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Mitochondrial COI gene-based phylogenetic and haplotype analysis of *Manouria emys* from Sumatra, Indonesia

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Abstract. *Ashrifurrahman, Syaifullah, Roesma DI, Tjong DH, Lestari I. 2025. Mitochondrial COI gene-based phylogenetic and haplotype analysis of* Manouria emys *from Sumatra, Indonesia. Biodiversitas 26: 6224-6231.* Genetic and phylogenetic analyses are essential for understanding the evolutionary relationships and genetic variation of the critically endangered *Manouria emys*, particularly in Indonesia, where molecular data remain limited. This study aimed to determine the phylogenetic placement and haplotype diversity of *M. emys* from Sumatra, Indonesia representing the first COI record from this region. A single sample collected from West Sumatra was analyzed through DNA extraction, PCR amplification of the COI (Cytochrome Oxidase Subunit I) gene, and sequencing. An 886 bp COI gene fragment was confirmed as *M. emys* through sequence similarity analysis and subsequently aligned with 29 global reference sequences for phylogenetic and haplotype analyses. Phylogenetic analysis using the Maximum Likelihood method showed that the Sumatran sample clustered within the *M. emys emys* clade, together with sequences from Borneo and the Taipei Zoo. Three subclades were observed within *M. emys*, corresponding to *M. emys emys*, *M. emys phayrei*, and one distinct genetic lineage of unconfirmed subspecies status. The analysis showed low genetic divergence within each subspecies but relatively high differentiation between *M. emys* and the outgroup. Haplotype analysis identified three main haplogroups, with the Sumatran sample showing close genetic affinity to *M. emys emys* from Borneo. The presence of multiple *M. emys emys* haplotypes reported from India may reflect population movement or human mediated translocation, highlighting the need for broader regional studies. This study provides the first molecular evidence of *M. emys* from Sumatra, offering a valuable genetic reference for future research, conservation management, and monitoring of wildlife trade involving this critically endangered tortoise.

Keywords: Brown tortoise, conservation genetics, DNA barcoding, haplotype diversity, Sumatra

INTRODUCTION

The Asian giant tortoise (*Manouria emys* (Schlegel & Müller, 1844)) is one of the largest and most evolutionarily primitive tortoise species in Southeast Asia, distributed across Bangladesh, India, Thailand, Myanmar, Malaysia, and Indonesia (Borneo and Sumatra), and is currently classified into two subspecies: *M. emys* subsp. *emys* (Schlegel & Müller, 1844) and *M. emys* subsp. *phayrei* (Blyth, 1854). Adults of *M. e. emys* are characterized by a dark brown carapace, while juveniles typically show lighter or pale brown coloration at the center of the scutes. This subspecies can reach a carapace length of up to 60 cm and a body weight of about 40 kg. In contrast, *M. e. phayrei* exhibits a uniformly black carapace in both juveniles and adults, with carapace lengths reaching up to 66 cm. A distinguishing characteristic is that the pectoral scutes of *M. e. phayrei* touch at the plastral midline, while those of *M. e. emys* are positioned far apart (Ernst and Barbour 1989; Iskandar 2000; Stanford et al. 2015; Kundu et al. 2018; Platt et al. 2018; Choudhury et al. 2019).

The conservation status of *M. emys* as critically endangered according to the International Union for Conservation of Nature (IUCN) is due to habitat degradation, low reproductive rates, and illegal trade (Choudhury et al. 2019). Furthermore, its inclusion in Appendix II of CITES highlights its vulnerability to international trade. In Indonesia, this species is legally protected under Indonesian national law (Permen LHK No. P.106/MENLHK/SETJEN/KUM.1/6/2018). Effective conservation efforts require accurate taxonomic and genetic information, making an understanding of the evolutionary relationships and genetic diversity among testudine populations crucial (Le et al. 2006; Kundu et al. 2022; Abedin et al. 2025).

Despite its importance, baseline genetic information for *M. emys* in Indonesia remains scarce, particularly in Sumatra. To date, there are no recorded COI gene sequences of *M. emys* from Indonesia in the National Center for Biotechnology Information (NCBI) genomic database. Similarly, the Barcode of Life Data (BOLD) Systems database contains only one sequence from Indonesia (Borneo) out of 33 available *M. emys* sequences. This lack of molecular data hampers effective monitoring and management, especially as individuals are sometimes found outside their expected ranges, likely due to anthropogenic factors such as wildlife trade or translocation (Kundu et al. 2018; Schultz et al. 2021). In such contexts, genetic tools are essential for subspecies identification, understanding population structure, and providing data for conservation enforcement (Hohenlohe et al. 2021).

DNA barcoding using the mitochondrial gene COI has become a powerful tool for species identification and phylogenetic studies (Kundu et al. 2013; Yang et al. 2018; Roesma et al. 2020; Ashrifurrahman et al. 2022; Mohd

Salleh et al. 2023). The COI gene is widely used due to its relatively high conservation level and its ability to distinguish closely related taxa (Hebert and Gregory 2005; Ward et al. 2005). Several studies have successfully used COI sequences to differentiate tortoise species and assess levels of genetic divergence and haplotype diversity across geographic regions, such as in some of tortoise species from northern region of India (Bhaskar and Mohindra 2018), southern river terrapin (Mohd Salleh et al. 2023) and big headed turtle (Gong et al. 2023), specially in *M. emys* (Kundu et al. 2013).

Phylogenetic analysis and haplotype diversity assessment of *M. emys*, particularly in Indonesia, are crucial not only for understanding genetic relationships but also for identifying the origin of collected samples. Recent studies on *M. emys* have reported cases that are difficult to explain, in which individuals were found outside their natural geographic range (Kundu et al. 2018). Such findings highlight the need for localized genetic studies in Indonesia, where the species distribution and subspecific composition remain poorly understood.

Therefore, this study aims to provide the first mitochondrial COI sequence of *M. emys* from Sumatra, determine its subspecies identity through phylogenetic placement, and haplotype variation from this location to enrich the existing global dataset for conservation purposes. Establishing this baseline genetic reference is a crucial first step to understanding genetic divergence, supporting conservation management, and addressing potential wildlife trade involving *M. emys* in Indonesia.

MATERIALS AND METHODS

Sampling site

Claw samples were obtained from a wild *Manouria emys* encountered in a forest in Padang, West Sumatra, Indonesia, in coordinate point 0°55'30.22"S 100°28'44.79"E (Figure 1). The location where the specimen was collected lies adjacent to the Bukit Barisan Mountains, this species has also been recorded several times in this surrounding area.

Sample collection

The sample was collected using the claw clipping method. Due to the critically endangered status and rarity of *M. emys* in the wild, only a single individual was encountered and sampled in this study. Consequently, all genetic analyses were performed using this specimen, supplemented with comparative COI sequences retrieved from GenBank and BOLD databases. Although the limited sample size precludes population level inferences for the Sumatran population, this study provides an important first reference for future molecular and conservation research on *M. emys* in Indonesia. The individual was released back into its natural habitat immediately after sampling. The samples were preserved at -20°C until analysis. DNA extraction and PCR were carried out at the Genetics and Biomolecular Laboratory, Department of Biology, Universitas Andalas, Indonesia.

DNA extraction

DNA extraction using Quick-DNA Miniprep Plus Kit protocol (Zymo Research). The claw sample was initially rinsed with sterile distilled water to remove surface contaminants and then ground into a fine powder using a mortar and pestle under liquid nitrogen. Approximately 25 mg of the powdered material was transferred into a 1.5 mL microcentrifuge tube for DNA extraction. DNA concentration and purity were assessed using a NanoPhotometer NP80 (IMPLEN). Polymerase Chain Reaction (PCR) was conducted to amplify a fragment of the COI gene. The PCR reaction mixture comprised 11 µL of Bioline Supermix, 9 µL of nuclease-free water, 1 µL each of the forward (MEPF 1) and reverse (MEPR 3) primers, and 3 µL of DNA template, yielding a total volume of 25 µL.

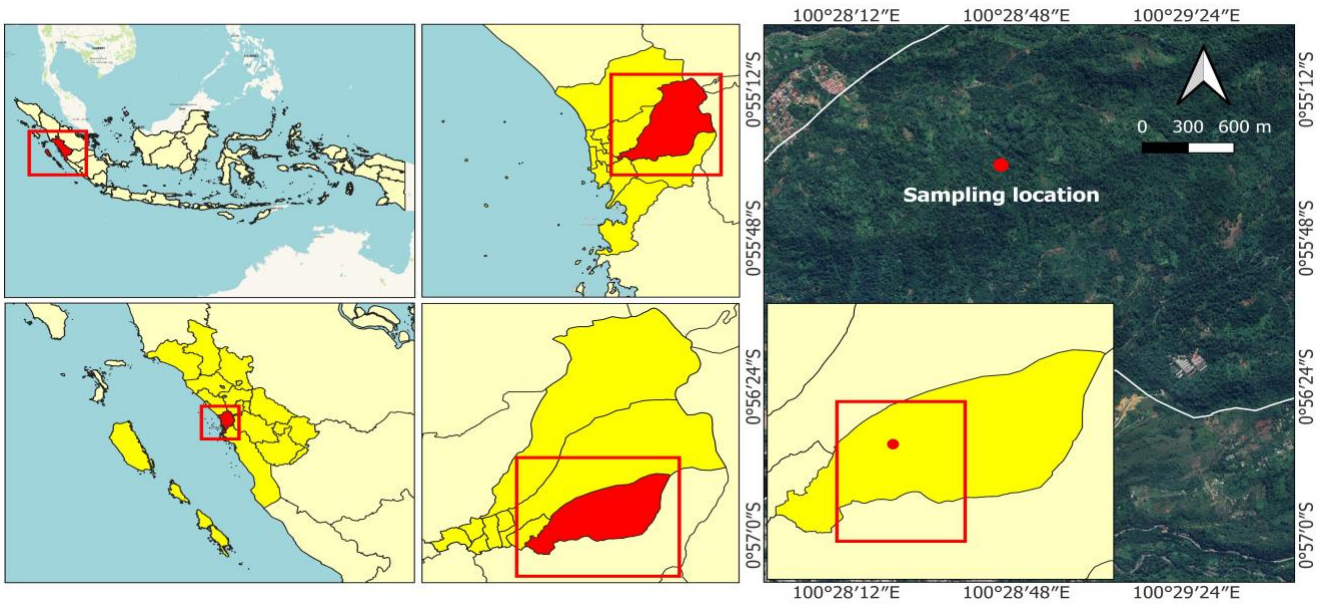

Figure 1. Location where *Manouria emys* was found in Padang, West Sumatra, Indonesia

PCR amplification

The primer pairs were designed and used, namely the forward primer MEPF1 5' TCAGCCACCTTACCTGTGTT 3' and the reverse primer MEPR3 5' ACAGTAAATATGTGGTGGGCTC 3'. The primers were designed using Primer3Plus, targeting the mitochondrial COI region. The design considered conserved regions across available *M. emys* sequences in GenBank to maximize amplification success. PCR thermal cycling was performed under the following conditions: an initial denaturation at 96°C for 1 min; 35 cycles of denaturation at 96°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 90 sec; followed by a final extension at 72°C for 3 min.

Sequence processing and alignment

The PCR amplification product was sent to First BASE Laboratories (Malaysia) for purification and sequencing. DNA sequencing was carried out using high-throughput capillary electrophoresis platforms from Applied Biosystems, and the same forward and reverse primers were used for PCR amplification. The resulting forward and reverse sequence reads were assembled and edited using the DNASTAR software package (Burland 2000). *M. emys* sequences were aligned using Clustal X version 1.8 with multiple alignment sequence together with reference sequences obtained from NCBI data and the BOLD system.

Phylogenetic analysis

A total of 29 comparison sequences were used, 27 sequences were all *M. emys* sequences available in the genome data bank, the other two were used as outgroups for phylogenetic analysis. Some sequences do not have associated reference articles but are available in the NCBI database (Table 1). *Astrochelys yniphora* was selected as an outgroup because it is phylogenetically distinct but closely related within Testudinidae, making it suitable for rooting the tree. Sequence polymorphism analysis was conducted with DnaSP version 5.10 to identify nucleotide variations (haplotypes) and to calculate haplotype and nucleotide diversity (Rozas 2009). MEGA (Molecular Evolutionary Genetics Analysis) version 11 was employed to analyze nucleotide differences among sequences with pairwise distance method and phylogenetic analysis with maximum likelihood method based on K2P model with the Neighbor-Joining algorithm, and BioNJ (Tamura et al. 2021).

RESULTS AND DISCUSSION

Phylogenetic and genetic divergence

In this study, a single sample from *M. emys* collected from West Sumatra, Indonesia, was used for DNA isolation, PCR amplification, and sequencing. An 899 bp fragment of the COI gene was obtained, then it was used as input for BLAST analysis, which confirmed with 100% validity that the sample collected from the Sumatran forest was *M. emys emys*. A total 588 bp after alingment was used for phylogenetic and haplotype diversity analyses, along with 29 comparison sequences (Table 1). Based on the information provided in the database, nine sequences were classified as *M. emys emys*, eight as *M. emys phayrei*, ten were identified only to the species level (*M. emys*), and two sequences (*A. yniphora*) were included as the outgroup.

Phylogenetic analysis was carried out using MEGA 11, two main clades are evident: clade I, which includes *M. emys*, and clade II, which consists of *A. yniphora*, serving as the outgroup in this analysis (Figure 2). The *M. emys* (MEPD001) is positioned within clade I, clustering together with other *M. emys* sequences, specifically residing in subclade I, which is predominantly composed of sequences from the subspecies *M. emys emys*. Subclade II is consistently occupied by sequences originating from the subspecies *M. emys phayrei*. In contrast, Subclade III comprises *M. emys* sequences whose subspecies classification remains unconfirmed.

This study provides the first COI sequence from *M. emys* in Sumatra, confirming its identity as *M. emys emys* and revealing its close genetic relationship with populations from Borneo. However, since only a single sample from Sumatra was analyzed, this study cannot assess local genetic diversity or population structure. Thus, the obtained sequence should be regarded as a preliminary reference point rather than a representative of the entire Sumatran population. Nevertheless, it provides valuable comparative data for understanding the evolutionary relationships among *M. emys emys, M. emys phayrei*, and other *M. emys* lineages. Phylogenetic analyses further demonstrated the presence of three distinct genetic groups within *M. emys*. Each clade formed in the phylogenetic tree represents a monophyletic group which may suggest limited gene flow and long-term isolation between subspecies (Figure 2). The presence of such monophyletic groups reflects close evolutionary relationships among individuals within *M. emys*, arising from shared, directly inherited genetic sequences. This observation aligns with the view of Nei and Kumar (2000), who emphasized that monophyletic clades serve as crucial evidence in phylogenetic analyses, as they indicate clear, uninterrupted lines of descent without mixing with taxa of different origins. The distribution of *M. emys* likely occurred during the Pleistocene, when the Sunda Shelf formed a continuous landmass, persisting until the early Holocene during the transition from the Last Glacial Maximum (LGM; ~26,000-21,000 years ago) (Bird et al. 2005). Similar biogeographic patterns have been documented for *Amyda cartilaginea* and Indotestudo species in Asia (Fritz et al. 2014; Ihlow et al. 2024).

The genetic distances (p-distance) among *M. emys* subspecies and the outgroup are presented in Table 2. The lowest genetic distance was observed within *M. emys phayrei* (0-0.3%) and *M. emys emys* (0-0.5%). In contrast, the overall genetic distance across *M. emys* was higher (0-3.1%). The genetic distances among all *M. emys* sequences and the outgroup *A. yniphora* ranged 10.2% to 11.2%.

Pairwise genetic distances, calculated using the pairwise difference method, are shown in Table 3. The pairwise differences between subclades ranged from 0.80861 (between subclades II and III) to 0.87391 (between subclades I and III), showing consistent levels of genetic divergence among subclade pairs.

Table 1. Comparative sequence data used in this study, including accession numbers and sources from NCBI GenBank

Accession/Id	Sample origin	Species/Subspecies	References
KM201283	India (Pet kept)	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KM201284	India (Pet kept)	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KM201285.1	India (Pet kept)	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KM201286	India (Pet kept)	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KP268841	India	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KP268842	India	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KP268843	India	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KP268844	India	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
MTDT_290	Indonesia (Borneo)	*Manouria emys* subsp. *emys* (Schlegel & Müller, 1844)	Bold system (2021)
KM201279	India (Pet kept)	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KM201280	India (Pet kept)	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KM201281	India (Pet kept)	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KM201282	India (Pet kept)	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KP268837	India	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KP268838	India	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KP268839	India	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KP268840	India	*Manouria emys* subsp. *phayrei* (Blyth, 1854)	Kundu et al. (2018)
KP268845	India	*Manouria emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KP268846	India	*Manouria emys* (Schlegel & Müller, 1844)	Kundu et al. (2018)
KF894791	India	*Manouria emys* (Schlegel & Müller, 1844)	-
KF894792	India	*Manouria emys* (Schlegel & Müller, 1844)	-
JN794084	India	*Manouria emys* (Schlegel & Müller, 1844)	-
GU563917	India	*Manouria emys* (Schlegel & Müller, 1844)	-
DQ080040	Museum of Vertebrate Zoology	*Manouria emys* (Schlegel & Müller, 1844)	Parham et al. (2006)
NC_007693.1	Museum of Vertebrate Zoology	*Manouria emys* (Schlegel & Müller, 1844)	Parham et al. (2006)
MG679403.1	Taipei Zoo	*Manouria emys* (Schlegel & Müller, 1844)	Chang et al. (2018)
OP741012	Bangladesh	*Manouria emys* (Schlegel & Müller, 1844)	-
MG679402.1	-	*Astrochelys yniphora* (Vaillant, 1885)	-
HQ329748.1	-	*Astrochelys yniphora* (Vaillant, 1885)	-

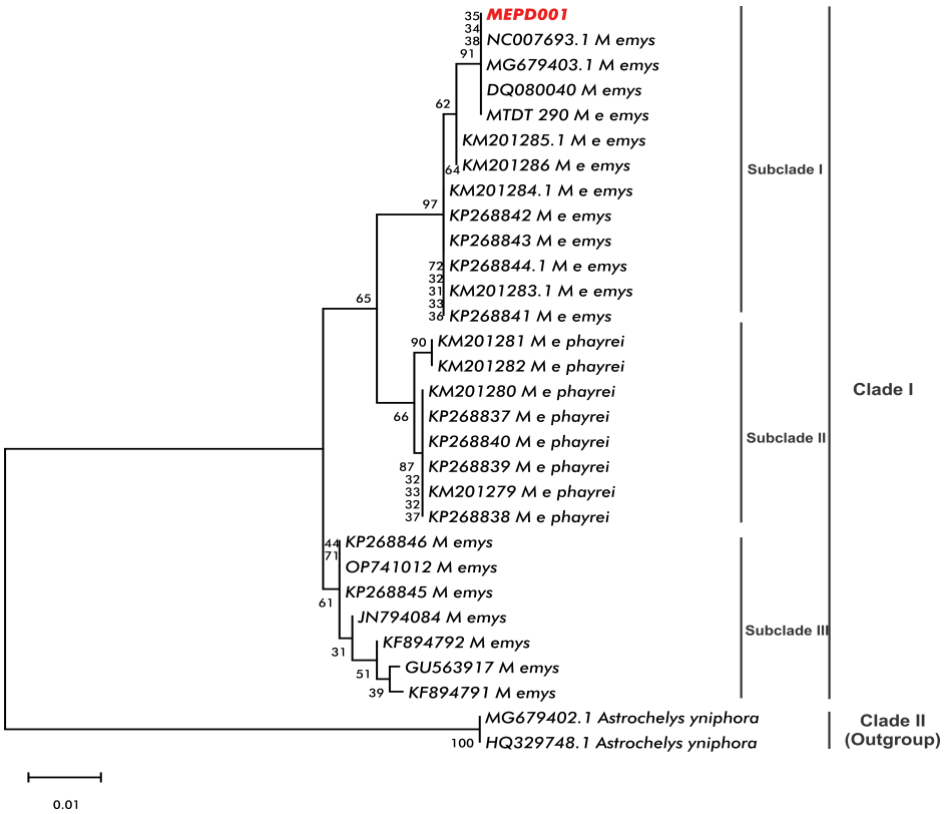

Figure 2. Maximum likelihood phylogenetic tree of *Manouria emys* and outgroup inferred from COI gene sequences, constructed using 1000 bootstrap replicates. Numbers at nodes indicate bootstrap support values. The tree shows the clustering of *Manouria emys emys*, *Manouria emys phayrei*, and unidentified subspecies *Manouria emys* lineages

Consistent with the phylogenetic tree topology, the genetic distance within *M. emys emys* was low, reflecting high genetic similarity among individuals of this subspecies (subclade I). Similarly, *M. emys phayrei* also showed minimal divergence, suggesting strong genetic homogeneity within this group (subclade II). In contrast, the overall genetic distance across *M. emys* (clade I) was higher, indicating substantial divergence among the subspecies (Table 2). Previous studies have also reported that the intraspecific genetic distance of *Emys orbicularis* ranges from 0.15% to 0.61% (Ciofi et al. 2017). Another study conducted by Fitriani (2018) on *M. emys* kept in a zoological garden in Indonesia, based on the Cyt b gene, also reported that the intraspecific genetic distance of this species ranged from 1.18% to 1.37%. In contrast, the genetic distance between *M. emys* and the outgroup species *A. yniphora* is substantially higher (Table 2). Kundu et al. (2018) also reported that the interspecific genetic distance between *M. emys* and *M. impressa* ranges from 9.4% to 10.6%. Bhaskar and Mohindra (2018) reported the species divergence of freshwater turtle was 13.3%. Among Testudines, the mitochondrial DNA sequence divergence rate is estimated to range between 0.4% and 0.6% per million years (Van der Kuyl et al. 2005).

Haplotype and nucleotide diversity

Haplotype and nucleotide diversity values for *M. emys* are summarized in Table 4. From 28 analyzed sequences, the haplotype diversity (Hd) was recorded at 0.881 and the nucleotide diversity value of *M. emys* was 0.01316. Based on haplotype network analysis, three major haplogroups were identified (Figure 3). Haplogroup I consists of *M. emys pharei* and includes two haplotypes. Haplogroup II comprises three haplotypes and includes individuals from *M. emys emys*, *M. emys* (MEPD001), and *M. emys*. Haplogroup III consists of five haplotypes and is composed of specimens documented as *M. emys*. Each haplogroup is characterized by specific nucleotide substitutions (Table 5). In haplogroup 1, there are only two haplotypes with two mutation points or two different bases, namely a thymine-to-cytosine substitution at position 144 and an adenine-to-cytosine substitution at position 231. In haplogroup 2, there are three haplotypes with three base substitutions: cytosine-to-adenine at position 231, guanine-to-adenine at position 243, and thymine-to-cytosine at position 366. In haplogroup 3, there are five haplotypes with four base substitutions: thymine-to-cytosine at position 10, guanine-to-thymine at position 25, cytosine-to-adenine at position 34, and cytosine-to-adenine at position 52.

The haplotype analysis was not based on the grouping of population origin, but rather on information derived from subspecies confirmation data. As indicated in the notes of Figure 3, the analyzed sequences are documented according to their respective subspecies designation. The yellow color represents the subspecies *M. emys phayrei*, the green indicates *M. emys emys*, the black refers to specimens recorded only as *M. emys*, and the red denotes the sample obtained in this study. Specific nucleotide bases that could clearly distinguish subspecies could not yet be confirmed, as seven sequences were documented only to the species level and did not show a clear tendency with any particular subspecies. However, three sequences with accession numbers MG679403, DQ080040, and NC_007693.1 originating from the Taipei Zoo and the Museum of Vertebrate Zoology were confirmed as the subspecies *M. emys emys*. All three sequences belong to haplotype 3 (Table 4, Figure 3) and cluster within subclade I of the phylogenetic tree (Figure 2), indicating a close genetic relationship. *M. emys* in haplogroup III is genetically closer to *M. emys pharei* in haplogroup I than to *M. emys emys* in haplogroup II. To date, no study has confirmed the existence of three distinct subspecies within this species. Therefore, the genetic data in haplogroup III suggest a closer affinity to *M. emys phayrei*, but morphological and more extensive genetic data are required to confirm its taxonomic status.

Table 3. Pairwise genetic distances between *Manouria emys* subclades based on COI sequences

	1	2	3
Subclade I	0.000		
Subclade II	0.872	0.000	
Subclade III	0.873	0.808	0.000

Table 2. Summary of genetic distances among *Manouria emys* and the outgroup sequence, based on 10 representative sequences showing the most contrasting values (from a total of 30 sequences analyzed)

Sequence	1	2	3	4	5	6	7	8	9
MEPD001									
KP268841. *Manouria emys emys*	0.005								
KM201280. *Manouria emys phayrei*	0.020	0.015							
KM201281. *Manouria emys phayrei*	0.017	0.015	0.003						
NC007693.1. *Manouria emys*	0.000	0.005	0.020	0.017					
KF894792. *Manouria emys*	0.029	0.024	0.012	0.015	0.029				
KP268846. *Manouria emys*	0.024	0.019	0.014	0.017	0.024	0.005			
GU563917. *Manouria emys*	0.031	0.026	0.010	0.014	0.031	0.002	0.007		
MG679402.1. *Astrochelys yniphora*	0.111	0.109	0.112	0.111	0.111	0.107	0.102	0.109	
HQ329748.1. *Astrochelys yniphora*	0.111	0.109	0.112	0.111	0.111	0.107	0.102	0.109	0.000

Table 4. Haplotype variation, haplotype diversity value (Hd) and nucleotide diversity (Pi) in the *Manouria emys* sequences

Haplotype	Sequences	Sample origin	Haplotype diversity (Hd)	Nucleotide diversity (Pi)
H_1	KM201279. *Manouria emys phayrei*	India (Pet kept)	0.881±0.032	0.01316±0.00083
	KM201280. *Manouria emys phayrei*	India (Pet kept)		
	KP268839. *Manouria emys phayrei*	India		
	KP268838. *Manouria emys phayrei*	India		
	KP268840. *Manouria emys phayrei*	India		
	KP268837. *Manouria emys phayrei*	India		
H_2	KM201281. *Manouria emys phayrei*	India (Pet kept)		
	KM201282. *Manouria emys phayrei*	India (Pet kept)		
H_3	MEPD001	Indonesia (West Sumatra)		
	MG679403.1. *Manouria emys*	Taipei Zoo		
	MTDT. 290. *Manouria emys emys*	Indonesia (Borneo)		
	NC007693.1. *Manouria emys*	Museum of Vertebrate Zoology		
	DQ080040. *Manouria emys*	Museum of Vertebrate Zoology		
H_4	KP268842. *Manouria emys emys*	India		
	KP268844.1. *Manouria emys emys*	India		
	KP268841. *Manouria emys emys*	India		
	KP268843. *Manouria emys emys*	India		
	KM201283.1. *Manouria emys emys*	India (Pet kept)		
	KM201284.1. *Manouria emys emys*	India (Pet kept)		
H_5	KM201285.1. *Manouria emys emys*	India (Pet kept)		
	KM201286. *Manouria emys emys*	India (Pet kept)		
H_6	KP268846. *Manouria emys*	India		
	OP741012. *Manouria emys*	Bangladesh		
	KP268845. *Manouria emys*	India		
H_7	KF894792. *Manouria emys*	India		
H_8	GU563917. *Manouria emys*	India		
H_9	KF894791. *Manouria emys*	India		
H_10	JN794084. *Manouria emys*	India		

Table 5. Variable nucleotide positions detected among *Manouria emys* COI sequences showing the base substitutions

Sequences	Variable site																	
	1	1	2	3	3	5	1	1	1	1	2	2	2	3	3	3	4	5
	1	1	2	3	3	5	4	5	6	9	3	4	6	1	2	6	3	1
	0	8	5	4	6	2	4	6	5	2	1	3	4	2	8	6	8	0
KM201279. *Manouria emys phayrei*	C	G	C	A	A	A	T	A	G	A	A	A	G	A	A	C	C	A
KM201280. *Manouria emys phayrei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP268839. *Manouria emys phayrei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP268838. *Manouria emys phayrei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP268840. *Manouria emys phayrei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KP268837. *Manouria emys phayrei*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KM201281. *Manouria emys phayrei*	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.
KM201282. *Manouria emys phayrei*	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	.	.	.
MEPD001	T	.	.	C	G	C	C	G	A	G	C	G	.	G	.	T	.	.
MG679403.1. *Manouria emys*	T	.	.	C	G	C	C	G	A	G	C	G	.	G	.	T	.	.
MTDT. 290. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	C	G	.	G	.	T	.	.
NC007693.1. *Manouria emys*	T	.	.	C	G	C	C	G	A	G	C	G	.	G	.	T	.	.
DQ080040. *Manouria emys*	T	.	.	C	G	C	C	G	A	G	C	G	.	G	.	T	.	.
KP268842. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KP268844.1. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KP268841. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KP268843. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KM201283.1. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KM201284.1. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	.	.	G	.	.	.	.
KM201285.1. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	G	.	G	.	.	.	.
KM201286. *Manouria emys emys*	T	.	.	C	G	C	C	G	A	G	.	G	.	G	.	.	.	.
KP268846. *Manouria emys*	T	A	.	C	.	C	.	.	.	.	.	.	A	.	G	.	T	G
OP741012. *Manouria emys*	T	A	.	C	.	C	.	.	.	.	.	.	A	.	G	.	T	G
KP268845. *Manouria emys*	T	A	.	C	.	C	.	.	.	.	.	.	A	.	G	.	T	G
KF894792. *Manouria emys*	.	A	T	.	.	C	.	.	.	.	.	.	A	.	G	.	T	G
GU563917. *Manouria emys*	.	A	T	.	.	.	.	.	.	.	.	.	A	.	G	.	T	G
KF894791. *Manouria emys*	.	A	.	.	.	.	.	.	.	.	.	.	A	.	G	.	T	G
JN794084. *Manouria emys*	T	A	.	.	.	C	.	.	.	.	.	.	A	.	G	.	T	G

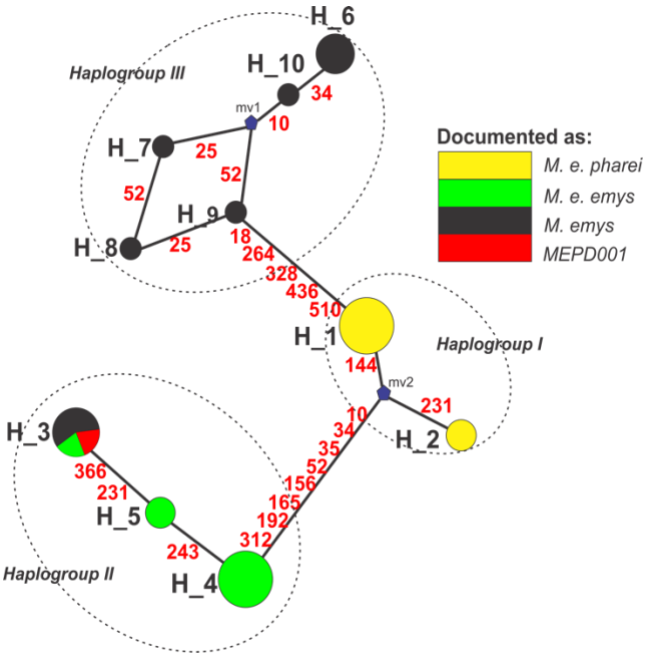

Figure 3. Haplotype network of *Manouria emys* based on COI gene, the size of the circle represents the number of samples, the color interprets the identity of the subspecies or species of the sample, yellow as *Manouria emys pharei*, green as *Manouria emys emys*, black as *Manouria emys*, and red as sample with code MEPD001

This interpretation is supported by the genetic distance data in Table 2, which shows that the genetic distance between *M. emys emys* (haplogroup II) and *M. emys* (haplogroup III) ranges from 1.4% to 3.1%, whereas the distance between *M. emys pharei* (haplogroup I) and *M. emys* (haplogroup III) is smaller, ranging from 0.9% to 1.7%. These results are consistent with the findings of Kundu et al. (2018) who proposed the recognition of each group as an Evolutionary Significant Units (ESUs), who reported genetic divergences of 1.7% between *M. emys emys* (ESU-1) and *M. emys pharei* (ESU-2), 2.0% between *M. emys emys* (ESU-1) and *M. emys* (ESU 3), and 0.8% between *M. emys pharei* (ESU-2) and *M. emys* (ESU-3).

The identification of three haplotypes in *M. emys emys* and three in *M. emys phayrei* suggests intrasubspecies genetics, although the number of haplotypes differs between the two. This may reflect differences in population size, distribution range, or historical demographic events affecting each subspecies (Van der Kuyl et al. 2005). Interestingly, the fifth sequence of *M. emys* with uncertain subspecies status exhibited a unique haplotype (H_6-H_10). This pattern may indicate the presence of a genetically distinct lineage, potential admixture between two recognized subspecies, or a population that has not yet been formally characterized.

Morphological evidence supports the molecular differentiation observed among the major clades, according to Kundu et al. (2018). In haplogroup I, the anterior and posterior medial edges of the pectoral scutes each contact the plastral midline independently a diagnostic trait of the *M. emys phayrei*. In contrast, in haplogroup II, the pectoral scutes do not reach the midline, which is characteristic of the *M. emys emys*. In haplogroup III, the medial edges of the anterior and posterior regions of the pectoral scutes form a distinctive cone-shaped pattern that converges at the plastral midline. These morphological patterns correspond well with the genetic groupings identified in this study.

Six sequences of *M. emys emys* in haplotype 4 and two sequences in haplotype 5, representing the southern subspecies *M. emys emys*, were found in the northern geographic region (India). Of these, four individuals were identified as specimens from the pet trade. This distribution pattern suggests possible natural dispersal or, more likely, human-mediated translocation, particularly through the wildlife trade (Perez et al. 2014; Koo et al. 2020; Esposito et al. 2022). This was also documented by Jenkins (1995), which recorded that between 1988 and 1993, a total of 450 *M. emys* individuals were exported abroad for the pet and zoo trade. In the same period, a large number of other exports were not properly recorded. Human-mediated translocation has also been reported to result in the presence of three non-native turtle species (*Amyda* sp., *Chitra* sp., and *Cyclemys* sp.) in northeastern India (Kundu et al. 2016), supporting the hypothesis that anthropogenic activities play a major role in the translocation of freshwater turtles beyond their native ranges. (Kundu et al. 2013) also reported the occurrence of a non-native Manouria subspecies and an Indotestudo species of Indonesian origin in the wild in Mizoram State. In addition, for *Indotestudo elongata* (Blyth, 1854) specimens from Myanmar, Thailand, Cambodia, and Vietnam, no statistically significant differences were found between biogeographic units, with a genetic distance of 1.2% based on the cytochrome b gene. Its current wide distribution is supported by archaeological evidence indicating extensive trade of this species during prehistoric and historic periods (Mudar and Anderson 2007; Ihlow et al. 2024).

In conclusion, this study provides the first COI sequence of *M. emys* from Sumatra, confirming its identity as *M. emys emys* and showing close genetic similarity to Bornean samples. While these results contribute valuable baseline information for conservation genetics, they should be regarded as preliminary due to the reliance on a single sample. As a preliminary contribution, these findings establish an essential molecular baseline for future taxonomic, conservation, and trade monitoring efforts involving this critically endangered species. To build upon this foundation, future studies should include larger sample sizes and broader geographic coverage across Sumatra and neighboring regions to better capture population level variation and clarify the phylogenetic relationships and subspecies boundaries within *M. emys*.

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