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Complete Mitochondrial Genomes of Two *Corydoras* (Siluriformes, Callichthyidae) and their Phylogenetic Implications

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Key words
Corydoradinae, *COII*, Dwarf *Corydoras*, Guanine-cytosine content, mtDNA

ABSTRACT

Mitochondrial DNA is the most reliable tool in species classification, genetic diversity, and phylogeny of fish studies. In this study, two new mitochondrial genomes in the genus *Corydoras* (Callichthyidae) were determined, specifically *C. hastatus* and *C. cruziensis*. Comparative and phylogenetic analyses were conducted using our data and those of 12 other mitochondrial genomes from *Corydoras*. The nucleotide diversity and genetic distance among the protein-coding genes of the *Corydoras* mitochondrial genomes showed that the most conserved gene was *COII*. Analysis of the selection pressures on each gene showed that *COI* was associated with the strongest purifying selection. The *Corydoras* mitochondrial genomes had similar AT and GC contents, AT and GC skew, genetic distances, nucleotide diversity, number of codons, and Ka/Ks values, supporting concerted evolution within this genus. The resulting phylogenetic relationship supports a sister-group relationship between *C. hastatus* and *C. pygmaeus* and between *C. cruziensis* and (*C. rabauti* + *C. aeneus*). The complete mitochondrial genomes of *C. hastatus* and *C. cruziensis* provide valuable resources for future studies on the molecular phylogeny and population genetics of Callichthyidae.

INTRODUCTION

Fish comprise the most primitive and dominant group of vertebrates in terms of the number of species and genera (Compagno, 1990; Lévêque *et al*., 2008). They include a wide range of species with widespread distribution and a complex origin. Studying their genetic differentiation and clarifying their evolutionary paths have always been interesting topics (Kelsh, 2004). In recent years, with the widespread adoption of molecular biology technology in various research fields, studying the genetics and evolution of fish at the molecular level has become increasingly attractive (Glasauer and Neuhauss, 2014; Hauser and Carvalho, 2008). At the molecular level, it is important to select appropriate molecular markers when studying the genetics and evolution of fish. DNA molecules contain a large amount of information on genetic variation, from which we can obtain a more objective understanding of the evolution of organisms. The biological characteristics of mitochondrial DNA render its haplotype tree more consistent than nuclear autosomal gene and species trees, and mitochondrial DNA is often used to estimate the evolutionary history of biological groups (Avise, 2009; Moore, 1995). Fish mitochondrial DNA, similar to that of many other vertebrates, comprises covalently closed, circular, and double-stranded molecules that are closely arranged (Boore, 1999; Hurst *et al*., 1999; Liu *et al*., 2015; Sun *et al*., 2021). The mitochondrial DNA of fish is generally 15–20 kb in size. Mitochondrial genomes vary considerably among different species. Tandem repeats and a few scattered repeats are present in their sequences, similar to those in other vertebrates (Boore, 1999). The mitochondrial genome of fish is composed of 13 protein-coding genes (PCGs), two ribosomal RNA genes (rRNAs), and 22 transfer RNA genes (tRNAs), as well as the control region (D-loop) and light chain replication initiation region related to the non-coding region of heavy chain replication initiation. With the gradual maturation of DNA sequencing technology, fish mitochondrial genomes have been widely used as molecular markers for fish germplasm resource protection, population polymorphism analysis, and phylogenetic development (Jia *et al*., 2020; Saha *et*

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al., 2021; Zheng *et al*., 2021).

Corydoras are members of the genera *Aspidoras* Ihering, 1907, *Brochis* Cope, 1871, and *Corydoras* Lacépède, 1803 in the subfamily Corydoradinae (Bernt *et al*., 2013). Owing to two small barbells on the sides of their mouths, the fish resemble mice swimming in water, hence the name Corydoras. The dwarf species *Corydoras hastatus* Eigenmann and Eigenmann, 1888 has a silvery white body and a black tail handle with a white frame, which is of great ornamental value (Britto, 2003; Menni *et al*., 1992). The fish are mostly located in the Mato Grosso Plateau in Brazil. *Corydoras hastatus* is not a benthic fish. It is characterized by an obvious cross-shaped black spot on its tail handle, and it often swims in groups of other fish species with a very similar appearance, such as *Serrapinnus kriegi* (Serra *et al*., 2018), in native waters, forming a symbiotic relationship. It is as small as a lampfish and the least hungry species of *Corydoras* (max length: 2.4 cm). *Corydoras cruziensis* is characterized by a bright orange head and back, metallic green body, short snout, and a round figure (Knaack, 2002).

Building upon the study on *C. aeneus* and *C. paleatus* (Sevilla *et al*., 2007; Sun *et al*., 2022), we sequenced, assembled, and annotated the complete mitochondrial genomes of *C. hastatus* and *C. cruziensis*. Using the newly sequenced genomes and 12 complete mitochondrial genomes of the genus *Corydoras* available in the NCBI database, we aimed to conduct a comprehensive analysis that will provide a reference for the taxonomy, evolutionary genetics, and interspecific identification of the genus *Corydoras*.

MATERIALS AND METHODS

Fish collection, identification, and DNA extraction

Single fresh specimens of the two target species were collected from a wholesale flower, bird, and fish market in Mudanjiang City, Heilongjiang Province (44°35′20.08″N, 129°36′31.87″E), in January 2022. After euthanization, specimens were immersed in absolute ethanol and stored in a freezer at −80 °C until use. Total DNA was extracted from muscle tissue using a Magen Hi Pure Inspect DNA Micro Kit following the manufacturer's instructions. The quality and purity of the extracted DNA were tested using agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Species identification was verified using morphological characteristics (Alexandrou *et al*., 2011; Burgess, 1992), Cytb (Sevilla *et al*., 2007), and the 16S rRNA gene (Alexandrou *et al*., 2011) combined. The animal study protocol was approved by the Ethics Committee of Mudanjiang Normal University (Jan 12, 2022).

Genome sequencing, assembly, and annotation

The qualifying total genomic DNA was sent to Wuhan Benagen Biotechnology Co., Ltd., where the whole-genome shotgun method was used to build the library and next-generation sequencing technology was used to conduct high-throughput sequencing on the Illumina NovaSeq 6000 sequencing platform. SPAdes v3.11.1 (Bankevich *et al*., 2012) was used to assemble high-quality second-generation sequencing data from scratch. Contigs and scaffolds were constructed using the default parameters. Using *C. aeneus* MZ571336 as the reference sequence, we conducted the collinearity analysis, determined the positional relationship between segment overlapping groups, and filled in the missing sequences between the overlapping groups in MUMmer v3.1 (Kurtz *et al*., 2004). Pilon v1.18 (Walker *et al*., 2014) was used to correct the results and obtain the final mitochondrial sequence. The complete mitochondrial genome sequence obtained through splicing was functionally annotated using the MITOS web server (http://mitos.bioinf.uni-leipzig.de/) (Bernt *et al*., 2013). The genetic code was set to vertebrate, and other settings followed the default parameters of MITOS. The annotation results were further verified after manual correction and using MitoFish (Iwasaki *et al*., 2013) (http://mitofish.aori.u-tokyo.ac.jp/), online tools, and tRNAscan-SE v1.3.1 (Lowe and Eddy, 1997). Circular mitochondrial genome maps were generated in MitoFish (Iwasaki *et al*., 2013).

Genome analysis

The two newly obtained mitochondrial genomes were combined with 12 published mitochondrial genomes from the genus *Corydoras* for genome analysis. Base compositions and genetic distances were determined using MEGA v7.0 (Kumar *et al*., 1994). PhyloSuite v1.2.2 (Zhang *et al*., 2020) was used to calculate the number of codons, AT and GC content, AT skew [AT skew = (A – T)/(A + T)], and GC skew [GC skew = (G – C)/(G + C)] in the mitochondrial genome (Perna and Kocher, 1995). The Ka/Ks ratio and nucleotide diversity for the 14 *Corydoras* species were calculated using DNAsp v5.1 (Librado and Rozas, 2009). These results were plotted in origin v2018 (Moberly *et al*., 2018).

Phylogenetic analysis

For the analysis, we created a dataset of 14 Callichthyidae mitochondrial genomes (12 *Corydoras*, one *Brochis*, and one *Hoplosternum* available in the NCBI database) and the two newly sequenced genomes; *Hyphessobrycon amandae* MT484069 (Sun *et al*., 2021) in the Characidae family was selected as an outgroup (Table I). IQ-TREE v1.6.8 (Nguyen *et al*., 2015) integrated in

PhyloSuite was used to build a maximum likelihood (ML) phylogenetic tree based on 13 PCGs and two rRNAs. The best partition model was screened using ModelFinder (Kalyaanamoorthy *et al*., 2017). Branch confidence was assessed by 200,000 ultrafast bootstrap replicates (Minh *et al*., 2013) and the Shimodaira–Hasegawa-like approximate likelihood-ratio test (Guindon *et al*., 2010). Mbayes v3.2.6 (Huelsenbeck and Ronquist, 2001) was used to construct a Bayesian inference (BI) phylogenetic tree under the partition model (two parallel runs of 20,000,000 generations each), in which the initial 25% of sampled data was discarded as burn-in. The optimal partition models for ML and BI are listed in Table II. iTOL (Letunic and Bork, 2016) was used to visualize the resulting phylogenetic trees.

Table I. Complete mitogenomes used in this study.

Family/ Taxa	Length	AT %	GenBank accession No.
Callichthyidae			
Brochis multiradiatus	16916	58	MN641874
Corydoras aeneus	16604	58.5	NC_063780
Corydoras agassizii	16562	58.4	MN641875
Corydoras arcuatus	16822	58.5	NC_049096
Corydoras cruziensis	16531	59.5	OP562096
Corydoras duplicareus	16667	59.4	NC_049095
Corydoras hastatus	16518	58.6	OP562095
Corydoras nattereri	16557	57.9	KT239009
Corydoras paleatus	16593	58.2	NC_063781
Corydoras panda	16611	58.8	NC_049097
Corydoras pygmaeus	16840	60.3	ON729306
Corydoras rabauti	16831	58.6	NC_004698
Corydoras schwartzi	16632	58.3	KT239007
Corydoras sterbai	16636	59	NC_048967
Corydoras trilineatus	16526	58.9	NC_049098
Hoplosternum littorale	16597	61	KX087170
Characidae			
Hyphessobrycon amandae	16701	57.2	MT484069

RESULTS AND DISCUSSION

Two new mitochondrial genomes

The total lengths of the mitochondrial genomes of *C. hastatus* and *C. cruziensis* were 16,518 bp (GenBank accession number: OP562095) and 16,531 bp (GenBank accession number: OP562096), respectively. Complete mitochondrial genomes are double-chained rings consisting of a heavy chain (J strand) and a light chain (N strand) (Fig. 1). Both mitochondrial genomes contained 37 genes (13 PCGs, 22 tRNAs, and two rRNAs) and one D-loop

Table II. Best substitution models for Bayesian inference (BI) and maximum likelihood (ML) analyses.

Gene	BI	ML
12S rRNA	GTR+F+I+G4	TIM2+F+I+G4
16S rRNA	GTR+F+I+G4	TIM2+F+I+G4
ND1	GTR+F+I+G4	GTR+F+I+G4
ND2	HKY+F+G4	TPM3u+F+G4
COI	GTR+F+I+G4	GTR+F+I+G4
COII	GTR+F+I+G4	GTR+F+I+G4
ATPase 8	HKY+F+G4	TPM3u+F+G4
ATPase 6	GTR+F+I+G4	GTR+F+I+G4
COIII	GTR+F+I+G4	GTR+F+I+G4
ND3	HKY+F+I+G4	K3Pu+F+I+G4
ND4L	GTR+F+I+G4	GTR+F+I+G4
ND4	GTR+F+I+G4	GTR+F+I+G4
ND5	GTR+F+I+G4	GTR+F+I+G4
ND6	HKY+F+I+G4	HKY+F+I+G4
Cyt b	GTR+F+I+G4	GTR+F+I+G4

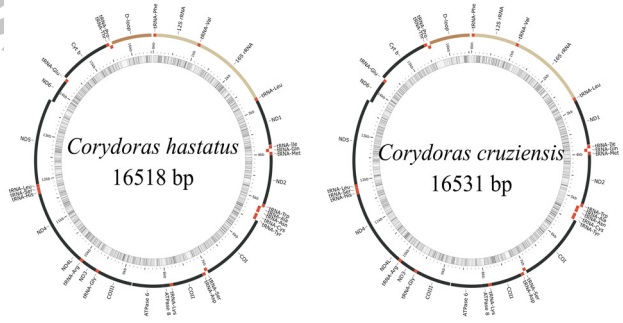

Fig. 1. Circular maps of *Corydoras hastatus* (left) and *Corydoras cruziensis* (right) mitochondrial genomes.

non-coding control region (Table III). There were nine genes on the N chain, namely *tRNA-Gln*, *tRNA-Ala*, *tRNA-Asn*, *tRNA-Cys*, *tRNA-Tyr*, *tRNA-Ser*, *tRNA-Glu*, *tRNA-Pro*, and *ND6*, and the remaining 28 genes were on the J chain. The length of most of the gene sequences and the interval repeats were identical between *C. hastatus* and *C. cruziensis* (Table III). There were 11 spacer regions in the mitochondrial genomes of *C. hastatus* and *C. cruziensis*, with spacer lengths of 69 and 70 bp, respectively. The largest spacers were between *tRNA-Asn* and *tRNA-Asn*, comprising 31 and 30 bp in the genomes of *C. hastatus* and *C. cruziensis*, respectively. Eight adjacent genes in the two genomes overlapped. The maximum overlap of 13 bp was between *COI* and *tRNA-Ser*, and the minimum overlap of 1 bp was between *tRNA-Gln* and *tRNA-Met* and between

Table III. Characteristic features of *Corydoras hastatus* (left) and *Corydoras cruziensis* (right) mitochondrial genomes.

Gene	Strand	Position		Intergenic nucleotides	Length (bp)	Start codons	Stop codons	Anticodon
		From	To					
tRNA-Phe	J	1/1	68/68	0/0	68/68			GAA
12S rRNA	J	69/69	1013/1013	0/0	945/945			
tRNA-Val	J	1014/1014	1085/1085	0/0	72/72			TAC
16S rRNA	J	1086/1086	2759/2758	0/0	1674/1673			
tRNA-Leu	J	2760/2759	2834/2833	0/0	75/75			TAA
ND1	J	2835/2834	3806/3805	8/8	972/972	ATG/ATG	TAG/TAG	
tRNA-Ile	J	3815/3814	3886/3885	-2/-2	72/72			GAT
tRNA-Gln	N	3885/3884	3955/3954	-1/-1	71/71			TTG
tRNA-Met	J	3955/3954	4024/4023	0/0	70/70			CAT
ND2	J	4025/4024	5069/5068	0/0	1045/1045	ATG/ATG	T/T	
tRNA-Trp	J	5070/5069	5140/5140	1/1	71/72			TCA
tRNA-Ala	N	5142/5142	5210/5210	1/1	69/69			TGC
tRNA-Asn	N	5212/5212	5284/5284	31/30	73/73			GTT
tRNA-Cys	N	5316/5315	5381/5381	-1/-1	66/67			GCA
tRNA-Tyr	N	5381/5381	5450/5450	1/1	70/70			GTA
COI	J	5452/5452	7011/7011	-13/-13	1560/1560	GTG/GTG	AGG/AGG	
tRNA-Ser	N	6999/6999	7069/7069	4/4	71/71			TGA
tRNA-Asp	J	7074/7074	7142/7142	4/4	69/69			GTC
COII	J	7147/7147	7837/7837	0/0	691/691	ATG/ATG	T/T	
tRNA-Lys	J	7838/7838	7911/7911	1/1	74/74			TTT
ATPase 8	J	7913/7913	8080/8080	-10/-10	168/168	ATG/ATG	TAA/TAA	
ATPase 6	J	8071/8071	8754/8754	15/17	684/684	ATG/ATG	TAA/TAA	
COIII	J	8770/8772	9553/9555	0/0	784/784	ATG/ATG	T/T	
tRNA-Gly	J	9554/9556	9624/9627	0/0	71/72			TCC
ND3	J	9625/9628	9973/9976	0/0	349/349	ATG/ATG	T/T	
tRNA-Arg	J	9974/9977	10043/10046	0/0	70/70			TCG
ND4L	J	10044/10047	10340/10343	-7/-7	297/297	ATG/ATG	TAA/TAA	
ND4	J	10334/10337	11714/11717	0/0	1381/1381	ATG/ATG	T/T	
tRNA-His	J	11715/11718	11784/11787	0/0	70/70			GTG
tRNA-Ser	J	11785/11788	11851/11854	1/1	67/67			GCT
tRNA-Leu	J	11853/11856	11925/11928	0/0	73/73			TAG
ND5	J	11926/11929	13752/13755	-4/-4	1827/1827	ATG/ATG	TAG/TAA	
ND6	N	13749/13752	14264/14267	0/0	516/516	ATG/ATG	TAG/TAA	
tRNA-Glu	N	14265/14268	14333/14336	2/2	69/69			TTC
Cyt b	J	14336/14339	15473/15476	0/0	1138/1138	ATG/ATG	T/T	
tRNA-Thr	J	15474/15477	15546/15549	-2/-2	73/73			TGT
tRNA-Pro	N	15545/15548	15614/15617	0/0	70/70			TGG
D-loop		15615/15618	16518/16531	0/0	904/914			

tRNA-Cys and *tRNA-Tyr*. The start and stop codons of the PCGs in *C. hastatus* and *C. cruziensis* were identical and similar to other *Corydoras* species. The start codons were ATG and GTG, and the stop codons were TAG, TAA, and the incomplete stop codon T-.

Comprehensive analysis of 14 Corydoras *mitochondrial genomes*

The two mitochondrial genomes obtained in this study were combined with 12 published mitochondrial genomes for a comprehensive analysis that included the base composition, base bias, paired genetic distance, nucleotide diversity, Ka/Ks ratio, and number of codons. The mitochondrial genomes with the 13 PCGs and two rRNAs of the 14 *Corydoras* species showed a positive AT skew but a negative GC skew, except for *ND6* (Fig. 2), which has also been reported in a mitochondrial genome study on other fish species (Ruan *et al*., 2020). Among the 13 PCGs and two rRNAs, the AT content of *ATP8* and *ATP6* was the highest, and that of *ND4L* was the lowest (Fig. 3). The GT content exhibited the opposite trend. Except for *ND4* of *C. agassizii* and *C. schwartzi*, the AT content of all other genes was greater than the GC content, which coincided with the fact that the mitochondrial base composition of teleost fish exhibits a preference for A and T (Broughton *et al*., 2001; Sun *et al*., 2021).

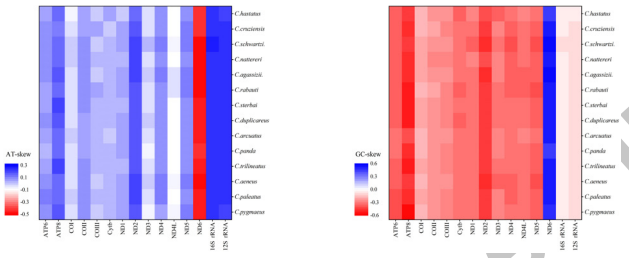

Fig. 2. AT and GC skews for protein-coding genes and ribosomal RNA genes of 14 *Corydoras* mitochondrial genomes.

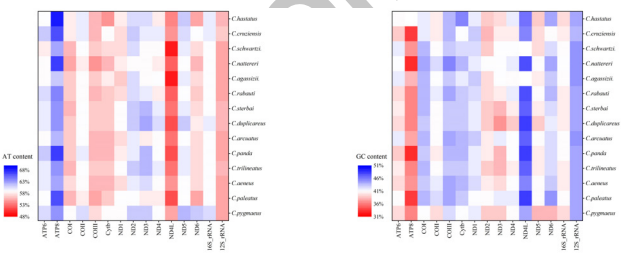

Fig. 3. AT and GC contents of protein-coding genes and ribosomal RNA genes in 14 *Corydoras* mitochondrial genomes.

To estimate the average divergence among the mitochondrial genomes of *Corydoras*, the overall mean K2P genetic distances were analyzed based on 13 PCGs (Fig. 4). Congruent results showed that both *COII* (0.073) and *COIII* (0.097) had the smallest genetic distance, whereas *ND4* (0.136) had the largest, thereby representing the most conserved and the most variable genes, respectively. The results of the nucleotide diversity analysis (Fig. 4) were consistent with those of genetic distance.

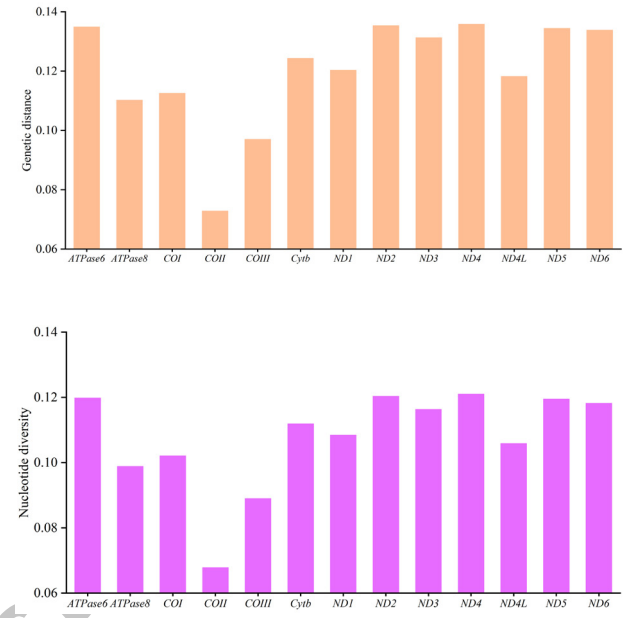

Fig. 4. Genetic distances and nucleotide diversity of protein-coding genes in 14 *Corydoras* mitochondrial genomes.

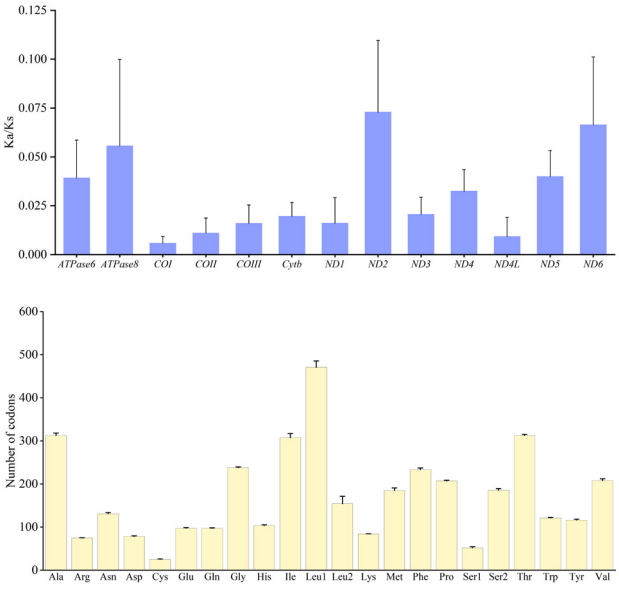

Fig. 5. Ka/Ks ratios and number of codons in protein-coding genes of 14 *Corydoras* mitochondrial genomes.

To evaluate selection pressure (Lemos *et al*., 2005; Sun *et al*., 2020) on the mitochondrial genome

of *Corydoras* fish, the Ka/Ks values of 13 PCGs in the mitochondrial genome were estimated, and a histogram of this ratio was constructed (Fig. 5). The Ka/Ks values of the 13 PCGs ranged from 0.006 to 0.072 and were less than one, indicating strong purification selection. The Ka/Ks values of *COI* (0.006) were the lowest, suggesting that this gene was under the greatest purifying selection pressure during evolution.

The PCGs of the 14 mitochondrial genomes of *Corydoras* were translated into 3,785–3,797 codons. Ile (307.35 ± 9.60 codons), Thr (312.58 ± 2.45 codons), Ala (312.52 ± 5.80 codons), and Leu1 (470.88 ± 14.61 codons) were the four predominant codon families (Fig. 5) and might be associated with the coding function of the chondriosome (Gu *et al*., 2022). In contrast, Cys (25.12 ± 0.83 codons) and Ser1 (52.82 ± 2.57 codons) were with the smallest number of codons.

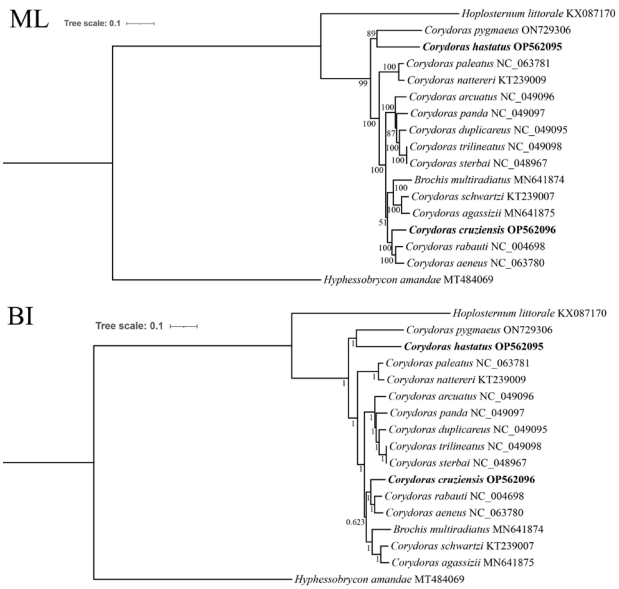

Fig. 6. Phylogenetic relationships of *Corydoras* based on complete mitochondrial genomes inferred using maximum likelihood (ML) and Bayesian inference (BI) analyses.

Phylogenetic analysis

The phylogenetic tree of *C. hastatus*, *C. cruziensis*, and 14 species of the family Callichthyidae, based on the tandem sequences of 13 PCGs and two rRNAs in the mitochondrial genome, was constructed using the ML and BI methods (Fig. 6). Consistent with previous studies (Alexandrou *et al*., 2011; Roxo *et al*., 2019; Sun *et al*., 2022), the ML and BI tree topologies were congruent, confirming the monophyly of the genus *Corydoras*; however, *Brochis multiradiatus* was clustered within this genus. *Corydoras hastatus* and *C. pygmaeus* formed a highly supported clade (BI potential probabilities, PP = 1; ML bootstrap, BS = 89), which is consistent with the results reported by Alexandrou *et al*. (2011). *Corydoras cruziensis* clustered with (*Corydoras rabauti* + *Corydoras aeneus*) in a highly supported clade (PP = 1; BS = 100). Fourteen species of the genus *Corydoras* clustered together quite well. Similar to Sun *et al*. (2022), we believe that the clustering of *C. trilineatus* and *C. sterbai* is attributable to identification errors, introgressive hybridization, or that the two names are homonyms.

CONCLUSIONS

In this study, the mitochondrial genomes of two *Corydoras* species were sequenced and assembled. The results showed that the sequenced gene arrangements were consistent with the putative ancestral fish mitochondrial genomes, as understood today. Comprehensive analysis of the two new and 12 published *Corydoras* mitochondrial genomes showed that the 14 mitochondrial genomes had similar AT and GC content, AT and GC skew, genetic distances, nucleotide diversity, number of codons, and Ka/Ks values.

The nucleotide diversity and genetic distance of PCGs in the *Corydoras* mitochondrial genomes showed that *ND2* and *ND4* were the most variable genes, whereas *COII* was the most conserved gene. An analysis of the selection pressures on each gene showed that *COI* was associated with the strongest purifying selection.

Phylogenetic analyses based on PCGs and rRNAs from the mitochondrial genomes of 17 species have thus clarified the phylogenetic relationships of *Corydoras*. The sister-group relationships between *C. hastatus* and *C. pygmaeus* and between *C. cruziensis* and (*C. rabauti* + *C. aeneus*) were well supported at the mitogenome level. Our findings also suggest that mitogenome sequences are effective molecular markers to study the phylogenetic relationships within *Corydoras* and *Callichthyidae*.

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Ethics statement

The animal study protocol was approved by the Ethics Committee of Mudanjiang Normal University.

Data availability statement

The original contributions presented in this study are publicly available. This data can be found in the GenBank repository under accession numbers OP562095 and

OP562096.

Statement of conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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