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DNA barcoding of bats from two selected forest reserves in Peninsular Malaysia

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Abstract. *Abdul Malek SNH, Azmir IA, Shafie NJ, Esa Y, Hanafi WNW. 2025. DNA barcoding of bats from two selected forest reserves in Peninsular Malaysia. Biodiversitas 26: 2843-2856.* Bats (order Chiroptera) are notable for their species diversity and a variety of distinct characteristics. However, as several molecular studies have shown, morphological similarities in some taxa of this group can result in neglected diversity. DNA barcodes can help by providing an accurate, rapid, and effective method of species recognition. In this study, we used DNA barcoding to identify bats captured from two selected regions in Peninsular Malaysia: Kenyir, Terengganu and Taman Alam Liar Negeri Kenaboi (TALNK), Jelebu, Negeri Sembilan. DNA barcoding analysis was performed on 38 bat specimens to further verify the species identification of the captured bats from morphological characteristics. The COI gene (603-604 bp) was successfully sequenced from the 38 bat species, representing 3 families, 5 genera, and 15 species. The phylogenies of NJ, ML, and Bayesian methods resulted in most of the specimens of the same species being clustered together. According to the IUCN Red List, *Rhinolophus trifoliatus* is listed as Near Threatened (NT), whereas 14 species were listed as Least Concern (LC). The findings reported in this study confirm the usefulness of DNA barcoding to identify bat species and to measure bat diversity in Kenyir and TALNK forest reserves. Improving habitat conservation in forest reserves is necessary to guard against the disruption and extinction of bat species.

Keywords: Chiroptera, Cytochrome Oxidase subunit I (COI), DNA barcoding, Kenyir, TALNK

INTRODUCTION

Bats are the second most highly diversified mammalian order worldwide (Benítez et al. 2021). Being the only mammals with powered flight, they are special. Traditionally, bats were classified into Megachiroptera and Microchiroptera (Gaudioso et al. 2020); however, newer molecular evidence supports their reorganization into Yinpterochiroptera and Yangochiroptera (Kammerer et al. 2017). Bats also differ in diet and can be carnivorous, omnivorous, nectarivorous, frugivorous, or insectivorous (Ingala et al. 2021). Insectivorous bats depend on echolocation for hunting and navigation since they have small eyes and poor vision (Buckles 2014). The dominant insectivorous bat groups in Peninsular Southeast Asia were identified from the family Hipposideridae, Rhinolophidae and Vespertilionidae (from the subfamily Murininae, and Kerivoulinae) (Lim et al. 2019). Bats inhabit a variety of ecosystems, including urban areas, forests, mangroves, and orchards (Nasir et al. 2021). The highest species diversity is found in forested habitats (Russo et al. 2016). Features like flight-related morphology and roosting behavior affect their presence in forests (Novella-Fernandez et al. 2022).

Bats play vital roles in tropical ecosystems, including pollination and pest control (Basri et al. 2022). Fruit bats (Family Pteropodidae) aid in seed dispersing for various ecological and economically important plants. While, insectivorous bats help regulate pest populations by consuming insects (Elangovan et al. 2018). Despite their ecological importance, bat diversity remains understudied in certain regions of Peninsular Malaysia. Although extensive research has been conducted in West Coast states and parts of Pahang, bat diversity in Terengganu and Negeri Sembilan remains relatively understudied (Pounsin et al. 2018). To address this gap, this study employs DNA barcoding as a molecular tool to assess bat diversity in two selected forest reserves in Peninsular Malaysia. Tasik Kenyir is the largest artificial lake in Southeast Asia, surrounded by the world's oldest rainforest, and has become home to various types of flora and fauna (Mohammad Noor et al. 2019). Since Dipterocarpaceae species dominated the area of Tasik Kenyir, it is classified as a dipterocarp forest (Basri et al. 2022). This forest support higher number of niches for unique species compositions, including bat species, due to denser forest area, higher structural diversity, and higher availability of food sources (Pounsin et al. 2018). Meanwhile, Taman Alam Liar Negeri Kenaboi (TALNK) is a lowland and hill dipterocarp forest located in Negeri Sembilan and was inhabited by clumps of bamboo, shrubs, and other pioneer species like *Macaranga* sp. (Fatma et al. 2020). Before TALNK was designated as a rehabilitated forest, the forest used to be a place for logging activities and had a significant impact on the local species due to the loss of habitat (Fatma et al. 2020).

Conventional methods, such as external measurements, have commonly been used to identify bat species through taxonomic keys. However, these methods have grown more challenging due to morphological, acoustics, and biometrics similarities among bat species (Mota et al. 2022). DNA sequencing has improved species resolution in taxonomically complex groups, and it is currently being used by many researchers in phylogenetic and molecular taxonomy studies (Benítez et al. 2021). DNA barcoding, a molecular technique that necessitates standard gene segment sequencing has proven to be an effective technique for species identification (Purty and Chatterjee 2016; Lim et al. 2017). The mitochondrial Cytochrome Oxidase I (COI) gene is the most common barcode used in animals, mainly due to the high rate of mutation and the conserved sequence within species, which makes the COI gene helpful for identifying closely related species (Purty and Chatterjee 2016; Sarvananda, 2018). The COI gene also offer fast and accurate method for species identification, especially when dealing with morphological ambiguity or confusion (Basith et al. 2021).

Unfortunately, like many other wildlife species, bat species are also under threat from human activities. Deforestation, farming, and urban development are destroying their natural habitats and roosting sites (Atagana et al. 2021). Malaysia is a critical country for global bats conservation (Nasir et al. 2021), having a total of 143 bat species reported with 113 species recorded in Peninsular Malaysia (Senawi and Norhayati, 2021). The extinction of the bat species is a possibility if these threats remain unaddressed and uncontainable. Several ecological functions that are essential to human societies, like pollination, seed dispersal, and pest control, may be negatively impacted by the extinction of some bat species (Frick et al. 2020). The results obtained in this study shows that DNA barcoding is a useful and reliable method for identifying bats in Peninsular Malaysia and mitochondrial DNA, particularly the COI gene, is a powerful tool for understanding genetic diversity and supporting conservation efforts (Bingpeng et al. 2018).

MATERIALS AND METHODS

Ethical and permit approvals

This study was conducted with ethical approval from the UMT Animal Ethics Committee (UMT/JKEPHMK/ 2021/60). Permits for entering the study site were obtained from the Forestry Department (JH/100Jld.29(76) and PHNT.100/58/2/33Bhg.3(6)) and the Development Authority of Terengganu Tengah (LKTT:060/1/4/3/5Jld.3-(28)). The permit to capture animals was obtained from the Department of Wildlife and National Park (UMT/JPHLTn.600-6/1/ 4JLD2(83)/210922).

Study area

Two forest reserves in Peninsular Malaysia were selected as the study sites for this research. The selected sites were Kenyir, Terengganu (5°08′32.5″N 102°45′39.7″E) and Taman Alam Liar Negeri Kenaboi (TALNK), Jelebu, Negeri Sembilan (3°10′39.6″N 101°59′12.8″E) (Figure 1). Kenyir is a dipterocarp forest that supports 698 species of small mammals, birds, and herpetofauna, and 113 species of flowers (Ramlee et al. 2020). The sampling in Kenyir was carried out within the area of Sungai Buweh Waterfall and the Universiti Malaysia Terengganu (UMT) Kenyir Research Station, where primary rainforests heavily surround the area. On the other hand, TALNK is a secondary forest made up of lowland and hill dipterocarp forests (Fatma et al. 2016). The sampling was done in an area of dry forest with clumps of bamboo surrounding it.

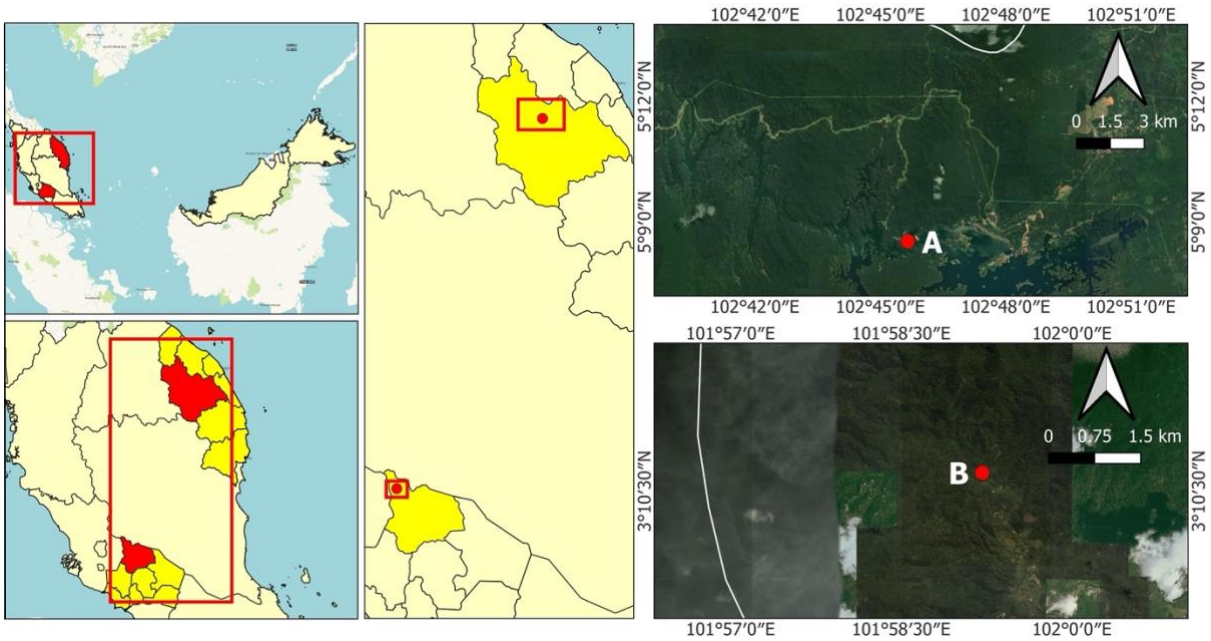

Figure 1. Map showing the geographical locations of the study areas in Peninsular Malaysia. A. Kenyir, Terengganu is a dipterocarp forest ecosystem, B. Taman Alam Liar Negeri Kenaboi (TALNK), Jelebu, Negeri Sembilan

Field methods

Two sampling sessions were carried out between June 2022 and July 2023 in a 1 ha plot at each selected study site. Three harp traps were installed and deployed randomly in the plot and placed next to flowing rivers or streams, as many species of bats utilize water sources to forage since there is a greater abundance of insects there (Korine et al. 2016). The traps were open for five consecutive nights during each sampling session (Hazwani et al. 2021). The traps were checked once in the morning and after sunset (07.00 hours and 21.00 hours, respectively) to ensure all bats captured were safe and collected. Captured bats were recorded for sex, weight, body length, and standard morphometric measurements (fur color, forearm, ear, tibia, and tail). Taxonomic keys from Kingston et al. (2006), Phillipps and Phillipps (2016), and Francis (2019) were used for species identification based on morphological traits.

A small tissue sample (~3 mm diameter) was excised from the wing membrane using a sterile single-hole puncher, immediately preserved in 95% alcohol solution, and stored at -20°C for DNA (Deoxyribonucleic Acid) extraction. The bats were held in a ventilated holding bag for up to 12 hours and were monitored for signs of stress or injury before being released at the capture site (Marina et al. 2021). To minimize handling stress, all sampling was conducted following established bat handling protocols approved by the UMT Animal Ethics Committee.

DNA extraction, PCR amplification, and sequencing

The total genomic DNA of the specimens was extracted from the wing membrane tissue using ReliaPrep™ gDNA Tissue Miniprep System Kit (Promega, Madison, United States) following the manufacturer's protocol. A 650 bp COI gene fragment was amplified by Polymerase Chain Reaction (PCR) using the Folmer et al. (1994) primer pair, Folmer_newF 5′-TNTCAACNAAYCAYAARGAYATYGG-3′ and Folmer_newR 5′-TANACYTCNGGRTGNCCRAARAAYCA-3′, utilizing a Bio-Rad T100 Thermal Cycler. A total of 25 µL reaction volume contained 50-100 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, and 2.5 U of TaKaRa Taq DNA polymerase (Sun et al. 2016). The reaction was set up with the following parameters: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 35 s, annealing at 50°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 5 min.

The PCR products were run on a 1% agarose gel (Sigma-Aldrich, USA) stained with 1 µL of GelRed dye (Thermo Fisher Scientific, USA) along with a 100 bp ready-to-load DNA ladder (Promega, USA). The electrophoresis conditions were set to 100 V, 400 A, and 45 min in 1× TBE buffer. Later, the gel was visualized under Ultra-Violet (UV) light using a UVIdoc HD2 gel documentation system (UVITEC, Cambridge, UK). The sequencing was carried out by a private company (First Base Laboratory Sdn. Bhd., Malaysia) using traditional Sanger sequencing.

Mitochondrial DNA sequence analysis

Chromas Lite software version 2.6.6 was used to view the trace files obtained from the sequencing of the bat specimens. The multiple sequence alignment and editing process were carried out using CLUSTAL X version 2.1 (Azmir et al. 2020). Once the sequences had been aligned, they were manually trimmed using BioEdit version 7.2.6.1 to achieve the desired length (Azmir et al. 2020). The acquired DNA sequences were searched against the Barcode of Life Data System (BOLD) and Basic Local Alignment Search Tool (BLAST) of GenBank for species identification. After that, the sequences were submitted to GenBank to obtain an accession number and to BOLD to obtain their own barcode status (Azmir et al. 2020).

Phylogenetic assessment

The query dataset of bat samples was assessed for their genetic distances among specimens caught, where the pairwise genetic distances among intraspecies, intragenus, and intrafamily were measured using the 'Distance Summary' command in BOLD (Azmir et al. 2020). The Kimura 2-Parameter (K2P) evolutionary model served as the foundation for the computation. The phylogenetic relationship was inferred using three different methods: Bayesian analysis, Maximum Likelihood (ML), and Neighbor-Joining (NJ). The bootstrap confidence of 1000 replicates was applied to NJ and ML analyses to estimate the highest likelihood of a tree. The ML tree was generated after selecting the best-fit substitution model in MEGA (Park et al. 2019). Mr Bayes version 3.2.2 was used to perform Bayesian phylogenetic analysis (Azmir et al. 2020).

Genetic diversity analysis

DNA Sequence Polymorphism (DnaSP) version 6 was used to estimate genetic diversity parameters such as number of polymorphic sites, number of haplotypes (h), Haplotypic diversity (Hd), and nucleotide diversity (π).

RESULTS AND DISCUSSION

Species assignment of bats

A total of 84 bats were captured, identified to species level in the field, and then utilized for molecular confirmation. After detailed observations based on morphological characteristics, the bats were classified into 3 families, 5 genera, and 13 species. The families are Hipposideridae (4 species), Rhinolophidae (3 species), and Vespertilionidae (6 species) (Table 1, Figure 2). The most abundant family of bats captured at Kenyir was Hipposideridae (n = 40), composed of 3 species, followed by Vespertilionidae (n = 3), composed of 2 species and Rhinolophidae (n = 2), composed of 1 species. Meanwhile, for TALNK, the most abundant family of bats found was Rhinolophidae (n = 16), grouped into 3 species, followed by Hipposideridae (n = 12), grouped into 3 species, and Vespertilionidae (n = 11), forming 5 species.

Table 1. Species assignment of 84 bats captured from Kenyir and TLNK forest reserve through morphological measurements

Family	Species	Common name	Locations	
			Kenyir	TALNK
Hipposideridae	*Hipposideros bicolor* (Temminck, 1834)	Temminck's Bicoloured Roundleaf Horseshoe Bat	34	0
	Hipposideros cervinus (Gould, 1854)	Fawn-Coloured Roundleaf Bat	2	7
	Hipposideros larvatus (Horsfield, 1823)	Large Roundleaf Horseshoe Bat	4	4
	Hipposideros diadema (É.Geoffroy Saint-Hilaire, 1813)	Diadem Roundleaf Bat	0	1
Rhinolophidae	*Rhinolophus affinis* (Horsfield, 1823)	Intermediate Horseshoe Bat	2	13
	Rhinolophus trifoliatus (Temminck, 1834)	Trefoil Horseshoe Bat	0	1
	Rhinolophus stheno (K.Andersen, 1905)	Lesser Brown Horseshoe Bat	0	2
Vespertilionidae	*Murina peninsularis* (Hill, 1964)	Peninsular Tube-Nosed Bat	1	0
	Kerivoula hardwickii (Horsfield, 1824)	Hardwicke's Woolly Bat	2	1
	Kerivoula titania (Bates, Struebig, Hayes, Furey, Mya Mya, Thong, Tien, Son, Harrison, Francis & Csorba, 2007)	Titania's Woolly Bat	0	2
	Kerivoula cf. *papillosa* (Temminck, 1840)	Papillose Woolly Bat	0	3
	Tylonycteris malayana (Chasen, 1940)	Mainland Greater Bamboo Bat	0	3
	Tylonycteris fulvida (Blyth, 1859)	Mainland Lesser Bamboo Bat	0	2
		Total number of individuals	45	39
		Total number of species	6	11

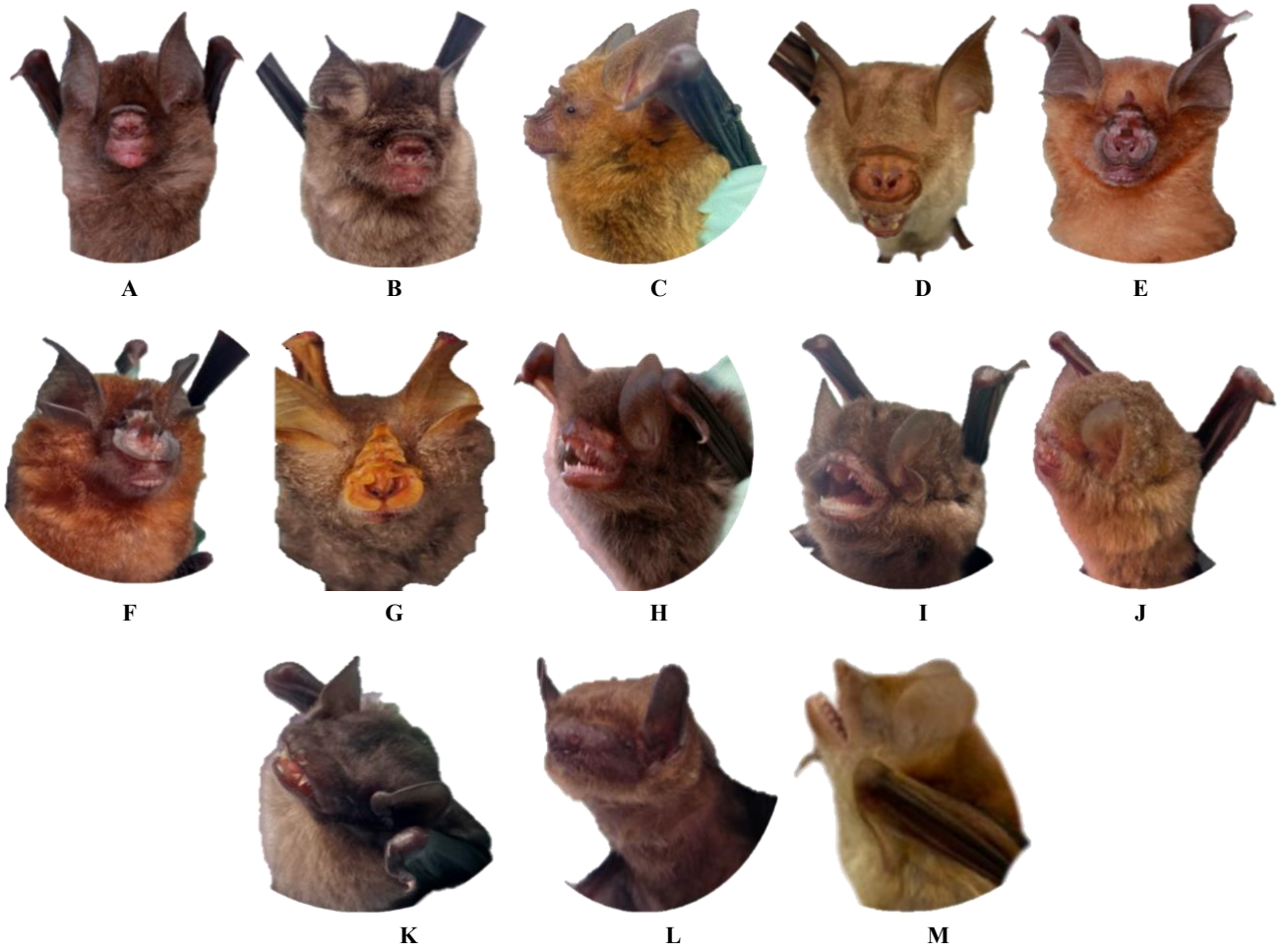

Figure 2. Bat species recorded in Tasik Kenyir, Terengganu and Taman Alam Liar Negeri Kenaboi, Jelebu, Negeri Sembilan. A. *Hipposideros bicolor*, B. *Hipposideros cervinus*, C. *Hipposideros larvatus*, D. *Hipposideros diadema*, E. *Rhinolophus affinis*, F. *Rhinolophus stheno*, G. *Rhinolophus trifoliatus*, H. *Kerivoula hardwickii*, I. *Kerivoula titania*, J. *Kerivoula* cf. *papillosa*, K. *Tylonycteris malayana*, L. *Tylonycteris fulvida*, M. *Murina peninsularis*

The captured bats were further identified using DNA barcoding approaches. The COI barcoding of bats was performed on 38 samples using wing membrane tissues. The 38 bat samples were selected from the total number of bats captured (n = 84) and representatives of each species identified morphologically were chosen with a minimum of 3 samples per species. All 38 bat samples were successfully barcoded, where the amplicons were first directly amplified and visualized on a 1% agarose gel (Sigma-Aldrich, USA) to ensure the presence of the desired size, which is 650 bp (Figure 3). The 38 bat species sequenced represent 3 families, 5 genera, and 15 species. The identity assignment of the 38 successfully barcoded sequence samples matched the available sequences in the database, with query coverage ranging from 91% to 100% (Table 2). According to the International Union for Conservation of Nature (IUCN) Red List of Threatened Species, out of 15 species captured, *Rhinolophus trifoliatus* (Temminck, 1834) was listed as Near Threatened (NT) while the other 14 were listed as Least Concern (LC).

Bats' COI gene assessment

All resulting 38 mitochondrial DNA (mtDNA) sequences of bats captured were assigned with GenBank accession number and a BOLDSYSTEM index number (Table 3). All amplified fragments of bat species specimens inhabiting the Kenyir and TALNK forest reserves contained 603 or 604 bp. The sequence showed good quality, with no evidence of insertions, deletions, or stop codons. The average base compositions of the COI gene among the examined samples were 28.80% T, 29.20% C, 25.10% A, and 16.90% G. The frequency of A + T content (53.90%) was slightly higher than that of G + C content (46.10%).

Table 2. Species assignment of 38 barcoded bats from Kenyir and TALNK forest reserve in Peninsular Malaysia, representing 3 families, 5 genera, and 15 species

Family	Species	%	
		GenBank identity	BOLD similarity
Hipposideridae	*Hipposideros bicolor*	99.48	99.49
	Hipposideros bicolor	100	100
	Hipposideros dyacorum	99.47	99.66
	Hipposideros cervinus	100	100
	Hipposideros cervinus	100	100
	Hipposideros cervinus	100.00	100
	Hipposideros cervinus	99.54	100
	Hipposideros cervinus	99.55	100
	Hipposideros cervinus	100	100
	Hipposideros cf. *larvatus*	100	100
	Hipposideros cf. *larvatus*	100	100
	Hipposideros cf. *larvatus*	100	100
	Hipposideros larvatus	99.84	99.83
	Hipposideros larvatus	100	100
	Hipposideros larvatus	98.95	100
	Hipposideros cf. *larvatus*	100	100
	Hipposideros diadema	100	100
Rhinolophidae	*Rhinolophus affinis*	99.85	100
	Rhinolophus affinis	100	100
	Rhinolophus affinis	94.63	99.66
	Rhinolophus affinis	99.66	99.32
	Rhinolophus stheno	97.66	97.46
	Rhinolophus stheno	97.65	97.63
	Rhinolophus trifoliatus	99.53	99.83
Vespertilionidae	*Kerivoula hardwickii*	99.68	99.83
	Kerivoula hardwickii	99.68	99.83
	Kerivoula hardwickii	99.21	99.32
	Kerivoula titania	99.48	99.46
	Kerivoula titania	99.48	99.46
	Kerivoula cf. *papillosa*	99.76	99.83
	Kerivoula cf. *papillosa*	99.84	100
	Kerivoula cf. *papillosa*	99.84	100
	Tylonycteris fulvida	94.48	93.91
	Tylonycteris fulvida	94.48	93.94
	Tylonycteris malayana	91.21	99.66
	Tylonycteris malayana	91.23	99.66
	Tylonycteris malayana	90.89	99.66
	Murina peninsularis	94.96	94.14

Note: N/A is not available

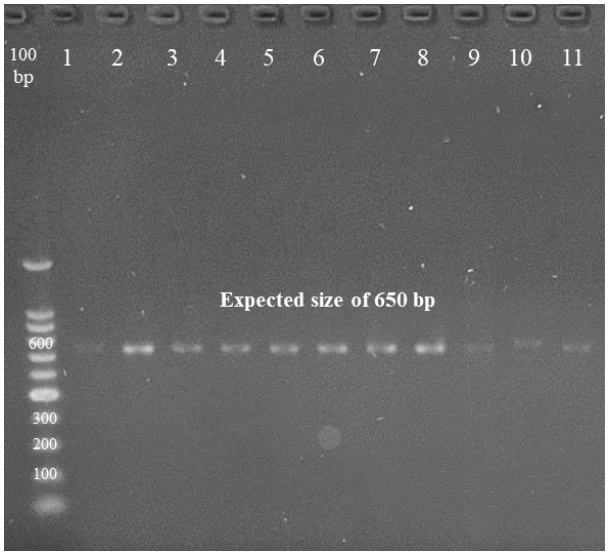

Figure 3. Gel electrophoresis (1% agarose gel) visualization of selected bat species specimens amplified using COI gene. Lane 1: 100 bp DNA ladder. Lane 2 until 12: The amplified PCR product of selected bat species specimens

The observed intraspecific Kimura 2-Parameter (K2P) (0%-1.00%) in Table 4 falls within the expected range typically exhibit less than 2% variation within species (Hebert et al. 2003; Azmir et al. 2020). The genetic divergence values were generated using the BOLD System engine and analyzed based on the available species records within the BOLD database. The high interspecific divergence (16.14% mean genetic distance) supports the effectiveness of COI barcoding in distinguishing closely related bat species. Notably, the genetic distance between *Hipposideros bicolor* and *Hipposideros dyacorum* (Thomas, 1902) (12.45%) suggests substantial evolutionary separation, potentially indicating a long-standing divergence between these species. These results align with global studies that report interspecific genetic distances exceeding 10% in bats (Clare et al. 2007).

Characteristics of COI dataset

The summary of the barcode gap was done by using the MUSCLE alignment program and Kimura 2-parameter. The mean intraspecific distances showed 0% of minimum divergence and 0.67% of maximum divergence. Approximately 73% (11/15) of species assignments successfully achieved divergent values of less than 2%. Meanwhile, the results show no intraspecific barcode gap value for four species: *Hipposideros diadema*, *Hipposideros dyacorum*, *Rhinolophus trifoliatus*, and *Murina peninsularis*, owing to a singleton. *Murina peninsularis* and *Kerivoula* cf. *papillosa* exhibited the highest distance to the Nearest Neighbor (NN), which is 26.97%.

The nearest neighbor's distance was calculated using a minimum distance of 0.5% and a maximum distance of 26.97% (Table 5). The 0.5% value of the nearest neighbor for *Hipposideros* cf. *larvatus* shows confirmation that *Hipposideros larvatus* and *Hipposideros* cf. *larvatus* are similar species. If this species is excluded from the calculation of nearest neighbor distance, then the range of minimum to maximum distance to nearest neighbor is 6.87%-26.97%. This range is accurate since genetic differences within the same species are typically indicated by a genetic distance value of less than 2%.

Phylogenetic analysis

The phylogenetic results (Figure 4) confirm that DNA barcoding provides a reliable means of distinguishing bat species in Malaysia. The distinct clustering of Hipposideridae, Rhinolophidae, and Vespertilionidae aligns with established taxonomic classifications (Sazali et al. 2011). The high bootstrap support (100%) for *R. affinis* and *R. stheno* further strengthens their close evolutionary relationship, supporting previous molecular studies (Lim et al. 2017). The phylogenetic structure within Vespertilionidae reveals four clades, suggesting potential cryptic diversity within this family, which warrants further investigation. These findings highlight the utility of molecular methods in resolving taxonomic ambiguities that arise from morphological similarities among bat species.

The General Time Reversible model (GTR+G) (-lnL: 6099.181889), the best-fit model evolution, was utilized to construct the ML tree. The ML analysis revealed distinct groupings both within and among the Hipposideridae, Rhinolophidae, and Vespertilionidae (Figure 5). The groupings within Hipposideridae (71% of bootstrap value) were like those obtained using the NJ, where two groups were formed, but there is a shift in the species order. For Rhinolophidae, all species were clustered together in Group 3 with a 94.6% bootstrap value. However, within Vespertilionidae, two groups were formed. Group 4 (52.8% of bootstrap value) consisted of *Kerivoula* cf. *papillosa*, *Kerivoula titania*, and *Kerivoula hardwickii*; Group 5 (35% of bootstrap value) consisted of *Murina peninsularis*, *Tylonycteris fulvida*, and *Tylonycteris malayana*. Bayesian analysis revealed a significant shift in the relative positions of the three families, with only 3 groups formed (Figure 6).

Molecular genetic diversity analysis

The mitochondrial genetic diversity of bat species from Kenyir and TALNK is indicated using various genetic diversity indices (Table 6). In terms of haplotype and nucleotide diversity of species, *H. bicolor* (s = 51, Hd = 1.000, π = 0.0050) showed the highest genetic variation, followed by *R. affinis*, *T. fulvida*, *R. stheno*, *K.* cf. *papillosa*, *K. hardwickii*, *H. larvatus*, and *T. malayana*. On the other hand, *H. cervinus*, *H.* cf. *larvatus* and *K. titania* showed the lowest genetic variation (s = 0, Hd = 0, π = 0). The haplotype or genetic diversity of *H. dyacorum*, *H. diadema*, *R. trifoliatus*, and *M. peninsularis* could not be quantified because only one sample was collected for each species.

Table 3. Summary of mitochondrial DNA data from 38 bat specimens collected in Kenyir and TALNK Forest Reserves, Peninsular Malaysia, representing 3 families, 5 genera, and 15 species

Species	GenBank accession number	BOLDSYSTEM index number (BIN)	Location	Sequence base pair (bp)
Hipposideros bicolor	PP216255	BOLD:ACE6229	Terengganu	604
Hipposideros bicolor	PP216256	BOLD:ACE6229	Terengganu	604
Hipposideros dyacorum	PP216258	BOLD:AAD0486	Terengganu	604
Hipposideros cervinus	PP216259	BOLD:AAB6249	Negeri Sembilan	604
Hipposideros cervinus	PP216260	BOLD:AAB6249	Negeri Sembilan	604
Hipposideros cervinus	PP216261	BOLD:AAB6249	Terengganu	604
Hipposideros cervinus	PP216262	BOLD:AAB6249	Negeri Sembilan	604
Hipposideros cervinus	PP216263	BOLD:AAB6249	Negeri Sembilan	604
Hipposideros cervinus	PP216264	BOLD:AAB6249	Terengganu	604
Hipposideros cf. *larvatus*	PP216265	BOLD:AAA4092	Terengganu	604
Hipposideros cf. *larvatus*	PP216266	BOLD:AAA4092	Terengganu	604
Hipposideros cf. *larvatus*	PP216267	BOLD:AAA4092	Terengganu	604
Hipposideros larvatus	PP216268	BOLD:AAA4092	Negeri Sembilan	604
Hipposideros larvatus	PP216269	BOLD:AAA4092	Negeri Sembilan	604
Hipposideros larvatus	PP216270	BOLD:AAA4092	Negeri Sembilan	604
Hipposideros cf. *larvatus*	PP216271	BOLD:AAA4092	Terengganu	604
Hipposideros diadema	PP216272	BOLD:AAB8310	Negeri Sembilan	604
Rhinolophus affinis	PP216273	BOLD:ACF0990	Terengganu	604
Rhinolophus affinis	PP216274	BOLD:ACF0990	Terengganu	604
Rhinolophus affinis	PP216275	BOLD:ACF0990	Negeri Sembilan	604
Rhinolophus affinis	PP216276	BOLD:ACF0990	Negeri Sembilan	604
Rhinolophus stheno	PP216278	BOLD:ABY8707	Negeri Sembilan	604
Rhinolophus stheno	PP216279	BOLD:ABY8707	Negeri Sembilan	604
Rhinolophus trifoliatus	PP216280	BOLD:AAB6408	Negeri Sembilan	604
Kerivoula hardwickii	PP216281	BOLD:AAA6722	Terengganu	604
Kerivoula hardwickii	PP216282	BOLD:AAA6722	Terengganu	604
Kerivoula hardwickii	PP216283	BOLD:AAA6722	Negeri Sembilan	604
Kerivoula titania	PP216284	BOLD:AAC1298	Negeri Sembilan	604
Kerivoula titania	PP216285	BOLD:AAC1298	Negeri Sembilan	604
Kerivoula cf. *papillosa*	PP216286	BOLD:AAC9529	Negeri Sembilan	604
Kerivoula cf. *papillosa*	PP216287	BOLD:AAC9529	Negeri Sembilan	604
Kerivoula cf. *papillosa*	PP216288	BOLD:AAC9529	Negeri Sembilan	604
Tylonycteris fulvida	PP216291	BOLD:AAC1211	Negeri Sembilan	604
Tylonycteris fulvida	PP216292	BOLD:AAC1211	Negeri Sembilan	604
Tylonycteris malayana	PP216293	BOLD:AFU5588	Negeri Sembilan	604
Tylonycteris malayana	PP216294	BOLD:AFU5588	Negeri Sembilan	604
Tylonycteris malayana	PP216295	BOLD:AFU5588	Negeri Sembilan	604
Murina peninsularis	PP291904	BOLD:AFW6475	Terengganu	603

Table 4. Genetic divergences based on Kimura 2-Parameter (K2P) distances at different taxonomic levels among bat specimens from Kenyir and TALNK Forest Reserves in Peninsular Malaysia, analyzed and compared based on the available species records in the BOLD database

Comparison within	Taxa	Comparison	Distance			
			Min (%)	Mean (%)	Max (%)	SE (%)
Species	15	43	0.00	0.22	1.00	0.01
Genus	5	152	0.50	16.14	20.00	0.04
Family	3	53	21.57	36.55	75.00	0.41

Discussion

In this study, the collected bat samples were initially identified using morphological characteristics, which included forearm length, ear length, tibia length, tail length, head-to-body length, weight, sex, facial features, and fur color. From this record, all species captured were insectivorous bats, accounting for 54% of bat species recorded in Kenyir, and another 46% were recorded in Taman Alam Liar Negeri Kenaboi (TALNK). From the collected data, the higher number of individuals found in Kenyir, the number of bat species in TALNK was found to be higher. This result can be influenced by a wide range of variables, including microclimate, topography, species abundance, species distribution, type of habitat, and food source (Basri et al. 2022). The forested area in Kenyir is one of the undisturbed primary forests that provides a home for many species. As mentioned in a previous study by Pounsin et al. (2018), the Hulu Terengganu rainforest has a higher diversity of bat species because of the dense forest area, greater structural diversity, and greater food availability.

These factors allow the rainforest to support more niches for a greater variety of unique species compositions (Pounsin et al. 2018). Therefore, catching a lot of bats in the Kenyir area is not impossible. Bat capture rates during our sampling, however, have been influenced by variables like sampling methods, environmental circumstances, and sampling periods. Unlike Kenyir, TALNK is a secondary forest due to habitat loss from logging activities decades ago. Although many habitats are destroyed when land is exploited, there is a benefit to the situation in that it creates new habitats for other highly adaptive species (Basri et al. 2022). Previous studies have demonstrated that bats are one of the species that can easily adapt to a wide range of habitats. Plus, the availability of more open water bodies in disturbed areas provides this species with drinking and foraging opportunities, resulting in increased bat activity (BakwoFils et al. 2021). Out of the 14 bat species captured, only *R. trifoliatus* is classified as near threatened. *Rhinolophus trifoliatus* is one of the bat species in the Rhinolophidae family and inhabits both primary and secondary forests (Kingston et al. 2006). Hughes et al. (2010) mentioned that Rhinolophids significantly contribute to the biodiversity of bats in Southeast Asian forests, thus, declines in their diversity or population size may indicate the decline or disappearance of significant rainforest habitats. A study of the diversity of insectivorous bat assemblages in Peninsular Malaysia revealed that the north of Peninsular Malaysia had fewer species richness than the south (Mohd-Hanif et al. 2015).

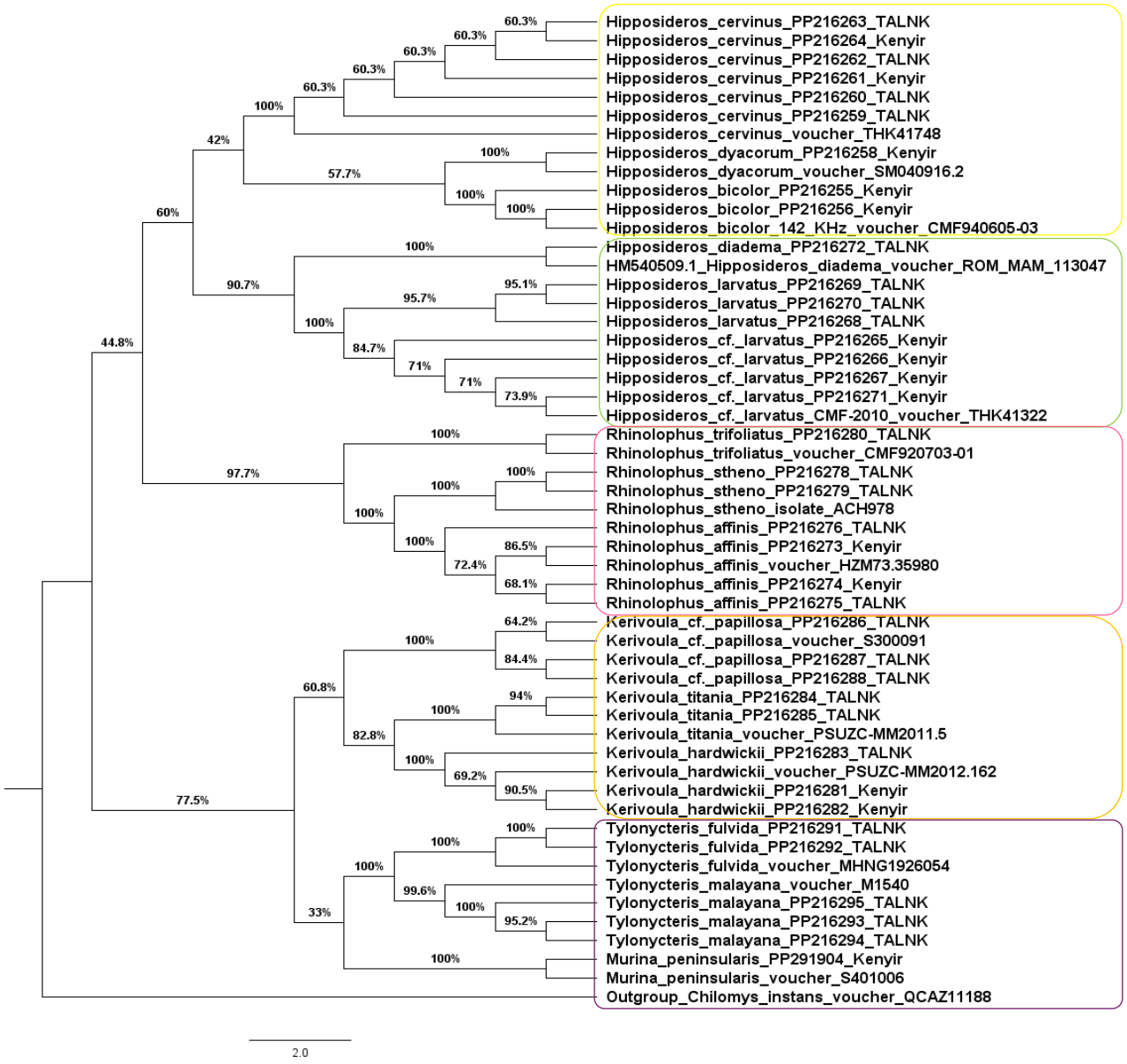

Figure 4. Neighbour-Joining (NJ) tree showing relationships among bats sampled from two selected forest reserve in Peninsular Malaysia

Table 5. DNA barcode gap between 15 bat species from Kenyir and TALNK forest reserve in Peninsular Malaysia, including four singleton species with N/A mean intra-specific value

Species	Mean intra-sp	Max intra-sp	Nearest species	Distance to NN
Hipposideros bicolor	0.5	0.5	*Hipposideros dyacorum*	14.74
Hipposideros cervinus	0	0	*Hipposideros dyacorum*	16.9
Hipposideros cf. *larvatus*	0	0	*Hipposideros larvatus*	0.5*
Hipposideros diadema	N/A	0	*Hipposideros* cf. *larvatus*	14.16
Hipposideros dyacorum	N/A	0	*Hipposideros bicolor*	14.74
Hipposideros larvatus	0.11	0.17	*Hipposideros* cf. *larvatus*	0.5*
Rhinolophus affinis	0.67	1.00	*Rhinolophus stheno*	**6.87**
Rhinolophus stheno	0.17	0.17	*Rhinolophus affinis*	**6.87**
Rhinolophus trifoliatus	N/A	0	*Rhinolophus affinis*	16.85
Kerivoula cf. *papillosa*	0.56	0.83	*Kerivoula hardwickii*	17.19
Kerivoula hardwickii	0.44	0.67	*Kerivoula titania*	16.18
Kerivoula titania	0	0	*Kerivoula hardwickii*	16.18
Murina peninsularis	N/A	0	*Kerivoula* cf. *papillosa*	**26.97**
Tylonycteris fulvida	0.5	0.5	*Tylonycteris malayana*	15.22
Tylonycteris malayana	0.33	0.5	*Tylonycteris fulvida*	15.22

Note: N/A is not available

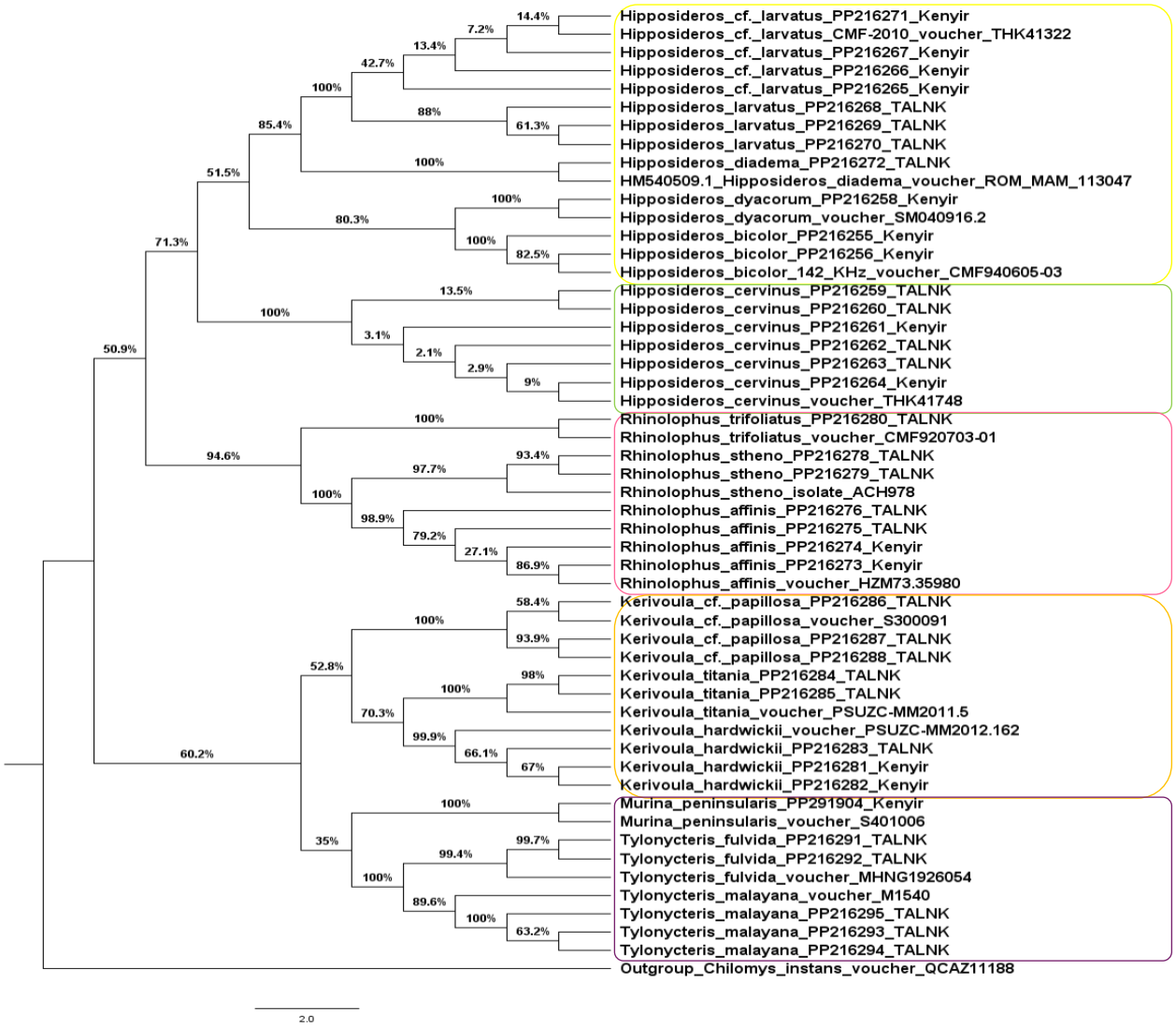

Figure 5. Maximum Likelihood (ML) tree showing relationships among bats sampled from two selected forest reserves in Peninsular Malaysia

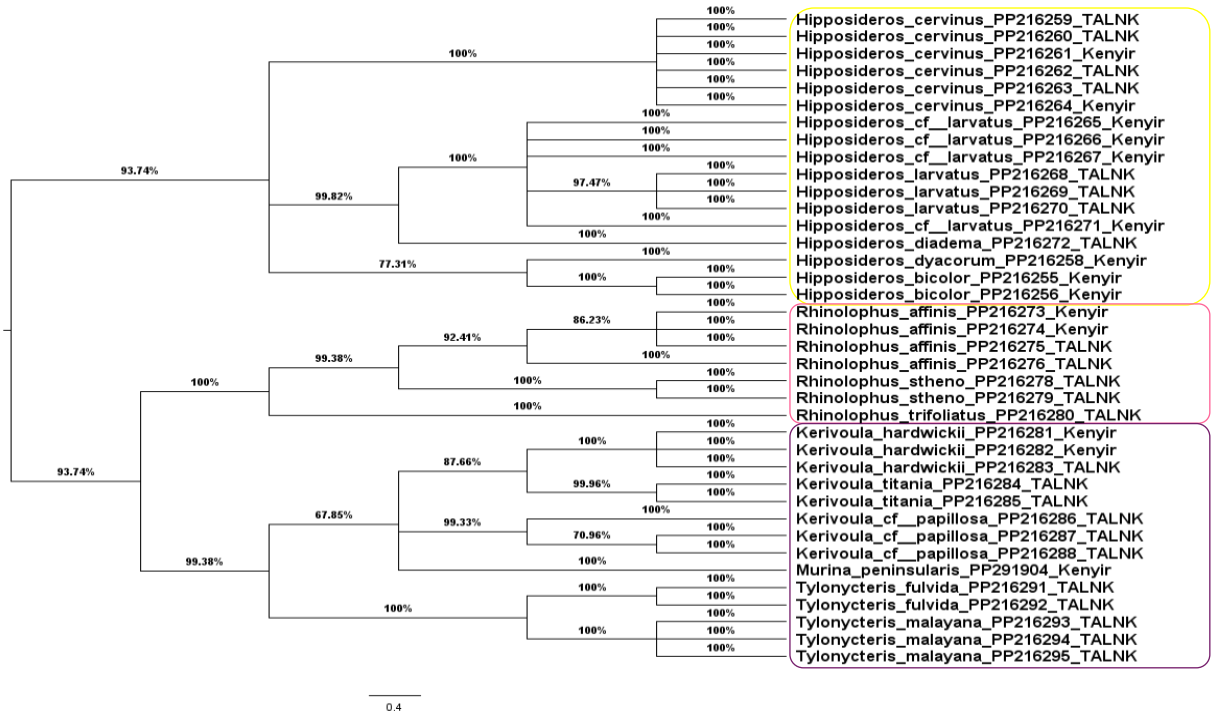

Figure 6. Bayesian Inference (BI) tree showing relationships among bats sampled from two selected forest reserve in Peninsular Malaysia

Table 6. Molecular diversity statistics of bats species from Peninsular Malaysia based on concatenated mitochondrial COI gene sequences

Species/locations	n	Polymorphism sites	h	Hd±SD	π±SD
Hipposideros bicolor	2	51	2	1.000±0.500	0.0050±0.0025
Hipposideros cervinus	6	0	1	0	0
Hipposideros larvatus	3	1	2	0.667±0.099	0.0011±0.0005
Hipposideros cf. *larvatus*	4	0	1	0	0
Hipposideros dyacorum	1	-	1	-	-
Hipposideros diadema	1	-	1	-	-
Rhinolophus affinis	4	8	4	1.000±0.177	0.0066±0.0018
Rhinolophus trifoliatus	1	-	1	-	-
Rhinolophus stheno	2	1	2	1.000±0.500	0.0016±0.0008
Kerivoula hardwickii	3	4	2	0.667±0.314	0.0044±0.0020
Kerivoula titania	2	0	1	0	0
Kerivoula cf. *papillosa*	3	5	2	0.667±0.314	0.0055±0.0026
Tylonycteris malayana	3	3	2	0.667±0.314	0.0033±0.0015
Tylonycteris fulvida	2	3	2	1.000±0.500	0.0049±0.0024
Murina peninsularis	1	-	1	-	-

Note: n: Number of individuals included in the dataset, h: Number of observed haplotypes, Hd: Haplotype diversity±1 SD, π: Nucleotide diversity±SD

The limitations of morphological identification have led to the use of DNA barcodes to identify bat species (Fahim et al. 2024). The species were identified using sequences of COI gene, and phylogenetic analysis was conducted for confirmation at the species level. The successful barcoding of bat samples in this study confirms that the mitochondrial COI gene is the standard barcoding marker for mammals and can be used to reliably identify most species (Kruskop and Artyushin 2021). Based on the comparison between the sequences and the available data in the GenBank and BOLD databases (Table 2), one member of the genus *Hipposideros* had differences between its molecular identification and field classification. Based on COI sequences, an individual that was initially identified morphologically as *H. bicolor* was reassigned to *H. dyacorum* through molecular identification. This shows that inadequate data could lead BLAST searches to yield ambiguous results. Hence, the availability of high-quality reference sequences in public sequence libraries (BOLD and GenBank) is crucial to prevent contradictions in species validation (Azmir et al. 2020).

The values of intraspecific and interspecific genetic distances computed (Table 4) are parallel to those stated by Hebert et al. (2003), where across a wide range of taxa, intraspecific genetic distances based on K2P are typically low (below 1%) and almost never greater than 2%. Moreover, sequence divergence analyses within bat species typically show a range of 1% to 2.5% (Foley et al. 2015). The mean interspecific distance was 16 times greater than the mean intraspecific distance. This finding is consistent with the 34-fold difference in Jamaican bats (Lim and Hernandez 2016) and the 13-fold difference in Guyana neotropical bats (Clare et al. 2007). Thus, this result aligns with the DNA barcoding principle, where interspecific divergence sufficiently outscores intraspecific divergence (Bingpeng et al. 2018). Less than 2% of genetic distances were found within a species, whereas the average genetic distances between genera and families exceeded 5%, significantly surpassing the genetic distances within species. Thus, the result of this study supports the requirements stated in the BOLD system, where it is essential to have a low sequence divergence within species compared to sequence divergence at higher taxonomic levels (Wan Zainal Azhar and Azmir 2019).

Based on the average intraspecific values (Table 5), most species (11/15) displayed less than 2% intraspecific divergence. According to Mota et al. (2022), in relation to COI genes in bats, genetic distance values of less than 2% indicate intraspecific variation, values between 2% and 11% indicate interspecific variation (requiring a thorough taxonomic analysis), and values greater than 11% indicate the existence of distinct species. The highest distance to the nearest neighbor was between *M. peninsularis* and *K.* cf. *papillosa* which is 26.97%. Despite being members of the same family (Vespertilionidae), *K.* cf. *papillosa* belongs to the subfamily Kerivoulinae, whereas *M. peninsularis* belongs to the subfamily Murininae. Moreover, this study supports the morphological differences between the two species, where the members of the genus *Kerivoula* are distinguished by their long woolly fur, funnel-shaped ear, and elongated rostrum (Hasan and Abdullah 2011), while the members of the subfamily Murininae are distinguished by their distinctive tubular nostrils, thick and woolly fur, and relatively well-developed anterior upper premolars (Yu et al. 2020). Besides, *Kerivoula* is known as an interior forest-dwelling bat that forages in cluttered environments and is often found in the understory of tall secondary forests (Mohd-Hanif et al. 2015). Meanwhile, *Murina* is found in a range of forest environments, including semi-evergreen, hill evergreen, and lowland dipterocarp forests (Soisook 2013). The lowest distance to the nearest neighbor recorded was between *H.* cf. *larvatus* and *H. larvatus*. *H.* cf. *larvatus* and *H. larvatus* are distinct taxa of the *H. larvatus* species complex. Although the specimens in question are very closely related, the "cf." indicates some uncertainty in the identification, suggesting that they are not unquestionably *H. larvatus* (Yuzefovich et al. 2022). The nearest neighbor value of 0.5% in this study, however, makes it abundantly evident that *H.* cf. *larvatus* and *H. larvatus* captured are the same species.

All the phylogenies of NJ, ML, and Bayesian methods, inferred from 603-604 bp of the mtDNA COI gene, resulted in most of the specimens of the same species being clustered together, which reflects the prior taxonomic assignment based on morphology. However, the current study was unable to reveal the ancestral lineage of the family, as they were supported by only moderate bootstrap values, which could be attributed to the short sequence length analyzed, small sample sizes, and incomplete representation of the entire Hipposideridae, Rhinolophidae, and Vespertilionidae species in Malaysia (Sazali et al. 2011). Although the morphology of the Rhinolophidae and Hipposideridae is very similar, Miller (1907) was the first to distinguish between the two families (Gu et al. 2008). According to some molecular findings, the Rhinolophidae and Hipposideridae are sister taxa, but the current study demonstrates that they diverged approximately 35 million years ago (Sazali et al. 2011). Foley et al. (2015) stated that there is still uncertainty regarding the taxonomic and phylogenetic relationships between the closely related Rhinolophidae and Hipposideridae. However, the constructed NJ, ML, and Bayesian trees in our results support the divergence of the families Rhinolophidae and Hipposideridae.

The grouping of *H. bicolor* and *H. dyacorum* (14.74% mean sequence divergence) a similar arrangement in every tree and is generally supported by a previous study (Sazali et al. 2011). According to Sazali et al. (2011), these species are similar in that they lack lateral leaflets and have a simple noseleaf for facial ornamentation. In addition, the clustering among *H. diadema* and *H. larvatus* (14.16% mean sequence divergence) is supported by Kingston et al. (2006), as these species have three or more lateral leaflets. Besides, these two species can be easily identified by the forearm length: *H. larvatus* forearm ranges from 52 to 65 mm, whereas *H. diadema* forearm ranges from 76 to 87 mm (Kingston et al. 2006). The remaining species, *H. cervinus*, is independently clustered, and this species has two lateral leaflets (Sazali et al. 2011). In the family Rhinolophidae, the superior connecting process and sella shape were used to distinguish the species within this family (Kingston et al. 2006). Based on the constructed trees, *R. trifoliatus* was grouped separately from *R. affinis* and *R. stheno* (6.87% mean sequence divergence), which were grouped. As recorded by Kingston et al. (2006), *R. affinis* has a round connecting process with a concave sella; *R. stheno* has a round connecting process with a sella having almost parallel sides. In comparison, the noseleaf of *R. trifoliatus* has lateral lappets at the base of the sella with broadly rounded superior connecting process. Vespertilionidae is the largest bat family, with approximately 500 species known to date and is divided into several subfamilies (Monadjem et al. 2021). It is hard to distinguish between species in the Vespertilionidae as they have the same features, and some species look identical to one another. The resulting phylogenetic tree makes a clear distinction between species within the family Vespertilionidae based on their subfamilies. The genus *Tylonycteris* was grouped separately from another subfamily in the resulting tree. Unlike other Vespertilionidae caught in this study, *Tylonycteris* can be easily identified because it has a

different face shape, which is a flattened head (Kingston et al. 2006). Another group in the tree consists of the genera *Kerivoula* and *Murina*. *Kerivoula* is characterized by its long woolly fur, funnel-like ear, and elongated rostrum (Hasan and Abdullah 2011), while *Murina* possesses prominent tubular nostrils and a rounded ear with a long-pointed tragus with a notch at the base of the posterior margin (Kingston et al. 2006).

All living things evolve through adaptive means, and genetic diversity enables them to adapt to changing environmental conditions (Richards et al. 2016). According to the genetic diversity levels found in this study (Table 6), 8 of the analyzed bat species most likely maintain genetic diversity levels that could aid in their conservation if changes to the environment continue at the current rate (Cruz-Salazar et al. 2018). Populations with a high level of genetic diversity are more likely to be able to adapt to environmental disturbances and survive (Basith et al. 2021). Hence, the high genetic diversity value recorded in this study suggests that the bat populations in the TALNK forest are in good condition and have a good chance of surviving. Furthermore, the high haplotype diversity seen in *H. bicolor*, which can inhabit a variety of habitats, particularly the primary forest, indicates a high level of genetic diversity. This species is thought to be resilient to environmental changes in any habitat (Mubarok et al. 2023). Furthermore, the absence of genetic diversity in *H. cervinus* and *K. titania* could be attributed to sweeping selection or a probable recent bottleneck, both of which would result in a sharp decline in genetic diversity levels (Cruz-Salazar et al. 2018). Genetic analysis of species provides useful information about the scales at which anthropogenic activities have an impact on wild species, as well as information about successful demographic management of wild species (Sovic et al. 2016). Advances in molecular techniques have transformed systematics and improved taxonomy for some more complex chiropteran species, where molecular genetics has revealed many discoveries in the taxonomy of understudied and species-rich tropical areas (Clare et al. 2007). Additionally, in temperate fauna where relative species diversity is low, molecular genetics has resolved taxonomic uncertainties (Idnan et al. 2021).

In conclusion, the COI barcode sequences for the selected 38 bat species from Kenyir and TALNK forest reserves were amplified successfully in this study. The universal primer pairs used in this study were able to amplify the target, implying that DNA barcoding could serve as a global standard for identifying bat species. Thus, this finding provides information regarding the use of DNA barcoding in identifying and measuring the diversity of captured bat species in Kenyir and TALNK forest reserves. The findings of this study have direct implications for bat conservation in Malaysia. Particularly in tropical regions with the highest biodiversity, land-use changes, habitat destruction, and deforestation pose a serious threat to bat populations. Accurate species identification is essential for monitoring population trends, determining conservation priorities, and evaluating biodiversity. DNA barcoding is a reliable and efficient way to identify bat species, especially for cryptic taxa that are difficult to distinguish by morphology alone. The finding of *Rhinolophus trifoliatus*, a species that is regarded as near threatened, highlights the need for continued monitoring in the Kenyir and TALNK forest reserves. As natural pest controllers, pollinators, and seed dispersers, bats are vital to maintaining ecosystem balance. The dynamics of insect populations, crop yields, and plant reproduction may all be impacted by a decline in bat populations, disrupting these ecological services. As a result, preserving forested habitats is crucial for both biodiversity preservation and sustainable agriculture. In future conservation efforts, DNA barcoding should be integrated into habitat restoration, community-based conservation programs, and long-term ecological monitoring. By enabling authorities to detect population declines and develop evidence-based protection strategies for these ecologically significant mammals, the establishment of genetic databases for Malaysian bat species will further enhance conservation initiatives.

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