The Complete Mitochondrial Genome of Schistura fasciolata: Phylogenetic and Evolutionary Implications within Tribe Nemacheilini

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Research Article

Keywords: Schistura fasciolata, Nemacheilini, mitogenome, phylogeny, evolution

Posted Date: March 1st, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3988578/v1

Additional Declarations: No competing interests reported.

Abstract

Background: The mitochondrial genome is widely used to understand the phylogeny and evolution of tribe Nemacheilini. This study aims to deepen the understanding of the mitochondrial genome of *Schistura fasciolata*, and the evolutionary implications within Nemacheilini.

Methods and Results: We conducted a comprehensive sequence analysis of the complete mitochondrial genome of *Schistura fasciolata*, which is comprised of 16,570 base pairs including 37 genes: 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), and two ribosomal RNA genes (rRNAs). The base composition analysis indicated an A+T content of 56.51% and a G+C content of 43.49%. Phylogenetic analysis based on the 13 PCGs revealed extensive polyphyly within Nemacheilini. Furthermore, accelerated evolutionary rates in *ND4*, *ND5*, and *CYTB* genes were observed in *Homatula* and *Troglonectes*, suggesting an adaptation to specific environmental conditions.

Conclusions: The mitochondrial genome analysis of *S. fasciolata* provides critical insights into the phylogeny and adaptive evolution within Nemacheilini. Our findings underscore the complex evolutionary dynamics within the tribe, with distinct evolutionary trajectories observed in mitochondrial genes among different genera.

Introduction

Nemacheilidae, an ecologically significant and taxonomically complex family of freshwater fish, mainly distributed across mainland Asia and its adjacent islands, Europe, and northeast Africa [1]. The species in this lineage live in various habitats, including swamps, rapids, rivers, streams, caves, and lakes [1, 2]. The family consists of nearly 760 species in 47 genera [3–5].

Prokofiev performed a detailed comparative morphological analysis [3], which divided Nemacheilidae into five tribes: Vaillantellini, Lefuini, Yunnanilini, Triplophysini, and Nemacheilini [4, 6–10]. However, the challenges of valid species identification exist due to morphological and genetic diversity, especially within the tribe Nemacheilini [9, 11]. Besides, the distribution also has led to taxonomic issues, especially in *Homatula*, *Nemacheilus* and *Schistura* [9, 11, 12]. Thus, the relationship has been always controversial within Nemacheilini. The polyphyly observed in some genera, such as *Homatula*, *Nemacheilus*, and *Schistura*, further complicates the taxonomy [12, 13]. Many studies focus on the complex phylogenetic relationships ususlly based on some mitochondrial gene fragments and/or morphological traits [14–16]. However, the confusion has been always discussed due to incomplete sample coverage, inadequate analysis methods, and lack of data, especially the short molecular fragments.

Mitochondrial DNA (mtDNA), known for its simple structure, maternal inheritance, rapid evolution, and short coalescence time, is crucial for species identification and phylogenetic analysis in fish [17], which helps assess genetic diversity, phylogeny, and evolution [18–20]. In this study, we obtained the complete mitochondrial genome of *S. fasciolata*, and then constructed the phylogeny of tribe Nemacheilini based on 13 PCGs, meanwhile, the evolutionary rate of each genus in Nemacheilini was evaluated. This study

aims to: (1) examine the mitogenomic structure of *S. fasciolata*; and (2) explore its phylogenetic and adaptation within this lineage.

Materials and methods

Sample collection and genomic DNA extraction

Adult specimens of *S. fasciolata* were collected from Jianchuan, Yunnan, China (26.25788065°N, 99.82247990°E) in May 25, 2023. Identification of adult specimens was based on morphological characteristics based on FishBase (https://fishbase.de/search.php). The samples were stored in 100% ethanol, and then were sent to Shanghai Personal bio technology Co. for genomic DNA extraction and sequencing. The Whole Genome Shotgun (WGS) strategy was used to construct a library of different insertions with a length of 400 bp. These libraries were Paired-end reads (PE, 150 bp in length) sequenced using Next-Generation Sequencing (NGS), based on the Illumina NovaSeq sequencing platform. Subsequently, the raw sequencing data was filtered to generate high-quality sequences. Then, the whole mitochondrial sequence was submitted to NCBI (Accession No. **OR797719.1**).

Sequence assembly, annotation and analyses

High quality second-generation sequencing data were reconstructed from scratch using A5-miseq v20150522 [21] and SPAdes v3.9.0 [22] to construct contig and scaffold sequences. Moreover, the sequences were extracted according to the sequencing depth of the splicing sequences, and the sequences with high sequencing depth were compared with the NT library on NCBI by blastn (BLAST v2.2.31 +), and the mitochondrial sequences of each splicing result were selected. Secondly, mummer v3.1 [23] software was used to carry out collinearity analysis of mitochondrial splicing results obtained by the above different software, so as to determine the position relationship between contigs and fill the gap between contigs. The results were corrected using pilon v1.18 [24] software to obtain the final mitochondrial sequence, the ".fasta" file. Subsequently, the complete mitochondrial genome sequence obtained by splices was uploaded to the MITOS web server (http://mitos.bioinf.uni-leipzig.de/) for functional annotation [25]. The Genetic Code selection is set to 02-vertebrate and the rest of the settings follow the default parameters set by MITOS. At the same time, in the MITOS web server, click Download each tRNA, structure download svg format file, you can view each predicted tRNA secondary structure.

In addition, PhyloSuite v1.2.3 software was used to compute the mitochondrial whole genome, the protein-coding genes, and the base composition of the rRNA genes, respectively [26, 27]. The strand asymmetry was calculated by using the formulas: AT-skew = (A - T) / (A + T), GC-skew = (G - C) / (G + C) [28, 29]. The whole mitochondrial genome was mapped using CGView visualization software [30].

Phylogenetic Analysis

To investigate the position of *S. fasciolata* within Nemacheilini and its phylogenetic relationships, we retrieved mitochondrial genome sequences of 12 Nemacheilini genera and used *Triplophysa bombifrons* and *T. labiata* as outgroups from NCBI. We utilized the sequences of 13 protein-coding genes (PCGs)

from 58 species to construct the phylogenetic tree (**Table S1**). Initially, sequences were processed using MEGA 7 software [31]. Following alignment of PCG sequences with the MAFFT algorithm [32], we reconstructed phylogenetic relationships using Maximum Likelihood (ML) methods in PhyloSuite v1.2.3 [26, 27]. ML phylogenetic trees were generated in IQ-TREE [33] using the ultrafast bootstrap (BS) algorithm with 1000 replications. ML bootstrap values (BS) ≥ 75 were deemed significant, indicating higher values correlate with increased topology reliability [34]. BI analyses, four independent runs were performed for 20 million generations. The phylogenetic trees were sampled every 1,000th generation, which resulted in 20,000 trees, and the first 25% were discarded as burn-ins, BI Posterior Probability (*PP*) ≥ 0.8 were deemed significant. Convergence of the BI analyses was assessed by the average standard deviation of split frequencies < 0.01 and used Tracer v1.5 [35] to investigate the convergence of the BI analyses (ESS > 200). Finally, the phylogenetic trees were visualized and refined using FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/software/Figtree/). This phylogenetic tree was used in next-step selective analysis.

Selection analyses

We used the CodeML program in PAML v4.9 [36] to assess the selection pressure on 13 PCGs sequences of mitochondria from 58 species, and calculated the evolution rate (namely, the ratio of nonsynonymous substitution and synonymous substitution, dN/dS) of each branch by using the free-ratio model. In order to evaluate whether there were significant differences in the evolution rate of 13 PCGs of each main genus in Nemacheilini, the dN/dS values of 56 extant species were extracted. In order to evaluate the discrepancy accurately, the data of dN or dS = 0, and dN/dS ≥ 1 were not considered, and then Kruskal-Wallis H test was implemented in SPSS to test the difference.

Results and Discussion

Mitogenome organisation and nucleotide composition

The mitochondrial genome of *S. fasciolata*, a double-stranded, circular molecule measuring 16,570 base pairs, aligns with the size range typical for the genus *Schistura* (**Supplementary Table S1**, from *S. incerta*: 16,561 bp to *S. geisleri*: 16,819 bp). This mitogenome consists of a canonical set of 13 PCGs, two rRNAs (12S rRNA and 16S rRNA), 22 tRNAs, and a control region known as the D-loop. Positioned between the tRNA-Pro and tRNA-Phe genes, the D-loop region serves as a crucial regulatory element for initiating replication and transcription. This organization is typical of metazoan mitochondrial genomes [37, 38]. Notably, the mitochondrial genome analysis reveals the presence of 23 intergenic spacers of variable lengths, including a significant − 10 base pair overlap between the *ATP8* and *ATP6* genes, indicative of direct genetic contiguity [38, 39]. Analysis of the *S. fasciolata* mitogenome nucleotide composition shows 30.14% adenine (A), 16.69% guanine (G), 26.37% thymine (T), and 26.80% cytosine (C). The A + T content (56.51%) predominates over the G + C content (43.49%) (**Table S2**), highlighting the genome's nucleotide bias and providing insights into its evolutionary adaptations and constraints.

Additionally, the mitogenome comprises two ribosomal RNAs, 12S rRNA (952 bp) and 16S rRNA (1,657 bp) (Table 1). Positioned between *tRNA-Phe* and *tRNA-Leu*, and separated by *tRNA-Val*, these rRNAs have high A / T contents of 50.32% and 55.94%, respectively (**Supplementary Table S2**). Their positioning and nucleotide composition, indicated by a positive AT-skew and a negative GC-skew, are essential for the structural and functional integrity of ribosomal RNA, crucial for protein synthesis [18].

Transfer RNAs, ribosomal RNAs

The *S. fasciolata* mitochondrial genome contains 22 tRNA genes, ranging in size from 66 bp (tRNA-Cys) to 76 bp (tRNA-Lys), each displaying the classic cloverleaf secondary structure (Table 1, Fig. 1). This set highlights the structural integrity and functional importance of mitochondrial tRNAs, particularly the trnA gene's retention of its dihydrouridine (DHU) arm, demonstrating these molecules' conservation across mitochondrial genomes. Despite some tRNAs lacking the D-arm, notably in trnS for AGY/N codons, this trait is widespread, signifying evolutionary adaptations for mitochondrial efficiency [40]. Further analysis of tRNA base-pairing shows mostly classical Watson-Crick A-U and G-C matches, with deviations like unmatched pairs U-U, U-C, A-A, and C-C in various tRNAs, indicating specific evolutionary adaptations. Additionally, a rare C-A pairing in trnF, trnH, trnM, and trnR was noted (Fig. 1). These observations highlight the nuanced balance between conserving structural features essential for function and introducing variations that may confer advantages in the mitochondrial context.

Table 1
Analysis of mitochondrial genome characteristics of *S. fasciolata.*

Genes	Strand	Position (start-end)	Length (bp)	Initiation codon	Stop codon	Anticodon	Intergenic nucleotide
trnF	N	1–69	69			GAA	
rrnS	N	70 – 1,021	952				2
trnV	N	1,024 – 1,095	72			TAC	19
rrnL	N	1,115-2,771	1,657				
trnL2	N	2,772-2,846	75			TAA	
nad1	N	2,847-3,821	975	ATG	TAA		7
trnI	N	3,829-3,900	72			GAT	-2
trnQ	J	3,899-3,969	71			TTG	1
trnM	N	3,971-4,039	69			CAT	
nad2	N	4,040 – 5,084	1,045	ATG	T(AA)		
trnW	N	5,085 – 5,155	71			TCA	2
trnA	J	5,158-5,226	69			TGC	1
trnN	J	5,228-5,300	73			GTT	1
OL	N	5,302-5,332	31				-2
trnC	J	5,331-5,396	66			GCA	
trnY	J	5,397-5,465	69			GTA	1
coI	N	5,467-7,017	1,551	GTG	TAA		
trnS2	J	7,018 – 7,088	71			TGA	2
trnD	N	7,091 – 7,163	73			GTC	13
coII	N	7,177-7,867	691	ATG	T(AA)		
trnK	N	7,868-7,943	76			TTT	1
atp8	N	7,945-8,112	168	ATG	TAA		-10
atp6	N	8,103-8,786	684	ATG	TAA		-1
coIII	N	8,786-9,570	785	ATG	TA(A)		-1
trnG	N	9,570-9,642	73			TCC	
nad3	N	9,643-9,992	350	ATG	T(AA)		-1

Genes	Strand	Position (start-end)	Length (bp)	Initiation codon	Stop codon	Anticodon	Intergenic nucleotide
trnR	N	9,992 – 10,061	70			TCG	
nad4l	N	10,062 – 10,358	297	ATG	TAA		-7
nad4	N	10,352 – 11,733	1,382	ATG	TA(A)		
trnH	N	11,734 – 11,803	70			GTG	
trnS1	N	11,804 – 11,870	67			GCT	1
trnL1	N	11,872 – 11,944	73			TAG	
nad5	N	11,945 – 13,783	1,839	ATG	TAA		-4
nad6	J	13,780 – 14,301	522	ATG	TAG		
trnE	J	14,302 – 14,370	69			TTC	4
cob	N	14,375 – 15,515	1,141	ATG	T(AA)		
trnT	N	15,516 – 15,586	71			TGT	-2
trnP	J	15,585 – 15,654	70			TGG	6
OH	N	15,661 – 16,401	741				168

Protein-coding genes and codon usage

The *S. fasciolata* mitochondrial genome contains 13 PCGs for mitochondrial function: seven NADH dehydrogenases (*ND1-6*, *ND4L*), three cytochrome c oxidases (*COI*, *COII*, *COIII*), two ATP synthase subunits (*ATP*6, *ATP8*), and one cytochrome b (*CYTB*), totaling 16,570 base pairs. All genes are similar in length and identical in gene arrangement to those in other *Schistura* species. This arrangement, with 12 PCGs on the major strand and *ND6* on the minor strand, exemplifies the coding efficiency and conservation of the mitochondrial genome. The PCGs employ standard start codons, primarily ATN and TTG, and their stop codons exhibit specificity for precise gene expression: TAA for *COI*, *ATP8*, *ATP6*, *ND1*, *ND4L*, and *ND5*; TAG for *ND6*; and a unique TA dinucleotide or a single T for the others, suggesting post-transcriptional modifications to complete the standard stop codons (Fig. 2).

Analysis of Relative Synonymous Codon Usage (RSCU) values provides further insight into the mitogenome's evolutionary dynamics, revealing codon usage biases that favor genes encoding Ala, Arg, Pro, Ser2, Thr, and Leu1 over those encoding Asn, Asp, and Cys (Fig. 3). This codon usage bias reflects adaptive evolutionary strategies aimed at enhancing translational efficiency and accuracy [42], underscoring the nuanced interplay between genetic code specificity and mitochondrial function optimization in *S. fasciolata.*

Phylogenetic relationships

In this study, we reconstructed phylogenetic relationships using ML methods and BI methods, yielding a phylogenetic tree with a single topology and high support rate (with Bootstrap Support (BS) values consistently above 75 and BI posterior probabilities all exceeding 0.85), significantly enhancing our understanding of their phylogenetic relationships (Fig. 4).

Phylogenetic analyses show that *Acanthocobitis*, *Barbatula*, *Nemacheilus* and *Homatula* form a distinct monophyletic lineage, respectively (Fig. 4), which was in line with previous studies [1, 9, 11, 13]. On the other hand, our study reveals a widespread paraphyly within the tribe Nemacheilini, which is also prevalent across the entire Nemacheilidae family [4, 10]. Specifically, *Schistura*, *Troglonectes*, and *Heminoemacheilus* form a paraphyletic lineage. Luo et al. uncovered paraphyly between *Heminoemacheilus* and *Paranemachilus*, recommending merging them to ensure the monophyly of *Paranemachilus* [41]. Conversely, our research challenges the classification of *Heminoemacheilus*, particularly highlighting *H. zhengbaoshani* placement within *Troglonectes*, which also questions *Troglonectes* previously accepted monophyly [7, 42]. The extensive paraphyly across tribe Nemacheilini, even family Nemacheilidae, might be related with the rapid evolution, extensive radiation, and remarkable adaptability of species, reflected in their diversity and wide ecological niche occupation [1, 2, 3], as well as geological event [43, 44], niche competition [45], hybridism [46], and rapid adaptive evolution in response to environmental pressures [47]. These comprehensive factors collectively contribute to the rapid evolution within the family Nemacheilidae. Additionally, the genus *Schistura* was clustered into two clades in this study (clade 1 and clade 2 in Fig. 4), among which the *S. incerta* (**No. MK361215.1**) was nested with *S. fasciolata* (clade 2 in Fig. 4), our results align with prior studies [12–14] which imply the paraphyletic origin for genus *Schistura*. Of cause, it possibly resulted from the complex gene flow [48] or need redefinition of species. Meanwhile, the same situation for *Acanthocobitis botia* in genus *Acanthocobitis*, and *Barbatula toni* in *Barbatula.*

Selection analyses

In this study, we examined the evolutionary dynamics of 13 mitochondrial PCGs of five genera in Nemacheilini: *Acanthocobitis*, *Barbatula*, *Homatula*, *Schistura*, and *Troglonectes*. By applying the M0 model, we found omega (ω) ratios significantly below 1, ranging from 0.00659 (*COI*) to 0.12080 (*ATP8*), across the tribe Nemacheilini. This result emphasizes the role of purifying selection in the oxidative phosphorylation (OXPHOS) pathway. Notably, the *ATP8* gene showed the highest dN/dS value, indicating

potential accelerated evolution, similar with prior research on species such as *Glyptothorax macromaculatus* [49], *Orthoptera insects* [50], *Desis jiaxiangi* [51], and *Lamprologus* [52]. In contrast, the *COI* gene displayed the lowest dN/dS value, suggests it may have faced much stronger evolutionary constraints, which consist with Singh et al. [53], Yang et al. [54], and Li et al. [55]. This evidence underlines the extensive use of the *COI* gene in phylogenetic reconstructions across species groups due to its low mutation rate. Furthermore, M1 model analysis revealed significant dN/dS ratio variability among mitochondrial PCGs, indicating a diversity of selective pressures in Nemacheilini (Table 2). This variability suggests that while some genes are highly conserved due to their critical roles in cellular metabolism and energy production, others may evolve more rapidly, possibly reflecting adaptations to different ecological niches or life-history strategies within the tribe.

In order to further evaluate the significance of evolutionary rates among different genus, the Kruskal-Wallis H test was implemented. The results uncovered significance of evolutionary rates for the *ND4*, *ND5*, *ND6*, *ATP6*, and *CYTB* genes (Table 3). Specifically, the *ND4*, *ND5*, and *CYTB* genes have undergone the accelerated evolution in cave-adapted genera *Homatula* and *Troglonectes.* It might be related to the environmental pressures of their cave-dwelling lifestyle, such as low temperatures, and low oxygen levels, where it needs to much more accurate energy consumption [55, 56]. The studies have confirmed that *ND5* interacts with protein subunits, playing a crucial role in regulating respiration [57]. And, the transmembrane helices in *ND4* and *ND6*, crucial for the proton transfer process in complex I, interact with at least one lipid molecule [57]. Consequently, the accelerated evolution of *ND4* in *Homatula* and *ND6* in *Troglonectes* may significantly influence mitochondrial regulation of lipid synthesis and metabolism, potentially conferring a survival advantage in environments with irregular resources. However, the *ATP6*, *ND4*, *ND5*, *ND6*, and *CYTB* genes exhibit lower evolutionary rates in *Acanthocobitis.* In summary, our findings underscore the complexity of adaptive evolution within the tribe Nemacheilini, with various taxa exhibiting unique evolutionary trajectories in mitochondrial genes, reflecting their diverse ecological adaptations (Fig. 5)

Table 2
The results of evolution rate of tribe Nemacheilini lineages detected by free ratio model.

Genes	Model	-lnL	2Δlnl	df	*P*-value	ω-value
COI	M0	-18431.620056	136.609028	112	0.056934002	0.00659
	M1	-18363.315542				
COII	M0	-7382.775398	131.968276	112	0.09574604	0.01357
	M1	-7316.791260				
COIII	M0	-8628.686080	139.28185	112	0.041230627	0.01570
	M1	-8559.045155				
ND1	M0	-14520.605528	199.603388	112	6.96069E-07	0.02220
	M1	-14420.803834				
ND2	M0	-17380.652518	174.248292	112	0.000151349	0.05310
	M1	-17293.528372				
ND3	M0	-5171.078323	157.301696	112	0.003095583	0.05282
	M1	-5092.427475				
ND4L	M0	-3594.222622	92.17911	112	0.914096146	0.02056
	M1	-3548.133067				
ND4	M0	-20760.870248	174.96119	112	0.000131825	0.03231
	M1	-20673.389253				
ND5	M0	-28335.202604	249.026302	112	2.02416E-12	0.05280
	M1	-28210.689453				
ND6	M0	-7610.482435	239.374544	112	2.95995E-11	0.04300
	M1	-7490.795163				
ATP6	M0	-9989.832989	204.373632	112	2.28909E-07	0.03085
	M1	-9887.646173				
ATP8	M0	-1974.880635	99.76198	112	0.78945838	0.12080
	M1	-1924.999645				
CYTB	M0	-15779.463851	304.378548	112	1.05877E-19	0.02112
	M1	-15627.274577				

Table 3
Statistical test of evolutionary rates in different genes.

Genes	Test of Homogeneity of Variances		Kruskal-Wallis Test	
	Levene Statistic	Levene Sig.	Chi-Square	Asymp. Sig.
ND1	2.9614	0.0394	6.7316	0.1508
ND2	1.7365	0.169	8.1148	0.0875
ND3	2.9061	0.0479	9.6162	0.0474
ND4L	0.5202	0.723	4.1544	0.3855
ND4	1.3608	0.2674	14.7231	0.0053
ND5	4.7105	0.0041	15.0192	0.0047
ND6	0.9982	0.4286	0.7011	0.9512
COI	1.0037	0.43	8.6007	0.0719
COII	1.2243	0.342	8.3019	0.0811
COIII	1.4934	0.2437	9.4803	0.0502
ATP6	1.8928	0.1459	12.5892	0.0135
ATP8	0.8693	0.5049	3.738	0.4426
CYTB	1.5131	0.2275	16.8024	0.0021

Conclusions

The mitochondrial genome of *S. fasciolata* was sequenced and analyzed in this study, shedding light on the mitochondrial genome of the tribe Nemacheilini. Notably, the phylogenetic analysis reveals widespread polyphyly within the tribe Nemacheilini. Furthermore, the evolutionary dynamics highlight the intricate nature of adaptive evolution within the tribe, and each taxon displaying distinct evolutionary trajectories in mitochondrial genes, indicative of their unique ecological adaptations. Our results have established a foundation for further exploration of evolutionary mechanisms in Nemacheilini, even for Nemacheilidae.

Declarations

Acknowledgements

The authors thanked the anonymous reviewers and the editor for their valuable comments.

Fundings

Grants supporting this research were received from the Erhai Watershed Ecological Environment Quality Testing Engineering Research Center of Yunnan Provincial Universities to WH and YM (No. DXDGCZX03), as well as the start-up projects on high-level talent introduction of Dali University to YM (No. KY1916101940).

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Author contributions

C.L. was responsible for data analysis and drafting the manuscript, ensuring accurate conveyance of research content and a coherent logical structure. C.Z. and Y.G. contributed to data collection, analysis, and organization, providing a solid data foundation for this study. W.H. offered critical guidance in research design and manuscript composition, and meticulously revised the draft to enhance the study's quality and depth. Y.M. oversaw the experimental design, directed the research focus, and participated in manuscript writing and revision, also securing project funding to ensure smooth progression. All authors meticulously reviewed the final manuscript, ensuring the integrity and precision of the research findings, and collectively consented to the publication of this article.

Ethical approval

This research project was conducted in accordance with the ethical guidelines. And the animal study was reviewed and approved by Laboratory Animal Welfare Ethics Review Committee, Dali University, for research project (No. DXDGCZX03). The ethical certification was shown in "Related files".

Supplementary information

The supporting materials in this paper will be obtained in Figshare (https://figshare.com/) based on this DOI: 10.6084/m9.figshare.25285282.

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Figures

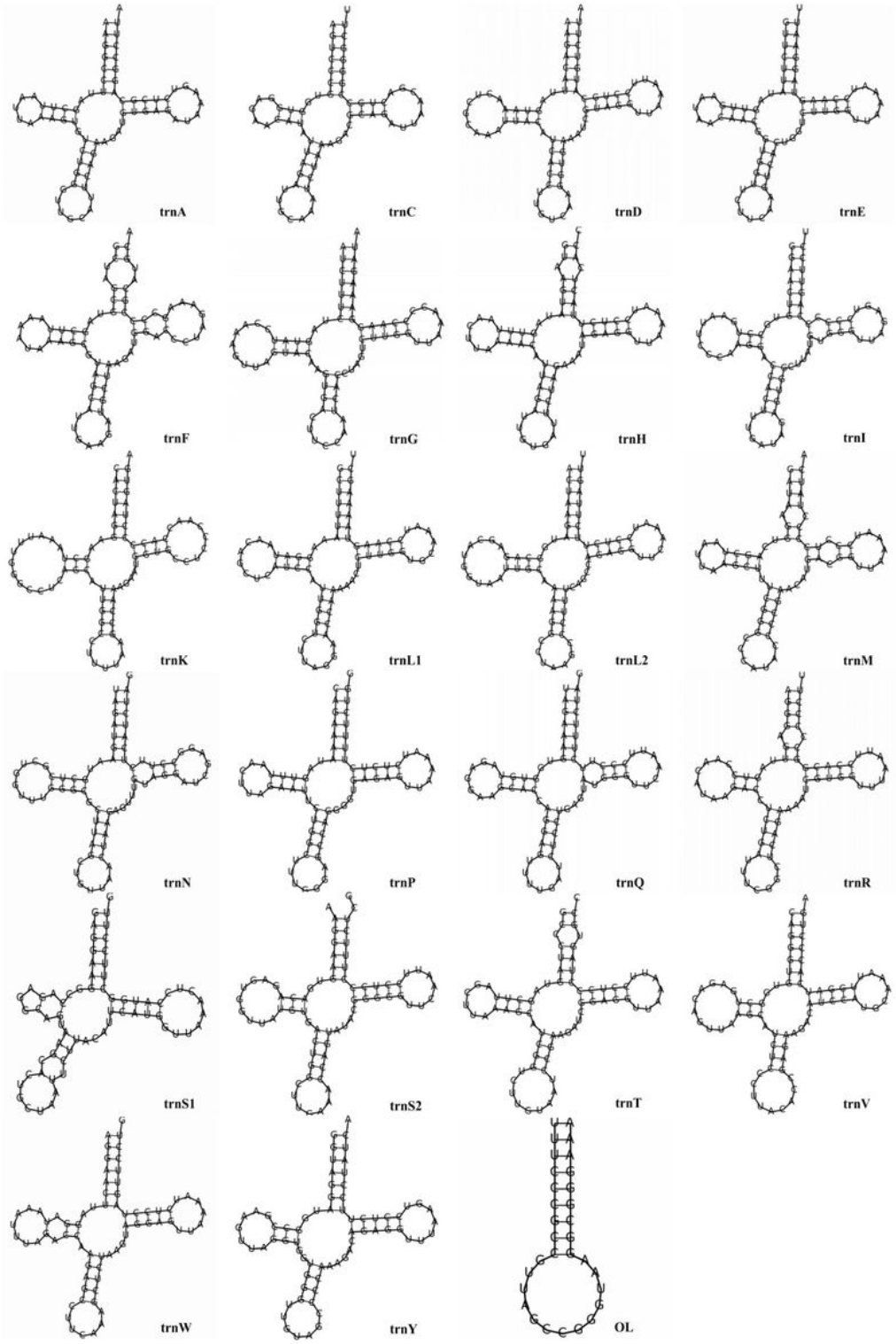

Figure 1

The predicted secondary structure of 22 tRNAs in the mitochondrial genome of *S. fasciolata*.

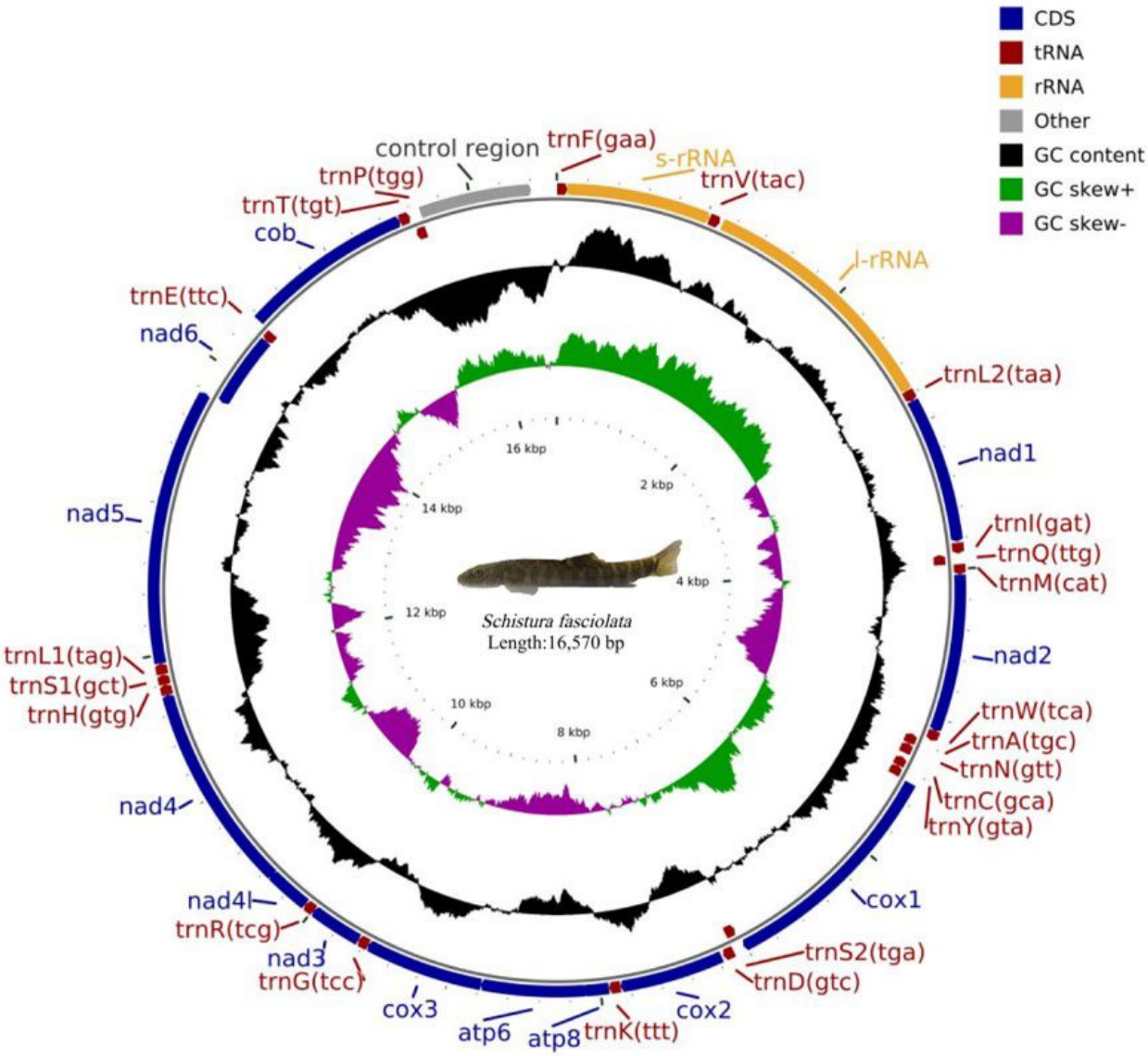

Figure 2

The complete mitochondrial genome circle map of *S. fasciolata*.

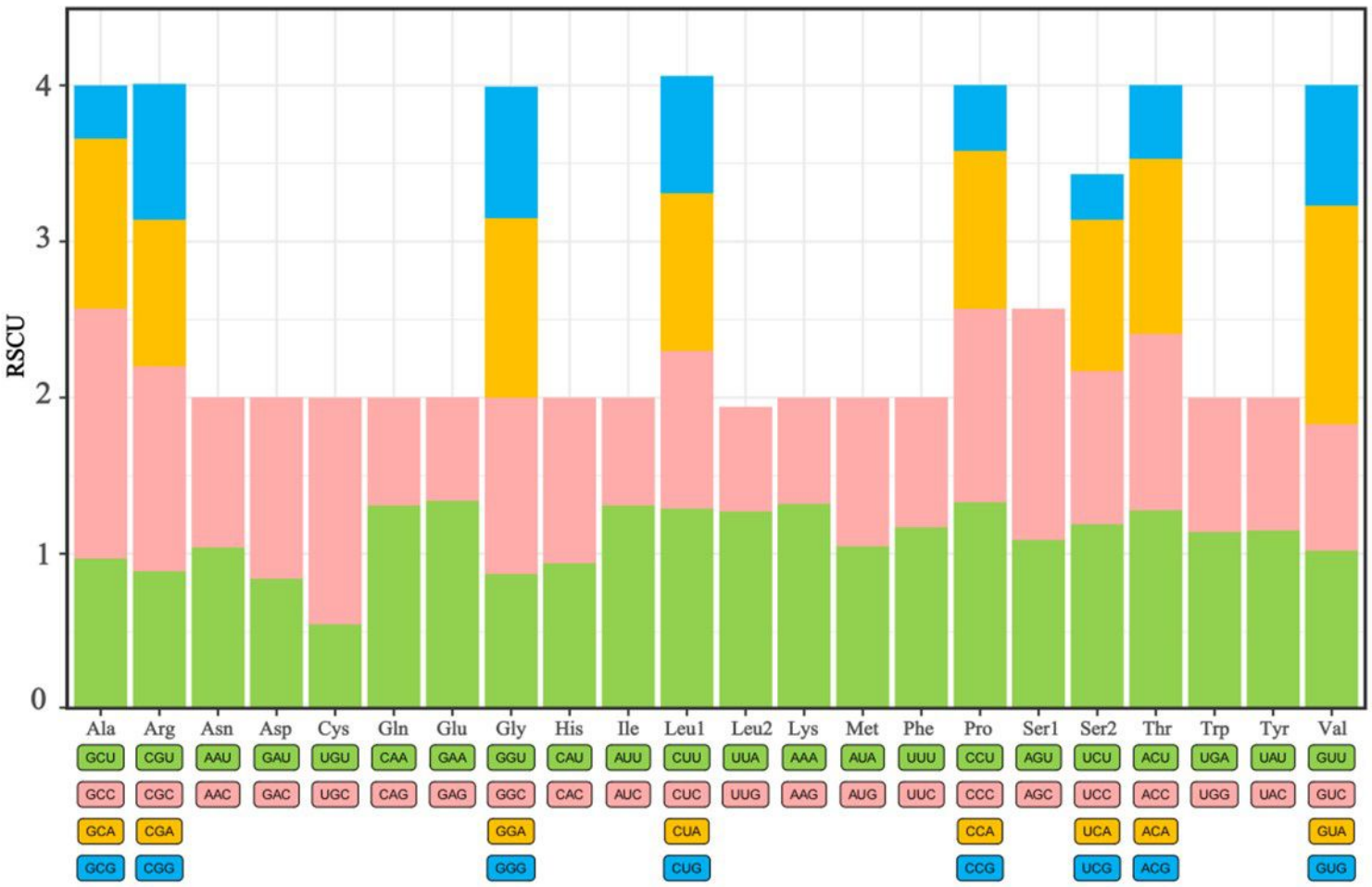

Figure 3

Relative synonymous codon usage (RSCU) in the mitochondrial genomes of *S. fasciolata.*

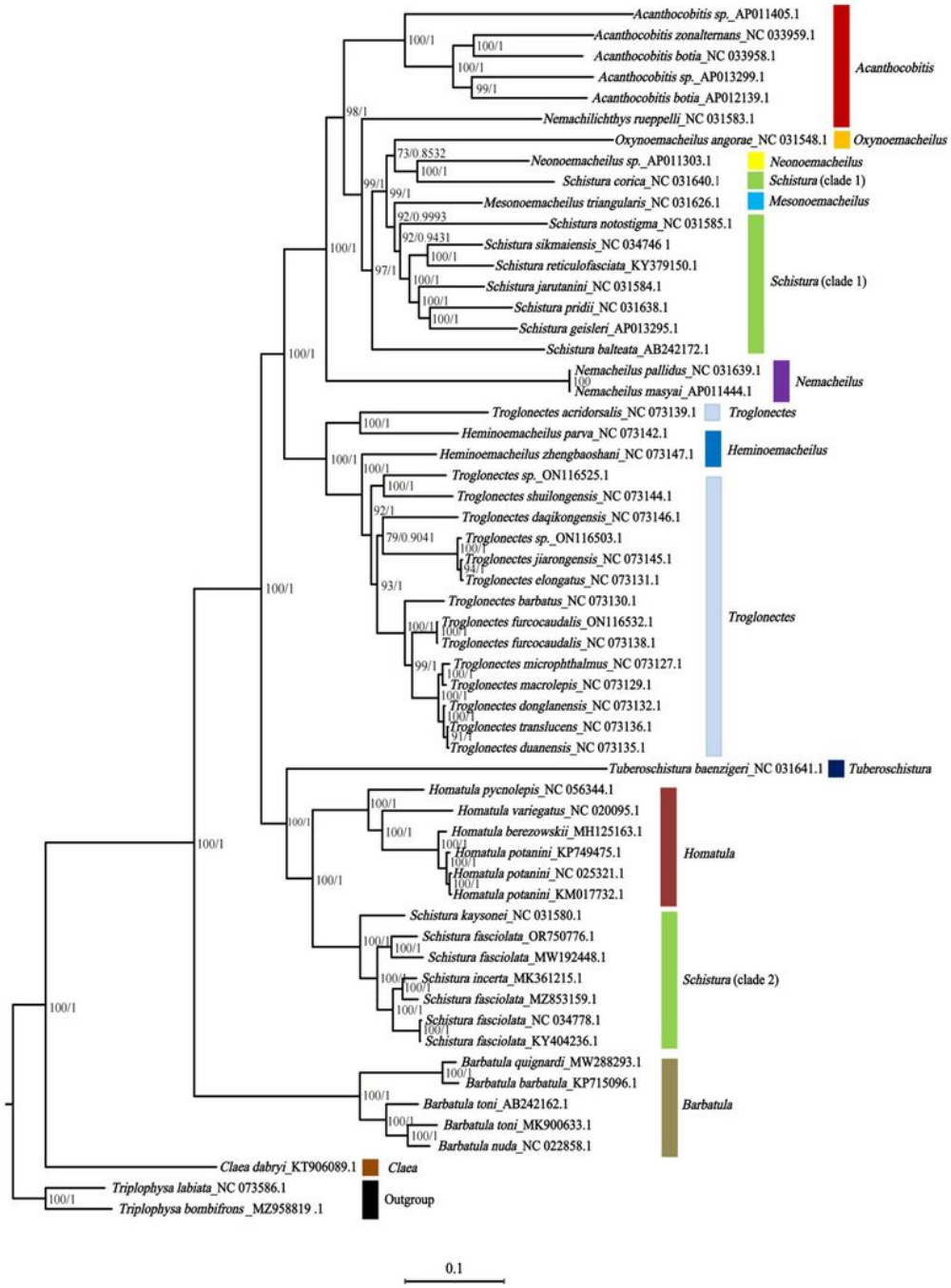

Figure 4

Phylogenetic analysis of 58 species using 13 PCGs via ML and BI, with node numbers indicating BS and *PP* values.

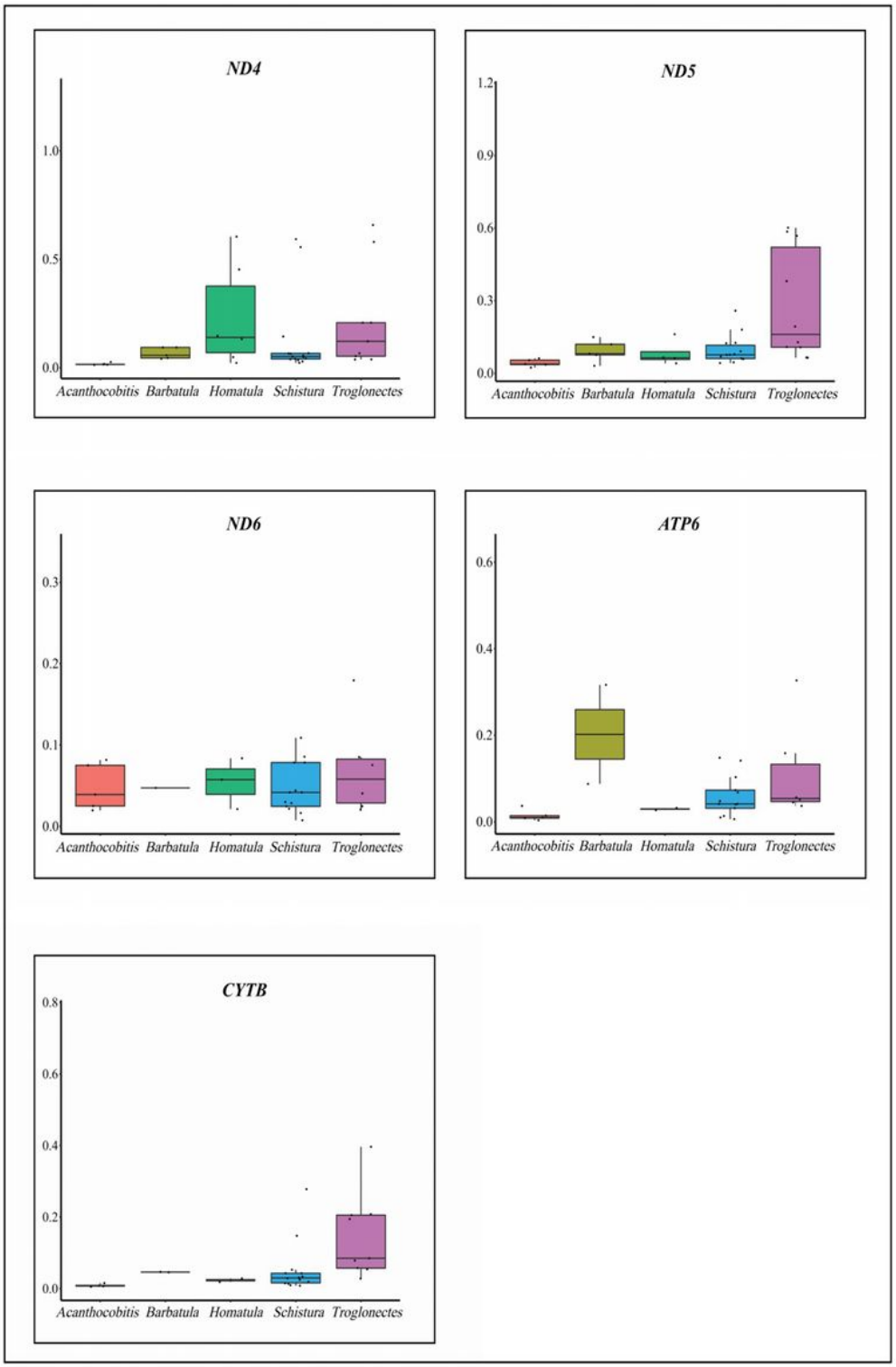

Figure 5

Boxplot of *dN/dS* of the13 PCGs for 5 main genus. These data were calculated with the free ratio model.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1SpeciesInformationandSelectionPressureAnalysisResultsfortheTribeNemacheilini.xlsx
- TableS2SchisturafasciolataGenomeStructureBaseComposition.xlsx