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Complete mitochondrial genome and phylogenetic analysis of *Dollfustrema vaneyi* (Trematoda: Bucephalidae)

Ye Hu[1], Tong Ye[1], Hong Zou[2], Gui-Tang Wang[2], Wen-Xiang Li[2*] and Dong Zhang[1,3*]

Abstract

Background The Bucephalidae is a large family of digenean trematodes but most previous analyses of its phylogenetic position have relied on a single mitochondrial gene or morphological features. Mitochondrial genomes (mitogenomes) remain unavailable for the entire family. To address this, we sequenced the complete mitogenome of *Dollfustrema vaneyi* and analyzed the phylogenetic relationships with other trematodes.

Results The circular genome of *Dollfustrema vaneyi* spanned 14,959 bp and contained 12 protein-coding genes, 22 transfer RNA genes, 2 ribosomal RNA genes, and a major non-coding region. We used concatenated amino acid and nucleotide sequences of all 36 genes for phylogenetic analyses, conducted using MrBayes, IQ-TREE and PhyloBayes. We identified pronounced topological instability across different analyses. The addition of recently sequenced two mitogenomes for the Aspidogastrea subclass along with the use of a site-heterogeneous model stabilized the topology, particularly the positions of Azygiidae and Bucephalidae. The stabilized results indicated that Azygiidae was the closest lineage to Bucephalidae in the available dataset, and together, they clustered at the base of the Plagiorchiida.

Conclusions Our study provides the first comprehensive description and annotation of the mitochondrial genome for the Bucephalidae family. The results indicate a close phylogenetic relationship between Azygiidae and Bucephalidae, and reveal their basal placement within the order Plagiorchiida. Furthermore, the inclusion of Aspidogastrea mitogenomes and the site-heterogeneous model significantly improved the topological stability. These data will provide key molecular resources for future taxonomic and phylogenetic studies of the family Bucephalidae.

Keywords *Dollfustrema vaneyi*, Bucephalidae, Mitochondrial genome, Phylogenetic analysis

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Background

The Bucephalidae Poche, 1907 (Platyhelminthes: Neodermata: Trematoda: Digenea: Plagiorchiida: Bucephalata: Bucephaloidea) is a large family of digenean trematodes, comprised of nine subfamilies [1]. Typically, Bucephalidae parasitize marine, brackish and freshwater fishes [2] and have a triple-host life history. They are one of only nine digenean families that utilize bivalves as their first intermediate hosts [3, 4]. The cercariae (a larval stage in trematodes, which develops within the germinal cells of the sporocyst or redia) emerge from the bivalves and infect the second intermediate hosts (Osteichthyes). Metacercariae develop from cercariae in the second intermediate host, and the life cycle is completed when the second intermediate host, or the part of it that contains a metacercaria, is ingested by the final host, a piscivorous Osteichthyes [1].

A species from this family, *Dollfustrema vaneyi* (Tseng, 1930) Nagaty, 1937 utilizes *Limnoperna fortunei* (Mollusca: Mytillidae) as the first intermediate host. It can utilize a variety of small Cypriniformes and Siluriformes fishes as its second intermediate hosts, and multiple sinipercid fishes (e.g., *Siniperca chuatsi*) as the terminal hosts. *Dollfustrema vaneyi* is widely distributed in China, where the adult worms mainly parasitize the intestines of *S. chuatsi* and many other freshwater fish species. Metacercariae commonly parasitize the gills, kidneys, liver, mouth, gall bladder, and heart of *Carassius auratus*, *Ctenopharyngodon idella*, and *Hemibarbus maculatus* [5].

Traditionally, morphology was the most common and widely used method for identifying and classifying parasites. Members of the family Bucephalidae differ from all other digeneans in the morphology of the digestive system and terminal genitalia. They have neither oral nor ventral suckers; instead, they have a rhynchus. Characterization of this organ is taxonomically important in Bucephalidae [6]. However, morphological methods have multiple limitations for species identification and phylogenetic studies in small parasitic animals, comprising the low resolution caused by a small number of distinguishing traits and host-induced morphological variation, often producing homoplastic traits [3, 7, 8]. Molecular data are increasingly employed to this end in helminths, but they remain unavailable or limited in scope for many lineages.

Furthermore, the systematic position of Bucephalidae remains unresolved It was initially hypothesized that Bucephalidae shared a common ancestor with Brachylaemidae due to the similarity of sporocyst and miracidium structures [9], but a recent study showed that Bucephalidae, Gymnophallidae and Fellodistomidae likely form a single clade [10]. In addition, the position of the suborder Bucephalata within the Digenea also remains unresolved. It was initially described as a suborder Strigeoidea [11], but early molecular phylogenetic studies indicated that Bucephalata is a distinct branch of Digenea, comprising Bucephaloidea and Gymnophalloidea [12]. A subsequent study found that Bucephalata was paraphyletic, because Bucephalidae did not cluster with Gymnophalloidea [13]. Further studies are needed to improve our understanding of the taxonomy and phylogeny of Bucephalata.

The Azygiidae family is also an important and controversial lineage within the Trematoda class. Previous studies have discussed the positioning of the Azygiidae family relative to other trematode families. Most authors currently recognize this lineage as a separate superfamily, Azygioidea [13–15], but there are differing views on its higher taxonomic placement, with some considering it a separate suborder (Azygiata) [16, 17], or even an order (Azygiida) [18, 19]. Analyses based on lsrDNA and nuclear *18S* and *28S* rRNA genes have indicated a close relationship between Azygiidae and the superfamily Hemiuroidea [13, 20]. In contrast, recent phylogenetic analyses using mitochondrial genome data found evidence that Azygiidae formed a distinct, early-diverging clade within the Digenea [21, 22]. To our knowledge, none of the previous studies found Azygiidae to be closely related to Bucephalidae.

Mitochondrial genome sequences are much more informative than short sequences of individual genes for phylogenetic reconstruction [23]. Along with a number of other comparative advantages (e.g. unilinear inheritance, the absence of recombination, etc.), mitogenomes are a powerful, albeit not flawless, phylogenetic marker [24, 25]. However, currently there are no complete mitochondrial genomes available for the Bucephalidae family. Therefore, the aim of this study was to sequence and characterize the complete mitochondrial genome of *D. vaneyi*, and use its coding regions to infer the phylogenetic relationships between the family Bucephalidae and other trematodes.

Methods

Specimen collection and DNA extraction

Dollfustrema vaneyi specimens were obtained from mandarin fish (*Siniperca chuatsi*) in Liangzi Lake (E114°37′, N30°11′), Hubei Province, China. The host fish were euthanized using 250 mg/L MS-222 (buffered with sodium bicarbonate for a pH between 7–7.5) and then immediately surgically dissected. The parasites were washed in physiological saline, and some of them were fixed in 4% formaldehyde, whereas others were stored in 99% ethanol at 4 °C. The specimens fixed in formaldehyde were later stained in carmine, and morphologically

identified based on the anterior rhynchus with triple crown of spines and the ventral mouth in the posterior half of body as described by Moravec et al. [26]. In order to further validate its identity, we extracted DNA from specimens stored in ethanol using the entire specimen and the TIANamp Micro DNA Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Finally, we sequenced the 18S ribosomal RNA (*18S*) gene and confirmed high similarity to orthologues of other samples from this species available in the GenBank (see Table S1).

DNA amplification and sequencing

Partial sequences of NADH dehydrogenase subunit 4 (*nad4*), 12S ribosomal RNA (*12S*), and cytochrome c oxidase subunit 2 (*cox2*) were preliminarily amplified by PCR using the following degenerate primer pairs (see Table S2). Based on the sequences of these fragments, we designed specific primers for subsequent PCR amplification (see Table S2). The PCR reaction was performed in a 20 µl reaction mixture consisting of 7.4 µl of double-distilled water, 10 µl of 2 × PCR buffer (Mg^{2+}, dNTP plus; Takara, Dalian, China), 0.6 µl of each primer, 0.4 µl of rTaq polymerase (250 U, Takara), and 1 µl of DNA template. The amplification conditions were as follows: predenaturation at 98 °C for 2 min; followed by 40 cycles at 98℃ for 10 s, 48 ~ 60℃ for 15 s, 68℃ for 1 min/kb; and the last extension at 68 °C for 10 min. The PCR products were sequenced bi-directionally at Sangon Company (Shanghai, China) using the primer-walking strategy as described previously [27].

Mitogenomic annotation and analyses

After the BLASTn [28] analysis, the mitochondrial genome sequence was assembled manually in a step-by-step manner. To identify gene boundaries, the mitochondrial genome of *D. vaneyi* was aligned with the mitochondrial genome sequences of other published digenean species using the MAFFT version 7.149 software [29]. The Open Reading Frame Finder [30] and MITOS Web Server [31] were used with the genetic codes specific to echinoderms and flatworms to predict the protein-coding genes (PCGs) [32]. The transfer RNA genes (tRNAs) were identified using ARWEN [33], DOGMA [34], and MITOS web servers. The two ribosomal RNA genes (rRNAs), *rrnL* and *rrnS*, were also preliminarily identified using MITOS, and their exact boundaries were then determined by comparing them to closely related orthologues. The sliding window analysis was conducted using DnaSP v5 [35] using a sliding window of 100 bp and a step size of 25 bp to estimate the nucleotide diversity (pi) between the mitochondrial genomes of *D. vaneyi* and *Azygia hwangtsiyui* (Azygiidae) [21], identified as the phylogenetically closest available mitogenome. The evolutionary rate analysis of 12 PCGs of *D. vaneyi* and *A. hwangtsiyui* was performed using KaKs_Calculator [36]. PhyloSuite was used to calculate and plot the codon usage and relative synonymous codon usage (RSCU) for PCGs [37, 38]. It was also utilized for the analysis of mitochondrial genomes of *D. vaneyi* and *A. hwangtsiyui*, including the calculation of genetic distances (identity) between sequences and statistical analysis. The genetic distances (identity) between mitochondrial genome sequences were calculated using the "DistanceCalculator" function in Biopython, utilizing the "identity" model. Tandem Repeats Finder was used to identify repetitive sequences in non-coding regions [39], and the MFOLD web server was used to predict their secondary structures [40].

Phylogenetic analyses

In addition to the newly sequenced mitogenome of *D. vaneyi*, we retrieved mitogenomes for all 52 available Trematoda species for phylogenetic analysis (for the full list, see Table S3). Two Cestoda species (*Didymobothrium rudolphii* and *Breviscolex orient*) were used as outgroups. Taxonomy is presented according to the WoRMS database as the main authority [41]. PhyloSuite was used to parse and extract mitogenomic annotations recorded in Word documents and create GenBank submission files and organization tables for the mitogenome. PhyloSuite was also used to extract gene sequences from GenBank files and import the extraction results into MAFFT [29] for multiple sequence alignment. Then, MACSE [42] was used to optimize PCG alignments. Alignments of nucleotide sequences of PCGs were trimmed using Gblocks [43], whereas amino acid (AA) and RNA sequences were pruned using trimAl [44]. All sequences were concatenated using PhyloSuite. ModelFinder [45] was used to select the optimal partitioning strategy and evolutionary models for concatenated datasets. To infer phylogenetic relationships, we applied two datasets and three different algorithms. Datasets were: (1) concatenated nucleotide sequences of 12 PCGs, 22 tRNAs, and two rRNAs (PCGsRNA dataset), and (2) concatenated amino acid sequences of 12 PCGs (PCGAA dataset). Maximum Likelihood (ML) in IQ-TREE version 2.2.0 [46] and Bayesian inference (BI) in MrBayes-3.2.7 [47] were conducted using plugins in PhyloSuite. For the PCGAA dataset, mtInv + F + I + R6 was chosen as the best model for ML analysis, and JTT + F + I + G4 for the BI analysis. For the PCGsRNA dataset, each partition was assigned its own optimal model in both ML and BI analyses (see Table S4). Finally, we also tested the perfomance of CAT-GTR model in PhyloBayes MPI 1.5a [48] (PB). Phylogenetic trees and gene orders were visualized

and annotated using iTOL [49] and annotation files generated by PhyloSuite. During the revision, we used the same methodological approach as described above to perform phylogenetic analysis on the following two datasets, in order to evaluate their impact on the topological structure:

(1) We added the mitochondrial genome sequence of *Azygia robusta*, which it was unavailable at the time when we conducted original analyses
(2) Aside from adding the *Azygia robusta*, we removed the mitochondrial genome sequences of two recently sequenced *Aspidogaster* species [50].

In addition, C10—C60 models were tested by ModelFinder to determine which 'site-heterogeneous model' best fit our data, with C50 emerging as the optimal choice. Ultimately, we analyzed the PCGAA to the dataset that included the mitochondrial genome sequence of *Azygia robusta* and *Aspidogaster* species using a profile mixture model (mtInv + I + C50 + F + R6).

Results

Mitochondrial genome characterization

The complete mitochondrial genome of *D. vaneyi* was a 14,959 bp-long circular molecule (Table 1 and Fig. 1). It contained 36 genes, comprising 12 PCGs, 22 tRNAs, two rRNAs, and a major non-coding region (NCR). Similar to other Neodermata, it lacked the *atp8* gene. We identified 22 intergenic regions (ranging from 1 to 46 bp in length), and four overlaps between pairs of genes (ranging from 1 to 40 bp in length). The largest overlap was identified between *nad4L* and *nad4* genes (Table 1). The nucleotide composition showed a strong A + T bias. AT skew was -0.426 (Table 2).

Protein-coding genes and codon usage

In the mitochondrial genome of *D. vaneyi*, the highest A + T content was exhibited by *nad2* (67.5%) and the lowest by *cox2* (59.5%). Among the 12 PCGs, the start codon of three genes (*cytb*, *nad3*, and *cox1*) was GTG, the start codon of *nad6* was TTG, and the start codon of all other genes was ATG. Four genes (*cytb*, *nad1*, *cox1*, *nad5*) terminated with the abbreviated T– stop codon, and all other genes used the TAG stop codon (Table 1 and Table 2). According to the amino acid usage and relative synonymous codon usage, among the 12 PCGs, the most commonly used codon, UUU (Phe), occurred 365 times. The least used codons, CGA (Arg) and GCA(Ala), occurred only three times. The most common amino acids in the 12 PCGs of *D. vaneyi* were leucine (Leu1 + Leu2) and Phe (Fig. 2).

Transfer RNAs, ribosomal RNAs, and non-coding region

The 22 tRNAs genes of *D. vaneyi* ranged in length from 54 bp (*trnS1*) to 68 bp (*trnC*) (Table 1 and Fig. S1). The *rrnL* gene was located between *trnT* and *trnC*, and *rrnS* was located between *trnC* and *cox2*. The major non-coding region was located between *cox3* and *trnG* (Fig. 1). It comprised 16 sequence repeats (90 bp each repeat), with the 16th repeat exhibiting two base deletions (Fig. 3).

Gene arrangement

Comparative analyses of gene arrangements among taxa in the order Plagiorchiida revealed an almost perfectly conserved mitogenomic architecture (Fig. S2). All selected taxa shared three gene blocks: *trnH-cytb-nad4L-nad4-trnQ-trnF-trnM-atp6-nad2-trnV-trnA-trnD*, *trnP-trnI-trnK-nad3*, and *trnT-rrnL-trnC-rrnS-cox2-nad6*. The gene order was nearly identical in Bucephalidae, Azygiidae, Notocotylidae, Cyclocoelidae, Plagiorchiidae, Prosthogonimidae, and Dicrocoeliidae, with the exception of translocations involving *trnE* and *trnG* genes. Contrary to this, Schistosomatidae displayed extensive genetic reorganization of protein-coding genes and tRNAs. A species in the family Paramphistomidae exhibited major inversions in the gene arrangement, but this mitogenome was incomplete so we strongly suspect assembly and annotation artefacts.

Phylogenetic inference

Except for the BI and ML analyses of the PCGAA dataset, the phylogenetic trees constructed using PCGAA and PCGsRNA datasets consistently demonstrated the division of Trematoda into three strongly supported orders (Fig. 4–5, Fig. S3-S5). The earliest diverging (or basal) order was Aspidogastrida (2 species—1 family), and the remaining majority of species was divided into Diplostomida (17 species—6 families) and Plagiorchiida (34 species—19 families). Azygioidea and Bucephaloidea formed the basal lineage within the Plagiorchiida. The remaining lineages were divided into two major clades; the smaller one comprising Pronocephaloidea and Paramphistomoidea, whereas the larger one comprised a range of superfamilies, including Echinostomatoidea, Microphalloidea, Plagiorchioidea, Opisthorchioidea, Brachycladioidea, Troglotrematoidea, and Gorgoderoidea. Most taxa were monophyletic, apart from Echinostomatoidea due to Eucotylidae clustering with Microphalloidea.

BI and ML analyses of the PCGsRNA dataset produced phylogenetic trees with fully congruent topologies (Fig. 4). The PB tree topology (Fig. 5) exhibited a few differences in comparison to the BI and ML topologies:

Table 1 Organization table of the mitochondrial genome of *Dollfustrema vaneyi*

Gene	Position		Size	Intergenic nucleotides	Codon		
	From	To			Start	Stop	Anti-codon
NCR	13314	14,959	1646	13,313			
cox3	1	651	651		ATG	TAG	
trnH	656	718	63	4			GTG
cytb	723	1797	1075	4	GTG	T–	
nad4L	1844	2104	261	46	ATG	TAG	
nad4	2065	3333	1269	-40	ATG	TAG	
trnQ	3352	3414	63	18			TTG
trnF	3419	3481	63	4			GAA
trnM	3492	3557	66	10			CAT
atp6	3561	4070	510	3	ATG	TAG	
nad2	4080	4967	888	9	ATG	TAG	
trnV	4981	5040	60	13			TAC
trnA	5041	5102	62				TGC
trnD	5103	5162	60				GTC
nad1	5167	6061	895	4	ATG	T–	
trnN	6062	6126	65				GTT
trnP	6127	6192	66				TGG
trnI	6192	6256	65	–1			GAT
trnK	6267	6332	66	10			CTT
nad3	6336	6698	363	3	GTG	TAG	
trnS1	6702	6755	54	3			GCT
trnW	6758	6818	61	2			TCA
cox1	6823	8386	1564	4	GTG	T–	
trnT	8387	8448	62				TGT
rrnL	8449	9409	961				
trnC	9408	9475	68	–2			GCA
rrnS	9467	10,233	767	–9			
cox2	10,234	10,821	588		ATG	TAG	
nad6	10,826	11,275	450	4	TTG	TAG	
trnY	11,276	11,339	64				GTA
trnL1	11,341	11,402	62	1			TAG
trnS2	11,405	11,468	64	2			TGA
trnL2	11,472	11,532	61	3			TAA
trnR	11,538	11,597	60	5			TCG
nad5	11,604	13,188	1585	6	ATG	T–	
trnE	13,189	13,249	61				TTC
trnG	13,251	13,313	63	1			TCC

(1) In the PB tree, the family Clinostomidae formed a cluster with Brachylaimidae and Schistosomatidae. However, in the BI and ML trees, Clinostomidae clustered together with Cyathocotylidae, Strigeidae, and Diplostomidae.
(2) In the PB tree, the family Dicrocoeliidae grouped with Brachycladiidae, Paragonimidae, Heterophyidae, and Opisthorchiidae. Conversely, in the BI and ML trees, Dicrocoeliidae clustered with Prosthogonimidae, Eucotylidae and Plagiorchiidae.

Regarding the PCGAA dataset, there were also some topological differences among the results produced by the three different algorithms (Fig. S3-S5). In the BI topology, the orders Diplostomida and Plagiorchiida were paraphyletic. At the family level, Brachylaimidae

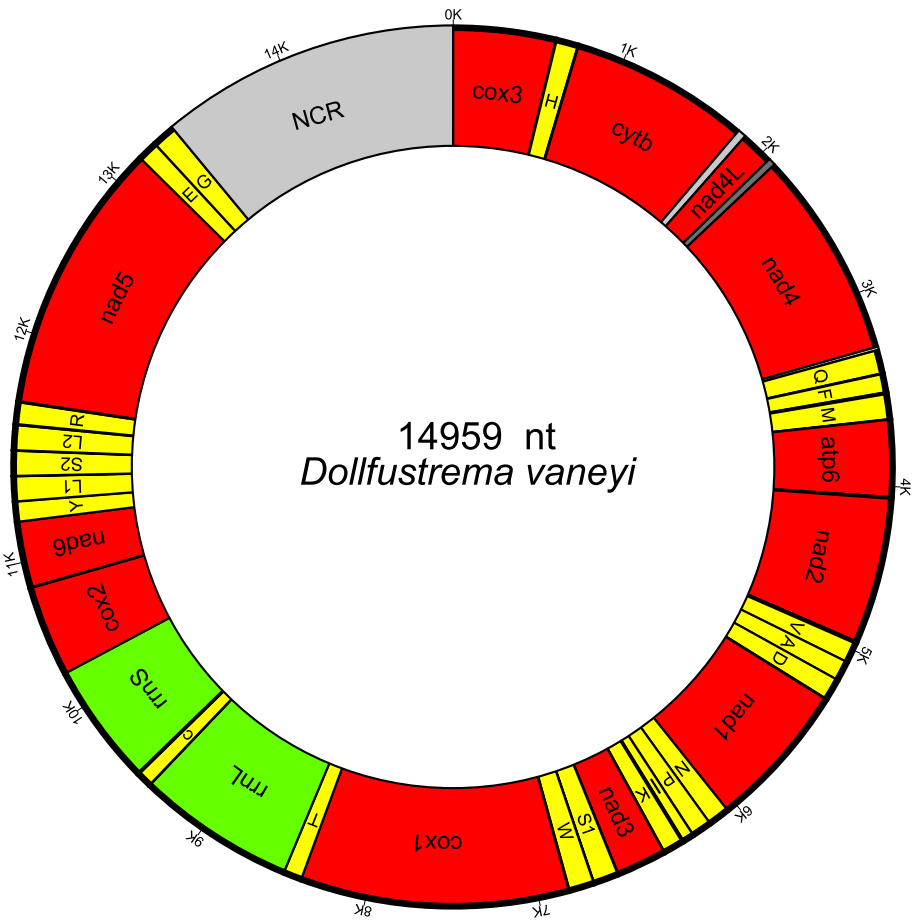

Fig. 1 The circular mitochondrial genome of *Dollfustrema vaneyi*. Protein-coding genes are shown in red, tRNAs in yellow, rRNAs in green, and non-coding regions in grey

clustered with Clinostomidae, and Dicrocoeliidae clustered with Prosthogonimidae and Eucotylidae. In contrast, in the PB topology, the order Diplostomida was a monophyletic group, Brachylaimidae clustered with Schistosomatidae, Prosthogonimidae and Eucotylidae clustered with Plagiorchiidae. In the ML topology, Azygioidea and Bucephaloidea clustered within the Diplostomida, resulting in paraphyletic Plagiorchiida. All results support the close phylogenetic relationship of Bucephalidae and Azygiidae.

The addition of *Azygia robusta* to PCGAA and PCGsRNA datasets did not affect the phylogenetic position of *Dollfustrema* and *Azygia* species (Fig. S6-S12). Azygiidae and Bucephalidae were consistently closely related to each other, and positioned at the base of the Plagiorchiida order in all analyses of PCGAA and PCGsRNA datasets, including the profile mixture model analysis of the PCGAA dataset. The only exception was ML analysis of the PCGAA dataset, where they were positioned at the base of the subclass Digenea. However, after the removal of *Aspidogaster* species in the PCGAA and PCGsRNA datasets (with *Azygia robusta*), the phylogenetic positions of *Dollfustrema* and *Azygia* species have changed (Fig. S13-S18). The topological structure of the phylogenetic tree became less stable, with three different phylogenetic positions inferred for *Azygia* species:

(1) In the BI and PhyloBayes analysis of the PCGsRNA dataset and PhyloBayes analysis of the PCGAA, Azygiidae was placed at the basal position within the Digenea.
(2) In the BI analyses of the PCGAA dataset, Azygiidae clustered together with a portion of Diplostomida species and the remaining Plagiorchiida species, thus rendering Diplostomida polyphyletic.
(3) In the ML analyses of the PCGAA and PCGsRNA dataset, Azygiidae was placed at the base of the Plagiorchiida order.

Table 2 Nucleotide composition and skewness of different elements of the mitochondrial genome of *Dollfustrema vaneyi*

Regions	Size (bp)	T(U)	C	A	G	AT(%)	GC(%)	GT(%)	AT skew	GC skew
PCGs	10,095	46.8	14.2	16.2	22.9	63	37.1	69.7	-0.486	0.234
1st codon position	3365	39.7	14.6	20.3	25.3	60	39.9	65	-0.323	0.268
2nd codon position	3365	48	14.8	15.6	21.6	63.6	36.4	69.6	-0.509	0.189
3rd codon position	3365	52.6	13.2	12.6	21.6	65.2	34.8	74.2	-0.613	0.242
atp6	510	48.2	14.1	14.3	23.3	62.5	37.4	71.5	-0.542	0.246
cox1	1564	43.7	15.3	18.7	22.3	62.4	37.6	66	-0.4	0.186
cox2	588	40.1	13.8	19.4	26.7	59.5	40.5	66.8	-0.349	0.319
cox3	651	49.8	12.1	16.4	21.7	66.2	33.8	71.5	-0.503	0.282
cytb	1075	45.5	14.8	18.1	21.6	63.6	36.4	67.1	-0.43	0.187
nad1	895	45	14.4	17.2	23.4	62.2	37.8	68.4	-0.447	0.237
nad2	888	51.5	11.7	16	20.8	67.5	32.5	72.3	-0.526	0.28
nad3	363	48.5	12.4	18.5	20.7	67	33.1	69.2	-0.449	0.25
nad4	1269	46.2	15.1	13.4	25.3	59.6	40.4	71.5	-0.55	0.251
nad4L	261	46.7	11.9	19.9	21.5	66.6	33.4	68.2	-0.402	0.287
nad5	1585	49.1	14.4	13.2	23.3	62.3	37.7	72.4	-0.575	0.236
nad6	450	50	16.2	12.7	21.1	62.7	37.3	71.1	-0.596	0.131
rrnL	961	41.7	14.3	22.4	21.6	64.1	35.9	63.3	-0.302	0.206
rrnS	767	42.1	14.7	22.6	20.6	64.7	35.3	62.7	-0.302	0.166
rRNAs	1728	41.9	14.5	22.5	21.2	64.4	35.7	63.1	-0.302	0.188
tRNAs	1379	40.3	13.9	22.4	23.4	62.7	37.3	63.7	-0.286	0.253
Full genome	14959	45.1	13.3	18.2	23.4	63.3	36.7	68.5	-0.426	0.275

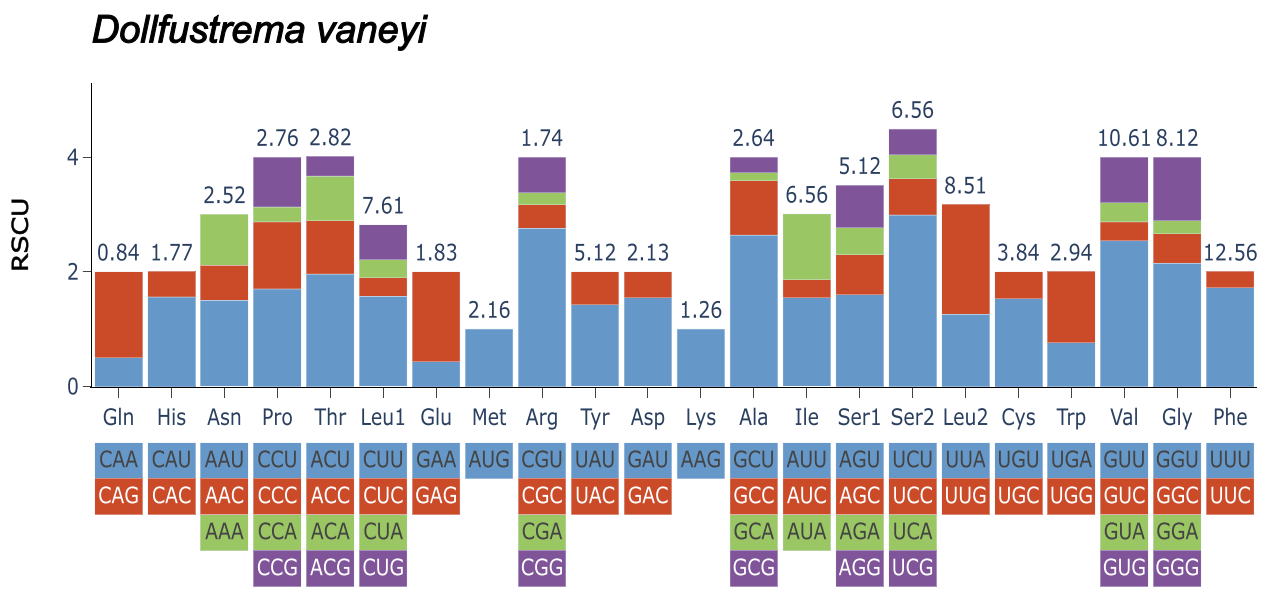

Fig. 2 Relative synonymous codon usage (RSCU) of *Dollfustrema vaneyi*. The values at the top of the bars indicate amino acid usage. Codon families are labeled on the x-axis

Discussion

Currently, there are no complete mitochondrial genomes available for the Bucephalidae family. Previous studies have primarily relied on a single mitochondrial gene or morphological features and have primarily focused on investigating intra-generic relationships within the Bucephalidae family. As a result, there remain significant gaps in our understanding of the evolution and classification of the Bucephalidae family. To address these knowledge gaps, we focused on characterizing and analyzing

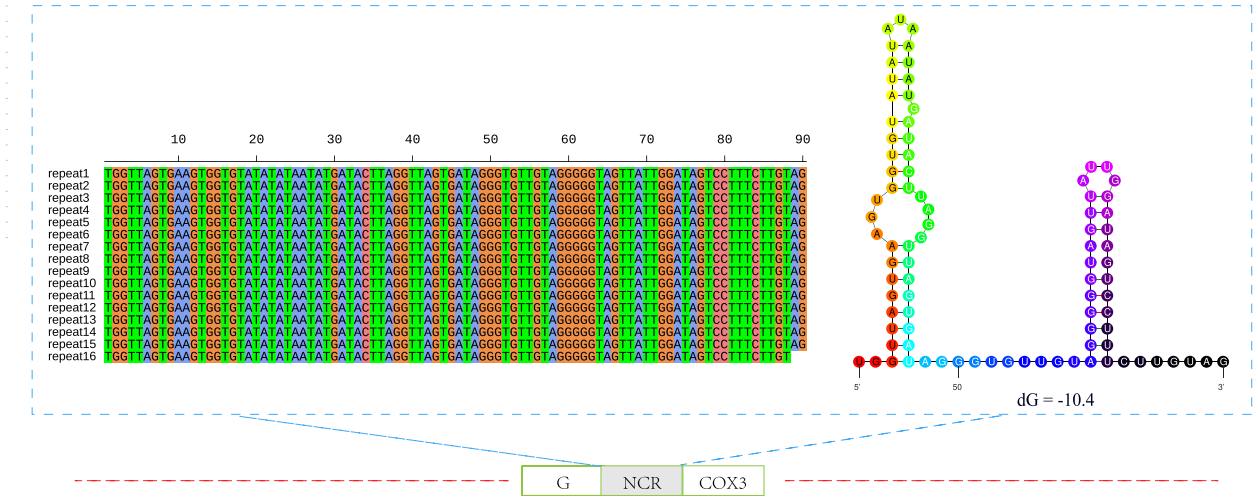

Fig. 3 Repeats and their structure in the major non-coding region of *Dollfustrema vaneyi*. Thermodynamic energy values (dG) are shown next to the secondary structures

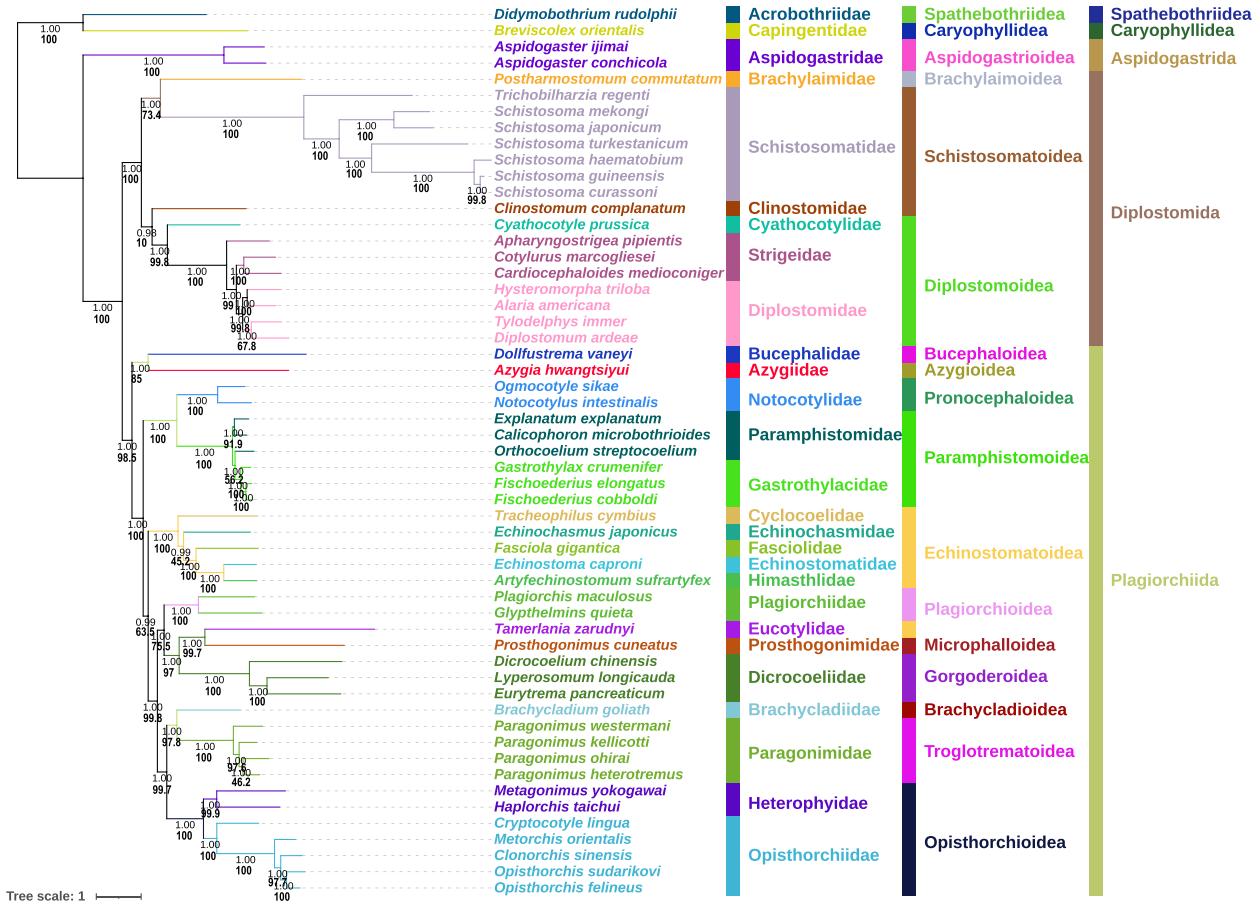

Fig. 4 Phylogeny reconstructed using the PCGsRNA dataset from representative species and families of Trematoda, and BI and ML algorithms. Statistical support values for BI are shown above the nodes, and below the nodes for ML. The taxonomic identity (families, superfamilies and orders) is shown to the right, with the family-level identity additionally indicated by different colors

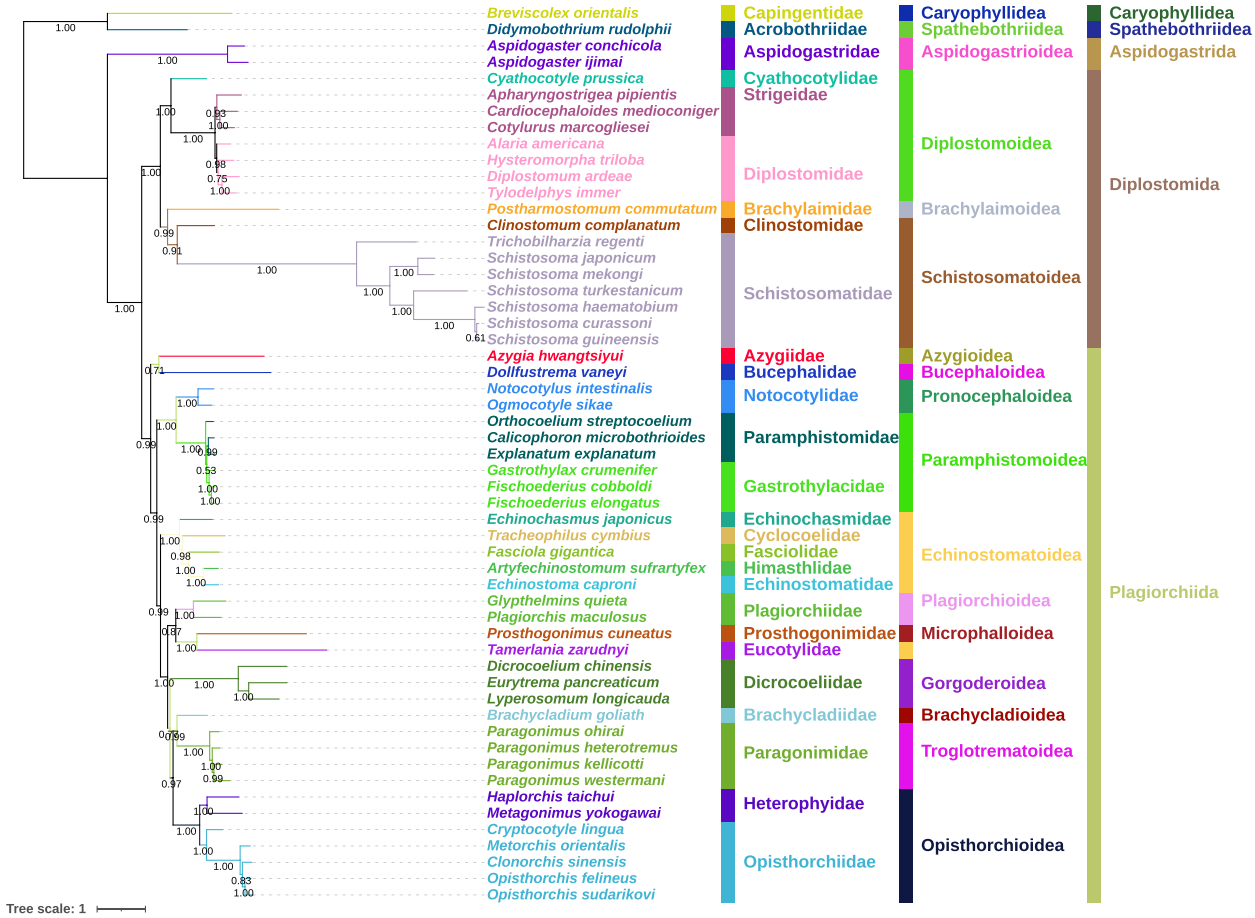

Fig. 5 Phylogeny reconstructed using the PCGsRNA dataset from representative species and families of Trematoda and PhyloBayes. The taxonomic identity (families, superfamilies and orders) is shown to the right, with the family-level identity additionally indicated by different colors

the mitochondrial genome of *D. vaneyi* and used it to infer the phylogenetic relationships between the family Bucephalidae and other trematodes.

The mitochondrial genome structure

The complete sequenced mitochondrial genome of *D. vaneyi* exhibited a standard architecture for trematodes. There was a major non-coding region, which has also been reported in some other species in the Plagiorchiida order. A very large overlap of 40 bp was identified between the *nad4L* and *nad4* genes, which is consistent with most Plagiorchiida species [51]. The nucleotide composition exhibited a strong A + T bias, similar to most other digeneans, such as *Plagiorchis multiglandularis* (65.17%) and *Echinostoma hortense* (63.03%) [52, 53].

Several genes putatively used the abbreviated T– termination codon, which was also reported in several other species from the order Plagiorchiida: *Eurytrema pancreaticum*, *Lyperosomum longicauda*, and *Plagiorchis maculosus* [54–56]. TTG was identified as a start codon for *nad6*. This is not a standard codon, but it has been reported in previous studies as an alternative start codon in the mitochondrial genomes of some flatworms [57].

Comparison of *D. vaneyi* with *A. hwangtsiyui*

In all six topologies, *A. hwangtsiyui* (Azygiidae) and *D. vaneyi* (Bucephalidae) were closely related lineages within the order Plagiorchiida (Fig. S19 and Table S5-S6). Among the species of the order Plagiorchiida included in this study, only *D. vaneyi* and *A. hwangtsiyui* are parasites of carnivorous fish: *D. vaneyi* is a parasite of *Siniperca chuatsi* (order Centrarchiformes), while *A. hwangtsiyui* parasitizes predatory fish species belonging to the order Anabantiformes, such as *Ophiocephalus argus* (Cantor, 1842) and *Channa asiatica* (Linnaeus, 1758) [21]. Morphologically, *A. hwangtsiyui* and *D. vaneyi* share certain characteristics. They both have a short esophagus and are characterized by the arrangement of two testes, one anteriorly and one posteriorly, located in the posterior 1/3 of their bodies. The ovaries are located before the anterior testes. The uterine ring in both species exhibits folds that open at the genital foramen. They also possess Lowe's

ducts and follicular yolk glands. However, there are notable differences between *D. vaneyi* and *A. hwangtsiyui* in terms of their body surface. *D. vaneyi* is densely covered with small spines, while *A. hwangtsiyui* lacks spines on its body surface. Furthermore, *A. hwangtsiyui* possesses both an oral sucker and a ventral sucker, whereas *D. vaneyi* does not.

Phylogeny

The systematic position of Bucephalidae within the Digenea has been a topic of debate for a long time due to initial studies suggesting that "gasterostomes" were distinct from the majority of other Digenea groups [58]. The structural similarity between the sporocyst and miracidium suggested a possible ancestral relationship between the families Bucephalidae and Brachylaemidae [59]. Additionally, the close resemblance of their cercariae has indicated a potential close relationship between the families Fellodistomidae and Brachylaimidae [60, 61]. However, further studies have shown that Bucephalidae is not closely related to Fellodistomidae [13, 62]. Subsequent research, particularly investigations into the life cycle of digeneans [16] and molecular analyses [20], have provided compelling evidence that Bucephalidae is not a basal lineage in this subclass.

In all topologies inferred in this study, Paramphistomidae and Gastrothylacidae were consistently resolved as sister lineages, and the formed sister group shares the most recent common ancestor with Notocotylidae. This pattern aligns with previous studies utilizing the mitochondrial genome [51, 63–65]. However, a previous phylogenetic study based on ITS2 showed a different relationship compared to these findings: Notocotylidae was closely related to Bucephalidae, rather than Paramphistomidae and Gastrothylacidae [66]. Contrary to this, in our analyses, Azygiidae and Bucephalidae formed a clade, rather than Notocotylidae and Bucephalidae.

In previous mitochondrial genome-based studies, the Azygiidae formed a distinct, early divergent lineage, supporting their identification as a separate order (Azygiia) [21, 22]. However, these studies used only standard ('homogenous') models for amino acids data and lacked the mitochondrial genomes of some other key lineages of Trematoda (e.g. Aspidogastrea and Bucephalidae). According to all phylogenetic analyses conducted herein, Bucephalidae is closely related to Azygiidae. With the exception of the ML analysis of PCGAA dataset, all results support the position of Azygiidae and Bucephalidae at the base of the Plagiorchiida order. To further resolve this discrepancy, we replaced the standard model with a profile mixture model in the ML analysis of the PCGAA dataset. This analysis resolved Azygiidae and Bucephalidae at the base of the Plagiorchiida order. This finding suggests that the topological instability observed in the ML analysis of the PCGAA dataset might be attributed to the base composition heterogeneity of the dataset. This observation aligns with previous research indicating that data heterogeneity can cause inaccuracies in phylogenetic reconstruction [67, 68]. Employing phylogenetic models designed to account for data heterogeneity, such as the profile mixture model, can (often) effectively address this issue [25].

Consistent with previous phylogenetic studies based on ITS1 [66], the Bucephalidae family diverged before Notocotylidae, Plagiorchiidae, Dicrocoeliidae, Heterophyidae, and Opisthorchiidae in all of our phylogenies. However, two studies based on different molecular markers have produced different results: one study based on lsrDNA and maximum-likelihood and Bayesian inference found that Bucephalidae was closely related to Haplosplanchnidae [13], and a study based on the nuclear *18S* and *28S* rRNA genes found that Bucephalidae was closely related to Fellodistomidae + Tandanicollidae [20]. These inconsistent results across different molecular markers and analytical methods highlight the need for further in-depth studies aimed at understanding the phylogenetic relationships of Bucephalidae [69, 70].

In all phylogenetic trees, the phylogenetic positions of most lineages of the order Plagiorchiida were consistent with previous studies. However, some differences were observed regarding the Echinostomatoidea superfamily. Our analyses show that Echinostomatidae and Himasthlidae clustered together. However, a phylogenetic analysis based on the mitochondrial genomes showed that Himasthlidae clustered with Echinochasmidae [66], and in a study based on ssrDNA and maximum-likelihood and Bayesian inference, Echinochasmidae was closely related to Philophthalmidae, and together they formed a clade with Cyclocoelidae, while Echinostomatidae was closely related to Fasciolidae [13]. This discrepancy may be attributed to the lack of mitochondrial genomic data for other species of the superfamily Echinostomatoidea. Therefore, further studies are needed to draw accurate conclusions about the relationship between Echinostomatidae and Himasthlidae in the superfamily Echinostomatoidea. Additionally, within the superfamily Echinostomatoidea, Eucotylidae did not cluster with other families, offering further evidence that the suborder Echinostomata is polyphyletic [51].

Regarding the taxonomic position of Paragonimidae, there have been disagreements in previous findings. In a study based on Bayesian inference using lsrDNA and ssrDNA, Paragonimidae were placed within the Gorgoderoidea [20]. However, subsequent studies classified Paragonimidae into the superfamily Troglotrematoidea [71]. In our results, Paragonimidae clustered with

Brachycladiidae, while Gorgoderoidea formed a separate clade. Therefore, our mitochondrial genome results are consistent with the findings of Vainutis et al. in indicating that Paragonimidae does not belong to the superfamily Gorgoderoidea; but rather belongs to the superfamily Troglotrematoidea [71].

The removal of *Aspidogaster* species in the PCGAA and PCGsRNA datasets destabilized the topology, with three different phylogenetic positions observed for Azygiidae. However, the inclusion of *Aspidogaster* species stabilized the phylogenetic placement of the Azygiidae and Bucephalidae at the base of the Plagiorchiida order. This finding suggests that the placement of Azygiidae was influenced by the inclusion of *Aspidogaster* species data. The two *Aspidogaster* species are the only sequenced representatives for the entire Aspidogastrea subclass of Trematoda [50, 72]. This finding further emphasizes the importance of key taxa in phylogenetic analysis [73–75], as their inclusion can affect the stability of the topology of the phylogenetic tree. The entire Aspidogastrea subclass (represented by *Aspidogaster* herein) has been absent from previous phylogenetic analyses of the Trematoda class, particularly those based on mitochondrial genomes, which may have produced erroneous results. Therefore, it is recommended that future phylogenetic studies of the class Trematoda should include *Aspidogaster* species data to improve the accuracy and stability of phylogenetic trees.

Conclusions

In summary, we conducted the sequencing and analysis of the mitochondrial genome of *D. vaneyi*, representing the first comprehensive description and annotation of mitochondrial genome for the Bucephalidae family. Phylogenetic reconstruction supports a close relationship between Azygiidae and Bucephalidae, and all results support the position of these two families at the base of the Plagiorchiida order. The inclusion of recently sequenced *Aspidogaster* species (Aspoidogastrea) improved the topological stability, so we infer that this is a crucial lineage for phylogenetic studies of Trematoda. Ignoring sequence heterogeneity can lead to incorrect clustering and inaccurate phylogenetic relationships. The use of a site-heterogeneous model effectively addressed this issue, resulting in a more robust and reliable phylogeny. However, it is important to acknowledge several limitations of our study. The low support for some nodes, highlights the need for additional studies with stronger datasets. In addition, some key lineages were missing from our dataset, which certainly affected the accuracy of our phylogenetic analyses. Future research should focus on obtaining complete mitochondrial genome sequences from unrepresented and underrepresented lineages of Trematoda to address this limitation.

Abbreviations

18S	18S ribosomal RNA
nad4	NADH dehydrogenase subunits 4
12S	12S ribosomal RNA
cox2	Cytochrome c oxidase subunits 2
ORFinder	Open Reading Frame Finder
PCGs	Protein-coding genes
AA	Amino acid
PCGsRNA	12 PCGs, 22 tRNAs, and two ribosomal RNA genes
PCGAA	12 Protein-coding genes
ML	Maximum Likelihood
BI	Bayesian inference
PB	PhyloBayes MPI 1.5a
NCR	Non-coding region
tRNAs	Transfer RNA genes
rRNAs	Ribosomal RNA genes
rrnS	Small ribosomal RNA
rrnL	Large ribosomal RNA
lsrDNA	Large subunit ribosomal RNA gene
ssrDNA	Small subunit ribosomal RNA gene

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Authors' contributions

D.Z., W. X. L, G.T.W and Y.H. designed the study. Y.H., W. X. L, T.Y. and H.Z. conducted the experiments. Y.H., D.Z. and T.Y. conducted the data analysis. Y.H. wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets for the conclusions of this paper are included in this paper and its supplementary material. The mitochondrial genome of *Dollfustrema vaneyi* has been deposited in GenBank (accession number: PP860916).

Data availability

The mitochondrial genome of Dollfustrema vaneyi has been deposited in GenBank (accession number: PP860916).

Declarations

Ethics approval and consent to participate

All animal experiments were approved and conducted in compliance with the experimental practices and standards of the Ethics Committee of the College of Ecology, Lanzhou University (ethics approval form No. EAF2024012).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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