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MITOGENOME REPORT

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Two complete mitochondrial genomes of *Boulenophrys* (Anura: Megophryidae: Megophryinae): characteristics and phylogenetic implications

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ABSTRACT

The Chinese horned toads, *Boulenophrys boettgeri* (Boulenger, 1899) and *Boulenophrys kuatunensis* (Pope, 1929), are two captivating species within the family Megophryidae, which inhabit the mountainous streams in the Eastern of China. In this study, two new complete mitochondrial genomes of *B. boettgeri* and *B. kuatunensis* were sequenced, assembled, and annotated using next-generation sequencing. The length of mitochondrial genomes of *B. boettgeri* and *B. kuatunensis* was 16,597 and 17,921 bp, respectively, with both containing 13 protein coding genes, 22 tRNA genes, two rRNA genes, and one putative control region. Phylogenetic relationships based on protein-coding mitochondrial genes showed that the two *Boulenophrys* species formed a cluster with other *Boulenophrys* species. The two new sequences provide valuable insights into the mitochondrial genomes of these two species, offering important data for understanding the phylogenetic relationships of the genus *Boulenophrys*.

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1. Introduction

The genus *Boulenophrys*, also known as the Chinese horned toad, was introduced as a newly discovered taxonomic group by Fei and Ye (2016), based on the absence of both vomerine teeth and vomerine ridge in its species. However, the validity of this genus had remained a contentious issue, and in various cases the genus had been placed variously as a subgenus of *Megophrys* (Mahony et al. 2017) or as an distinct genus (Chen et al. 2017). The genus *Boulenophrys* from its synonymy with *Megophrys* was formally separated by Qi et al. (2021). Lyu et al. (2023) subsequently recognized it as one of the 10 genera within the subfamily Megophryinae, relying on insights from phylogenetic relationships and morphological distinctions.

Up to date, a total of 68 identified species had been documented within the genus *Boulenophrys*, with distribution spanning most regions of China except Xizang region, and stretching southward into Vietnam, Laos, Thailand, and Myanmar (Frost 2024). Notably, over 90% of these species were discovered in China (AmphibiaChina 2024). However, within the GenBank database, only four complete mitochondrial genome sequences for this genus have been publicly released, indicating a significant lack of available data (Liu et al. 2016; Wu et al. 2024). *Boulenophrys boettgeri* (Boulenger 1899) and *Boulenophrys kuatunensis* (Pope 1929) are among the relatively early-discovered species within the genus *Boulenophrys*, dating back to the late eighteenth century and early nineteenth century, and they inhabit stream habitats at different elevations within the Wuyi Mountain range in eastern China (AmphibiaChina 2024).

In this study, we successfully assembled two high-quality complete mitochondrial genomes from the aforementioned species from the genomic data obtained through the next-generation sequencing technology. Additionally, we investigated the features of these two mitochondrial genomes and their phylogenetic relationships within the genus *Boulenophrys*.

2. Materials and methods

2.1. Specimen identification and collection

During the herpetological biodiversity survey in 2020 and 2021, a male *B. boettgeri* specimen was captured in Jiulongshan (N 28.3383°, E 118.8813°, altitude: 1199 m), Suichang, Zhejiang Province, China and a male *B. kuatunensis* was captured in Tongmuguan (N 27.6980, E 117.6599, altitude: 1000 m), Wuyishan, Fujian Province, China. Both specimens matched the identification features described by Ding et al. (2022). *B. boettgeri* has a light-colored semicircular spot on the dorsal shoulder area (Figure 1(A)), while *B. kuatunensis* is typically reddish-brown with X-shaped back markings and distinct white temporal folds (Figure 1(B)). After morphological identification, the specimens were preserved in 70%

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Figure 1. Male adult specimen of (A) *Boulenophrys boettgeri* and (B) *Boulenophrys kuatunensis* used in this study, photographed by Guo-Hua Ding.

ethanol and later transported for permanent storage at the Museum of Laboratory of Amphibian Diversity Investigation, Lishui University (voucher: LSU20200727JLS007 for *B. boettgeri* and LSU20210606WYS001 for *B. kuatunensis*; Contact: Guo-Hua Ding; email: guwoding@lsu.edu.cn).

2.2. Sequencing, genome assembly, and annotation

DNA was extracted from muscle tissue using the EasyPure genomic DNA kit (TransGen Biotech Co., Beijing, China) and two DNA libraries were constructed with the Illumina PE Cluster Kit (Illumina, San Diego, CA). Next-generation sequencing was conducted in paired-end mode on the Illumina NovaSeq 6000 platform (Novogene Bioinformatics Technology Co. Ltd., Tianjin, China), yielding approximately 20 million of raw reads per sample with a read length of 150 bp. NOVOPlasty 3.7 (Dierckxsens et al. 2017) was used for the *de novo* assembly of the mitochondrial genomes of the two *Boulenophrys* species, utilizing a partial reference sequence from *Boulenophrys omeimontis* (KP728257) (Liu et al. 2016). Coverage assessment following Ni et al. (2023) showed average depths of ×874.18 for *B. boettgeri* and ×2465.46 for *B. kuatunensis* (Figure S1), indicating high accuracy and completeness. Gene annotation was conducted using MitoZ v2.4 (Meng et al. 2019) and MITOS WebServer (Matthias et al. 2013), with consistency checks. Mitochondrial genome maps were generated using Proksee (Grant et al. 2023).

2.3. Phylogenetic analysis

In our phylogenetic analysis, we employed previously released mitochondrial genomes (OR063945, KP728257, ON646614, MZ364157, and JX458090) from five species within the subfamily Megophryinae (Xiang et al. 2013; Liu et al. 2016; Wu et al. 2024), along with newly obtained sequences from two *Boulenophrys* species, to investigate phylogenetic relationships. *Leptobrachella alpina* (MW487804; Shu et al. 2021) from the subfamily Leptobrachiinae served as the outgroups. All species belong to the family Megophryidae. The nucleotide sequences of the 13 protein-coding genes (PCGs) were extracted using PhyloSuite v1.2.1 (Zhang et al. 2020), subsequently aligned them with MAFFT v7.388 (Katoh and Standley 2013). These aligned sequences were concatenated into a single dataset for phylogenetic analysis using Bayesian inference (BI) in MrBayes 3.2.7 (Ronquist and Huelsenbeck 2003). The optimal substitution model (GTR + I + G) was identified using MrModelTest 2.3 (Nylander 2004). Four parallel MCMC runs were conducted for 1,000,000 generations, sampling every 1000 generations and discarding the initial 1000 trees as burn-in. Convergence was confirmed by ensuring the average standard deviation of split frequencies was below 0.01.

3. Results

3.1. Characteristics of the mitochondrial genomes

The full mitochondrial genome sequence of *B. boettgeri* and *B. kuatunensis* was 16,597 bp and 17,921 bp in length, with GC content of 40.6% and 39.8%, respectively (Figure 2). Both genomes contained 13 PCGs, two rRNA genes, 22 tRNA genes, and a putative control region. Of the 37 genes, 28 were encoded on the heavy strand, while nine were on the light strand in both species. The putative control region showed greater variability, measuring 1146 bp in *B. boettgeri* and 2471 bp in *B. kuatunensis*, while other gene lengths were similar.

Notable differences in codons were observed between the species. The stop codons in *COX1* differed (AGA in *B. boettgeri* and TAA in *B. kuatunensis*), while the remaining 12 PCGs shared identical start and stop codons. The start codon in *COX1* was GTG in both species. The other PCGs used ATT in *ND2* and *ND3*, and ATG in the remaining 10. Among the stop codons, four variations were identified: AGG in *ND6*, TAA in *ND4L*, *ND5*, and *ATP8*, TA in *ND1*, *COX3*, *ATP6*, and *CYTB*, and T in *ND4*, *COX2*, *ND2*, and *ND3*.

3.2. Phylogenetic relationship

Figure 3 shows that the BI tree indicated: (1) the two *Boulenophrys* species clustered with other *Boulenophrys* species, with *B. kuatunensis* more closely related to *B. baishanzuensis* compared to *B. boettgeri* and (2) the genera *Boulenophrys* and *Atympanophrys* formed a sister clade within the subfamily Megophryinae.

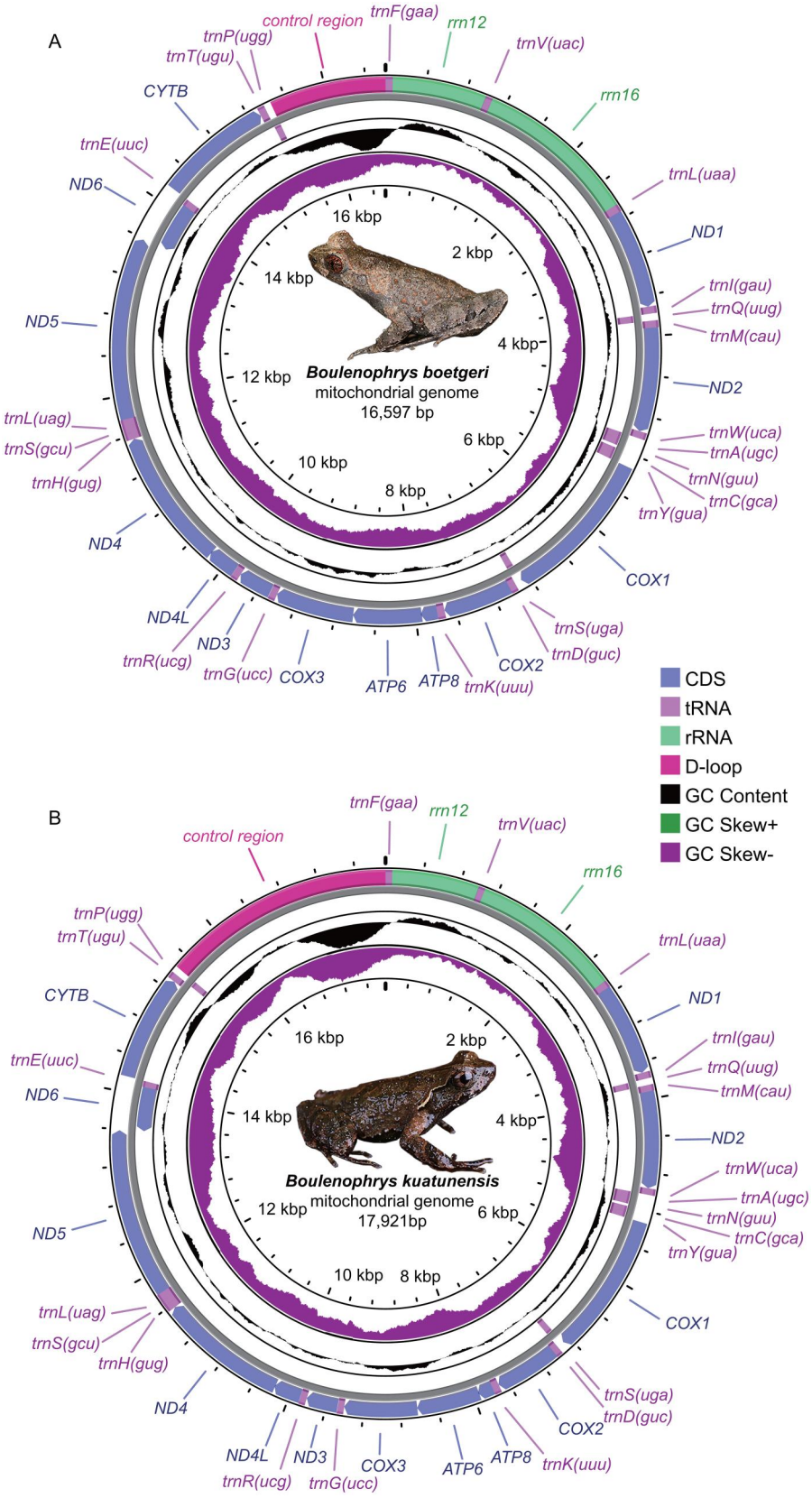

Figure 2. Circular maps depicting the mitogenomes of (A) *Boulenophrys boettgeri* and (B) *Boulenophrys kuatunensis*.

4. Discussion and conclusions

Whole-genome sequencing data are available resource for detecting variations in mitochondrial genomes (Chen et al. 2021). In this study, we obtained the complete mitochondrial genomes of two *Boulenophrys* species. Compared to the recently reported mitochondrial sequence of *B. baishanzuensis* (17,040 bp; Wu et al. 2024), *B. kuatunensis* was more similar, differing by only 881 bp from *B. boettgeri*. The gene

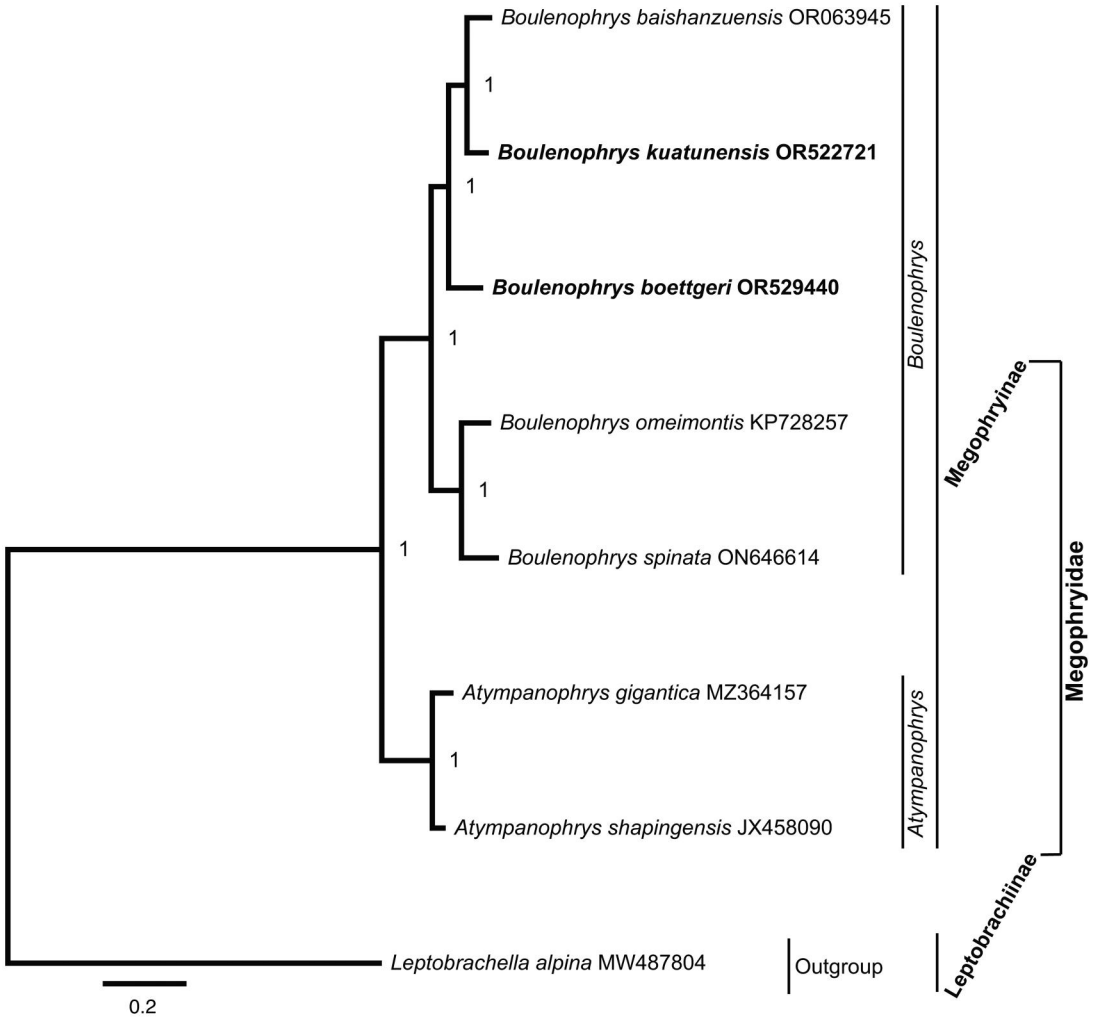

Figure 3. Phylogenetic analysis based on Bayesian's inference method of eight mitogenome sequences, including the newly sequenced *Boulenophrys boettgeri* and *Boulenophrys kuatunensis* using 13 protein-coding genes. Numbers at the nodes represent Bayesian's posterior probabilities. Samples sequenced in the present study are highlighted in bold. The following sequences were used: OR063945 (Wu et al. 2024), KP728257 (Liu et al. 2016), ON646614 (unpublished), MZ364157 (unpublished), JX458090 (Xiang et al. 2013), and MW487804 (Shu et al. 2021).

arrangement and composition of the two *Boulenophrys* species closely resembled those of other reported *Boulenophrys* species (Liu et al. 2016; Wu et al. 2024). The ATG start codon, common in the mitochondrial PCGs of these species, was also found in *Boulenophrys baishanzuensis* (Wu et al. 2024) and *B. omeimontis* (Liu et al. 2016). Our phylogenetic results, aligning with Wu et al. (2024), showed that the two *Boulenophrys* species clustered with other species of the genus, supporting the validity of *Boulenophrys*.

In summary, this study presents the first complete mitochondrial genome sequences of *B. boettgeri* and *B. kuatunensis*, providing valuable data for understanding the phylogenetic relationships within the genus *Boulenophrys*.

Author contributions

ZYW, KH, and GHD designed the study. ZYW, YW, HLH, LM, and GHD carried out the experiments and drafted the manuscript. ZYW and GHD analyzed the data. LM and GHD modified the final manuscript. All authors read and approved the final manuscript.

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 supporting the findings of this study are openly accessible in the GenBank of NCBI at https://www.ncbi.nlm.nih.gov, with the following accession numbers: OR529440 (*Boulenophrys boettgeri*) and OR522721 (*Boulenophrys kuatunensis*). Additionally, the associated BioProject, SRA, and Biosample numbers for each species are as follows: PRJNA695083, SRR13590461 (*B. boettgeri*), and SRR15178600 (*B. kuatunensis*), along with SAMN17711364 (*B. boettgeri*) and SAMN20286340 (*B. kuatunensis*).

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