Kit was employed for the extraction of DNA from the feces of fishing cat, as depicted in Plate (2B). The DNA extraction procedure followed the guidelines provided by the Silica Extraction Kit. Observations and analysis were conducted in the Molecular Laboratory at the University of Yangon.

(A) (B)

Plate 2 (A) Preservation of the fecal samples in the plastic container containing 75% ethanol
(B) Silica Extraction Kit

PCR amplification

To amplify the mitochondrial cytochrome gene, we designed universal primers targeting the cyt b region (Table 1). The PCR reaction consisted of 17.5 µL of extracted DNA template, 7.5 µL of each primer, 25 µL of Gold Taq DNA polymerase.

The PCR conditions involved an initial activation step at 95°C for 15 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 40 seconds, extension at 72°C for 40 seconds, and a final extension at 72°C for 15 minutes (Rana, 2021). Subsequently, PCR products (around 559bp) were separated and visualized via electrophoresis on a 1% agarose gel stained with Gel green stain.

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 excise the fragment from an agarose gel using a clean scalpel. Transfer up to 300 mg of the gel slice into a microcentrifuge tube, add 500 µl of binding buffer GP1, vortex, and incubate at 55°C for 10-15 minutes, inverting the tube every 2-3 minutes. Next, apply up to 800 µl of the sample mixture from previous step into the FastGene® GP Column and centrifuge at 13,000 rpm for 30 seconds. Then, add 600 µl Wash Buffer GP2, and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through, return the column to the tube, and centrifuge again for 2 minutes to dry the column. Finally, add 20-50 µl elution buffer GP3 to the column, let it stand for 2 minutes, and centrifuge at 13,000 rpm for 2 minutes to elute the purified DNA.

Table 1. Primer details for the PCR detection of the fishing cat

Primers	Direction	Sequences	Tm
PrionCytb 2F	Forward	(5'-GGCTGAATCATCCGATACATAC-3')	52°C
PrionCytb 2R	Reverse	(5'-GAGGATTAGCGGGGATGTAGTTA-3')	52°C

Tm = melting temperature

Analysis of DNA Sequence and constructing of phylogenetic tree

Purified DNA products were quantified and directly used for DNA sequencing with an ABI gene sequencer employing the PCR primers. The sequences are then aligned, edited and deleted whether there are stop codons or not that by using MEGA 11 software (Tamura *et al*., 2013). The fishing cat's gene sequence was compared with that of other fishing cat 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 b (cyt b) region. The obtained sequence was then compared to mitochondrial cyt b sequences from the GenBank database, specifically those associated with the Felidae family. The analysis confirmed the species from Yangon Zoologial Garden belonging to the genus *Prionailurus,* including *Prionailurus viverrinus*, *Prionailurus bengalensis.*

A phylogenetic analysis of the mitochondrial cytochrome b gene (mtDNA cyt b) 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. viverrinus* species is compared with various species of *Prionailurus* with reference GenBank samples.

Results

Morphological description

The fishing cat has a broad, rounded head with relatively small, rounded ears set low on the head. The eyes are large, forward-facing, and typically greenish-yellow. Its coat is gray-brown, adorned with distinct black spots arranged in longitudinal rows along the body. It has short legs with strong muscular limbs. The toes are partially webbed, an adaptation that aids in swimming and hunting in water. Its tail is short and thick, less than half the body length. It has powerful jaws and sharp canines suited to grasp slippery prey like fish. Its carnassial teeth are well developed. According to morphological characters, the fishing cat was tentatively identified as belonging to the species *Prionailurus viverrinus.*

Concentration of DNA extraction from collected fecal samples

Total genomic DNA concentration ranged from 54.2 ng/µl of FC1,53.2 ng/µL of FC2 and 8.3 ng/µl of FC3 from Yangon Zoological Garden. The purity (260/280 nm) were from 0.03-0.09 nm (Table 2).

Table 2. Concentration and purity of extracted genomic DNA from fecal samples

Sr.No	Sample code	Nucleic acid conc-ng/µL	A260/280nm	A260/230nm
1.	FC1	54.2	1.9	0.09
2.	FC2	52.3	1.82	0.03
3.	FC3	8.3	1.9	0.05

DNA sequencing and constructing of phylogenetic tree

The PCR analysis performed on the fishing cat specimen successfully amplifies a mitochondrial DNA of the cyt b region about 559 base pairs (bp) (Figure 2). Figure 4 illustrates DNA sequences of a 1140 bp fragment were obtained from the green mussel. Blast results revealed that our sample is 99% similar to *Prionailurus viverrinus* with the accession numbers KF754958.1 and KF754956.1 (Table 3). These accession numbers with sequence submissions originated in India. The findings revealed that it is a *Prionailurus viverrinus* species that clustered together with members of the Felidae family. Moreover, 99.03 % identities with the species *Prionailurus viverrinus* (KF754958.1) from (India) and 99.03 % to *Prionailurus viverrinus* (KF754958.1) from (Indochina and sundaland).

Phylogenetic analysis was conducted using the Neighbor-Joining (NJ) method, based on the aligned sequences of 522 bp. Each cluster in the phylogenetic tree represented distinct fishing cat species derived from the analyzed fecal samples, aligned against reference sequences from GenBank. The phylogenetic tree was constructed with the 12 similar sequences obtained from the GenBank database; the accession numbers are shown in Fig (5). The graphic blast visualization is shown in Figure 5.

A notable clade in the NJ tree comprised a single cluster containing haplotypes from two fishing cats (FC1 PrionCytbF2 and FC2 PrionCytbF2) collected from the Yangon Zoological

Garden, which clustered closely with another haplotype (FC3 PrionCytbF2) from the same facility. The fishing cat populations from this study area, particularly samples FC1, FC2, and FC3, showed close genetic relationships with populations from India, Thailand, Southeast Asia, Indochina, and Sundaland. The phylogenetic tree illustrated strong support for these relationships, as indicated by high confidence values in the NJ analysis.

Notably, the haplotype from India (KF75496.1) clustered closely with the haplotype from Thailand (AB210239.1), and also related haplotypes from Indochina and Sundaland (KF754958.1). Furthermore, a haplotype from Indochina (OR126345.1) clustered with a haplotype from Southeast Asia (AB210238.1), reflecting connections between populations across these regions.

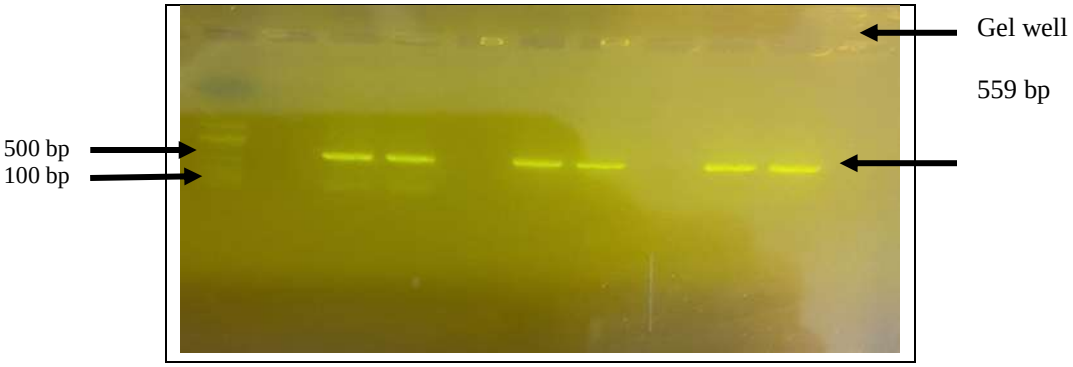


Figure 2 PCR amplification of mitochondrial DNA of the cyt b region of fishing cats after examination with agarose gel exhibits a fragment of 559 bp

GAGCGGGGACTCATATTCTTTATCTGTCTATACATGCACGTGGGCCGAGG……50
AATATATTAGGTTCCTACACCTTCTCAGAAACATGAAACATTGGAATCAT…… 100
AAAATGAAAGGTTATATAATGTTGTAGTAACAACTCATGCATTAGTAATA……150
ACTGCTATTCGCAACCATAGCCACAGCTTTCATAGGTTACGTACTACCATG……200
GGGCCAAATATCCTTCTGAGGGGCAACCGTAATCACCAACCTCCTATCAG… …… 250
CAATCCCATAATGGAACCAACTTAGTAGAATGGGTCTGAGGGGGTTTCTC…… 300
AGTAGACAAAGCCACCTAACACGATTCTTCGCTCTCCATTTCATTCTCCCA……350
TTCATTATTTCAGCCTTAGCAGCAGTACACCTCCTATTCCTCCATGAAACAG…… 400
GATCCAATAACCCCTCAGGAGTCACATCCGACTCGGACAAAATCCCATTCC…… 450
ACCCATATTATACAATCAAGGACATCCTGGCCTTCTAGTACTAATCTCAACAC…… 500
TTATACTACTTGTTCTATTCTCACCAGACCTGTTAGGAGACCCAGATAACTACA…… 550
TCCCGCTTAAATCCCCAAAAAAGCCTTCGATCCTTGCTTGGTTATTATTCCTGG…… 600
ATAAACTAGAGTCGAGCATTAAACTGCGCGTGCCACGACGTAGCATTATGGAC……650
TGACTGGATTGACCCCGCCATGTAATATGCACAAGTATTTCTCTCGAAAGAGTA…… … 700
ATGCGCCAGTCTAGTTATAACTTGCCCTCAATCAGTCTGTCACACTCAGCCGTC……750
AGAACTCTGCAGGCGATGGGGAATAACCATCCTGACAGTCAGTGTCCGGTAGC……800
TCATTAGTTAGGAGCAGATCGAAACGTCAGAATTGATTCCATCGGTAGCTCACAT……900
CACTCTTGTTCGTACGAGCACGGGATAGTGAGCCTGCATAGCTCGTCCACAAAG……950
TGGAGTCATTTCCGAGCTGGACTTATGCGGATTCGGAGCGGGACACGCTAAGA……1000
GGGAAACGTAGAGACTGAACGGGAACACACTTTTCGTACTGAGAACTGATACG……1050
TGACTTAACAAGCGCAGGGACGAAAATGGGGGCGCGTGGTACGCGATCTG……1100
ACACTCATGTCTGTAATAATGTCCGGAGATCAGGGTGATGTGTCATGCGT……1140

Figure 3 Partial sequences of mitochondrial cytochrome gene of the studied fishing cat

Table 3 Mitochondrial cytochrome gene partial sequence identities of the studied fishing cat to other species in the Gene Bank

Species	Locality	Percent Identity (%)	Assessor number in NCBI
Prionailurus viverrinus			
FC1-PrionCytb F2	Myanmar	99	This study
FC2_PrionCytb F2		98	
FC3_PrionCytb F2		98	
Prionailurus viverrinus	India	99.03	KF754956.1
Prionailurus viverrinus	Thailand	97.63	AB210239.1
Prionailurus viverrinus	Indochina & sundaland	99.03	KF754958.1
Prionailurus bengalensis	Indochina	94.94	OR126345.1
Prionailurus viverrinus	Southeast Asia	95.21	AB210238.1
Prionailurus bengalensis	Continental Far East	94.55	AB210226.1
Prionailurus viverrinus	Thailand	97.47	AB210240.1
Prionailurus bengalensis	Thailand	94.41	AB210234.1
Prionailurus bengalensis	Tsushima islands	94.55	AB194818.1
Panthera tigris	Bangladesh	100	JQ409474.1
Panthera tigris	Bangladesh	100	JQ409475.1

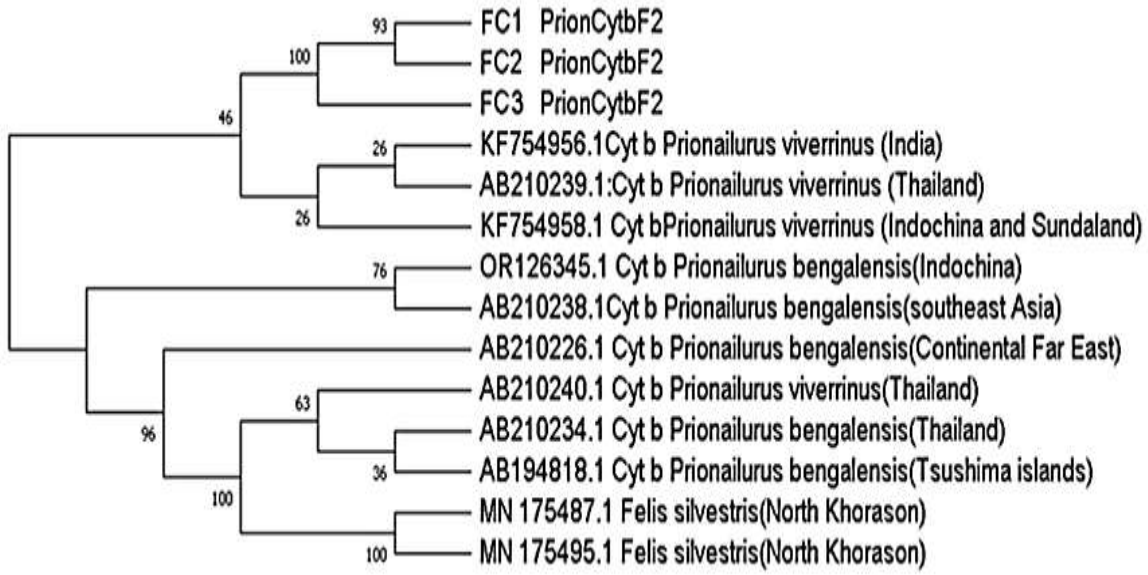


Figure 4 Phylogenetic trees are constructed using the Mitochondrial DNA of the cyt b region

Discussion

In the present study, morphological identification along with molecular identification using the mitochondrial cytochrome b gene sequencing for the identification of fishing cat was conducted. Based on morphological inspection, the specimen closely resembles the fishing cat (*Prionailurus viverrinus*) as described by Rajagopal *et al*. (2006).

The fishing cat (*Prionailurus viverrinus*) is a globally threatened small cat categorized as "Vulnerable" in the IUCN Red List (Mukherjee *et al*., 2016). However, very little is known about

the genetic status of different parts of the country. Therefore, the present research was conducted to assess the genetic status of fishing cats at Yangon Zoological Garden.

Partial cytochrome b (cyt b) gene of the mitochondrial DNA was used as a genetic marker sequence to analyze and compare the haplotypes from study sites. Genetic characterization is essential for understanding the genetic diversity of a species, which is crucial for conservation and management efforts. It can help identify species boundaries and distinguish between fishing cats and other felid species.

A significant finding was that the cyt b sequences from the study showed a high genetic similarity-ranging from 98% to 99%-to the fishing cat species, *Prionailurus viverrinus*. This high level of similarity indicates a close genetic relationship, although some evidence of admixture within the haplotypes of *P. viverrinus* was also observed.

Further examination of the phylogenetic relationships revealed that haplotypes from India (KF75496.1) clustered tightly with those from Thailand (AB210239.1), indicating a shared genetic background. Focusing on Clade I in the NJ tree, a distinct cluster composed of two specific haplotypes (FC1 and FC2) from the Yangon Zoological Garden were noted, which grouped with another haplotype (FC3) from the same location. Haplotypes FC1, FC2, and FC3 from Yangon Zoological Garden formed a distinct cluster (Clade I) in the Neighbor-Joining (NJ) tree. This cluster showed strong genetic similarity and bootstrap support, indicating that the fishing cat populations in Yangon Zoological Garden, Myanmar are closely related to those from India, Thailand, Southeast Asia, Indochina, and Sundaland. The high bootstrap support values associated with this cluster lend strong confidence to the evolutionary relationships proposed by the NJ analysis.

Overall, the results of the NJ phylogenetic analysis not only elucidate the genetic relationships among fishing cat populations but also provide insights into their potential evolutionary history and geographic distribution. These findings underscore the importance of conservation efforts for this species, particularly in managing gene flow and preserving the unique haplotype diversity present across its range.

High-quality samples as blood or tissue are ideal for generating genomic data. Non-invasive samples like hair, feces, and antlers though comparatively easy to obtain are generally poor in quality because of on-invasive samples like fecal samples often have an abundance of bacterial DNA apart from the DNA of the host and the prey species. Using the specific primers in PCR amplification allows for targeted amplification of particular DNA sequences, enhancing the specificity and sensitivity of the process.

In conclusion, the results of the Neighbor-Joining (NJ) phylogenetic analysis clarify the genetic relationships among fishing cat populations and offer valuable insights into their evolutionary history and geographic distribution. These findings emphasize the critical need for conservation efforts aimed at managing gene flow and preserving the unique haplotype diversity found across the species' range. Future research should consider expanding on these results by incorporating additional genetic markers or exploring the ecological factors that impact these populations.

Conclusion

The DNA extraction from fecal samples of fishing cats was successfully achieved, underscoring the effectiveness of non-invasive methods that minimize pain and stress for the animals involved. the genetic clustering analysis revealed a close genetic relationship among the samples from Yangon Zoological Garden, indicating a genetic connection to fishing cat populations in India, Thailand, Southeast Asia, Indochina, and Sundaland. These findings highlight the potential for genetic exchange among fishing cat populations and underscore the importance of conservation efforts to maintain their genetic diversity.

Acknowledgements

We would like to express our most profound thanks to Rector Dr. Tin Maung Tun, Pro-rector Dr. Khin Chit Chit, Pro-rector Dr. Cho Cho, and Pro-rector Dr. Thidar Aye from the University of Yangon, for permission to conduct this research, as well as to the Myanmar Academy of Arts and Sciences for their support in writing this paper. I would like to express many thanks to all of my professors, colleagues in the Molecular Laboratory of Zoology, University of Yangon.

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