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Molecular Identification and Phylogenetic Analysis of the Indian Leaf-Nosed Bat (*Hipposideros speoris*) from Tamil Nadu, India

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ABSTRACT
Accurate species identification is essential for understanding biodiversity, evolutionary relationships, and conservation of bats. However, morphological similarities among closely related species often limit traditional taxonomic approaches. The present study employed mitochondrial cytochrome c oxidase subunit I (COI) gene sequencing to investigate the molecular identity and phylogenetic relationship of the Indian leaf-nosed bat, *Hipposideros speoris*, from Tirunelveli District, Tamil Nadu, India. Genomic DNA was extracted from wing membrane tissue samples using the phenol–chloroform method, and a partial COI gene fragment of approximately 650 bp was amplified using universal Folmer primers. The amplified products were sequenced and compared with reference sequences available in the NCBI GenBank database. Phylogenetic analysis was performed using the Neighbour-Joining method based on Kimura 2-Parameter genetic distances with 1,000 bootstrap replications. The obtained COI sequence was successfully identified as *H. speoris* and clustered within the genus *Hipposideros* with strong bootstrap support. Genetic distance analysis revealed a divergence of 0.1638 between the present study sequence and the reference *H. speoris* sequence, while interspecific divergence with other *Hipposideros* species ranged from 0.1463 to 0.1891. The highest divergence was observed with the outgroup species *Taphozous melanopogon* (0.2477). The findings demonstrate the effectiveness of COI-based DNA barcoding for molecular identification and phylogenetic assessment of bats and provide valuable insights into the genetic diversity and evolutionary relationships of *H. speoris*.

Keywords: *Hipposideros speoris*, COI gene, DNA barcoding, molecular identification, phylogenetic analysis, Chiroptera.

1. INTRODUCTION

Bats (Order: Chiroptera) are the second-largest group of mammals, comprising more than 1,400 species worldwide and playing vital ecological roles as pollinators, seed dispersers, and biological pest controllers. Their ecological importance contributes significantly to ecosystem functioning and biodiversity conservation. However, accurate identification of bat species remains a major challenge because many species exhibit similar morphological characteristics, resulting in taxonomic ambiguities and the existence of cryptic species complexes (Clare *et al.,* 2007; Mayer *et al.,* 2007).

Traditional morphological methods often fail to distinguish closely related species due to overlapping external features and intraspecific variation. To overcome these limitations, molecular approaches have become indispensable tools in modern taxonomy and biodiversity research. Among various molecular markers, mitochondrial DNA (mtDNA) has been widely used because of its high mutation rate, maternal inheritance, and lack of recombination, making it highly informative for species-level identification and phylogenetic studies (Avise, 2000). Beyond species identification, COI sequences provide valuable information on evolutionary relationships and genetic diversity. Phylogenetic analyses using mitochondrial markers help clarify lineage divergence, species relationships, and historical biogeographic patterns. Molecular studies have revealed substantial cryptic diversity within several bat genera, demonstrating the importance of integrating genetic evidence with morphological and ecological data for accurate species delimitation (Mayer & Von Helversen, 2001; Juste *et al.,* 2013).

Accurate species identification is fundamental for effective conservation strategies. Genetic identification methods offer a reliable solution by enabling precise species recognition, even when voucher specimen collection is restricted by ethical, legal, or conservation considerations (Wilson *et al.,* 2014). Non-lethal tissue sampling techniques, such as wing membrane biopsies and tail membrane samples, have further facilitated molecular research while minimizing impacts on natural populations (Chen *et al.,* 2010).The development of international initiatives such as the Barcode of Life Data System (BOLD) has greatly expanded the availability of reference DNA sequences for species identification and comparative studies (Ratnasingham & Hebert, 2013). Advances in computational biology and phylogenetic software, including MEGA, BEAST, and MrBayes, have further strengthened molecular phylogenetic research (Drummond & Rambaut, 2007; Ronquist *et al.,* 2012).

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Therefore, the present study focuses on the molecular identification and phylogenetic analysis of selected chiropteran bats using the mitochondrial cytochrome c oxidase subunit I (COI) gene. The study aims to assess genomic DNA quality, amplify COI gene fragments through PCR, and infer phylogenetic relationships using Neighbour-Joining and Maximum Likelihood methods. By integrating molecular techniques with evolutionary analyses, this study contributes to improved species identification, taxonomic resolution, and the conservation of bat biodiversity.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The study was conducted in Tirunelveli District, Tamil Nadu, India. Adult individuals of *Hipposideros speoris* were captured using mist nets and hand-capture methods near roosting and foraging sites. All procedures followed the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes, 2016). Wing membrane tissue samples (approximately 5 mm) were collected aseptically from healthy bats and preserved in 95% ethanol at –20°C until further analysis.

2.2 Genomic DNA Extraction

Genomic DNA was isolated from preserved wing membrane tissues using the phenol-chloroform extraction method described by Sambrook and Russell (2001). Approximately 20–30 mg of tissue was digested overnight in extraction buffer containing Proteinase K at 55°C. Following complete tissue digestion, phenol-chloroform-isoamyl alcohol extraction was performed to separate DNA from proteins and other cellular components. The extracted DNA was precipitated using chilled ethanol, washed with 70% ethanol, and dissolved in nuclease-free water. DNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometric analysis. High-quality genomic DNA showing distinct bands without degradation was selected for downstream molecular analyses.

2.3 PCR Amplification of the COI Gene

A partial fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using universal barcode primers LCO1490 (5′ GGTCAACA AATCATAA AGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) developed by Folmer *et al.* (1994). These primers amplify approximately 650 base pairs of the COI gene and are widely used in DNA barcoding studies.PCR reactions were performed in a total reaction volume of 25 µL containing template DNA, PCR buffer, $MgCl_2$, dNTPs, forward and reverse primers, Taq DNA polymerase, and nuclease-free water. Amplification was carried out using a thermal cycler under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 48–50°C for 45 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Amplified products were separated on 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. A DNA ladder was used to verify the expected amplicon size.

2.4 Purification and DNA Sequencing

PCR products showing clear and distinct amplification were purified using the Bionteq Gel Elution Kit according to the manufacturer's instructions. Purification removed residual primers, nucleotides, enzymes, and other contaminants that could interfere with sequencing reactions. The purified PCR products were subjected to bidirectional sequencing using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit. Sequencing reactions were analysed using an ABI automated capillary DNA sequencer. Both forward and reverse sequence reads were obtained to ensure sequence accuracy and reliability.

2.5 Sequence Editing and Alignment

Raw chromatogram files obtained from sequencing were manually inspected and edited using Chromas Pro software. Ambiguous bases, low-quality regions, and sequencing artefacts were removed. Forward and reverse sequences were assembled to generate a consensus sequence for each specimen. The edited sequences were aligned using Clustal X version 2.0 software to identify conserved and variable nucleotide sites. To verify the authenticity of the mitochondrial COI sequences, all nucleotide sequences were translated into amino acid sequences to ensure the absence of stop codons, insertions, deletions, or frame-shift mutations.

2.6 Molecular Identification

The consensus COI sequences obtained from *Hipposideros speoris* were compared with available sequences in the NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST). Species identification was confirmed based on the highest percentage similarity, maximum query coverage, and lowest E-value. The generated sequences were further compared with previously published COI sequences of related species to evaluate genetic similarity and divergence.

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2.7 Phylogenetic Analysis

Phylogenetic relationships were analysed using Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura *et al.,* 2021). The aligned COI sequences of *Hipposideros speoris* and related reference sequences retrieved from GenBank were used for tree construction. Genetic distances were calculated using the Kimura 2-Parameter (K2P) model. Phylogenetic trees were constructed using the Neighbour-Joining (NJ) method to evaluate the evolutionary relationships among taxa. The resulting tree topology was examined to determine species clustering and genetic affinity with related Hipposideridae bats.

2.8 Bootstrap Analysis

The reliability and statistical support of the phylogenetic tree were assessed using bootstrap analysis following the method of Felsenstein (1985). A total of 1,000 bootstrap replicates were performed in MEGA version 11. Bootstrap values were displayed at the corresponding nodes of the phylogenetic tree. Branches with bootstrap support values greater than 70% were considered well supported, indicating a high level of confidence in the inferred evolutionary relationships. The bootstrap analysis provided an assessment of the robustness and stability of the phylogenetic placement of *Hipposideros speoris* within the family Hipposideridae.

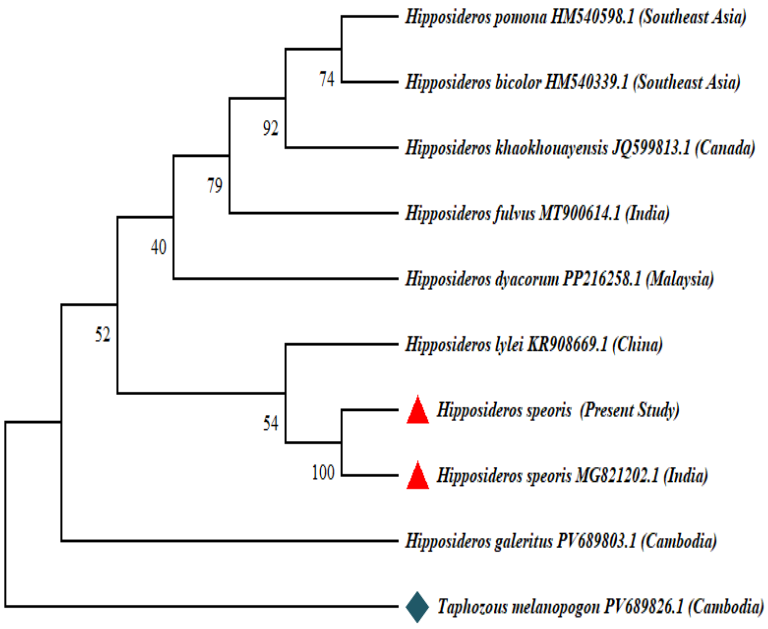
3. RESULT

The mitochondrial cytochrome c oxidase subunit I (COI) gene fragment of *Hipposideros speoris* was successfully amplified and sequenced. The amplified fragment produced a clear PCR band of approximately 650 bp, indicating successful amplification of the target mitochondrial region. After sequence editing and trimming of low-quality terminal regions, a high-quality consensus sequence was obtained and used for subsequent molecular analyses.

The obtained COI sequence was compared with homologous sequences available in the NCBI GenBank database. BLAST analysis confirmed that the sequence belonged to the genus *Hipposideros*, showing high similarity with previously reported *H. speoris* sequences. The sequence generated in the present study was deposited and analysed together with representative COI sequences of closely related species within the family Hipposideridae.

Phylogenetic reconstruction based on the Neighbour-Joining (NJ) method using the Kimura 2-Parameter (K2P) model produced a well-resolved tree topology (Figure 3.1). The sequence obtained in the present study clustered within the *Hipposideros* lineage and formed a distinct branch associated with *H. speoris*. The overall phylogenetic structure clearly separated species belonging to the genus *Hipposideros* from the outgroup taxon *Taphozous melanopogon*. All major clades received strong bootstrap support values, with several nodes showing 100% bootstrap support, indicating a high level of confidence in the inferred evolutionary relationships.

Figure 3.1 Phylogenetic relationship among the partial cytochrome c oxidase subunit I (COI) sequences of the family Hipposideridae.



The pairwise genetic distance matrix revealed considerable genetic divergence among the analysed taxa (Table 3.1). The genetic distance between the present study sequence and the reference *H. speoris* sequence (MG821202.1, India) was 0.1638. This divergence indicates measurable genetic differentiation between the studied population and the

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reference population. Despite this divergence, both sequences were placed within the same phylogenetic assemblage, supporting their taxonomic association within *H. speoris*.Among the analysed congeners, the genetic distance values between *H. speoris* and other *Hipposideros* species ranged from 0.1463 to 0.1891. The lowest divergence was observed between *H. speoris* and *H. galeritus* (0.1463), followed by *H. lylei* (0.1512) and *H. dyacorum* (0.1572). Moderate divergence values were observed with *H. fulvus* (0.1688) and *H. bicolor* (0.1755). The highest divergence among congeners was recorded with *H. pomona* (0.1891) and *H. khaokhouayensis* (0.1805). These results indicate substantial genetic differentiation among species of the genus *Hipposideros*. The genetic distance matrix further demonstrated that all examined species formed genetically distinct entities. No overlap was observed between the divergence values of the analysed taxa, indicating clear species boundaries based on COI sequence variation. The observed levels of divergence are consistent with species-level differentiation reported for other members of the family Hipposideridae.

The outgroup species, *Taphozous melanopogon*, exhibited the highest genetic divergence from *H. speoris*, with a pairwise K2P distance of 0.2477. Similarly high divergence values were observed between *T. melanopogon* and all other *Hipposideros* species included in the analysis. In the phylogenetic tree, the outgroup was clearly separated from the Hipposideridae clade and occupied a basal position, confirming its suitability for rooting the tree and validating the phylogenetic relationships observed among the ingroup taxa.

The clustering pattern observed in the NJ tree corresponded closely with the genetic distance data. Species with relatively lower genetic distances tended to occupy neighbouring positions within the phylogeny, whereas highly divergent taxa formed separate branches. This congruence between phylogenetic topology and pairwise distance estimates supports the reliability of the COI marker for assessing evolutionary relationships within the genus *Hipposideros*. The present molecular analysis demonstrated that the COI gene possesses sufficient discriminatory power to distinguish *H. speoris* from other closely related species. The combination of distinct phylogenetic placement, measurable genetic divergence, and strong bootstrap support provides robust evidence for the molecular identification of the studied specimen. Furthermore, the observed genetic differentiation highlights the existence of evolutionary divergence within the genus and contributes valuable information toward understanding the genetic diversity and phylogeographic structure of *Hipposideros* bats.

Species	*Hipposideros speoris* (Present Study)	*Hipposideros speoris* MG821202.1	*Hipposideros pomona* HM540598.1	*Hipposideros dyacorum* PP216258.1	*Hipposideros fulvus* MT900614.1	*Hipposideros khaokhouayensis* JQ599813.1	*Hipposideros bicolor* HM540339.1	*Hipposideros galeritus* PV689803.	*Hipposideros lylei* KR908669.1 (China)	*Taphozous melanopogon*
Hipposideros speoris (Present Study)	0									
Hipposideros speoris MG821202.1 (India)	0.1638	0								
Hipposideros pomona HM540598.1 (Southeast Asia)	0.1891	0.1616	0							
Hipposideros dyacorum PP216258.1(Malaysia)	0.1572	0.1534	0.1388	0						
Hipposideros fulvus MT900614.1 (India)	0.1688	0.1547	0.1322	0.1490	0					
Hipposideros khaokhouayensis JQ599813.1 (Canada)	0.1805	0.1583	0.1112	0.1517	0.1507	0				
Hipposideros bicolor HM540339.1 (Southeast Asia)	0.1755	0.1638	0.0843	0.1326	0.1348	0.0958	0			
Hipposideros galeritus PV689803.1 (Cambodia)	0.1463	0.1335	0.1566	0.1357	0.1480	0.1539	0.1566	0		
Hipposideros lylei KR908669.1 (China)	0.1512	0.1415	0.1588	0.1379	0.1529	0.1561	0.1616	0.0104	0	
Taphozous melanopogon PV689826.1 (Cambodia)	0.2477	0.2120	0.2452	0.2295	0.2508	0.2300	0.2211	0.2428	0.2459	0

Table 3.1 Kimura 2-Parameter (K2P) Genetic pairwise distance (%) Matrix for Selected Hipposideridae Species Based on COI Sequences

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4. DISCUSSION

The mitochondrial cytochrome c oxidase subunit I (COI) gene analysis conducted in the present study provided valuable insights into the taxonomic identity and phylogenetic position of *Hipposideros speoris*. The generated COI sequence clustered within the genus *Hipposideros* and formed a well-supported lineage in the Neighbour-Joining phylogenetic tree. The high bootstrap support values obtained for the major nodes indicate that the inferred phylogenetic relationships are robust and statistically reliable. Similar observations have been reported in previous molecular studies, where COI sequences successfully discriminated species of Hipposideridae and resolved taxonomic relationships among closely related taxa (Clare *et al.,* 2007; Francis *et al.,* 2010).

The genetic distance between the *H. speoris* sequence generated in the present study and the reference sequence (*H. speoris*, MG821202.1) was 0.1638 (16.38%). This value is considerably higher than the levels of intraspecific divergence generally reported for mammalian COI sequences, which are often below 2–5% (Hebert *et al.,* 2003; Baker *et al.,* 2009). Such elevated divergence may indicate substantial genetic differentiation between geographically separated populations. Geographic isolation, restricted gene flow, local adaptation, and historical demographic processes may contribute to the accumulation of genetic variation within populations of *H. speoris*. Despite the relatively high genetic divergence, both sequences were recovered within the same major lineage in the phylogenetic tree. This pattern suggests that the analysed specimens share a common evolutionary origin while exhibiting notable genetic differentiation. Similar patterns have been documented in several bat taxa, where geographically isolated populations exhibit deep mitochondrial divergence despite retaining morphological similarities (Mayer & von Helversen, 2001; Clare, 2011). Such divergence may represent population structuring, phylogeographic differentiation, or the early stages of lineage diversification.

Comparison with other species of the genus *Hipposideros* further demonstrated the distinctiveness of *H. speoris*. The interspecific genetic distances observed between *H. speoris* and other congeners ranged from 14.63% to 18.91%, indicating substantial evolutionary separation among species. The relatively high divergence values are consistent with previous studies reporting considerable mitochondrial differentiation among members of Hipposideridae (Francis *et al.,* 2010; Lim *et al.,* 2017). The clear separation of *H. speoris* from *H. fulvus*, *H. bicolor*, *H. pomona*, *H. galeritus*, *H. dyacorum*, *H. lylei*, and *H. khaokhouayensis* supports its recognition as a genetically distinct taxon. The phylogenetic tree topology also demonstrated that *H. speoris* occupied a separate branch within the genus, reflecting its independent evolutionary history. The concordance between the pairwise genetic distance matrix and phylogenetic clustering pattern provides strong evidence for the effectiveness of the COI gene in resolving species-level relationships within Hipposideridae. Such congruence between genetic distance and phylogenetic reconstruction has been considered an important criterion for reliable molecular taxonomy (Nei & Kumar, 2000; Dayrat, 2005).

The highest genetic divergence was observed between *H. speoris* and the outgroup species *Taphozous melanopogon* (approximately 24.6%). This result reflects the distant evolutionary relationship between the families Hipposideridae and Emballonuridae and confirms the suitability of *T. melanopogon* as an outgroup in the present phylogenetic analysis. The clear separation of the outgroup from the *Hipposideros* clade further supports the validity of the tree topology. The substantial genetic divergence observed within *H. speoris* may have important taxonomic implications. Increasing evidence from molecular studies suggests that many bat species comprise genetically structured populations or cryptic evolutionary lineages that remain undetected through morphology alone (Clare *et al.,* 2007; Francis *et al.,* 2010; Clare, 2011). Therefore, the divergence observed in the present study may reflect previously unrecognized genetic diversity within *H. speoris*. However, confirmation of such taxonomic status requires additional evidence from morphology, morphometrics, acoustics, ecology, and nuclear genetic markers.

Overall, the present study demonstrates that the mitochondrial COI gene is an effective molecular marker for the identification and phylogenetic assessment of *Hipposideros speoris*. The observed genetic divergence, distinct phylogenetic placement, and strong bootstrap support collectively indicate the presence of significant evolutionary differentiation within the species. These findings contribute to a better understanding of the genetic diversity, evolutionary history, and taxonomic relationships of *H. speoris* and highlight the importance of integrative approaches in bat systematics and conservation biology.

5. CONCLUSSION

The present study demonstrated the effectiveness of mitochondrial cytochrome c oxidase subunit I (COI) gene sequencing for the molecular identification and phylogenetic assessment of *Hipposideros speoris*. The obtained COI sequences successfully confirmed the taxonomic placement of the studied specimens within the family Hipposideridae and provided reliable genetic data for evaluating evolutionary relationships. Phylogenetic analyses based on the Neighbour-Joining method and Kimura 2-Parameter genetic distance model revealed a distinct lineage of *H. speoris* with strong bootstrap support, indicating the robustness of the inferred relationships. Genetic distance analysis revealed substantial divergence between the present study sequence and available reference sequences, suggesting the presence of considerable genetic variation within *H. speoris* populations. The clear separation of *H. speoris* from other congeners further highlights the utility of COI barcoding as a powerful tool for species discrimination and molecular taxonomy in bats. Overall, the findings contribute to the understanding of genetic diversity and evolutionary relationships within Hipposideridae and emphasize the importance of integrating molecular approaches into bat biodiversity studies. Further

investigations involving broader geographic sampling, larger population sizes, and additional molecular markers are recommended to better understand the phylogeographic structure and taxonomic status of *H. speoris* across its distribution range.

6. REFERENCE

1. Avise, J. C. (2000). *Phylogeography: The history and formation of species*. Harvard University Press.
2. Baker, R. J., Bininda-Emonds, O. R. P., Mantilla-Meluk, H., Porter, C. A., & Van Den Bussche, R. A. (2009). Species boundaries and genetic diversity in New World bats. *Occasional Papers, Museum of Texas Tech University*, 282, 1–16.
3. Chen, S. F., Jones, G., Rossiter, S. J., & Zhang, S. Y. (2010). Non-destructive tissue sampling methods for bats and their application in molecular studies. *Acta Chiropterologica, 12*(1), 151–158.
4. Clare, E. L., Lim, B. K., Engstrom, M. D., Eger, J. L., & Hebert, P. D. N. (2007). DNA barcoding of Neotropical bats: Species identification and discovery within Guyana. *Molecular Ecology Notes*, **7**(2), 184–190. https://doi.org/10.1111/j.1471-8286.2006.01657.x
5. Clare, E. L. **(**2011). Cryptic species? Patterns of maternal and paternal gene flow in bats. *Molecular Ecology*, 20(15), 3197–3199. https://doi.org/10.1111/j.1365-294X.2011.05183.x
6. Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85(3), 407–415. https://doi.org/10.1111/j.1095-8312.2005.00503.x
7. Drummond, A. J., & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology, 7*, 214. https://doi.org/10.1186/1471-2148-7-214
8. Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*(4), 783–791. https://doi.org/10.2307/2408678
9. Francis, C. M., Borisenko, A. V., Ivanova, N. V., Eger, J. L., Lim, B. K., Guillén-Servent, A., Kruskop, S. V., Mackie, I., & Hebert, P. D. N. (2010). The role of DNA barcodes in understanding and conservation of mammal diversity in Southeast Asia. *PLoS ONE*, **5**(9), e12575. https://doi.org/10.1371/journal.pone.0012575
10. Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3*(5), 294–299.
11. Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R**.** (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, **270**(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218
12. Juste, J., Benda, P., García-Mudarra, J. L., & Ibáñez, C. (2013). Phylogeny and systematics of Old World bats. *Acta Chiropterologica, 15*(1), 1–14.
13. Lim, B. K., Engstrom, M. D., Reid, F. A., Simmons, N. B., Voss, R. S., & Fleck, D. W. (2017). Molecular phylogenetics and species delimitation in bats. *Zootaxa*, 4254**(**1), 1–42.
14. Mayer, F., Dietz, C., & Kiefer, A. (2007). Molecular species identification boosts bat diversity. *Frontiers in Zoology, 4*, 4. https://doi.org/10.1186/1742-9994-4-4
15. Mayer, F., & von Helversen, O. (2001). Cryptic diversity in European bats. *Proceedings of the Royal Society B: Biological Sciences, 268*(1478), 1825–1832. https://doi.org/10.1098/rspb.2001.1744.
16. Nei, M., & Kumar, S. (2000). *Molecular evolution and phylogenetics*. Oxford University Press.
17. Ratnasingham, S., & Hebert, P. D. N. (2013). A DNA-based registry for all animal species: The Barcode Index Number (BIN) system. *PLoS ONE, 8*(7), e66213. https://doi.org/10.1371/journal.pone.0066213
18. Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology, 61*(3), 539–542. https://doi.org/10.1093/sysbio/sys029
19. Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor Laboratory Press.
20. Sikes, R. S., & the Animal Care and Use Committee of the American Society of Mammalogists. (2016). Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. *Journal of Mammalogy, 97*(3), 663–688. https://doi.org/10.1093/jmammal/gyw078
21. Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Molecular Biology and Evolution, 38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120
22. Wilson, J. J., Sing, K. W., & Sofian-Azirun, M. (2014). Building a DNA barcode reference library for bats. *PLoS ONE, 9*(10), e110060. https://doi.org/10.1371/journal.pone.01100.