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MITOGENOME REPORT

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The complete mitochondrial genome of the Baishanzu horned toad *Boulenophrys baishanzuensis* (Anura: Megophryidae)

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ABSTRACT

The mitochondrial genome (mitogenome) of *Boulenophrys baishanzuensis* (Anura: Megophryidae) was sequenced by the Illumina platform. The assembled circular mitogenome of *B. baishanzuensis* had a total length of 17,040 bp, with a GC content of 41.25%. It consisted of 13 protein-coding genes (PCGs), two rRNA genes, 22 tRNA genes, and a D-loop region. The majority of the PCGs were encoded by the H-strand, while one PCG (*nad6*) and eight tRNA genes (tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser2, tRNA-Glu, and tRNA-Pro) were encoded in the L-strand. Phylogenetic analysis revealed that the newly sequenced species formed a clade with other *Boulenophrys* species, while the genus *Boulenophrys* itself formed a sister group with the genus *Atympanophrys*.

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1. Introduction

The genus *Boulenophrys*, belonging to the order Anura and family Megophryidae of the class Amphibia, encompasses a total of 65 known species worldwide (Frost 2023). These species primarily inhabit various regions in China, with some extending their range southwards to Vietnam, Laos, Thailand, and Myanmar (Frost 2023). In 2021, the discovery of *Boulenophrys baishanzuensis* (Wu et al. 2020), a newly reported species exclusive to the high-altitude areas of the southwestern mountains in Zhejiang Province, China, added to the diversity of the genus *Boulenophrys* (Wu et al. 2020). However, our understanding of *B. baishanzuensis* remains limited as the available genetic information is confined to partial mitochondrial DNA sequences of the 16S rRNA and *cox1* genes (Wu et al. 2020), and a complete mitochondrial genome has yet to be obtained. Only the near-complete mitogenome of *Boulenophrys omeimontis* (Liu 1950) has been reported (Liu et al. 2016), while the GenBank database contains the complete mitogenomes of only two other *Boulenophrys* species. To address this knowledge gap, we utilized next-generation sequencing technology to successfully acquire and analyze the complete mitochondrial genome (mitogenome) of *B. baishanzuensis*. We gained insights into phylogenetic relationships by comparing their sequence with those of other reported species within the family Megophryidae.

2. Materials and methods

2.1. Sample collection

In late July 2020, a male adult *B. baishanzuensis* specimen (Figure 1) was collected from a mountain stream by the roadside in Dongshan'ao (27.72598° N, 119.62780° E; altitude: 1233 m), Jingning County, Zhejiang Province, China. The collected specimen underwent morphological identification and was subsequently preserved in 70% ethanol. It was then deposited at the Museum of Laboratory of Amphibian Diversity Investigation at Lishui University (voucher number: LSU20200721JNJC01, contact person: Guo-Hua Ding; email: guwoding@lsu.edu.cn).

2.2. Methods

The muscle tissue of the specimen was used to extract total DNA. The extraction of whole genomic DNA was performed using an EasyPure genomic DNA kit (TransGen Biotech Co., Beijing, China) following the manufacturer's instructions. The whole genomic DNA sequences were generated on an Illumina NovaSeq 6000 platform (Novogene Bioinformatics Technology Co. Ltd., Tianjin, China) using 150 bp paired-end reads. A total of 21.73 G of raw data were obtained and deposited in the NCBI's Sequence Read Archive database under the accession number SRR13558088.

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Figure 1. The male adult specimen of *Boulenophrys baishanzuensis* used in this study. (A) Side view and (B) back view. Photographed by Guo-Hua Ding.

The de novo assembly of the mitogenome was carried out using NOVOPlasty 3.7 (Dierckxsens et al. 2017). The raw sequencing reads are first aligned to the assembled mitogenome of *B. baishanzuensis* using BWA to generate a SAM alignment file. This SAM file is then converted to BAM format and sorted using samtools for efficient downstream processing. Per-position coverage statistics across the mitogenome were calculated from the sorted BAM file using samtools depth and outputted to a text file. This coverage file is imported into R and processed with tidyverse to group the data by position and calculate the mean coverage per position. Gene annotation of the assembled mitogenome was performed using the MITOS WebServer (http://mitos2.bioinf.uni-leipzig.de/) (Matthias et al. 2013) and tRNAscan-SE version 2.0 (http://trna.ucsc.edu/tRNAscan-SE/) (Lowe and Chan 2016). The annotated mitogenome was deposited in GenBank with the accession number OR063945.

We used DNA barcoding technology to further confirm the accuracy of species identification in this study. We downloaded partial sequences of 16S rRNA and *cox1* genes of six *B. baishanzuensis* individuals from NCBI's GenBank and compared them with the corresponding sequences from our study using MEGA 5.05. Genetic distances, specifically represented by p-distances, were used using MEGA 5.05 to validate the precision of species identification in this study.

To investigate the phylogenetic relationship of *B. baishanzuensis*, we retrieved 18 closely related mitogenomes from the GenBank database, with the mitogenome of *Bombina bombina* (Linnaeus 1761) (accession number: MH893761) selected as the outgroup. The Bayesian inference (BI) method in MrBayes 3.2.7 (Ronquist and Huelsenbeck 2003) was employed to construct a phylogenetic tree using concatenated sequences comprising 13 protein-coding genes (PCGs) and two rRNA genes, resulting in a total length of 14,047 bp. Four parallel runs of Markov Chain Monte Carlo (MCMC) were performed for 1,000,000 generations, with sampling every 1000 generations and discarding the initial 1000 trees as burn-in. The best-fit substitution model (GTR + I + G) was determined using MrModelTest 2.3 (Nylander 2004) in PAUP 4.0.

3. Results

3.1. Species identification via DNA barcoding

Based on comparisons of partial sequences of mitochondrial 16S rRNA and *cox1* genes, the genetic distances between the object specimen and six *B. baishanzuensis* individuals ranged from 0.01 to 0.011 and 0.016 to 0.018, respectively. Consequently, we can conclusively identify the specimen in this study as *B. baishanzuensis*.

3.2. Characteristics of B. baishanzuensis mitogenome

The complete circular mitogenome of the target species covered the reference sequence entirely, and the average read coverage depth was 1633.2× (Figure 2). The complete mitogenome sequence of *B. baishanzuensis* is 17,040 bp long and has a GC content of 41.25% (27.2% A, 31.5% T, 14.6% G, and 26.6% C). It consisted of 13 PCGs, two rRNA genes (12S rRNA and 16S rRNA), 22 tRNA genes, and a control region (D-loop) (Figure 3). The 12S rRNA (924 bp) is between tRNA-Phe and tRNA-Val, while the 16S rRNA (1591 bp) is between tRNA-Val and tRNA-Leu2. Among the 37 genes, 28 are encoded on the H-strand, while nine genes (*nad6*, tRNA-Gln, tRNA-Ala, tRNA-Asn, tRNA-Cys, tRNA-Tyr, tRNA-Ser2, tRNA-Glu, and tRNA-Pro) are located on the L-strand. The *cox1* gene was initiated with GTG, the *nad2* and *nad3* genes started with ATT, and the other 10 PCGs started with ATG. The PCGs contained five types of stop codons: TAA (*cox1*, *atp8*, *nad4L*, and *nad5*), TAG (*nad1*, *nad2*, *nad3*, and *cytb*), AGG (*nad6*), TA (*atp6* and *cox3*), and T (*cox2* and *nad4*).

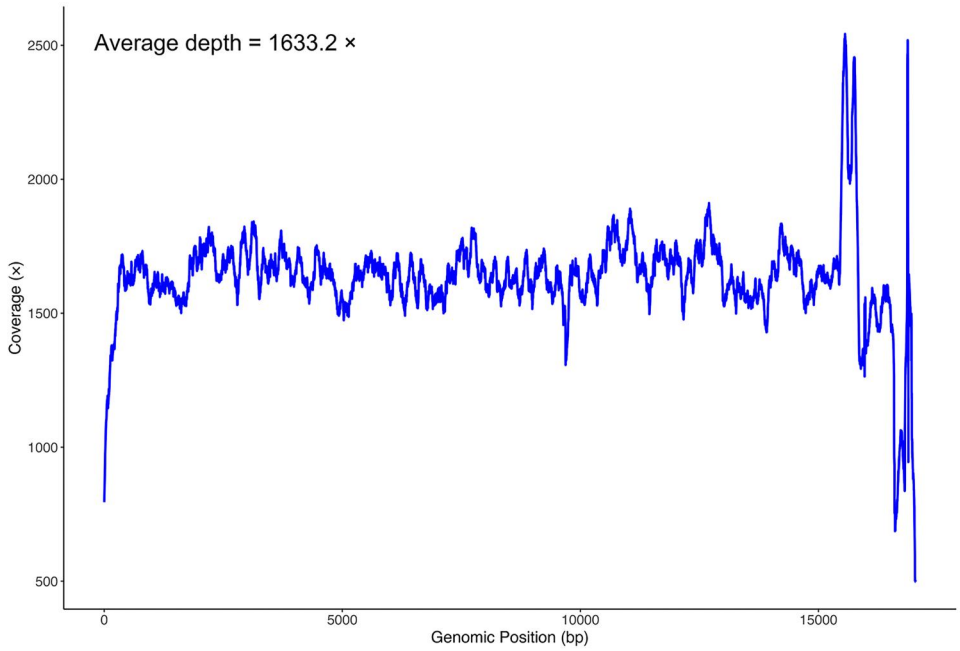

Figure 2. Coverage map of the mitogenome assembly.

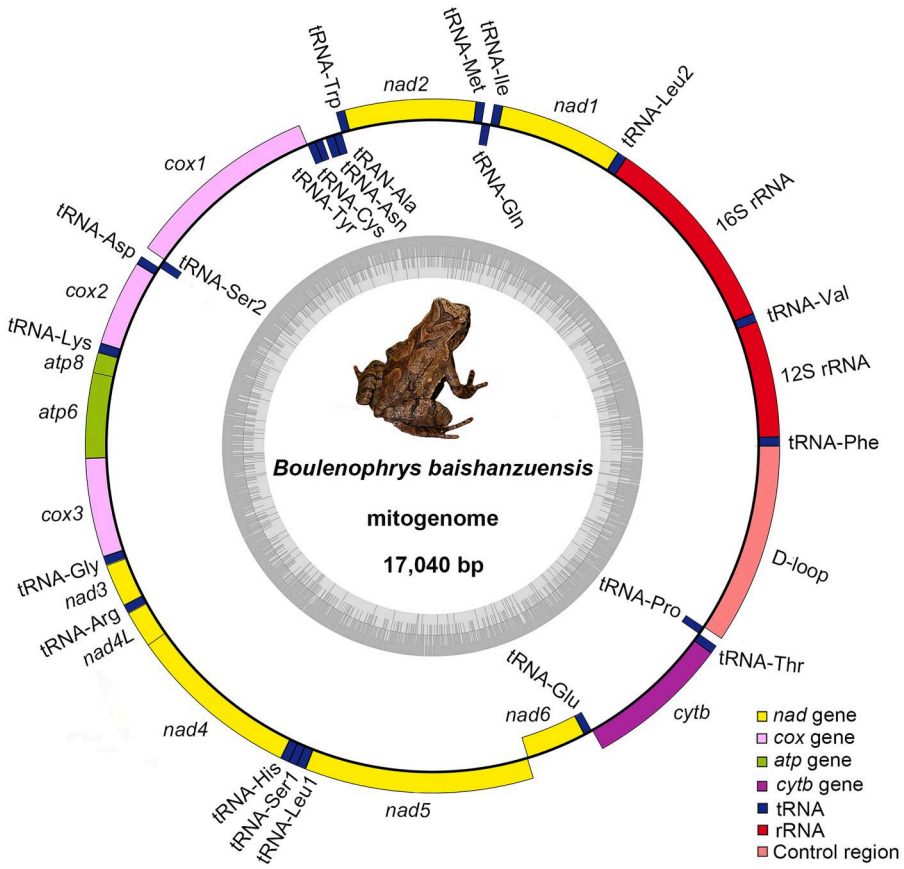

Figure 3. Circular map of the mitogenome of *Boulenophrys baishanzuensis*.

3.3. Phylogenetic position

Phylogenetic analysis revealed that (1) *B. baishanzuensis* formed a cluster with other *Boulenophrys* species; (2) the genus *Boulenophrys* was a sister clade to the genus *Atympanophrys*; and (3) the phylogenetic relationships among the six genera within the family Megophryidae were as follows: (*Boulenophrys* + *Atympanophrys*) + (((*Oreolalax* + *Scutiger*) + *Leptobrachium*) + *Leptobrachella*) (Figure 4).

4. Discussion

The DNA barcoding technology for species identification is an accurate and effective approach, especially when

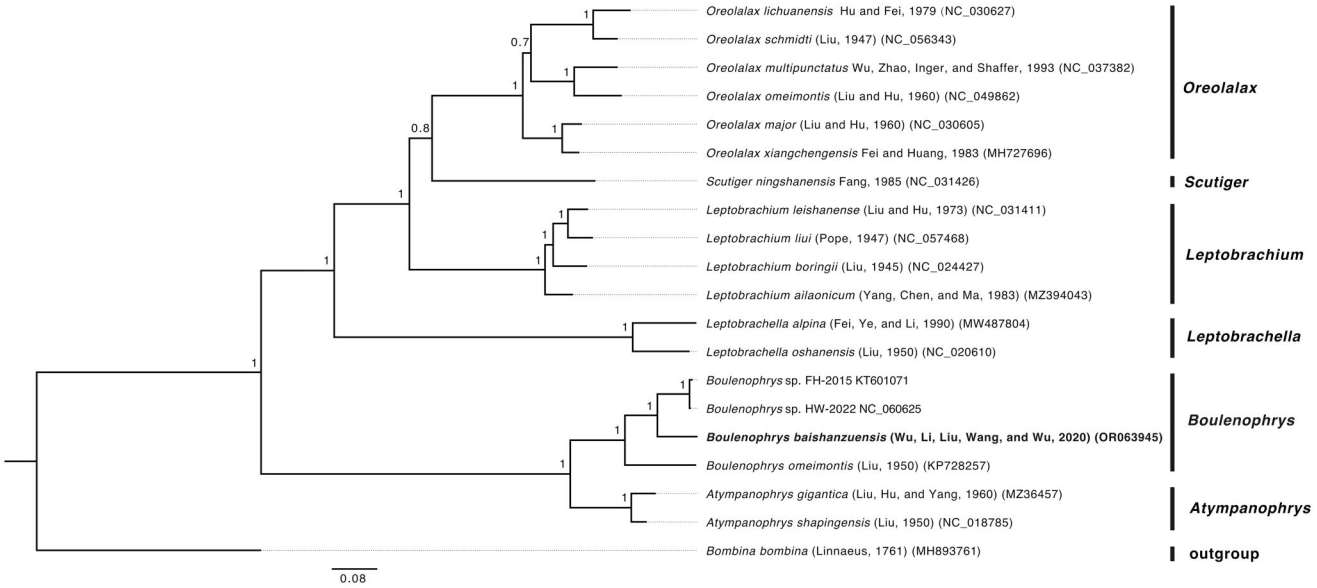

Figure 4. The Bayesian tree based on 13 PCGs and two rRNAs of 19 species from the family Megophryidae. *Bombina bombina* was selected as the outgroup. Numbers at the nodes represent Bayesian posterior probabilities. Samples sequenced in the present study are highlighted in bold.

encountering situations during field surveys where species cannot be precisely identified based on morphological traits. In this study, we confirmed that the collected amphibian specimen was *B. baishanzuensis*. The gene arrangement and composition of the mitogenome of *B. baishanzuensis* were found to be similar to other reported species of the genus *Boulenophrys* (Liu et al. 2016). The subfamily Megophryinae, which includes the genus *Boulenophrys*, was reclassified into 10 genera based on phylogenetic reconstruction and morphological comparisons by Lyu et al. (2023). However, before this reclassification, *Boulenophrys* species were previously classified under the genus *Xenophrys* (e.g. *Boulenophrys lishuiensis*) (Wang et al. 2017), *Megophrys* (e.g. *Boulenophrys baishanzuensis*), or *Panophrys* (e.g. *Boulenophrys sanmingensis*) (Lyu et al. 2021). Therefore, increasing the number of mitogenomes from species within the subfamily Megophryinae will contribute to the taxonomic classification of this subfamily. Our study presented the first complete mitogenome sequence of *B. baishanzuensis*, providing fundamental data for resolving phylogenetic and genetic issues related to the genus *Boulenophrys*.

Author contributions

Jian-Ping Wu: conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft. Jia-Lian Wu: collected data, species identification, and approved the final draft. Yun-Bo Chen: collected data, analyzed the data, and approved the final draft. Wei-Hua Xu: collected data, analyzed the data, and approved the final draft. Wen-Qi Xie: analyzed the data, prepared figures and/or tables, and approved the final draft. Zi-Qiang Tang: collected data, prepared figures and/or tables, and approved the final draft. Guo-Hua Ding: conceived and designed the experiments, and approved the final draft. Li Ma: conceived and designed the experiments, species identification, and approved the final draft.

Ethical approval

The experimental procedures used in this study complied with the current laws related to animal welfare and research in China and were specifically approved by the Animal Research Ethics Committee of Lishui University (#LSU-AREC-202006001). No specific permission was required for the collection site.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are openly available in the GenBank of NCBI at https://www.ncbi.nlm.nih.gov under the accession number OR063945. The associated BioProject, SRA, and Biosample numbers are PRJNA695083, SRR13558088, and SAMN17600862, respectively.

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