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Characterization of the Complete Mitogenome of *Mikrogeophagus altispinosus* (Cichliformes: Cichlidae) and Phylogenetic Analysis of New World Cichlids

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YL, JH, CS and QZ designed the study. XH and TW executed experimental work. YL and CS analyzed the data. YL and JH wrote the paper. QZ provided the laboratory equipment. QZ supervised the research.

Key words
Mitochondrial genome, Cichlidae, Bolivian butterfly cichlid, Dwarf butterfly cichlid, mtDNA

ABSTRACT

Following rapid advances in complete mitochondrial genome determination, researchers have published the mitogenomes of many New World cichlids. However, few phylogenetic analyses of New World cichlids have been conducted. In this study, we determined the complete mitogenome of *Mikrogeophagus altispinosus*. After sequencing with the Illumina HiSeq 4000 platform, SPAdes v3.10.1 was used for assembly, and MITOS2 and MitoFish were used for annotation. We then successfully annotated the mitogenome of *M. altispinosus*, which has a total length of 16,767 bp. The gene composition and base preferences of *M. altispinosus* are similar to those of other New World cichlids. Based on the concatenated sequences of 13 protein-coding genes and two rRNAs from the mitogenomes of 37 New World cichlids and one African cichlid, we reconstructed phylogenetic trees using maximum likelihood and Bayesian methods and verified the classification of the target species *M. altispinosus*. According to our analysis, further studies should focus on the relationships between *Amphilophus* and *Symphysodon*. By determining the complete mitogenome of *M. altispinosus*, this study improves the phylogenetic resolution of New World cichlids. This new resource is highly important for the classification of New World cichlids and provides valuable data for subsequent studies on Cichlidae evolution.

INTRODUCTION

The first complete mitochondrial genome reported in the literature was that of the most advanced population of vertebrates: human beings (Anderson *et al*., 1981). Advances in molecular technology have subsequently enabled mitochondrial genome sequencing for diverse species, with data for new taxa regularly being published (Cooper *et al*., 2001; Macaulay *et al*., 2005). Fish are the most ancient group of vertebrates, characterized by substantial variation in developmental and evolutionary diversification. In this regard, the phylogenetics and evolutionary development of fish are major areas of research. Most fish species can be identified according to their morphological characteristics. However, mitochondrial DNA analysis has also become an important tool for identifying fish species and populations and for further exploring the origin of fish taxa and hierarchical differentiation with respect to regional water patterns (Stepien and Kocher, 1997).

The Bolivian Butterfly Cichlid or Dwarf Butterfly Cichlid, *Mikrogeophagus altispinosus* (Haseman, 1911), belongs to the subfamily Geophaginae in the New World cichlid family Cichlidae, order Cichliformes. These aquarium fish, which have a mild temperament and are considered middle-bottom fish, are primarily distributed from Bolivia to northern Trinidad and Tobago (Haseman, 1911; Kullander, 2003; DoNascimiento *et al*., 2017; Staeck *et al*., 2022) and are characterized by striking red tails. *M. altispinosus* have important ecological and ornamental value. This species was first recorded as *Crenicara altispinosa* by Haseman (1911), then named *Mikrogeophagus altispinosa* and *Papiliochromis*

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altispinosus, and is now designated *M. altispinosus* (Kullander, 2003; DoNascimiento *et al*., 2017; Staeck *et al*., 2022).

The complete mitogenome sequences of many New World cichlids have already been published (Chen *et al*., 2020; Nam and Rhee, 2022). However, the phylogenetic relationships of new world cichlids according to their mitogenomes remain unclear. In this study, we determined and characterized the complete mitogenome sequence of *M. altispinosus*. We then evaluated the genetic relationships between *M. altispinosus* and other New World cichlid species by constructing a molecular phylogenetic tree, which provides a basis for germplasm identification and phylogenetic analyses of the Cichlidae family.

MATERIALS AND METHODS

Sample collection and ethics statement

The experimental samples were collected from the ornamental fish base in Guangzhou, Guangdong Province, in January 2022 (23° 3′ 57.33″ N, 113° 11′ 34.13″ E). After morphological identification, the pectoral fins were removed, stored in 95% ethanol, transported to the laboratory, and stored in a refrigerator at -20 °C. All experiments were conducted in accordance with Chinese laws, and all experiments were approved by the Animal Ethics Committee of the Department of Ecology of Jinan University and conducted in compliance with relevant animal welfare and protection laws.

DNA extraction, detection, and sequencing

Total DNA was extracted from 25 mg of *M. altispinosus* fin tissue samples using the Ezup Column Animal Genome DNA Extraction Kit produced by Shanghai Sangong Biological Engineering Co., Ltd. (Shanghai, China), following the instructions provided. After separation by 1% agarose gel electrophoresis, the NanoDrop Lite ultra-micro spectrophotometer (Thermo, USA) was used to detect the quality and concentration of total DNA. Wuhan Banner Technology Co., Ltd. constructed the library and performed sequencing using the Illumina HiSeq 4000 platform to obtain 2×150 bp reads. To ensure the accuracy of morphological identification, *COI* and *Cytb* were used for DNA barcoding.

Mitogenome assembly

Trimmomatic v0.39 (Bolger *et al*., 2014) was used for quality control, and SPAdes v3.10.1 (Bankevich *et al*., 2012) was used for assembly. Sequences with sufficient coverage depth and long assembly lengths were selected as candidate sequences and compared with those in the NCBI taxonomy database to confirm mitochondrial scaffold sequences. The matched clean reads were then assembled to obtain scaffolds. According to the paired-end reads and overlap relationship, gap closer was used to fill and optimize gaps in the newly assembled results. The parameters were set to default. The reference genome was used to correct the starting position and direction of mitochondrial assembly sequences, and the complete mitochondrial genome was obtained.

Mitogenome annotation and analyses

MITOS2 and MitoFish (Iwasaki *et al*., 2013) were used to predict protein-coding genes, tRNAs, and rRNAs in the complete mitogenome (Table I). The start and stop codon positions of genes were artificially corrected to obtain highly accurate conservative genomes. DNAStar software (Lasergene, DNAStar, Madison, WI, USA) was used to determine the total length of the mitogenome. MEGA7.0 (Kumar *et al*., 1994) was used to analyze the base composition, proportion of each part of the genome, and start and end positions of each gene. The asymmetry of the base content of the complete mitogenome was evaluated by AT skew and GC skew (Perna and Kocher, 1995), which were calculated as follows: GC skew = (G−C)/(G+C) and AT skew = (A−T)/(A+T).

Phylogenetic analysis

In total, 38 complete mitogenomes were included in the phylogenetic analysis (Table II), which included the newly obtained mitogenome, 36 complete mitogenome sequences of New World cichlids published in GenBank, and one complete African cichlid mitogenome as an outgroup. As implemented in PhyloSuite v1.2.1 (Zhang *et al*., 2020), based on 13 protein-coding genes and two rRNA genes, the maximum likelihood method and Bayesian inference method were used for tree construction using IQ-TREE v1.6.8 (Nguyen *et al*., 2015) and MrBayes v3.2.6 (Huelsenbeck and Ronquist, 2001), respectively. The best-fit partition model (Edge-linked) was selected using the Bayesian information criterion in ModelFinder. Branch support in the maximum likelihood tree was calculated by the self-expanding ultrafast bootstrapping method with 200,000 replicates. Bayesian inference analyses were run with 1,000,000 generations, where the initial 25% of sampled data were discarded as burn-in.

RESULTS AND DISCUSSION

Mitogenome features

The length of the mitogenome sequence of *M. altispinosus* was 16,767 bp, the base composition was 24.65% T, 30.92% C, 27.82% A, and 16.61% G, and the GC content was 47.53%, showing a distinct AT preference,

Table I. Organization of genes in the *Mikrogeophagus altispinosus* mitogenome with anticodon, direction, position, and length. '+' denotes forward direction and '–' denotes reverse direction.

Name	Start	Stop	Strand	Length	Intergenic nucleotides	Start/stop Codons	Anticodon
tRNA-Phe	1	69	+	69	0	/	GAA
12S RNA	70	1020	+	951	0	/	/
tRNA-Val	1021	1092	+	72	0	/	TAC
16S RNA	1093	2801	+	1709	0	/	/
tRNA-Leu2	2802	2875	+	74	0	/	TAA
ND1	2876	3850	+	975	2	ATG/TAA	/
tRNA-Ile	3853	3922	+	70	-1	/	GAT
tRNA-Gln	3922	3992	-	71	-1	/	TTG
tRNA-Met	3992	4060	+	69	0	/	CAT
ND2	4061	5105	+	1045	0	ATG/T	/
tRNA-Trp	5106	5177	+	72	1	/	TCA
tRNA-Ala	5179	5247	-	69	1	/	TGC
tRNA-Asn	5249	5321	-	73	34	/	GTT
tRNA-Cys	5356	5422	-	67	-1	/	GCA
tRNA-Tyr	5422	5489	-	68	1	/	GTA
COI	5491	7050	+	1560	27	GTG/TAA	/
tRNA-Ser2	7078	7149	-	72	3	/	TGA
tRNA-Asp	7153	7225	+	73	5	/	GTC
COII	7231	7921	+	691	0	ATG/T	/
tRNA-Lys	7922	7994	+	73	1	/	TTT
ATP8	7996	8163	+	168	-10	ATG/TAA	/
ATP6	8154	8836	+	683	0	ATG/TA	/
COIII	8837	9620	+	784	0	ATG/T	/
tRNA-Gly	9621	9692	+	72	0	/	TCC
ND3	9693	10041	+	349	0	ATG/T	/
tRNA-Arg	10042	10110	+	69	0	/	TCG
ND4L	10111	10407	+	297	-7	ATG/TAA	/
ND4	10401	11781	+	1381	0	ATG/T	/
tRNA-His	11782	11850	+	69	0	/	GTG
tRNA-Ser1	11851	11917	+	67	8	/	GCT
tRNA-Leu1	11926	11998	+	73	0	/	TAG
ND5	11999	13837	+	1839	-4	ATG/TAA	/
ND6	13834	14355	-	522	0	ATG/TAG	/
tRNA-Glu	14356	14424	-	69	4	/	TTC
Cyt b	14429	15569	+	1141	0	ATG/T	/
tRNA-Thr	15570	15641	+	72	-1	/	TGT
tRNA-Pro	15641	15710	-	70	0	/	TGG
D-loop	15711	16767	+	1057	0	/	/

Table II. Taxon, GenBank accession number, and base composition information for the available mitogenomes of 38 Cichlidae species included in this study.

Taxon (Species)	Size (bp)	AT %	AT- Skew	GC- Skew	GenBank
New World Cichlids					
Astronotinae					
Astronotus ocellatus	16569	55	0.049	-0.342	NC_009058.1
Chaetobranchopsis bitaeniatus	16610	58.4	0.042	-0.351	NC_033542.1
Cichlasomatinae					
Aequidens metae	16541	53.6	0.037	-0.315	NC_033544.1
Amphilophus amarillo	16521	54.1	0.053	-0.34	KY315559.1
Amphilophus citrinellus	16522	54.2	0.054	-0.34	NC_023827.1
Andinoacara pulcher	16513	56.8	0.011	-0.299	NC_033547.1
Andinoacara rivulatus	16585	56.9	-0.019	-0.259	NC_025671.1
Bujurquina mariae	16540	58.8	0.004	-0.286	NC_033543.1
Bujurquina oenolaemus	16532	57.5	0.012	-0.301	KX397358.1
Cichlasoma dimerus	16617	54.5	0.041	-0.327	NC_033551.1
Cryptoheros cutteri	16528	52.9	0.04	-0.328	NC_033552.1
Herichthys cyanoguttatus	16540	53.4	0.059	-0.344	NC_033546.1
Heros severus	16577	56.9	0.03	-0.221	MT363636.1
Hypselecara temporalis	16544	53.9	0.021	-0.316	NC_011168.1
Krobia guianensis	16539	54.3	0.045	-0.324	NC_031440.1
Nannacara anomala	16502	53.4	0.025	-0.301	NC_031183.1
Parachromis managuensis	16526	53.6	0.049	-0.339	NC_026918.1
Petenia splendida	16518	53.2	0.053	-0.338	NC_024835.1
Pterophyllum altum	16495	54.2	0.014	-0.325	NC_028723.1
Pterophyllum scalare	16491	54.2	0.016	-0.317	NC_026535.1
Rocio octofasciata	16539	54.4	0.041	-0.34	NC_033548.1
Symphysodon aequifasciata	16545	54.9	0.049	-0.335	NC_028182.1
Symphysodon discus	16544	54.9	0.052	-0.337	NC_026689.1
Symphysodon haraldi	16543	54.9	0.051	-0.336	NC_027965.1
Thorichthys aureus	16530	52.1	0.042	-0.325	NC_031182.1
Thorichthys meeki	16527	53.2	0.052	-0.339	MZ427899.1
Uaru amphiacanthoides	16549	54.4	0.044	-0.326	NC_033550.1
Vieja melanura	16543	52.6	0.058	-0.335	NC_023526.1
Cichlinae					
Cichla ocellaris	16526	54.3	0.076	-0.35	NC_030272.1
Geophaginae					
Apistogramma cacatuoides	16870	54.3	0.025	-0.302	KR150874.1
Geophagus brasiliensis	16559	54.1	0.044	-0.319	NC_031181.1
Geophagus steindachneri	16594	53.8	0.063	-0.339	NC_033545.1
Gymnogeophagus balzanii	16587	56	0.018	-0.306	KR150864.1
Mikrogeophagus altispinosus	16767	52.4	0.06	-0.301	OP595704
Mikrogeophagus ramirezi	16526	55.4	0.033	-0.294	NC_031439.1
Taeniacara candidi	16581	57.2	-0.005	-0.29	KR150873.1
Retroculinae					
Retroculus lapidifer	16537	52.8	0.058	-0.314	NC_033549.1
African cichlid (outgroup)					
Pseudocrenilabrinae					
Tylochromis polylepis	16976	54.5	0.041	-0.319	NC_011171.1

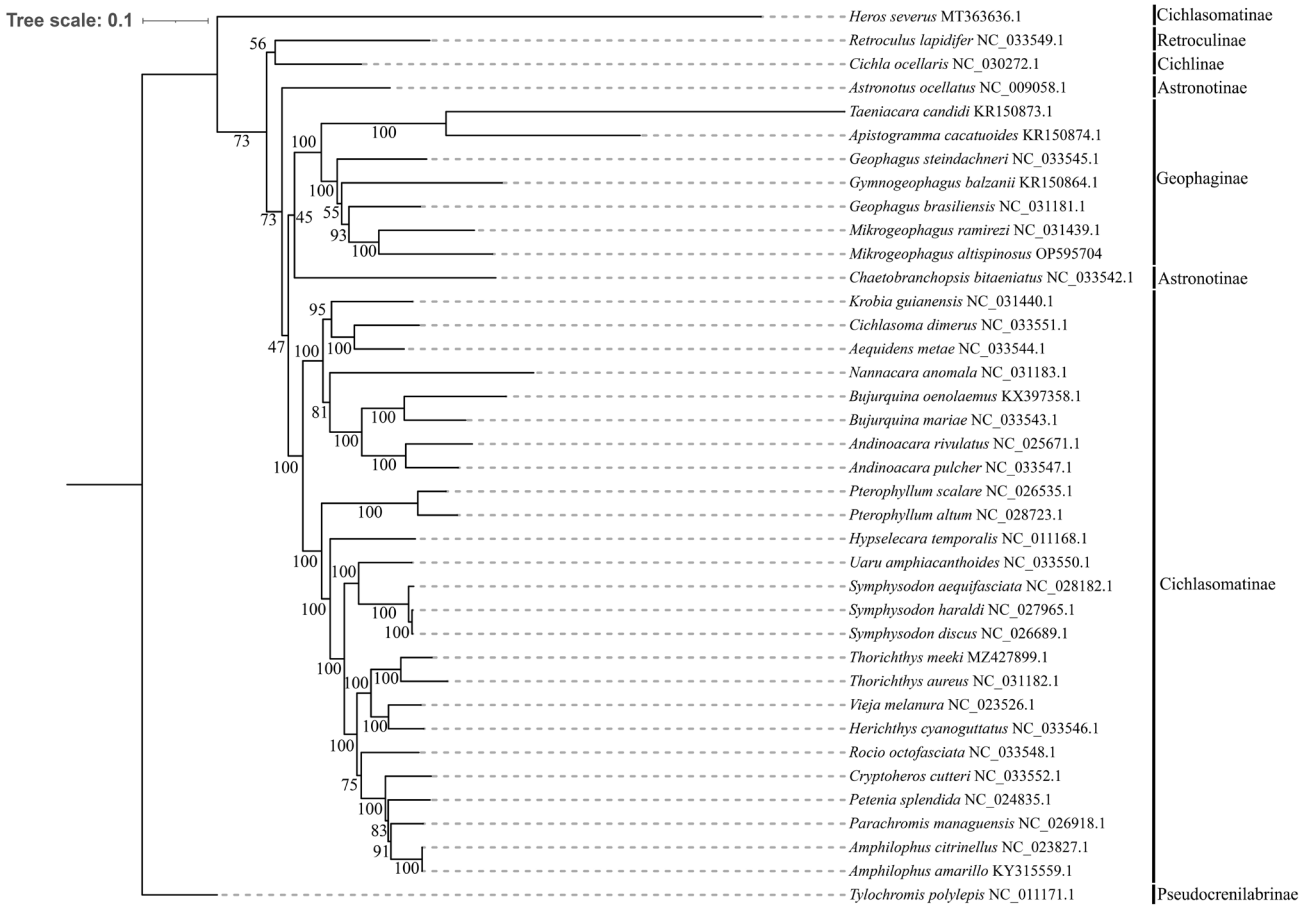

Fig. 1. Phylogenetic tree of 38 Cichlidae species based on the concatenated nucleotide sequences of 13 protein-coding genes and two rRNAs constructed using the maximum likelihood method. Numbers represent ultrafast bootstrap support values.

similar to the base composition of vertebrate genomes. The *M. altispinosus* G base content was similar to that of other teleost fishes, indicating a substantial anti-guanine skew (Gong *et al*., 2017).

Because of differences in selection pressure and natural mutation rates between DNA strands, the distributions of bases and mutations are often uneven (Brown *et al*., 1982). In the mitogenome of *M. altispinosus*, except for ND6 and eight tRNA coding genes located on the light strand, the other 26 coding genes were located on the heavy strand, similar to those in the mitogenomes of other fish (Ma *et al*., 2015; Yu and Kwak, 2015). The length of overlapping fragments in the genome of fish is generally only 7–10 bp, compared with 40–46 bp fragment length in mammals (Broughton *et al*., 2001; Zhu *et al*., 2013). In this study, we found that the length of *M. altispinosus* overlapping fragments was 1–34 bp, with a maximum overlap of 34 bp between tRNA-Asn and tRNA-Cys because of the light-strand replication origin between the two genes and minimum overlapping fragments (1 bp) between tRNA-Trp/tRNA-Ala, tRNA-Ala/tRNA-Asn, tRNA-Tyr/COI, and tRNA-Lys/ATP8. The control region showed substantial variation and a high rate of evolution. In this study, the A+T content (60.08%) was also relatively high in the *M. altispinosus* control region, which regulates mitochondrial replication and transcription.

According to the start and stop positions, length, and base composition of the 37 genes in the mitogenome of *M. altispinosus*, the mitogenome characteristics are highly similar to those of *M. ramirezi*, another species in the same genus. However, there are also differences, for example, the 16S RNA of *M. altispinosus* was 17 bp longer than that of *M. ramirezi*. Among the protein-coding genes, the *ATP6* of *M. altispinosus* was 1 bp shorter, the *Cytb* was 1 bp longer, and the *COI* was 3 bp longer than those of *M. ramirezi.*

The protein-coding genes of the complete mitogenome had an identical starting codon in *M. altispinosus* and *M.*

ramirezi; however, the *ATP6* and *Cytb* of *M. ramirezi* had the termination codon TAA, in contrast to the incomplete terminator T/TA in *M. altispinosus*. The 3 end of the post-transcriptional product of the incomplete termination codon is U, the mitochondrial mRNA is polyadenylated after transcription, and the termination codon T finally forms the UAA termination codon (Ojala *et al*., 1981). Among the 37 New World cichlids mitogenomes published to date, the AT% was greater than 50% (range: 52.1%–58.8%), and the length range was 16,491–16,870 bp. The GC skew showed evident negative values, and the AT skew was positive, except in the genomes of *Andinoacara rivulatus* and *Taeniacara candidi*. These characteristics were similar to those reported previously in the mitogenomes of Cichlidae (Chen *et al*., 2020; Nam and Rhee, 2022).

Phylogenetic analysis

To understand the evolutionary relationships of New World cichlids, maximum likelihood (Fig. 1) and Bayesian inference (Fig. 2) trees were constructed based on 13 protein-coding genes and two rRNAs from 38 species. Phylogenetic trees constructed using these two methods revealed the same topological structure, except for the location of *Chaetobranchopsis bitaeniatus*. The target species *M. altispinosus* and *M. ramirezi* were clustered with high support (bootstrap value of 100% and Bayesian posterior probability value of 1.0), and the monophyly of Geophaginae was well supported. All species in Cichlasomatinae, except *Heros severus*, were closely clustered. Among the eight multi-species genera (i.e., those including two or more species), we observed consistent classification and morphological results for seven genera, *Amphilophus, Andinoacara, Bujurquina, Pterophyllum, Symphysodon, Thorichthys,* and *Mikrogeophagus*; only two *Geophagus* species failed to form a branch. The two Astronotinae species also failed to form a single branch. At present, few complete mitogenomes are available for Astronotinae, Cichlinae, and Retroculinae, indicating that more research is required.

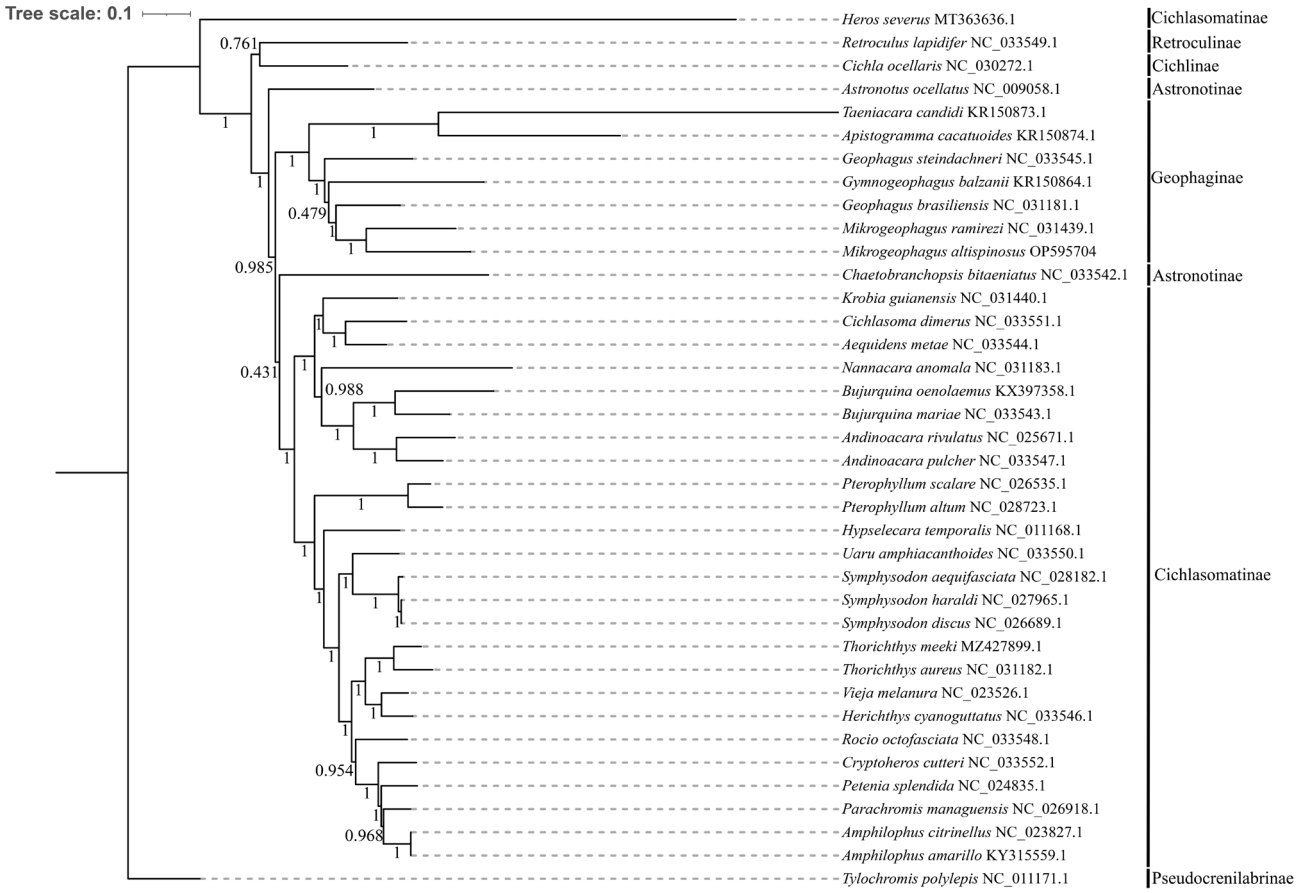

Fig. 2. Phylogenetic tree of 38 Cichlidae species based on the concatenated nucleotide sequences of 13 protein-coding genes and two rRNAs constructed using the Bayesian inference method. Applicable posterior probability values are shown.

Notably, the tree lengths of *Amphilophus* and *Symphysodon* were very short or nearly zero in both phylogenetic trees. Comparing the complete mitogenome sequences of *Amphilophus amarillo* and *A. citrinellus* revealed only four substitutions. The complete mitogenome sequences of *Symphysodon aequifasciata*, *S. discus,* and *S. haraldi* were also compared, which revealed differences between *S. aequifasciatus* and the other two species, with 243 variant sites as well as 41 variant sites between *S. discus* and *S. haraldi*. We propose at least three reasons for this result: morphological identification error, co-evolution, or interspecific hybridization. We expect that the novel mitogenome sequenced in this study will be useful for detailed analyses of *M. altispinosus* genetics as well as further phylogenetic studies, particularly as more data become available.

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IRB approval and ethical statement

All experiments were approved by the Animal Ethics Committee of the Department of Ecology of Jinan University and conducted in compliance with relevant animal welfare and protection laws.

Statement of conflict of interest

The authors have declared no conflict of interest.

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