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Analysis of the complete mitochondrial genome sequence of *Hipposideros pratti*

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ABSTRACT

In order to explore the characteristics of the mitochondrial genome sequence of Pratt's leaf-nosed bat (*Hipposideros pratti* Thomas 1891) and understand their phylogenetic status in Chiroptera, this study determined the mitochondrial genome sequences of *H. pratti* from five regions in China using high-throughput sequencing technology, sequence assembly, and genome annotation. The results showed that these sequences contained 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and 1 non-coding region, all exhibiting a significant AT bias. Based on the phylogenetic tree constructed using 13 protein-coding genes from 15 Chiroptera species, the study found that *H. pratti* from the five regions clustered together, and then clustered with *H. lylei* into a single clade. Meanwhile, *H. pratti* from Jiangxi, Fujian, and Guangdong regions of China showed closer genetic relationships, while *H. pratti* from Yunnan and Henan regions of China exhibited closer genetic relationships. This study not only supplemented the mitochondrial genome database of *H. pratti* but also laid a foundation for genetic variation, molecular classification, and evolutionary studies of *H. pratti*.

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Introduction

Hipposideros pratti, belonging to the genus *Hipposideros* in the family Hipposideridae of the order Chiroptera, is a large bat species in its family, second only to the *H. armiger* in size. It is mainly distributed in countries such as Myanmar, Thailand, Vietnam, Malaysia and China, with a notable presence particularly in southern China (Robinson et al. 2003). *H. pratti* exhibits strong selectivity toward its habitat, typically inhabiting caves for over half of its time. It prefers to roost near the entrance of caves that are deep and wide and often contain underground rivers (Figure 1). Current research on *H. pratti* mainly focuses on its distribution range, taxonomic status, habitat selection, echolocation mechanisms (Yang 2018), embryonic development (Wang et al. 2010), the evolution of functional genes (Li et al. 2006), and gastrointestinal bacteria (Yuan et al. 2017). However, there are no reported studies on the complete mitochondrial genome of this bat. This study primarily analyzed and compared the complete mitochondrial genomes of *H. pratti* from Fujian, Guangdong, Henan, Jiangxi, and Yunnan provinces in China, aiming to provide fundamental data for subsequent in-depth research on this species. Additionally, phylogenetic analysis was conducted based on 13 protein-coding genes to explore the relationships between *H. pratti* from these five regions and other bats of the genus *Hipposideros*.

Materials and methods

Sample collection and preservation

The study collected samples of *H. pratti* from caves in five distinct regions of China: Fujian (26°15′15″N, 118°16′55″E), Guangdong (24°32′19″N, 116°09′31″E), Henan (33°34′24.13″N, 112°06′14.42″E), Jiangxi (28°59′37.6″N, 114°12′19.2″E), and Yunnan (28°7′24.87″N, 104°14′25.67″E) (Figure S1). Using non-invasive sampling techniques, mist nets or scoop nets were strategically placed at the entrances of these caves, where the bats resided. As the bats emerged from their roosts, they were gently captured, and wing membrane tissue was extracted using a puncher. Following the sampling process, the bats were immediately released back into their natural habitats. The collected specimens were subsequently preserved in 95% ethanol and stored at a controlled temperature of −20 °C within the College of Life Sciences at Henan Normal University, awaiting further analysis and utilization. (Accession number: JT325; JT326; JT327; JT328; JT329.Tiantian Jiang, 2801317102@qq.com).

DNA sequencing and genome assembly

The sample was fragmented by sonication to a size of 350 bp. The fragments were polished, A-tailed, and ligated

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Figure 1. Image of an adult *hipposideros pratti*, photo was taken by Xinping He (unpublished photo), permission has been obtained from the photographer.

with full-length adapters for Illumina sequencing, followed by PCR amplification (primer sequences in Table S1). PCR products were purified using the AMPure XP system (Beckman Coulter, Beverly, USA), and DNA concentration was measured by Qubit®3.0 Flurometer (Invitrogen, USA). Library size distribution was analyzed by NGS3K/Caliper and quantified by real-time PCR (3 nM). Clustering was done on a cBot system with Illumina PE Cluster Kit (Illumina, USA). Sequencing on Illumina platform yielded 150 bp paired-end reads, generating 65.02 G of raw data, filtered to 64.50 G of clean data. SPAdes (version v3.14.1) (Bankevich et al. 2012) assembled and spliced the data (Figure S2 for sequencing depth maps). MITOS (http://mitos.bioinf.uni-leipzig.de/index.py) predicted mitochondrial coding proteins, tRNAs, and rRNAs. Initial predictions were refined and corrected for high accuracy.

Figure 2. The mitochondrial gene structure map of *Hipposideros pratti*.

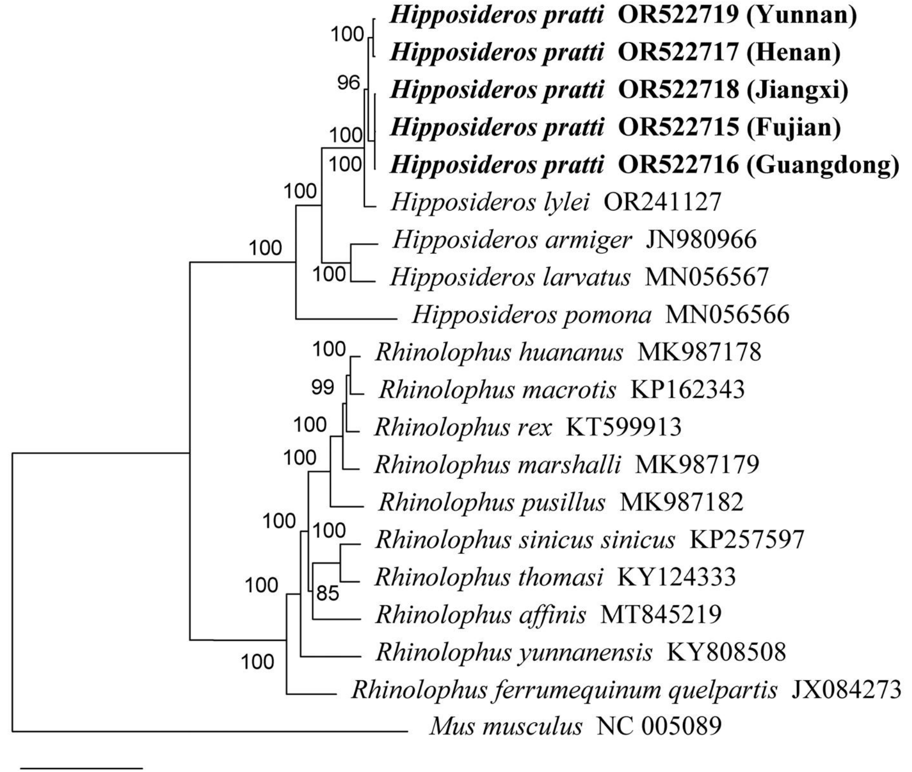

Figure 3. Phylogenetic tree construction using ML method based on 13 protein coding genes with *Mus musculus* as an outgroup. The following sequences were used: OR241127 (He et al. Unpublished), JN980966 (Xu et al. 2012), MN056567 (Liu 2019), MN056566 (Liu 2019), MK987178 (Zhang et al. 2021), KP162343 (Zhang et al. 2016), KT599913 (Shi et al. 2016), MK987179 (Zhang et al. 2021), MK987182 (Zhang et al. 2021), KP257597 (Sun et al. 2015), KY124333 (Xing and Mao 2016), MT845219 (Ding et al. 2021), KY808508 (Huang et al. 2017), NC_005089 (Bayona-Bafaluy et al. 2003).

Phylogenetic tree construction

In order to determine the phylogenetic relationships among *H. pratti* from five regions, this study retrieved mitochondrial genome sequences of bats from four species of the genus *Rhinolophus* and ten species of the genus *Hipposideros* from the NCBI database (Table S2). Combined with the gene sequences measured in this study, and using the sequence of *Mus musculus* as the outgroup, we employed MAFFT 7.037 (Katoh and Standley 2016) to align the 13 protein-coding genes across 15 species. The aligned data were then imported into the PhyloSuite software (Zhang et al. 2020), where Model Finder (Kalyaanamoorthy et al. 2017) was used to calculate and select the most suitable model: the TPM2 + F + I + G4 model. Maximum likelihood analysis was performed using IQ-TREE (Nguyen et al. 2015), and a bootstrap test with 5000 repetitions was conducted to evaluate the nodal support values within the phylogenetic tree. Finally, the phylogenetic tree was then visualized using FigTree v1.4.4 (Andrew Rambaut, University of Edinburgh, http://tree.bio.ed.ac.uk/software/figtree/).

Result

The genomes of *H. pratti* from the five regions all displayed a closed-loop double-stranded circular structure with lengths ranging from 16,833 bp to 16,836 bp (Figure 2). These genomes shared similar components, including 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes and a D-loop (Table S3 for Mitochondrial Genome Coding Gene Content of *Hipposideros Pratti*), while the adenine (A) content was the highest at 31.6% to 31.7%, followed by cytosine (C) at 27.8% to 27.9%, thymine (T) at 26.5% to 26.6%, and guanine (G) was the least abundant at 13.9% to 14.0%. Among the 13 protein-coding genes, 10 started with ATG, 3 with ATA (CYTB, ND5, and ND2), and different termination codons were observed, including TAA in six genes (COX1, COX2, ND4L, ND5, ND6, and ATP6), T in three genes (ND4, COX3, and ND2), AGA in one gene (CYTB), TA in one gene (ND1), and TAG in one gene (ND3).

The phylogenetic tree constructed in this study obtained high support degrees at most nodes (Figure 3). The phylogenetic analysis results showed that the bat species of *Hipposideros* and *Rhinolophus* formed two independent monophyletic groups with a support rate of 100% (Figure 3). In the *Hipposideros*, all five regional samples of *H. pratti* clustered together and then clustered with *H. lylei* into a single clade; the *H. armiger* and *H. larvatus* clustered together into a single clade; and *H. pomona* formed a separate clade. This suggests that *H. pratti* is more closely related to *H. lylei*, followed by *H. armiger* and *H. larvatus*, and more distantly related to *H. pomona*.

Discussion

Analysis of these five mitochondrial genome sequences revealed that their gene arrangement is consistent with that

of mitochondrial genomes in Chiroptera and other mammalian animals (Lei et al. 2010; Chiou et al. 2011; Vargas-Trejo et al. 2023). Furthermore, our measurement and analysis of their base content revealed a clear preference for AT, with the content of A + T being higher than that of G + C. This characteristic is similar to that of other Chiroptera animals that have been previously reported (Meganathan et al. 2012).

The phylogenetic tree suggests that *H. pratti* from the five regions should belong to the same subspecies, with closer genetic relationships among those from Jiangxi, Fujian, and Guangdong regions, and closer relationships between those from Yunnan and Henan regions. This finding is consistent with the results reported by He. (2016), who constructed a phylogenetic tree based on Cytochrome b (Cytb) and Cytochrome Oxidase I (COI) genes. Although *H. pratti* and *H. lylei* have relatively close phylogenetic relationships, there still exists significant genetic differentiation. Lu et al. (1965) and Zhang (1997) believed that the *H. lylei* in western Yunnan should be considered as a subspecies *H. p. lylei* of the *H. pratti*. However, Wang. (2003), Smith and Xie (2009) and Corbet and Hill (1992) argue that it should be considered as an independent species. The taxonomic status of *H. pratti* and *H. lylei* remains controversial. To more accurately reflect their evolutionary relationships, comprehensive analyses combining zoological geography, morphology, paleontology, and other methods are needed in future studies.

Ethical approval

The Study involving laboratory animals all follow the ARRIVE guidelines (https://arriveguidelines.org/), and all animal sample collection protocols were in accordance with current Chinese laws. All animal experimental procedures conducted in this study were by the ethical standards approved by the Ethics Committee for the Use of Animal Subjects of Henan Normal University (HNSD-SMKX-2119BS0524).

Author contributions

Conceived and designed the experiments: TJ, JH, JL, LZ, HN and YB. TJ, JL, LZ performed specimen collection, and experimented. Analyzed the data: TJ, LZ and JL. YB and TJ revised it critically for intellectual content, all authors revised the manuscript for intellectual content. All authors are accountable for all aspects of the work.

Disclosure statement

No potential conflicts of interest were reported by the authors.

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Data availability statement

The genome sequence data with the results of this research can be publicly available in NCBI GenBank at https://www.ncbi.nlm.nih.gov/. The registration number is: OR522715 (Fujian), OR522716 (Guangdong), OR522717 (Henan), OR522718 (Jiangxi), OR522719 (Yunnan). The associated BioProject, SRA, and Bio-Sample numbers are PRJNA1057368 (Fujian), SRR27558960 (Fujian), SAMN39137165 (Fujian); PRJNA1057368 (Guangdong), SRR27558959 (Guangdong), SAMN39137166 (Guangdong); PRJNA1057368 (Henan), SRR27558958 (Henan), SAMN39137167 (Henan); PRJNA1057368 (Jiangxi), SRR27558957 (Jiangxi), SAMN39137168 (Jiangxi); PRJNA1057368 (Yunnan), SRR27558956 (Yunnan), SAMN39137169 (Yunnan).

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