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The Complete Mitochondrial Genome Sequence and Phylogenetic Analysis of *Squalidus nitens* (Cypriniformes: Cyprinidae: Gobioninae)

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ABSTRACT

Squalidus nitens is a common small-bodied freshwater fish endemic to the middle and lower reaches of the Yangtze River. Genetic information on the species is quite scarce, as well as scientists have long disagreed on how to dispose of *Squalidus* and its related genera. In this study, we primarily reported the complete mitochondrial DNA genome of *S. nitens* by high-throughput sequencing, and explored the phylogenetic position of *S. nitens* within the subfamily Gobioninae. The entire length of the mitochondrial genome is 16,606 bp, containing 13 protein-coding genes (PCGs), 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes, and a control region (D-loop). The nucleotide composition was made up of 29.97% A, 27.26% C, 16.93% G, and 25.84% T, respectively, indicating an A + T (55.81%)-rich feature in the *S. nitens*. *Squalidus* shares close skinship with *Hemibarbus*, and both were found at the base of the evolutionary tree of subfamily Gobioninae by creating sister groups in a well-supported way, according to ML and BI analyses. The closest relatives to *S. nitens* are *S. chankaensis* and *S. gracilis*, not *S. argentatus*.

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LM collected the samples. LM and JG conducted the experiments. YSZ analysed the results and wrote the article. JL, YZ modified the final manuscript.

Key words
Cyprinidae, Gobioninae, Mitochondrial genome, Phylogenetic relationship, *Squalidus nitens*

INTRODUCTION

Squalidus nitens (Günther, 1873) is an endemic and primary freshwater species in the subfamily Gobioninae, which was identified as the synonym of *Gobio sihuensis* Chu (Yue, 1995) in 1995. The species mainly inhabited the middle or lower reaches of the Yangtze River and were of vital economic value. However, the phylogenetic relationship of the genus to which *S. nitens* belongs is still being debated. With the difference in analytical methods and the development of technical means, the current research on this genus has obtained different results in morphology and molecule. Based on morphological traits, it is considered that the closely related groups of *Squalidus* are *Gnathopogon* (Bănărescu and Nalbant, 1965; Hosoya, 1986) or *Gobio* (Yue, 1995). In recent years, molecular studies have shown that *Squalidus* has a close kinship with *Hemibarbus* genera. Yang *et al*. (2006) discovered that *Squalidus* and *Hemibarbus* are located at the most basic part of the phylogeny of the subfamily Gobioninae based on evidence from the cytb gene, as well as Tang *et al*. (2011) proved the same with the cytb gene, COI gene, Rag1 gene, and RH gene. To summarize, more in-depth research is needed to determine the phylogenetic relationship of this genus.

Mitochondrial DNA is a covalently closed circular double-stranded DNA molecule that functions independently of the nucleus in the areas of autonomous replication, transcription, and translation. Mitochondrial genes have a high level of coding efficiency, are simple to copy, and closely adhere to maternal inheritance (Avise *et al*., 1984; Duchene *et al*., 2012). As a result, mitochondrial DNA is commonly used in studies of fish evolutionary relationships. Among the researches related to mitochondrial DNA, in comparison to single gene data, complete mitochondrial genomes provide significant comparative advantages for resolving shallow-level phylogenetic and taxonomy problems (Mao *et al*., 2021). Nowadays, there is no complete mitochondrial genetic information available for *S. nitens*. The paucity of sequence data hampered *S. nitens* research significantly. Meanwhile, data on the mitogenomes of *S. nitens* is crucial for phylogenetic analysis and molecular evolution of *Squalidus* mitogenomes.

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Therefore, in this paper, we characterized the complete mitochondrial genome from this species for the first time, and obtained all 33 complete mitogenomes accessible for the subfamily Gobioninae to utilize in phylogenetic analysis. The complete mitochondrial gene sequencing of *S. nitens* not only complemented the species' molecular genetic data, but also offered significant information for comprehending the Gobioninae's evolutionary link.

MATERIALS AND METHODS

Sampling, DNA extraction and sequencing

The specimen of *Squalidus nitens* used in this study was collected from Cang Xi section of the Jialing River (31°42′N, 105°54′E) in May 2021. Morphological identification of *S. nitens* was performed according to Ding (Ding, 1994) as reference. Total DNA was extracted from alcohol-preserved muscle tissue using Marine Animals DNA Kit. Subsequently, the complete mitochondrial genome was sequenced by delivering to the Tsingke Biological Technology Company (Chengdu, China).

Sequence assembly, mitogenome annotation and sequence analysis

A shotgun DNA library was constructed and sequenced by Illumina NovaSeq. Using Fastp 0.20.0 to control the quality of raw Illumina reads A de novo assembly of a complete circular mitochondrial genome from clean data was performed with GetOrganelle1.7.5 (Jian *et al*., 2019). BLAST 2.7.1 was used to compare the assembly results with the reference genome of closely related species. The candidate sequences were determined based on the comparison. MITOS2 (http://mitos2.bioinf.uni-leipzig.de/index.py), a special platform for mitochondrial genomes, was employed to annotate the genome structure. The composition of nucleotides and relative synonymous codon usage (RSCU) were analyzed with MEGA X (Kumar *et al*., 2018). The skewness of the strands was calculated using the following formulas: AT-skew = (A-T)/(A+T) and GC-skew = (G-C)/(G+C) (Perna and Kocher, 1995).

Phylogenetic analysis

To investigate the phylogenetic position of *S. nitens* and phylogenetic relationships within the Gobioninae, we obtained mitogenomes from 33 species belonging to the subfamily Gobioninae from GenBank (http://www.ncbi.nlm.nih.gov) (Table I). Outgroups were chosen from the Acheilognathinae subfamily, which is closely related to Gobioninae: *Acheilognathus intermedia* and *Rhodeus notatus*. PhyloSuite (Zhang *et al*., 2020) was used to conduct, manage, and streamline the analyses.

Table I. List of the 33 Cyprinidae species in phylogenetic analysis, with their GenBank accession numbers.

Subfamily/ Species	GenBank No	Size (bp)
Gobioninae		
Abbottina obtusirostris	NC_026900	16,599
Acanthogobio guentheri	MF787799	16,604
Biwia springeri	NC_022188	16,606
Coreius heterodon	JF906110	16,611
Coreoleuciscus splendidus	EU848546	16,566
Gnathopogon herzensteini	MT295103	16,597
Gobio cynocephalus	NC_032294	16,605
Gobiobotia pappenheimi	NC_032293	16,605
Gobiocypris rarus	JN116719	16,601
Hemibarbus labeo	DQ347953	16,612
Ladislavia taczanowskii	NC_024634	16,613
Microphysogobio liaohensis	NC_032290	16,609
Paracanthobrama guichenoti	NC_024430	16,607
Platysmacheilus longibarbatus	NC_032289	16,615
Pseudogobio vaillanti	NC_032292	16,607
Pseudopungtungia nigra	EU332752	16,605
Pseudorasbora elongata	KF051938	16,587
Pungtungia herzi	NC_008664	16,600
Rhinogobio ventralis	NC_022720	16,604
Romanogobio ciscaucasicus	NC_031558	16,603
Sarcocheilichthys kiangsiensis	JX401522	16,672
Saurogobio dabryi	KU314696	16,609
Squalidus argentatus	NC_023336	16,607
Squalidus chankaensis	NC_050647	16,611
Squalidus gracilis	NC_024561	16,605
Squalidus longifilis	NC_051941	16,607
Squalidus mantschuricus	NC_051940	16,605
Squalidus multimaculatus	NC_029387	16,597
Squalidus nitens	This study	16,606
Squalidus wolterstorffi	NC_022190	16,602
Xenophysogobio boulengeri	KU314699	16,615
Acheilognathinae		
Acheilognathus intermedia	NC_013705	16,610
Rhodeus notatus	NC_029718	16,735

13 sequences were aligned in batches with MAFFT (Kazutaka *et al*., 2013) using auto strategy and codon alignment mode. The alignments were refined using the codon-aware program MACSE v. 2.03 (Vincent *et al*., 2018). Ambiguously aligned fragments of 13 alignments

were removed in batches using Gblocks (Gerard and Jose, 2007). ModelFinder (Kalyaanamoorthy *et al*., 2017) was used to select the best-fit model using the AIC criterion. The IQ-TREE (Nguyen *et al*., 2015) model was used for 20000 ultrafast (Minh *et al*., 2013) bootstraps, as well as the Shimodaira Hasegawa like approximate likelihood-ratio test (Guindon *et al*., 2010). Bayesian Inference (BI) phylogenies were inferred using MrBayes 3.2.6 (Huelsenbeck, 2012) under the GTR+I+G+F model. Phylogenetic trees were visualized through the online tool Interactive Tree of Life (Letunic and Bork, 2019).

RESULTS

Mitogenome organization

The complete mitochondrial DNA sequence of *S. nitens* was a 16,606 bp circular molecule with two ribosomal RNA (rRNA) genes (16S and 12S), 22 transfer RNA genes (tRNA), 13 protein-coding genes (PCGs) and one non-coding regulatory region (D-loop) (Table I). The overall base nucleotide makeup was 29.97% A, 27.26% C, 16.93% G, and 25.84% T, with a comparatively higher AT content (55.81%). The composition was skewed away from A in favor of T (the AT-skew was +0.074) but was almost balanced for G and C (the GC-skew was −0.234). The mitochondrial genome map and gene content were shown in Figure 1, Table II, respectively.

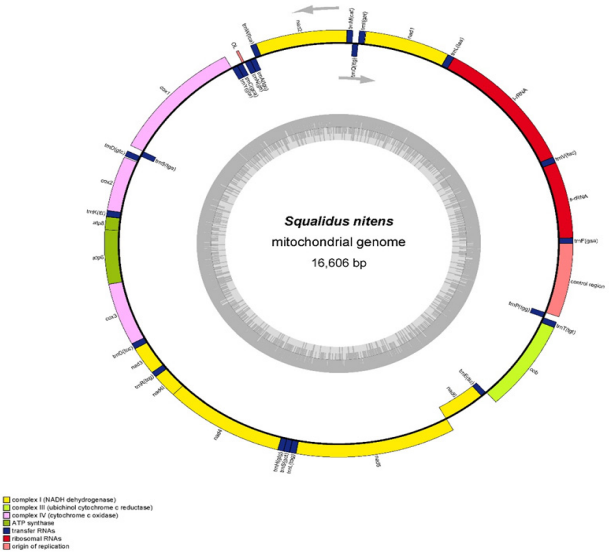

Fig. 1. The map of the mitochondrial genome of *S. nitens*. Genes encoded on the light and heavy strand are shown inside and outside the circle respectively. The gray histogram represents the GC content of the genome.

All 13 of the typical PCGs were encoded on the heavy strand except ND6. Twelve PCGs used ATG as the initiation codon, whereas COI began with GTG. Notably, ten PCGs terminated with the complete stop codons TAA (ND1, COI, ATPase8, ATPase6, ND4L and ND5) or TAG (ND2, ND3, ND4 and ND6), while the remaining ended with an incomplete stop codon T (COII and Cytb) or TA– (COIII). Twenty-two tRNA genes were interspersed across the mitogenome of *S. nitens*, ranging in size from 69 to 76 bp, which was typical for this subfamily (Mao *et al*., 2021). Fourteen tRNAs were found on the heavy strand, while the remaining eight were found on the light strand. Among the two rRNA genes, the 12S rRNA gene and the 16S rRNA gene were separated by tRNA-Val (Table II).

In the whole mitogenome, there were four pairs of adjacent PCs, ATP8–ATP6, ATP6–COIII, ND4L–ND4, and ND5–ND6 had the overlapping genes, and they overlap by seven, one, seven and four nucleotides, respectively. The D-loop was 925 bp in length and was located between the tRNA-Phe and tRNA-Pro.

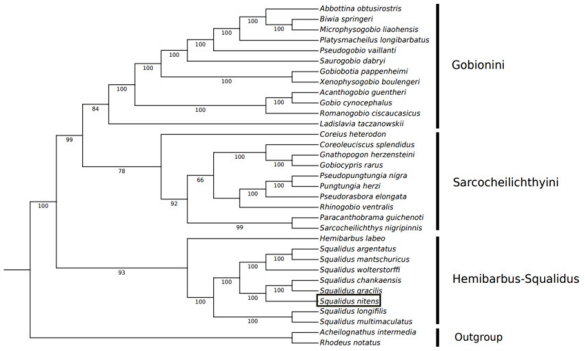

Fig. 2. Maximum likelihood (ML) tree showing the phylogenetic position of *Squalidus nitens* (black frame) among Gobioninae species. Only Bootstrap support (BP) greater than 50% are shown.

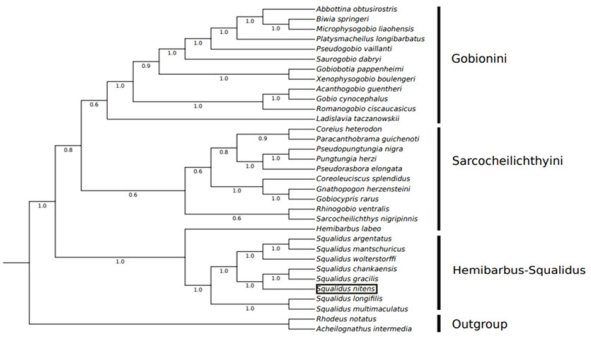

Fig. 3. Bayesian Inference (BI) tree showing the phylogenetic position of *Squalidus nitens* (black frame) among Gobioninae species. Only Bootstrap support (BP) greater than 50% are shown.

Table II. Characteristics of the complete mitochondrial genome of *S. nitens.*

Gene	Start	End	Length (bp)	Amino acid	Codon Start/ Stop	Anti Codon	Strand	Intergenic region
tRNA-Phe	1	69	69			GAA	+	0
12S rRNA	70	1026	957				+	2
tRNA-Val	1029	1100	72			TAC	+	0
16S rRNA	1101	2791	1691				+	0
tRNA-Leu	2792	2867	76			TAA	+	1
ND1	2869	3843	975	324	ATG/TAA		+	4
tRNA-Ile	3848	3919	72			GAT	+	-2
tRNA-Gln	3918	3988	71			TTG	-	1
tRNA-Met	3990	4058	69			CAT	+	0
ND2	4059	5105	1047	348	ATG/TAG		+	-2
tRNA-Trp	5104	5174	71			TCA	+	1
tRNA-Ala	5176	5244	69			TGC	-	1
tRNA-Asn	5246	5318	73			GTT	-	2
rep_origin	5321	5350	30				+	0
tRNA-Cys	5351	5418	68			GCA	-	1
tRNA-Tyr	5420	5490	71			GTA	-	1
COI	5492	7042	1551	516	GTG/TAA		+	0
tRNA-Ser	7043	7113	71			TGA	-	3
tRNA-Asp	7117	7188	72			GTC	+	13
COII	7202	7892	691	230	ATG/T		+	0
tRNA-Lys	7893	7968	76			TTT	+	1
ATP8	7970	8134	165	54	ATG/TAA		+	-7
ATP6	8128	8811	684	227	ATG/TAA		+	-1
COIII	8811	9595	785	261	ATG/TA		+	-1
tRNA-Gly	9595	9666	72			TCC	+	0
ND3	9667	10017	351	116	ATG/TAG		+	-2
tRNA-Arg	10016	10084	69			TCG	+	0
ND4L	10085	10381	297	98	ATG/TAA		+	-7
ND4	10375	11757	1383	461	ATG/TAG		+	-1
tRNA-His	11757	11825	69			GTG	+	0
tRNA-Ser	11826	11894	69			GCT	+	1
tRNA-Leu	11896	11968	73			TAG	+	0
ND5	11969	13804	1836	611	ATG/TAA		+	-4
ND6	13801	14322	522	173	ATG/TAG		-	0
tRNA-Glu	14323	14391	69				-	4
CYTB	14396	15536	1141	380	ATG/T		+	0
tRNA-Thr	15537	15608	72			TGT	+	-1
tRNA-Pro	15608	15677	70			TGG	-	0
D-loop	15678	16606	929				+	0

+ and - represent heavy strand and light strand, respectively. In the intergenic region column, negative numbers indicate an overlap between two adjacent genes. T/TA represent incomplete stop codons.

Phylogenetic analysis

To better understand the relationships among the *Squalidus* and other related species, phylogenetic trees were created using ML and BI techniques based on concatenated nucleotide sequences of 13 PCGs, 22 tRNA, and 2 rRNA from 31 Gobioninae minnows and 2 Acheilognathidae fishes. The results of the phylogenetic trees exhibited two similar topological structures with strong supports by both methods, which displayed three major clades: Gobionini, Sarcocheilichthyini, and Hemibarbus + Squalidus (Figs. 2 and 3). The two trees showed a marked consistency, disregarding the different support values for certain nodes. *Hemibarbus* and *Squalidus*, formed a separate clade at the base of the tree. Meanwhile, Gobionini and Sarcocheilichthyini formed sister-groups.

DISCUSSION

The mitogenome of *S. nitens* is structurally organized in a manner that is strikingly similar to that of other Gobioninae species (Chai and Fu, 2020). COI, like other bony fish, began with GTG, whereas the other twelve PCGs began with ATG (Miya *et al*., 2003). Ten PCGs featured conventional stop codons (TAA and TAG), whereas the remaining three PCGs had two types of incomplete stop codons (T- and TA-), which could be completed by post-transcriptional polyadenylation (Ojala *et al*., 1981). The non-coding region of *S. nitens* mitogenome, which ranged from 1 to 929 bp, might be important as splicing recognition sites during the process of transcription. Similar to other bony fish, the second largest spacer was located between tRNA-Asn and tRNA-Cys genes and was 30 bp in length. In conclusion, this report could enrich the mitogenome resource of *S. nitens* and seemed to be useful for evolutionary and conservation studies on subfamily Gobioninae fish species.

The results of ML and BI analyses in the current study showed similar topologies and both presented three major clades (Gobionini, Sarcocheilichthyini, and Hemibarbus + Squalidus group), which was in accordance with previous molecular studies (Tang *et al*., 2011; Mao *et al*., 2021). However, it contradicted the claim of Yang *et al*. (2006) that four lineages exist. The close relationship of *Squalidus* to *Hemibarbus* was supported by sequence data for Yang *et al*. (2006) using a single gene cytb and Tang *et al*. (2011) using four genes (cytb, COI, RAG1 and rhodopsin), whereas morphological data indicated that *Squalidus* was close to *Gnathopogon* (Bănărescu and Nalbant, 1965; Hosoya, 1986) or *Gobio* (Yue, 1995). Our study was based on whole sequence evidence of mitochondrial genes. It also indicated that *Squalidus* has close skinship with *Hemibarbus* and that both were located at the base of the phylogenetic tree of subfamily Gobioninae by forming sister groups. It's different from early research (Jeon *et al*., 2018) that the closest relation to *S. nitens* is not the *S. argentatus*, but *S. chankaensis* and *S. gracilis*. As Jeon *et al*. (2018) relied on a single mitochondrial gene to infer the phylogeny (NJ, ML and BI analyses based on the complete cytb gene dataset), it is likely that incomplete markers did not provide sufficient phylogenetic information for analysis (Mao *et al*., 2021).

In conclusion, the newly obtained *S. nitens* complete mitochondrial genome sequence would provide valuable molecular information fundamental to protect subfamily Gobioninae species and be useful for species delimitation and phylogenetic reconstruction.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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