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The First Complete Mitogenome of *Hipposideros diadema* (Diadem Leaf-nosed Bat)

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ABSTRACT

This study presents the first complete mitochondrial genome of the *Hipposideros diadema* (Diadem Leaf-nosed Bat) from Hulu Terengganu Permanent Forest Reserve, Terengganu, Malaysia. The mitochondrial genome was 16,912 bp in length featuring 37 functional genes. It consists of 13 protein-coding genes, 22 transfer RNA genes and 2 ribosomal RNA genes including control region (*D-loop*). Moreover, the genome nucleic acid consists of 31.6% A, 26.2 % T, 28.2 % C, and 14% G. Mitogenome analyses revealed a distinct lineage of *H. diadema* that was previously unrepresented in existing records. Consequently, this study provides a novel complete mitochondrial genome for the species.

Keywords: Bat, Hipposideridae, mitochondrial genome, Terengganu

INTRODUCTION

The Diadem Leaf-nosed Bat (*Hipposideros diadema* É. Geoffroy Saint-Hilaire in 1813) was first described from the Andaman Islands, India (Srinivasulu *et al*., 2016). This species is widely distributed across Southeast Asia, including mainland regions such as Myanmar, Thailand, Vietnam, Laos, Cambodia and Peninsular Malaysia (Kitchener *et al*., 1992; Francis, 2019). In insular Southeast Asia, it is found on the islands of Sumatra, Java, Borneo and several eastern Indonesian islands extending to New Guinea (Tjong *et al*., 2014). Additionally, the species has been recorded in the Philippines (Lucidos *et al*., 2020) and parts of the Western Pacific, including regions of northern Australia and the Solomon Islands (Hourigan, 2011). This species typically roosts in large colonies within caves, often alongside other species, but has also been observed in tree hollows or roosting alone beneath palm fronds (Francis, 2019; Monadjem *et al*., 2019). *Hipposideros diadema* hunts by hanging from a perch and ambushing passing prey, and it forages across a variety of forested habitats, including heavily disturbed areas (Kingston *et al*., 2006). *H. diadema* is classified as a species of Least Concern on the IUCN Red List of Threatened Species for Chiroptera (Waldien & Aguilar, 2021). However, the absence of a complete mitochondrial genome sequence for *H. diadema* reveals a notable gap in the existing genetic data for this species. Thus, the objective of this study was to characterize the first complete mitogenome of a Malaysian *H. diadema* lineage to expand the available genomic data for the genus. The complete mitochondrial genome of *H. diadema* offers crucial insights for resolving phylogenetic relationships within the order Chiroptera and serves as a foundation for future evolutionary and genetic studies.

MATERIALS AND METHODS

Sample Collection and Identification

One male specimen of *H. diadema* was collected during the Scientific Expedition on the Biodiversity of the Hulu Terengganu Permanent Forest Reserve (HTPFR) in Terengganu, Malaysia (4.969°N, 102.951°E), conducted from 7th to 11th October 2024. The specimens were identified based on their morphological characteristics, using the taxonomic key for bats of Peninsular Malaysia by Kingston *et al*. (2006). HTPFR is a forest reserve located in the Hulu Terengganu District in the state of Terengganu, Malaysia. It shares boundaries with the Tembat Forest Reserve, Hulu Terengganu Forest Reserve, Pasir Raja Barat Forest Reserve, and Taman Negara (National Park). The HTPFR was gazetted on 31 December 1958, covering an area of 11,260.30 hectares (Jabatan Perhutanan Negeri Terengganu, 2015). For forest management, it is fully supervised and protected by the Terengganu State Forestry Department through the Western Terengganu District Forestry Office under the National Forestry Act 1984. This forest reserve comprises a lowland dipterocarp forest, hill dipterocarp forest, and upper dipterocarp forest.

Ethical Approval

Ethical clearance for this study was obtained from the Institutional Animal Care and Use Committee (IACUC) at Texas Tech University (Protocols IACUC 10014-04 and IACUC 18011-03), granted to Juliana Senawi. The research was conducted with permission from the Terengganu State Forestry Department during the Scientific Expedition on the Biodiversity of the Hulu Terengganu Permanent Forest Reserve in Terengganu, Malaysia (Ref: PHNT.100/58/2/15 Bhg.3(15)). *H. diadema* is not classified as a globally endangered species, and all animal procedures were conducted in accordance with the ethical standards set out in the ARRIVE guidelines.

Tissue and DNA Extraction

A male *Hipposideros diadema* specimen was preserved in 97% ethanol and deposited in the Mammal Collection of the Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, under the voucher number JS241008.13. For genomic analysis, a tissue biopsy was obtained via a small incision in the pectoral muscle. Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's protocol with minor modifications to the lysis incubation time to ensure complete tissue digestion. DNA integrity was further confirmed through 1.0% agarose gel electrophoresis.

Mitogenome Sequencing and Analysis

A DNA sequencing library was constructed and paired end reads (150 bp) were sequenced by Illumina NovaSEQ6000 (Illumina, Malaysia). The Paired-end reads were adapter-trimmed using fastp v0.21 (Chen *et al*., 2018) and was assembled *de novo* using MEGAHIT v1.29 assembler (Li *et al*., 2015) and followed by Mitogenome-derived contigs were identified using the mitoZ v3.5 mitogenome assembly pipeline. Mitogenome annotation, including identification of protein-coding genes, tRNAs, and rRNAs, was performed using the mitoZ v3.5 annotation pipeline (Meng *et al*., 2019).

Phylogenetic Tree Analysis

The phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method in MEGA 12 (Kumar *et al*., 2024). The General Time Reversible (GTR) substitution model was selected as the optimal model based on the lowest Bayesian Information Criterion (BIC) and Akaike Information Criterion (AICc) scores. To accommodate rate heterogeneity across sites, a discrete Gamma distribution (+G) with five categories (α = 1.3461$) and a proportion of evolutionarily invariant sites (+I = 52.62%) were incorporated into the model. Quality Control (QC) involved the manual inspection of the 11,402-position alignment to remove ambiguous base calls and ensure the exclusion of misaligned gaps. The statistical robustness of the inferred tree topology was assessed through adaptive bootstrap of 5% threshold method. To determine the phylogenetic position of the newly sequenced mitogenome, 15 comparative *Hipposideros* sequences including *Rhinolophus* were retrieved from GenBank (Supplementary Table 2).

RESULTS AND DISCUSSION

Morphological taxonomic examination confirmed the specimen as a male *Hipposideros diadema*, based on diagnostic features of the noseleaf and forearm length. The fur on the underparts is dark brown with a pale base, and distinct white patches are present on the shoulders and sides. No other Asian species of *Hipposideros* displays pale shoulder patches (Kingston *et al*., 2006). The nose leaf features three or four lateral leaflets, with the posterior portion being large and rounded (Figure 1).

Figure 1. *Hipposideros diadema* collected on 9th October 2024 from Hulu Terengganu Permanent Forest Reserve

The complete mitochondrial genome was 16,912 bp in length, comprising 37 genes (13 protein-coding genes, 22 transfer RNAs, and 2 ribosomal RNAs) and a non-coding control region of *D-loop* (Figure 2). Moreover, the genome nucleic acid consists of 31.6% A, 26.2% T, 28.2% C, and 14% of G. The PCGs utilized the standard mitochondrial start codon (10 with ATG, 3 with ATA) and the regular stop codons (TAA or TAG) except for *CYTB* (AGA). The incomplete stop codon was observed in *ND4* (T*) as stop codons with asterisk indicate incomplete stop codons that are presumably polyadenylated after transcription to form complete TAA stop codons (Supplementary Table 1).

Phylogenetic analysis of the *H. diadema* mitogenome was performed based on 15 *Hipposideros* spp. and *Rhinolophus luctus* as an outgroup, as in (Figure 3). We concatenated 13 PCG sequences from 16 sequences of bats (Supplementary Table 2) and performed multiple alignments using MUSCLE (Edgar, 2004). Subsequently, a maximum-likelihood (ML) phylogenetic tree was constructed by Mega 12 (Kumar *et al*., 2024) with adaptive bootstrap of 5% threshold. The evolutionary rate differences among sites were modelled using a discrete Gamma distribution across 5 categories (+G, parameter = 1.3461), with 52.62% of sites deemed evolutionarily invariant (+I). The analytical procedure encompassed 16 nucleotide sequences with 11,402 positions in the final dataset. The results revealed that *H. diadema* formed a monophyletic lineage clearly separated from other *Hipposideros* species. Within the family Hipposideridae, this lineage was positioned as a sister clade to the Rhinolophidae (Figure 3).

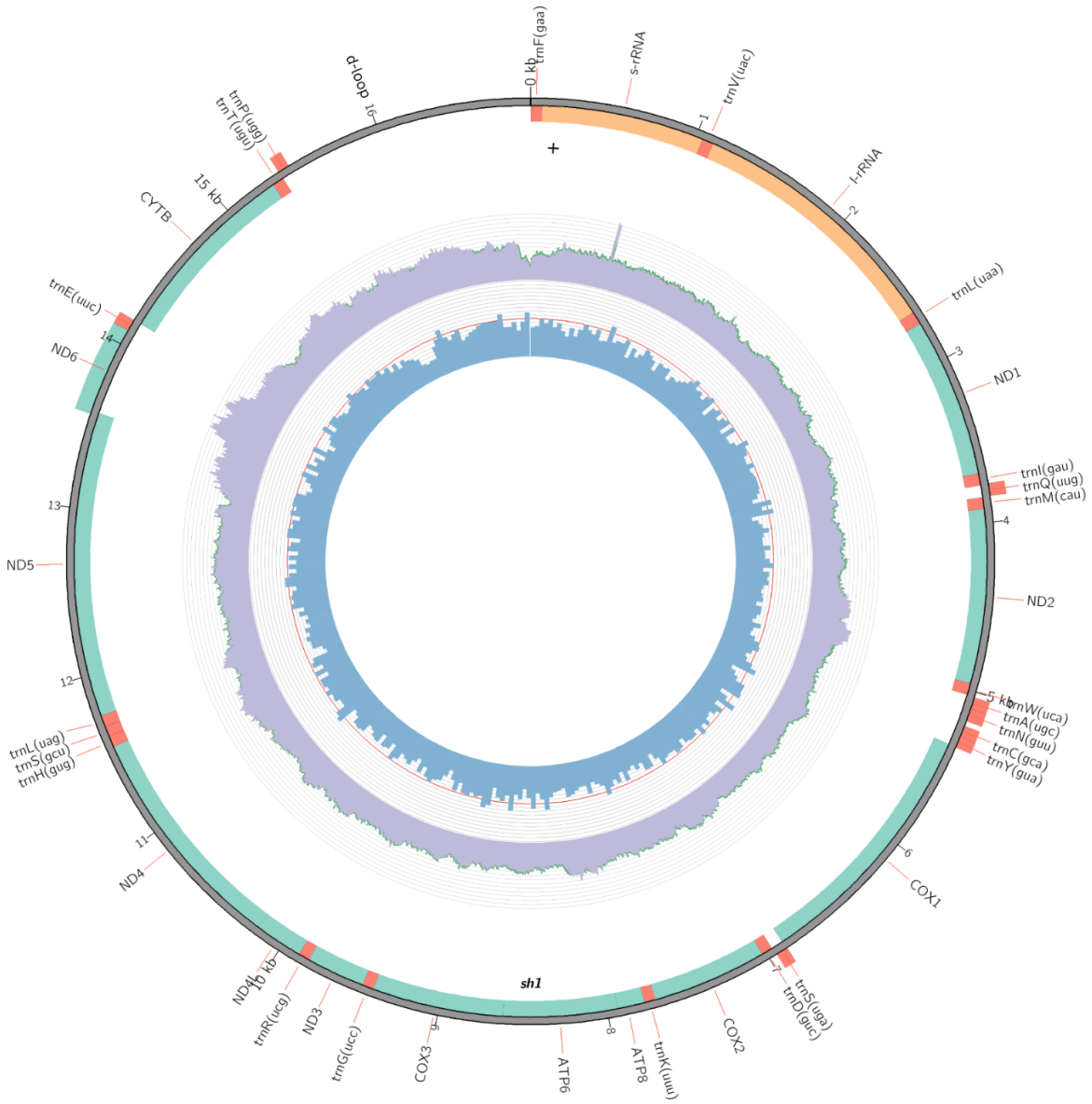

Figure 2. Circular map of the complete mitochondrial genome of *Hipposideros diadema* (16,912 bp). The mitogenome comprised 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes, together with a non-coding control region (D-loop). Gene orientation is indicated on the outer circle, while the inner rings represent GC content and GC skew across the mitochondrial genome

This arrangement is consistent with the expected taxonomic placement of *H. diadema* as a distinct species within the *Hipposideros* genus. Although our analysis included only a single *H. diadema* mitogenome, its phylogenetic position matches the expected taxonomy of the family. The specimen clustered within the *Hipposideros* clade, separate from species such as *H. armiger* and *H. larvatus*. This confirms that morphological identification is supported by molecular data. Furthermore, the high-level divergence between the Hipposideridae and Rhinolophidae clades reflects the deep evolutionary split between these two families, which is a well-documented feature of rhinolophoid evolution (Teeling, 2005).

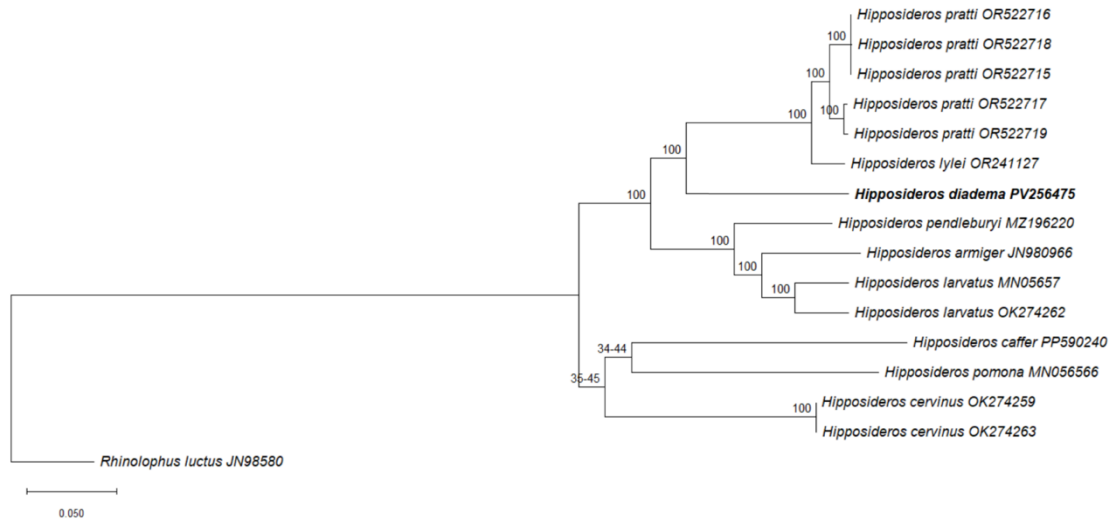

Figure 3. Maximum likelihood phylogenetic tree based on concatenated nucleotide sequences of 13 mitochondrial protein-coding genes from 15 *Hipposideros* species, with *Rhinolophus luctus* (JN98580) used as the outgroup

CONCLUSION

This study presents the first complete mitochondrial genome of *H. diadema* from Malaysia. The genome is 16,912 bp long, with 37 genes including D-loop, and has a nucleotide composition of 31.6% A, 26.2% T, 28.2% C, and 14.0% G. The phylogenetic analysis confirmed that *H. diadema* forms a distinct lineage within the *Hipposideros* genus, consistent with morphological classifications. This study reports a novel mitochondrial lineage of *H. diadema*, providing the first complete genomic record for this specific genetic group. These findings serve as a foundation for resolving the broader evolutionary history of the *H. diadema* species complex and the family Hipposideridae.

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