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Phylogenetic Relationships of the Pseudogobionini Group (Teleostei: Cyprinidae) with Selection Pressure Analyses to Genes of Mitochondrial Genome

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Abstract: We newly sequenced complete mitochondrial genomes (mitogenome) of two gudgeon species *Saurogobio dabryi* and *S. punctatus*, and we downloaded 49 gudgeon mitogenomes from GenBank to investigate the phylogeny of the Pseudogobionini group and analyze selection pressure of the genes. With genera *Gobio*, *Acanthogobio*, and *Romanogobio* as outgroups, the phylogeny of the Pseudogobionini group was revealed as ((*Xenophysogobio* + *Gobiobotia*) + (*Saurogobio* + (*Abbottina* + (*Pseudogobio* + *Biwia* complex)))) based on the concatenated nucleotide sequences of 13 protein-coding genes (PCGs). Based on the molecular phylogeny and morphological or osteological characters, we proposed a classification system of the Pseudogobionini group. Moreover, five pairs of sister taxa were selected for gene selection pressure analyses to explore the link of mitochondrial gene evolution to group differentiation and adaptations. We detected significantly different dN/dS values in 11 out of 13 (excluding *ND3* and *ND4L*) PCGs in five pairs of clades, significantly different mean dN/dS, dN, and/or dS values in 8 out of 13 PCGs (excluding *ND2*, *ATP8*, *ND3*, *ND4L*, and *ND6*) in three pairs of sub-clades and seven positively selected sites in another three pairs of sub-clades. These results indicated that mitochondrial gene evolution might have contributed to group differentiation and adaptations especially for river or lake environments.

Keywords: Gobioninae; *Pseudogobio esocinus*; amino acid substitution; adaptive evolution

Key Contribution: This work reveals the molecular phylogeny of the Pseudogobionini group based on mitogenomic data, and provides evidence that mitochondrial gene evolution may have contributed to group differentiation and adaptations to river and lake environments.

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1. Introduction

Due to its compactness (16–17 kb), absence of paralogs, and ease of sequencing [1–3], vertebrate mitogenomes have been used as an effective tool for molecular phylogenetic and evolution analysis in fish [4–8]. Mitochondria are the centers of energy metabolism in eukaryotic organisms and provide 95% of cellular energy via oxidative phosphorylation [9]. The evolution of mitochondrial genes may have played important roles in organism adaptive evolution and diversification [10–12]. Adaptive evolution is usually detected as higher nonsynonymous to synonymous substitution rate ratios (dN/dS, denoted omega) in protein coding genes (PCGs) [13]. Previous studies reported higher dN/dS ratios in some PCGs in loaches living in fast-flowing rivers or subtropical waters [10] and higher evolutionary rates in vertebrates living in high altitudes [12]. Therefore, analysis of mitochondrial DNA evolution may help us understand the mechanism of organism differentiation and adaptation to different habitats.

The family Cyprinidae is the most diverse family of freshwater fishes in the world, with about 367 genera and about 3006 species [14]. The Pseudogobionini is a group of rheophilic and benthic freshwater fishes [15,16], belonging to the group Cypriniformes, Cyprinidae, Gobioninae, with approximately 77 species [17,18]. They are distributed mainly in East Asia, extending to Europe, and exhibit great morphological, ecological, and behavioral variations [19–21]. They may have a pair of barbels, or they may be absent [22,23]; some species possess four pairs of barbels [19–21]. These fishes predominantly inhabit freshwater ecosystems, with a few that enter brackish environments [24].

There are a few reports on the phylogeny of the Pseudogobionini group based on morphological and molecular data. Hosoya [25] analyzed variations of the cephalic lateral line system and osteological characters of the Gobioninae and proposed Pseudogobionini as "true bottom dwellers", with the relationship suggested as (*Gobiobotia* + ((*Pseudogobio* + *Abbottina*) + (*Saurogobio* + (*Microphysogobio* + *Biwia*)))), while Yu and Yue [16] revealed Pseudogobionini phylogeny as (*Pseudogobio* + (*Saurogobio* + (*Abbottina* + (*Biwia* + (*Rostrogobio* + (*Microphysogobio* + (*Platysmacheilus* + *Huigobio*))))))). Based on mitogenomic data, the phylogeny was elucidated as ((*Xenophysogobio* + *Gobiobotia*) + (*Saurogobio* + (*Abbottina* + (*Pseudogobio* + *Biwia* complex)))) by Chen [26] or as ((*Xenophysogobio* + *Gobiobotia*) + (*Saurogobio* + (*Pseudogobio* + (*Abbottina* complex + *Biwia* complex)))) by Zhang et al. [27]. Thus far, due to insufficient taxon sampling, phylogenetic investigations of the Pseudogobionini are rather preliminary and controversial.

In the present study, we first sequenced and annotated five complete mitochondrial genomes of two gudgeon species, *Saurogobio dabryi* and *S. punctatus*. Then, we reconstructed the phylogeny of the Pseudogobionini group based on the concatenated nucleotide sequences of 13 PCGs. Finally, we analyzed the selection pressure and investigated the contributions of mitochondrial gene evolution to group differentiation and adaptations of the Pseudogobionini fishes.

2. Materials and Methods

2.1. Samples Collection and DNA Extraction

In this study, five individuals of two gudgeon species *S. dabryi* and *S. punctatus* were newly sequenced for complete mitogenomes. Forty-nine mitogenomes of gudgeon species, including six outgroups species, were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 12 November 2021). Detailed information, including the GenBank accession numbers, is listed in Table 1. Four individuals of *S. dabryi*1–4 (samples of *S. dabryi* in the Yangtze River Basin were composed of four highly divergent lineages based on mitochondrial Cyt *b* gene, corresponding to *S. dabryi*1–4 (unpublished data)) were collected from Hukou County, Jiangxi Province (116°11′39″ E, 29°43′02″ N); Chishui City, Guizhou Province; Yibin City, Sichuan Province (104°32′32″ E, 28°41′31″ N); and Panzhihua City, Sichuan Province (101°30′18″ E, 26°35′38″ N). One *S. punctatus* sample was collected from Chishui City, Guizhou Province (105°41′21″ E, 28°34′19″ N). Muscle tissues from these samples were preserved in 95% ethanol and stored at 4 °C until DNA extraction. Total genomic DNA was extracted from muscle tissues following the salt-extraction procedure of Aljanabi and Martinez [28] as modified in Tang et al. [29]. All experimental protocols with animals were approved by the Institutional Animal Care and Use Committee of the Institute of Hydrobiology, Chinese Academy of Sciences (Approval code: IHB/LL/2022028).

Table 1. The detailed information of species used in the present study.

Genus	Species	GenBank Accession Number	Length (bp)
Pseudogobio	*Pseudogobio esocinus*	NC013759	16,609
	Pseudogobio vaillanti	KU314695	16,607
	Pseudogobio guilinensis	MN883565	16,609
Saurogobio	*Saurogobio dumerili*	KF151214	16,604
	Saurogobio dabryi	KF612272	16,601
	Saurogobio immaculatus	AP012074	16,988
	Saurogobio lissilabris	MK860912	16,594
	Saurogobio gracilicaudatus	MK860909	16,608
	Saurogobio xiangjiangensis	MK860910	16,600
	Saurogobio gymnocheilus	MK860911	16,604
	*Saurogobio dabryi*1 *	ON533885	16,601
	*Saurogobio dabryi*2 *	ON533886	16,601
	*Saurogobio dabryi*3 *	ON533887	16,600
	*Saurogobio dabryi*4 *	ON533888	16,600
	Saurogobio punctatus *	ON533884	16,604
Abbottina	*Abbottina rivularis*	KM081703	16,597
	Abbottina obtusirostris	KF955012	16,605
	Abbottina liaoningensis	KU314691	16,608
	Abbottina binhi	NC048988	16,609
Biwia	*Biwia zezera*	AB250108	16,599
	Biwia springeri	NC022188	16,606
Gobiobotia	*Gobiobotia pappenheimi*	KU314697	16,605
	Gobiobotia filifer	NC029187	16,613
	Gobiobotia brevibarba	FJ515919	16,594
	Gobiobotia macrocephala	NC014877	16,610
	Gobiobotia naktongensis	NC020464	16,609
	Gobiobotia intermedia	NC022931	16,608
	Gobiobotia meridionalis	MW442088	16,609
Microphysogobio	*Microphysogobio brevirostris*	NC022704	16,608
	Microphysogobio fukiensis	NC024930	16,600
	Microphysogobio tungtingensis	NC051965	16,627
	Microphysogobio yaluensis	AP012073	16,603
	Microphysogobio kiatingensis	NC037402	16,603
	Microphysogobio koreensis	NC014880	16,606
	Microphysogobio tafangensis	NC023461	16,605
	Microphysogobio longidorsalis	NC022191	16,603
	Microphysogobio amurensis	AP012155	16,599
	Microphysogobio chenhsienensis	MZ853165	16,602
	Microphysogobio alticorpus	NC021451	16,568
	Microphysogobio elongatus	MN832777	16,612
	Microphysogobio liaohensis	NC032290	16,609
	Microphysogobio jeoni	MN581867	16,602
	Microphysogobio rapidus	NC045250	16,603
	Microphysogobio sp.	NC040303	16,607
Platysmacheilus	*Platysmacheilus exiguus*	KF926823	16,604
	Platysmacheilus nudiventris	KM502565	16,603
Xenophysogobio	*Xenophysogobio boulengeri*	KU314699	16,615
	Xenophysogobio nudicorpa	KU314698	16,617
Gobio	*Gobio gobio*	AB239596	16,607
	Gobio cynocephalus	KU314700	16605
	Gobio macrocephalus	MT632636	16,609
	Gobio acutipinnatus	MT632635	16,609
Acanthogobio	*Acanthogobio guentheri*	MF787799	16,604
Romanogobio	*Romanogobio ciscaucasicus*	AP011259	16,603

* newly sequenced.

2.2. PCR Amplification and Sequencing

We designed 16 pairs of primers to amplify the complete mitogenomes of *S. dabryi* and *S. punctatus* based on the published complete mitochondrial genome sequence of *S. dabryi* (GenBank accession number: KF612272) [30]. Furthermore, we designed four species-specific primer pairs to amplify gaps in the mitogenome of *S. punctatus*. Primers used in

this study are listed in Table S1. Polymerase Chain Reaction (PCR) amplifications were performed in a 30 μL reaction volume containing 22.5 μL sterile H_2O, 1 μL dNTPs (each 2.5 mM), 3 μL of 10× reaction buffer, 1 μL of each primer (each 10 μM), 0.5 μL (5 U/μL) of Taq polymerase, and 1 μL template DNA. The cycling parameters were set as follows: 94 °C for 4 min; 35 cycles of 94 °C denaturation for 45 s, annealing temperate for 45 s (Table S1), 72 °C extension for 1 min, and a final 72 °C extension for 10 min. The PCR products were checked by electrophoresis through 1% agarose gel, and the target products were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

2.3. Sequence Assembly and Analysis

For each individual, the nucleotide sequences were aligned, calibrated, and edited using the MEGA X program [31] with the published complete mitogenome of *S. dabryi* as reference. Mitogenomes were assembled by the SeqMan software [32] of DNASTAR's Lasergene. Nucleotide composition was analyzed using the MEGA X program. The following formulas were used to calculate the values of AT-skew = (A − T)/(A + T) and GC-skew = (G − C)/(G + C) [33].

2.4. Phylogenetic Analysis

Based on the principle of out-group selection [34] and previous molecular phylogenetic studies on the Gobioninae [24,35], the genera *Gobio*, *Acanthogobio*, and *Romanogobio* were selected as outgroups. We reconstructed the Bayesian Inference (BI) and Maximum Likelihood (ML) trees of the Pseudogobionini group in the PhyloSuite v1.2.2 program [36] based on the concatenated nucleotide sequences of 13 PCGs. The best-fit nucleotide substitution models were estimated by ModelFinder [37] for MrBayes and IQ-TREE with the Bayesian Information Criterion (BIC) [38] and Corrected Akaike Information Criterion (AICc) [37]. The BI tree was performed in MrBayes 3.2.6 [39]. For BI analyses, two independent analyses with four simultaneous Markov Chain Monte Carlo (MCMC) chains were run for 5,000,000 generations with tree sampling every 1000 generations, and runs were stopped after the standard deviation of split frequencies fell below 0.01. The first 25% of trees were discarded as burn-in. A 50% majority-rule consensus tree was obtained from the remaining 75% of trees. Posterior probabilities (PP) values of phylogenetic inferences were determined from the remaining trees. The ML tree was performed using IQ-TREE v1.6.8 [40]. The support values of each node were estimated using the ultrafast bootstrapping algorithm with 10,000 replicates [41].

2.5. Selection Pressure Analysis

Based on the molecular phylogeny, five pairs of sister taxa (between *Gobiobotia* subgroup and *Pseudogobio* subgroup, between *Saurogobio* tribe and *Pseudogobio* tribe, between *Saurogobio* branch A and *Saurogobio* branch B, between *Abbottina* branch and *Pseudogobio* branch, and between *Pseudogobio* subbranch and *Biwia* complex subbranch) were selected for genes selection pressure analysis. We used the branch model in the paml X [42,43] and the site model in the EasyCodeML [44] program for analyzing gene selection pressure, respectively. The following four steps were performed: (1) dN/dS values were estimated for each of the 13 PCGs, where one-ratio (all branches have the same dN/dS values) and two-ratio ('foreground' branch and 'background' branch have different dN/dS values) models were employed to conduct likelihood ratio tests (LRTs) [45] assessing the significant variation between each of the five pairs clades, and in total 65 tests were conducted (13 PCGs are multiplied by five pairs of clades; (2) genes detected with the significant difference in dN/dS values in step one were subsequently estimated further for this pair using the free-ratio model; (3) the mean dN/dS, dN, and dS values from step two were compared between so-called 'foreground' and 'background' pair comparison to assess possible changes in selection pressure; (4) furthermore, the site model [46] was applied to detect the potential selection among sites and allow for different ω ratios in different sites, codons, or amino acids [47]. We also used LRTs to assess these models and Bayes Empirical

Bayes (BEB) method [13] to evaluate the posterior probability of positively selected sites. For analysis of changes in dN/dS, only those that were without dS = 0 were used in the analysis or were discarded if dS = 0 [48]. All statistical analyses were finished in SPSS 20.0.

3. Results

3.1. Mitogenome Characteristics and Sequence Variation of the Pseudogobionini Group

In this study, the length of the mitogenomes of the Pseudogobionini group varied from 16,568 (*Microphysogobio alticorpus*) to 16,988 (*Saurogobio immaculatus*) base pairs (bp) (Table 1). All of them were composed of 13 PCGs, 22 transfer RNA genes, two ribosomal RNA genes, and one control region (CR). The average nucleotide composition was 30.0%, 26.3%, 17.0%, and 26.7% for A, T, G, and C, respectively, with higher A + T content (56.3%) than G + C (43.7%) and exhibited positive (0.066) AT-skew and negative (−0.222) GC-skew. The newly sequenced five gudgeon complete mitogenomes of two species were deposited in GenBank under the accession numbers ON533884-ON533888 (Table 1). Gene arrangements and organization were displayed in Table S2. Our newly sequenced mitogenomes were 16,600–16,604 bp in length, and the average total base composition was 29.7%, 26.4%, 16.8%, and 27.1% for A, T, G, and C, respectively. Among the Pseudogobionini species, most of the PCGs used ATG as the start codon except for the *COXI* gene, which used GTG as the initiation codon. In addition, stop codons varied among the 13 PCGs, some PCGs terminated with complete stop codons, including TAA or TAG, and other PCGs ended with incomplete stop codons, either TA or T.

3.2. Phylogenetic Analysis

We reconstructed BI and ML trees of the Pseudogobionini group based on the concatenated nucleotide sequences of 13 PCGs. The topological structures of BI and ML trees were almost consistent and supported by high support values. The bootstrap values and posterior probabilities were displayed on the nodes (Figure 1). The results showed that the genera *Biwia*, *Microphysogobio*, and *Platysmacheilus* were not monophyletic, but mixed, which was named as *Biwia* complex. The Pseudogobionini group was monophyletic, and the phylogeny was revealed as ((*Xenophysogobio* + *Gobiobotia*) + (*Saurogobio* + (*Abbottina* + (*Pseudogobio* + *Biwia* complex)))). The genus *Saurogobio* was divided into two major lineages, which we named *Saurogobio* branch A and *Saurogobio* branch B, respectively. The phylogeny among the species of *Saurogobio* was (((*S. dabryi* + (*S. gracilicaudatus* + *S. xiangjiangensis*)) + *S. punctatus*) + (((*S. gymnocheilus* + *S. immaculatus*) + *S. lissilabris*) + *S. dumerili*)) (Figure 1).

Finally, based on the molecular phylogeny and morphological or osteological characters (Table 2), we proposed a classification system for the Pseudogobionini group.

Table 2. The morphological or osteological characters for classification of the Pseudogobionini group.

***Gobiobotia* subgroup** four pairs of barbels	***Gobiobotia* tribe** urohyal vertical plate is considerably high		
***Pseudogobio* subgroup** one pair of barbels or be absent	***Saurogobio* tribe** urohyal vertical plate is medium high; pre-dorsal length/post-dorsal length < 1; the air-bladder anterior chamber enclosed by the bony capsule	***Saurogobio* branch A** lips smooth or with degenerated papillae ***Saurogobio* branch B** lips with developed papillae	
	***Pseudogobio* tribe** urohyal vertical plate is medium high or low; pre-dorsal length/post-dorsal length ≥ 1; the air-bladder anterior chamber enclosed by the membranous capsule	***Abbottina* branch** supraorbital bones absent; curve rob-shape end of 4th vertebral pleural rib	
		***Pseudogobio* branch** supraorbital bones present; wing-like lateral expansion end of 4th vertebral pleural rib	***Pseudogobio* subbranch** snout long and prominent; two rows of pharyngeal teeth ***Biwia* complex subbranch** snout short and blunt; one row of pharyngeal teeth

Pre-dorsal length: the distance from the origin of the dorsal to the snout; post-dorsal length: the horizontal distance between the end of the dorsal-fin base to the end of the caudal vertebrate.

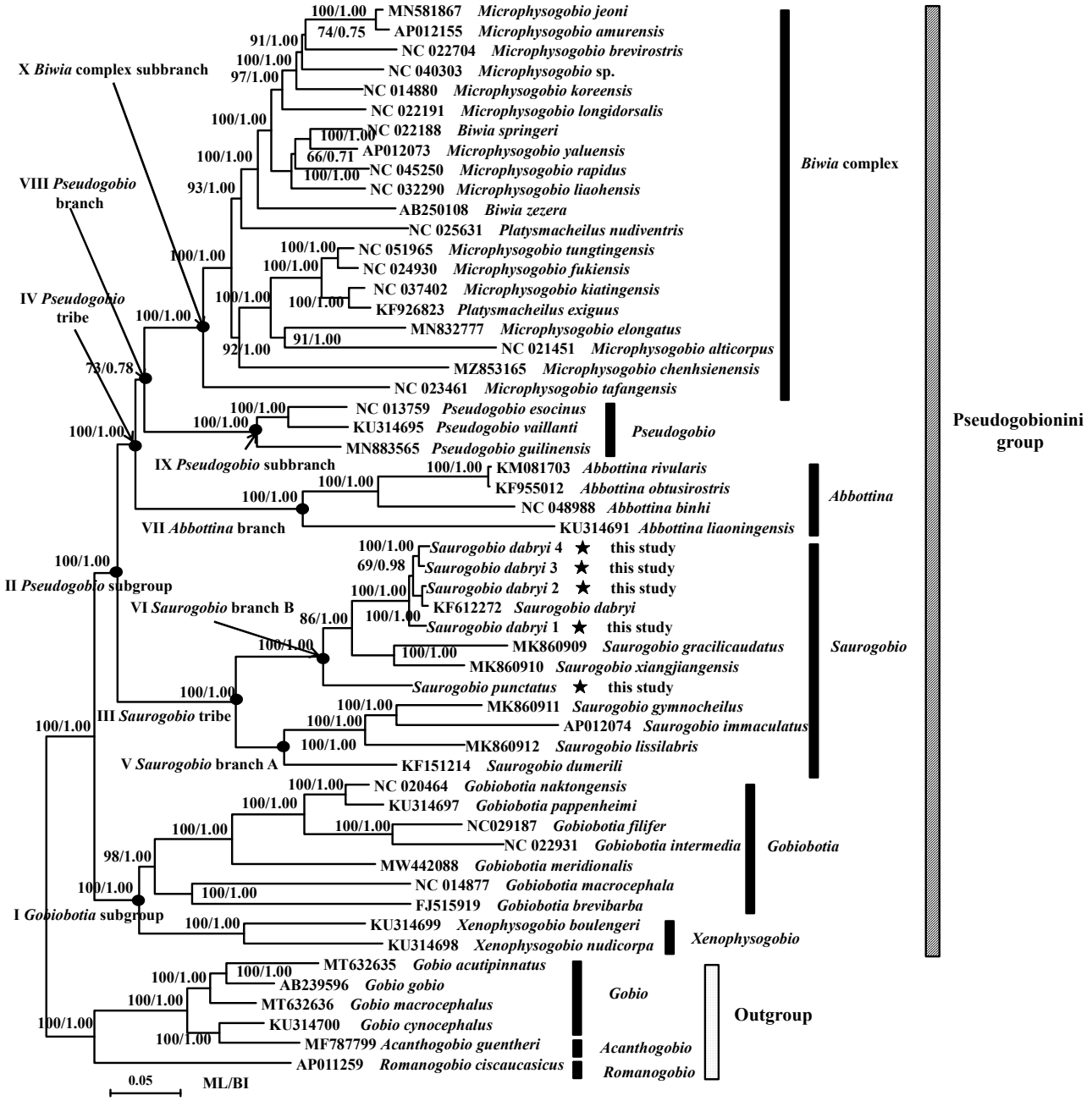

Figure 1. Phylogenetic relationships of the Pseudogobionini group in ML and BI analysis based on the concatenated nucleotide sequences of 13 PCGs. Numbers on nodes before/after represent bootstrap values and posterior probabilities, respectively. The asterisks (★) represent sequences determined in this study. The black dots (●) indicate that nodes were used for selection pressure analysis. Definitions of the classification system and sequences of GenBank accession numbers were shown in the figure. *Saurogobio* branch A contains four species: *S. dumerili*, *S. immaculatus*, *S. lissilabris*, and *S. gymnocheilus*; *Saurogobio* branch B includes four species: *S. dabryi*, *S. gracilicaudatus*, *S. xiangjiangensis*, and *S. punctatus*.

3.3. Selection Pressure Analyses

The one ratio model M0 analysis revealed that the average ω values of the 13 PCGs were less than 0.10, indicating that the average selection pressure affecting each gene has been negative (or purifying). Comparisons of LRTs detected significant variations in dN/dS values in 20 of 65 tests. Among them, 15 were with lower values in the foreground branch,

and 5 with higher values in the foreground branch (detailed information is displayed in Tables S3–S7).

The further analysis detected significant differences in mean dN/dS, dN, and/or dS values in 8 out of 13 PCGs (excluding *ND2*, *ATP8*, *ND3*, *ND4L*, and *ND6*) in three pairs of sub-clades (between *Gobiobotia* subgroup and *Pseudogobio* subgroup, between *Saurogobio* branch A and *Saurogobio* branch B as well as between *Abbottina* branch and *Pseudogobio* branch). No significant differences were detected between *Saurogobio* tribe and *Pseudogobio* tribe as well as between *Pseudogobio* subbranch and *Biwia* complex subbranch (Table 3). Among the detected significant differences, the mean dS value of the *ND4* gene in the *Gobiobotia* subgroup was significantly higher than that of *Pseudogobio* subgroup ($p = 0.032$). The mean dN/dS value for the *COXII* gene in the *Saurogobio* branch A was remarkably greater than that of *Saurogobio* branch B ($p = 0.001$). The mean dN values for *ND1*, *COXII*, *ND5*, and Cyt *b* genes, and mean dS values for *ND1*, *ATP6*, *COXII*, *ND5*, and Cyt *b* genes in the *Saurogobio* branch A were notably greater than that of *Saurogobio* branch B (Table 3). The mean dN/dS values of the *COXI*, *COXII*, and Cyt *b* genes in the *Abbottina* branch were notably higher than that of *Pseudogobio* branch. The mean dN values of *COXI*, *COXII*, *COXIII*, and Cyt *b* genes, and the mean dS value of the *COXII* gene in the *Abbottina* branch were significantly greater than that of *Pseudogobio* branch (Table 3).

Table 3. Statistical analyses of mean dN/dS, dN, and dS values under branch model for genes among five pairs of clades in the Pseudogobionini group.

Gene	Parameters	Node I #	Node II	Node III #	Node IV	Node V #	Node VI	Node VII #	Node VIII	Node IX #	Node X
ND1	dN/dS					0.0280	0.0376			0.0267	0.0533
	dN					0.0079 **	0.0019			0.0041	0.0029
	dS					0.3053 **	0.0889			0.2542	0.1753
ND2	dN/dS	0.0646	0.0687							0.0470	0.0609
	dN	0.0136	0.0084							0.0091	0.0070
	dS	0.2246	0.1531							0.2775	0.1596
COXI	dN/dS			0.0082	0.1560			1.0465 **	0.0270		
	dN			0.0006	0.0023			0.0150**	0.0003		
	dS			0.1007	0.0986			0.1537	0.0946		
COXII	dN/dS			0.0184	0.0288	0.0386 **	0.0092	0.2157 **	0.0061		
	dN			0.0022	0.0041	0.0060 **	0.0003	0.0341 **	0.0006		
	dS			0.0957	0.0984	0.1782 **	0.0451	0.1987 *	0.0896		
ATP8	dN/dS			0.0793	0.1924					0.0301	0.1227
	dN			0.0101	0.0203					0.0038	0.0082
	dS			0.1314	0.1116					0.1421	0.0990
ATP6	dN/dS					0.0328	0.0405			0.0236	0.0547
	dN					0.0082	0.0019			0.0027	0.0031
	dS					0.2250 **	0.0642			0.2254	0.1489
COXIII	dN/dS							0.0213	0.0136		
	dN							0.0042 *	0.0011		
	dS							0.1396	0.0962		
ND4	dN/dS	0.3164	0.0466	0.0456	0.0385						
	dN	0.0071	0.0058	0.0072	0.0052						
	dS	0.2256 *	0.1489	0.1557	0.1534						
ND5	dN/dS					0.0544	0.0459				
	dN					0.0109 *	0.0036				
	dS					0.2182 **	0.0730				
ND6	dN/dS	0.0411	0.0531								
	dN	0.0090	0.0077								
	dS	0.2511	0.1695								
Cyt *b*	dN/dS					0.0281	0.0148	0.0379 **	0.0076		
	dN					0.0063 **	0.0008	0.0103 **	0.0014		
	dS					0.2722 *	0.1066	0.2408	0.1608		

foreground branch, * $0.01 < p < 0.05$, ** $p < 0.01$.

Under the site model applied for detecting the positively selected sites from 13 PCGs, seven positively selected sites were detected in the *ND2*, *ND4*, and *ND5* genes. The residue 193 S (0.952 *) in the *ND4* gene between *Saurogobio* branch A and *Saurogobio* branch B (Table 4); residues 34 Q (0.972 *) and 525 H (0.997 **) in the *ND5* gene between *Abbottina* branch and *Pseudogobio* branch; residue 274 D (0.960 *) in the *ND2* gene; residue 26 A (0.975 *) in the *ND4* gene; and residues 34 P (0.952 *) and 525 S (0.988 *) in the *ND5* gene between *Pseudogobio* subbranch and *Biwia* complex subbranch (Tables S8 and S9).

Table 4. Parameter estimates and log-likelihood values under models among sites for *ND4* gene between *Saurogobio* branch A and *Saurogobio* branch B.

Model	Ln L	Estimates of Parameters			Model Compared	LRT *p*-Value	Positively Selected Sites
M3	−5078.253651	p_0 = 0.84992 ω_0 = 0.0062	p_1 = 0.14517 ω_1 = 0.19028	p_2 = 0.00491 ω_2 = 1.62404	M0 vs. M3	0.00000	[-]
M0	−5130.798044		ω_0 = 0.03624				Not Allowed
M2a	−5096.161404	p_0 = 0.96931 ω_0 = 0.02251	p_1 = 0.03069 ω_1 = 1.00000	p_2 = 0.00000 ω_2 = 36.02152	M1a vs. M2a	1.00000	[-]
M1a	−5096.161404	p_0 = 0.96931 ω_0 = 0.02251	p_1 = 0.03069 ω_1 = 1.00000				Not Allowed
M8	−5081.175068	p_0 = 0.98839 (p_1 = 0.01161)	p_1 = 0.04111 ω = 1.00000	q = 0.36593	M7 vs. M8	0.00013	186 F 0.706, 193 S 0.952 *
M7	−5090.122897	p = 0.04073	q = 0.32459				Not Allowed
M8a	−5081.175065	p_0 = 0.98839 (p_1 = 0.01161)	p = 0.04101 ω = 1.00000	q = 0.36457	M8a vs. M8	0.99804	Not Allowed

* $0.01 < p < 0.05$.

4. Discussion

4.1. Structural Features of Mitogenomes of the Pseudogobionini Group

All mitogenomes of the Pseudogobionini group are with the same gene arrangements and organization as found in other Cypriniformes species [5,26]. The average A + T content of these mitogenomes in this group was 56.3%, such an A + T rich pattern reflects the typical sequence feature of the vertebrate mitogenome [49]. Among the PCGs of this group, 12 out of 13 PCGs used ATG as the start codon except for the *COXI* gene, whose initiation codon was GTG. This is a typical phenomenon in fish mitogenomes [26,50]. In addition, incomplete stop codons (T or TA) were commonly found in these mitogenomes, which would be completed as TAA by post-transcriptional polyadenylation [51].

4.2. Phylogenetic Relationships of the Pseudogobionini Group

Our results supported that the Pseudogobionini group is monophyletic. This conclusion was consistent with previous studies on Gobioninae [8,24,26,27,35,52]. In our study, molecular phylogenetic relationships of the Pseudogobionini group showed that *Pseudogobio* had a closer relationship with *Biwia* complex, and that the *Saurogobio* was branch sister to the branch comprising three genera *Pseudogobio*, *Abbottina*, and *Biwia* complex. Our result was similar to a molecular phylogeny [26] but inconsistent with another molecular work [27] in which the *Abbottina* and *Biwia* complex were monophyletic. Although the *Pseudogobio* and *Biwia* complex share morphological characters (supraorbital bones present and wing-like lateral expansion end of 4th vertebral pleural rib) supporting our results, when considering the higher support value (97/1.00) on the node containing *Abbottina* complex and *Biwia* complex by Zhang et al. [27] than in our study (73/0.78) on the node involving *Pseudogobio* and *Biwia* complex, we suggest that further work is needed to resolve these relationships. Our phylogenetic relationships of the genus *Saurogobio* were congruent with the molecular evidence [53] in which the phylogeny was revealed by the mitochondrial Cyt *b* gene and strongly supported the monophyly of the genus *Saurogobio*.

4.3. Classification of the Pseudogobionini Group

Based on the molecular phylogeny and morphological or osteological [16,21,25] characters (Table 2), we proposed a classification system for the Pseudogobionini group as follows:

Pseudogobionini group

- *Gobiobotia* subgroup
- *Pseudogobio* subgroup
  - *Saurogobio* tribe
    - *Saurogobio* branch A
    - *Saurogobio* branch B
  - *Pseudogobio* tribe
    - *Abbottina* branch
    - *Pseudogobio* branch
      - *Pseudogobio* subbranch
      - *Biwia* complex subbranch

Our classification results showed that the Pseudogobionini group is monophyletic. This conclusion supported Hosoya's [25] and Yu and Yue's [16] views. However, the generic relationships within the Pseudogobionini are significantly different from previous studies [16,25]. Hosoya [25] proposed that the *Biwia* complex was the most specialized group, followed by *Saurogobio*, and they together formed sister groups with the branch composed of the genera *Abbottina* and *Pseudogobio*. Yu and Yue [16] revealed that the *Biwia* complex was the most specialized group, followed by *Abbottina*, then *Saurogobio*; *Pseudogobio* was the most primitive genus. We found that the *Biwia* complex has the highest phylogenetic position, followed by *Pseudogobio*, then *Abbottina*, and *Saurogobio* located at the base position based on mitogenomic data. The differences in the generic relationships within the Pseudogobionini suggested that the diversification and complication of this group and its phylogeny need to be resolved in the future via more extensive sampling and more comprehensive characters analysis. Although the *Gobiobotia* subgroup (including *Gobiobotia* and *Xenophysogobio*) was not included in Yu and Yue's [16] study, based on our results and Hosoya's [25] results, we proposed that the *Gobiobotia* subgroup was the most primitive genus among the Pseudogobionini genera.

4.4. Mitochondrial Gene Evolution and Group Differentiation and Adaptations of the Pseudogobionini Fishes

Mitochondrial gene evolution has been recognized playing important roles in animal adaptation to different environments and showing different dN/dS values [10–12,54]. In this study, we detected significantly different dN/dS values in 11 out of 13 (excluding *ND3* and *ND4L*) PCGs in five pairs of clades (Tables S3–S7), and significantly different mean dN/dS, dN, and/or dS values in 8 out of 13 PCGs (excluding *ND2*, *ATP8*, *ND3*, *ND4L*, and *ND6*) in three pairs of sub-clades (Table 3). We suggested that those mitochondrial gene evolution may have contributed to group differentiation to different habitats. For example, the *Abbottina* branch and *Biwia* complex subbranch mainly lives in lakes (standing water) spawning very sticky eggs [55] with very short and blunt snout [21–23], while the *Pseudogobio* subbranch mainly inhabits rivers (flowing water) spawning adhesive eggs [56] with a relatively depressed and elongated snout [21,57]. The *Saurogobio* branch A mainly lives in flowing water and smooth lips or with degenerated papillae, while the *Saurogobio* branch B mainly inhabits relatively standing water and lips with developed papillae [21,53]. We think that living in different habitats may result in the adaptation to different environments with different dissolved oxygen concentrations leading to different feeding and breeding methods.

Positive selections over the mitochondrial genes have been found contributing to high-altitude birds [54] and vertebrates [12] adaptation to the harsh environment. In our study, we detected one positively selected site in the *ND4* gene between *Saurogobio* branch A and *Saurogobio* branch B, two positively selected sites in the *ND5* gene between *Abbottina*

branch and *Pseudogobio* branch, one positively selected site in the *ND2* gene and one in the *ND4* gene, as well as two positively selected sites in the *ND5* gene between *Pseudogobio* subbranch and *Biwia* complex subbranch. (Table 4, Tables S8 and S9). These three genes belong to NADH dehydrogenase, which is the first and the largest enzyme complex in the respiratory chain [9,58]. Positively selected sites may change or affect the electron transport of the respiratory chain and thereby change the mitochondrial oxidative phosphorylation process. We speculated that positive selection over the mitochondrial gene might be associated with group differentiation and adaptations to lake or river environments in our investigated groups.

5. Conclusions

This study provides information on newly sequenced complete mitochondrial genomes of two gudgeon species *S. dabryi* and *S. punctatus*, well-supported phylogeny of the East Asia predominant fish group of Pseudogobionini, and gives hints that mitochondrial gene evolution might have contributed to group differentiation and adaptations of the Pseudogobionini fishes. This study promotes our understanding of the molecular phylogeny of the Pseudogobionini group and can serve as a valuable reference for further analysis of selection pressure in different taxa.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes8040201/s1. Table S1: Primers used for the amplification in the mitochondrial genomes of *Saurogobio dabryi* and *S. punctatus* in this study; Table S2: Characteristics of the mitochondrial genomes of two gudgeon species *S. dabryi*1–4 and *S. punctatus*; Table S3–S7: Likelihood ratio tests and parameter estimates under branch model for genes between *Gobiobotia* subgroup and *Pseudogobio* subgroup, between *Saurogobio* tribe and *Pseudogobio* tribe, between *Saurogobio* branch A and *Saurogobio* branch B, between *Abbottina* branch and *Pseudogobio* branch, as well as between *Pseudogobio* subbranch and *Biwia* complex subbranch; Table S8: Parameter estimates and log-likelihood values under models among sites for *ND5* gene between *Abbottina* branch and *Pseudogobio* branch; Table S9: Parameter estimates and log-likelihood values under models among sites for *ND2*, *ND4*, and *ND5* genes between *Pseudogobio* subbranch and *Biwia* complex subbranch.

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Data Availability Statement: The five newly sequenced data have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on 25 March 2023) under the accession numbers ON533884-ON533888, the other published mitogenomic data can be downloaded from GenBank, and the remaining data was contained in the article and Supplement Materials.

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