Characterization of the Complete Mitochondrial Genome of Leptobotia elongata and Its Phylogenetic Implications in Cobitidae

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Article

Characterization of the Complete Mitochondrial Genome of *Leptobotia elongata* and Its Phylogenetic Implications in Cobitidae

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Simple Summary: The complete mitochondrial genome has been widely used in phylogenetics-related studies, as it offers valuable insights into phylogenetic relationships. In this study, we reported the complete mitogenome of L. elongata and conducted a detailed analysis of its s characteristics, which was employed to infer phylogenetic relationships. These findings reveal that both the gene arrangement and composition of mitochondrial genes in L. elongata are comparable to those found in other bony fishes. Our study further demonstrated that the Cobitidae species under investigation could be grouped into two distinct clades, with L. elongata showing a sister relationship with L. microphthalma. Collectively, our research enhanced the understanding of the mitochondrial genome structure and contributed to the phylogenetic analysis of L. elongata.

Abstract: Elongate loach (*Leptobotia elongata*) is an endemic fish in China. Previous studies have provided some insights into the mitochondrial genome composition, and the phylogenetic relationships of *L. elongata* inferred using protein-coding genes (PCGs). However, the detailed information about is limited. Therefore, in this study, we sequenced the complete mitochondrial genome of *L. elongata* and analyzed its structural characteristics. The PCGs and mitochondrial genome were used for selective stress analysis and genomic comparative analysis respectively. The complete mitochondrial genome of the *L. elongata*, together with those of 36 Cyprinidae species, was used to infer the phylogenetic relationships of the Cobitidae family through maximum likelihood (ML) reconstruction. The results showed that the genome sequence has a full length of 16,591 bp, which includes 13 PCGs, 22 transfer RNA genes (tRNA), two ribosomal RNA genes (rRNA), and two non-coding regions (CR D-loop and light chain sub-chain replication origin OL). Overall, *L. elongata* shared the same gene arrangement and composition of the mitochondrial genes with other teleost fishes. The Ka/Ks ratios of all mitochondrial PCGs were less than 1, indicating that all the PCGs were evolving under purifying selection. Genome comparison analyses showed a significant sequence homology of species of Leptobotia. A significant identity between *L. elongata* and the other 5 Leptobotia species was observed in the visualization result, except for *L. mantschurica*, which lacked the tRNA-Arg gene and had a shorter tRNA-Asp gene. The phylogenetic tree revealed that the Cobitidae species examined here can be grouped into two clades, with *L. elongata* forming a sister relationship with *L. microphthalma.* This study could provide additional inferences for a better understanding of the phylogenetic relationships among Cobititdae species.

Keywords: elongate loach; cobititdae; mitochondria genome; phylogenetic analysis

1. Introduction

Elongate loach (*Leptobotia elongata*), belonging to Cobitidae of Cypriniformes, is indigenous to the middle and upper reaches of the Yangtze River in China [1]. It is characterized by rapid growth and exceptional ornamental value [2,3]. However, the wild population resources of *L. elongata* have experienced a significant decline since the 1980s due to overfishing, dam construction, destruction of feeding, and spawning grounds [4]. As a result, it has been classified as vulnerable grade (VU) in China Red Book of Endangered Animals-Fish [5].

The family Cobitidae was originally proposed by Regan [6]. In this family, extensive research focused on morphological characteristics and mitochondrial genes has been conducted for over a century [7–11]. Currently, many scientists tend to divide Cobitidae into three subfamilies: Nemacheilinae, Botiinae, and Cobitinae [12]. In order to maintain consistency between the phylogenetic relationship and the natural classification of Cobitidae fishes, Tang et al [13] elevated these three subfamilies to the family level, which aligns with the classification of Liu et al [9]. As the second-largest group of Cypriniformes, Cobitidae is a key element in resolving the phylogenetic relationships of Cypriniformes. Investigating the phylogenetic relationships of the *L. elongate*, one of the youngest species in the Cobitidae, in family Cobitidae is beneficial to resolve the taxonomic ambiguity of Cobitidae fishes. Previous studies only focused on biological characteristics [14], artificial breeding [15], embryo development, and genetic diversity [16,17]. However, its research on the phylogenetic relationships of *L. elongate* is limited [18]. Therefore, a reevaluation of the phylogenetic relationships of *L. elongata*, involving additional genes and a broader range of taxa, could provide more data for the conservation of *L. elongata*'s wild population resources.

Mitochondrial DNA (mtDNA) is present in the cells of all eukaryotes and possesses several genetic characteristics. It is primarily inherited maternally and exhibits conservation of coding regions, rapid evolution of the control region (CR), a high mutation rate, and a relatively independent genetic transcription system [19,20]. Compared to nuclear genes, mtDNA evolves at a faster rate, allowing for a more accurate representation of phylogenetic relationships. Therefore, mtDNA is widely utilized as a molecular marker in phylogenetic studies [21–23]. In fish phylogeny research, genes such as cytochrome b (*cytb*), cytochrome oxidase (*cox*), and 16SrRNA are commonly employed at species-to-family level [22,24,25]. However, when investigating higher taxonomic categories, relying solely on a single mitochondrial gene may lead to misleading phylogenetic data due to limited information capacity and homogenization effects [26]. In contrast, utilizing the complete mitochondrial genome could provide a more comprehensive set of phylogenetic information [27].

In this study, we sequenced the mitochondrial genome, analyzed the structural information of *L. elongata*, and compared the structures and complete mitochondrial genome with some of the determined Leptobotia species. Additionally, we reconstructed phylogenetic trees using complete mitochondrial genome sequences to analyze the evolutionary relationships *L. elongata* in the Cobitidae family. These study might provide futher insight into the structural of *L. elongate*, and improve understanding of evolutionary relationships of *L. elongate* within the Cobitidae.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The *L. elongata* sample was collected from Chengdu, Sichuan Province, China, in October 2020. The pectoral fin was collected and stored in 95% ethanol at −20°C. Genomic DNA was isolated from the pectoral fin using the phenol-chloroform method and DNA degradation and contamination were estimated using agarose gel electrophoresis.

2.2. Mitochondrial Genome Sequencing and Assembly

After qualifying the DNA sample, the DNA was mechanically fragmented using ultrasonic interruption. The fragmented DNA underwent fragment purification, end-repair, addition of A at the 3' end, connection of sequencing adapters, and selection of fragments of different sizes using agarose gel electrophoresis. PCR amplification was then performed to generate a sequencing library [18]. The qualified PCR products were sequenced on the Illumina HiSeq 2500 platform.

Prior to assembly, low-quality data, including the reads of average quality value< 5 or N content>5, were filtered using Fasta software (version 0.20.0), and the sequences linker and primer sequence were trimmed from the reads. The mitochondrial genome assembly was carried out using the following methodology. First, clean reads were assembled using SPAdes (version 3.10) [28]. Second, contigs were connected to generate scaffold sequences using SSPACE (version 2.0), and Gaps in the scaffold sequences were filled using Gapfiller (version 2.1.1) until a complete pseudo genome sequence was assembled. Lastly, the sequencing results were mapped onto the assembled pseudo genome sequence to identify and correct any incorrect bases, and the complete mitochondrial circular genome sequence was obtained by coordinate remaking.

2.3. Mitochondrial Genome Annotation and Analysis

The newly assembled sequences were annotated in the Mitos web server (http://mitos2.bioinf.uni-leipzig.de) [29] with the following parameters: E-value Exponent=5, Maximum Overlap=100, ncRNA overlap=100. The annotation results were then compared with those of closely related species. Finally, after manual correction, the final annotation results were obtained.

The secondary structure of tRNAs was obtained from the annotation results. The circular map of the mitochondrial genome was generated using OGDRAW (version 1.3.1) [30]. The relative synonymous codon usage (RSCU) values were analyzed with MEGA (version 6.0). The mitochondrial genome skew values were calculated using the following formula: ATskew=(A-T)/(AT); GCskew=(G-C)/(GC)[31]. Mafft (version 7. 310) [32] software was used for gene sequences comparative between *L. elongata* and six Leptobotia fishes (*Leptobotia mantschurica*, *Leptobotia taeniops*, *Leptobotia microphthalma*, *Leptobotia rubrilabris*, *Leptobotia punctata*, and *Leptobotia pellegrini*), and the evolutionary rate (Ka/Ks, ω) was calculated using KaKs_Calculator (version 2. 0) [33]. If the evolutionary rate is equal to 1, >1, or, <1, the PGCs are expected to be under no selection, positive selective constraint (purifying selection), or diversifying selection. The mitochondrial genome structure was compared between *L. elongata* and six Leptobotia fish species in CGVIEW server [34] with default parameters (http://stothard.afns.ualberta.ca/cgview_server/), and the alignment results were visualized using mauve software (version 2.4.0).

2.4. Phylogenetic Analyses

The phylogenetic tree was reconstructed using the complete mitochondrial genome sequences of 37 Cypriniformes species, with *Myxocyprinus asiaticus* and *Danio rerio* used as outgroups (Table 1). All the genome sequences were set to the same start points in the circular sequence. Multiple sequence alignment was performed in MAFFT software (version 7.42) with auto model, and the alignment sequences were trimmed using trimAl (version 1.4. rev15). Subsequently, the RaxML (version 8.2.0) software was used to conduct the rapid bootstrap analysis (bootstrap=1000) to construct the maximum likelihood evolution tree.

Table 1. Taxonomic information and GeneBank assession numbers of all species used in phylogenetic analysis.

Family	Genus	Species	Assession
Myxocyprinae	Myxocyprinus	*Myxocyprinus asiaticus*	NC_006401.1
Cyprinidae	Danio	*Danio rerio*	NC_002333.2
Cobitidae	Leptobotia	*Leptobotia microphthalma*	KY307846.1
	Leptobotia	*Leptobotia elongata*	
	Leptobotia	*Leptobotia pellegrini*	NC_031602.1
	Leptobotia	*Leptobotia taeniops*	AP013304.1
	Leptobotia	*Leptobotia rubrilabris*	KY307847.1
	Leptobotia	*Leptobotia punctata*	MH644033.1
	Leptobotia	*Leptobotia mantschurica*	AB242170.1
	Parabotia	*Parabotia fasciata*	AP011437.1
	Parabotia	*Parabotia banarescui*	NC_026127.1
	Parabotia	*Parabotia kiangsiensis*	MT850132.1
	Parabotia	*Parabotia lijiangensis*	MT323118.1
	Botia	*Botia udomritthiruji*	AP011349.1
	Botia	*Botia lohachata*	KP729183.1

	Chromobotia	*Chromobotia macracanthus*	AB242163.1
	Yasuhikotakia	*Yasuhikotakia morleti*	NC_031600.1
		Yasuhikotakia modesta	KY131962.1
	Sinibotia	*Sinibotia robusta*	NC_027853.1
		Sinibotia pulchra	KT362179.1
		Sinibotia reevesae	NC_030322.1
		Sinibotia superciliaris	JX683724.1
		Sinibotia superciliaris	KY307848.1
	Acanthocobitis	*Acanthocobitis botia*	AP012139.1
		Acanthocobitis botia	AP012138.1
	Triplophysa	*Triplophysa pappenheimi*	NC_033972.1
		Triplophysa siluroides	NC_024611.1
	Pangio	*Pangio kuhlii*	NC_031599.1
		Pangio cuneovirgata	NC_031594.1
	Cobitis	*Cobitis lutheri*	NC_022717.1
		Cobitis melanoleuca granoei	NC_023473.1
		Cobitis nalbanti	MH349461.1
		Cobitis elongatoides	NC_023947.1
		Cobitis hankugensis	MN841275.1
		Cobitis macrostigma	MK156771.1
		Cobitis sinensis	NC_007229.1
		Cobitis matsubarai	NC_029441.1

3. Result and Discussion

3.1. Mitochondrial Structural Characteristics

The complete mitochondrial genome of *L. elongata* was obtained through high-throughput sequencing technology, with a total length of 16,591bp (Figure 1). It consists of 37 typical animal mitochondrial genes, including 22 tRNA genes, 13 PCGs, 2 rRNA genes, and two non-coding regions (D-Loop and OL). Among the mitochondrial genes, nine genes (*trnQ, trnP, trnE, nad6, trnS2, trnY, trnC, trnN, trnA*) were encoded by the light (L) strand, while the remaining genes are encoded by the heavy (H) strand. The arrangement and content of the mitochondrial genome in *L. elongata* were similar to those reported in teleost fishes [20,35,36]. The entire base composition of the *L. elongata* mitochondrial genes is as follows (Table 2): 30.79 %A, 24.77 %T, 16.17 %G, and 28.27 %C, and the AT and GC percentages are 55.56 % and 44.44 % respectively, which results in a positive skew value for AT and a subtractive skew value for CG. It was suggested that the occurrence of A and C bases was more frequent in the genome. Previous studies have shown that the bias in base composition plays a crucial role in the replication and transcription of mitochondrial genomes [37].

Table 2. Nucleotide composition and skewness values of *L. elongata* mitogenome.

Leptobotia_elongata	Size (bp)	A%	T%	G%	C%	A+T%	G+C%	AT-skew	GC-skew
Mitogenome	16591	30.79	24.77	16.17	28.27	55.56	44.44	0.108	-0.272
PCGs	11430	28.56	26.77	15.55	29.13	55.33	44.67	0.032	-0.304
tRNAs	1558	28.18	25.8	23.49	22.53	53.98	46.02	0.044	0.021
rRNAs	2638	34.04	19.48	21.04	25.44	53.53	46.47	0.272	-0.095
Dloop	926	35.64	31.75	13.71	18.9	67.39	32.61	0.058	-0.159

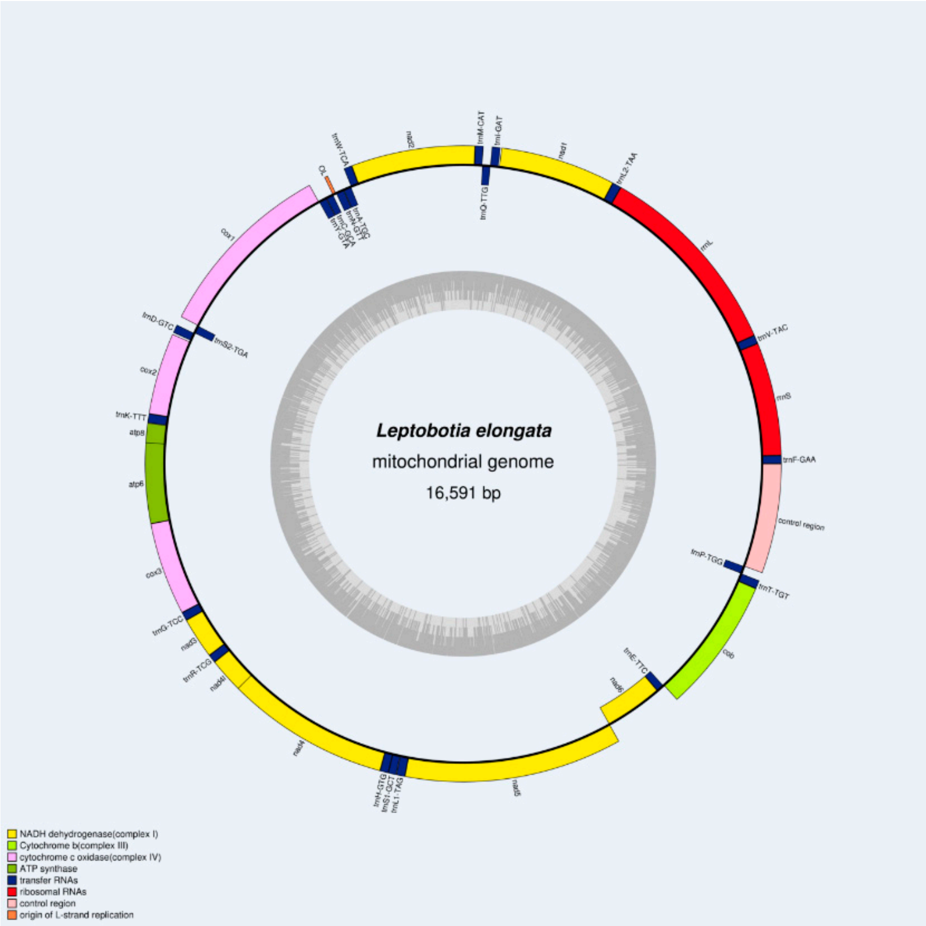

Figure 1. Mitochondrial genome map of *L. elongata*.

3.2. Protein Coding Genes (PCGs)

The PCGs account for 68.89% of the total length of the *L. elongata* mitochondrial genome. As expected (Table 3), most PCGs started with the regular codon ATG, except for the *cox1*, started with GTG. Among the PCGs, there were 7 genes that shared the complete stop codon TAA. While six genes shared incomplete stop codons (TA- or T--), which existed in many teleostean as numerous studies: *L. microphthalma* with seven incomplete stop codons [38], *Cobitis macrostigma* with seven incomplete stop codons [39], *Pelteobagrus fulvidraco* with five incomplete stop codons [20], *Parabotia kiangsiensis* with three incomplete stop codons [40], etc. The presence of tRNA sequences at the 3' end of these genes is responsible for the incomplete stop codons [41], and these incomplete stop codons can be converted to TAA through post-transcriptional polyadenylation [42].

Three overlapping regions between certain PCGs: ATPase8-ATPase6, ND4-ND4L, and ND5-ND6 were also identified in this study. These overlapping regions were 4-10bp in length, with the largest overlapping occurring between ATP8 and ATP6, which was common among Cobitidae species [43]. These overlapping regions contribute to the variation in mitochondrial genome length among closely related species [44]. The relative synonymous codon usage (RSCU) values of PCGs are revealed in Table 4 and Figure 2. In the protein-coding region, a total of 2012 codons were used. According to the degeneracy of codons, serine and leucine were encoded by six codons, while the remaining amino acids were encoded by either four or two codons. In the coded passwords, CUA (leucine), AUU (isoleucine), GCC (Aminopropanoic), and GCA (Aminopropanoic) are the most common, while AAA (Lysine) and CUA (leucine) have the highest RSCU values. Therefore, PCGs preferred the codons using adenine at the third codon. The codon usage varied between different species, which was more prominent between species with further phylogenetic relationship [45]. It is relevant to gene length, mutation bias, GC composition, amino acid composition, tRNA abundance, and translational selection [46–51].

Table 3. Summary of *L. elongata* mitogenome.

		Position				codon	
Gene	stand	From	To	size	Intergenic length	start	stop
tRNA-*phe*	H	1	69	69	0		
12s-rRNA	H	70	1024	955	0		
tRNA-*val*	H	1025	1096	72	0		
16S-rRNA	H	1097	2779	1683	0		
tRNA-*leu*	H	2780	2854	75	0		
nd1	H	2855	3829	975	0	ATG	TAA
tRNA-*ile*	H	3838	3909	72	8		
tRNA-*gln*	L	3908	3978	71	-2		
tRNA-*met*	H	3980	4048	69	1		
nd2	H	4049	5094	1046	0	ATG	TA-
tRNA-*trp*	H	5095	5163	69	0		
tRNA-*ala*	L	5166	5234	69	2		
tRNA-*asn*	L	5236	5308	73	1		
OL	L	5310	5340	31	1		
tRNA-*cys*	L	5339	5404	66	-2		
tRNA-*tyr*	L	5406	5476	71	1		
cox1	H	5478	7028	1551	1	GTG	TAA
tRNA-*ser*	L	7030	7100	71	1		
tRNA-*asp*	H	7103	7174	72	2		
cox2	H	7188	7878	691	13	ATG	T--
tRNA-*lys*	H	7879	7954	76	0		
ATPase8	H	7956	8123	168	1	ATG	TAA
ATPase6	H	8114	8797	684	-10	ATG	TAA
cox3	H	8797	9581	785	1	ATG	TA-
tRNA-*gly*	H	9582	9653	72	0		
nd3	H	9654	10002	349	0	ATG	T--
tRNA-*arg*	H	10003	10072	70	0		
nd4l	H	10073	10369	297	0	ATG	TAA
nd4	H	10363	11744	1382	-7	ATG	TA-
tRNA-*his*	H	11745	11814	70	0		
tRNA-*ser*	H	11815	11881	67	0		
tRNA-*leu*	H	11883	11955	73	1		
nd5	H	11956	13794	1839	0	ATG	TAA
nd6	L	13791	14312	522	-4	ATG	TAA
tRNA-*glu*	L	14313	14381	69	0		
Cytb	H	14386	15526	1141	4	ATG	T--
tRNA-*thr*	H	15527	15598	72	0		
tRNA-*pro*	L	15597	15666	70	-2		
CR	H	15666	16591	926	0		

Table 4. Relative synonymous codon usage and codon numbers of *Leptobotia_elongata* mitochondrial PCGs.

Codon	No.	RSCU	Codon	No.	RSCU	Codon	No.	RSCU
UAA(*)	7	1	AAA(K)	38	1.8536	CGG(R)	5	0.5
GCA(A)	68	1.4468	AAG(K)	3	0.1464	CGU(R)	5	0.5
GCC(A)	94	2	CUA(L)	123	2.271	AGC(S)	24	1.161

GCG(A)	3	0.064	CUC(L)	57	1.0524	AGU(S)	4	0.1938
GCU(A)	23	0.4892	CUG(L)	22	0.4062	UCA(S)	50	2.4192
UGC(C)	7	1.1666	CUU(L)	53	0.9786	UCC(S)	22	1.0644
UGU(C)	5	0.8334	UUA(L)	51	0.9414	UCG(S)	1	0.0486
GAC(D)	25	1.3158	UUG(L)	19	0.351	UCU(S)	23	1.113
GAU(D)	13	0.6842	AUA(M)	60	2.0226	ACA(T)	72	1.87
GAA(E)	36	1.44	AUG(M)	28	0.9438	ACC(T)	59	1.5324
GAG(E)	14	0.56	GUG(M)	1	0.0336	ACG(T)	1	0.026
UUC(F)	63	0.9618	AAC(N)	54	1.4594	ACU(T)	22	0.5716
UUU(F)	68	1.0382	AAU(N)	20	0.5406	GUA(V)	52	1.5524
GGA(G)	48	1.4116	CCA(P)	47	1.6348	GUC(V)	23	0.6864
GGC(G)	31	0.9116	CCC(P)	47	1.6348	GUG(V)	24	0.7164
GGG(G)	39	1.1472	CCG(P)	6	0.2088	GUU(V)	35	1.0448
GGU(G)	18	0.5296	CCU(P)	15	0.5216	UGA(W)	45	1.6364
CAC(H)	42	1.7142	CAA(Q)	45	1.9148	UGG(W)	10	0.3636
CAU(H)	7	0.2858	CAG(Q)	2	0.0852	UAC(Y)	26	0.963
AUC(I)	59	0.792	CGA(R)	23	2.3	UAU(Y)	28	1.037
AUU(I)	90	1.208	CGC(R)	7	0.7			

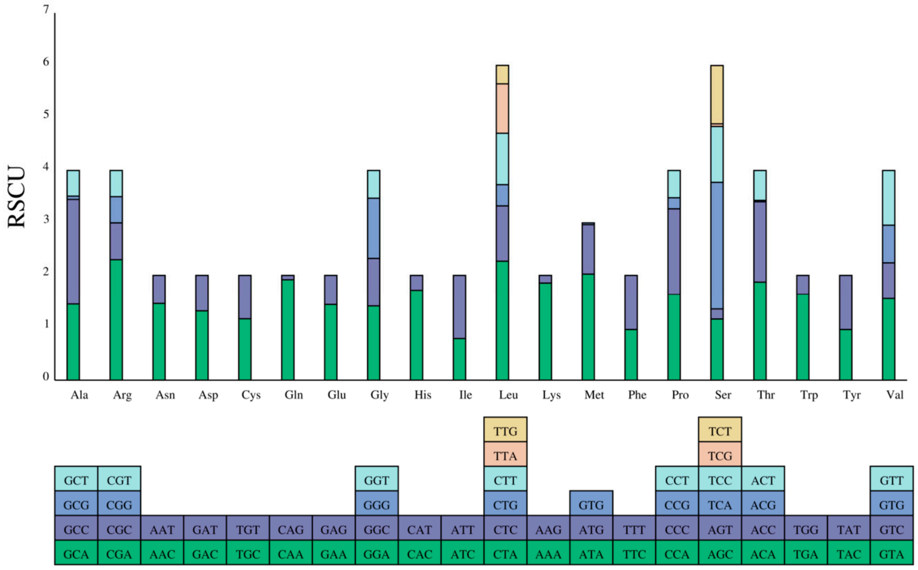

Figure 2. The relative synonymous codon usage (RSCU) in the mitogenome of *L. elongata.*

3.3. Genome Comparative Analysis

The nonsynonymous substitution ratio (Ka) and synonymous substitution ratio (Ks) were calculated to evaluate selective pressures during the evolutionary process of PCGs among Leptobotia species. It was shown that the average Ka was similar among the 6 fishes (0.0089-0.0114), with *nd5* exhibiting the highest average Ka (Figure 3A; Table 5), indicating that it might be under positive selection across species. The Ks of *Leptobotia microphthalma* was significantly lower than the other species (Figure 3B; Table 6). There were more synonymous substitutions per synonymous sites in *nd*4 and *atp6*, exhibiting the high polymorphic nature of the genes in these fishes. *nd4* has also been confirmed to be polymorphism among sharks [52]and blue-spotted maskray [45]. The Ka/Ks ratio (ω) is a means to examine molecular adaption [53,54], which could be used to estimate the evolutionary rate among Cobitidae species. In this study, the Ka/Ks ratios of all PGCs were less than 1, indicating that purifying selection possesses the leading role in the evolution of these PGCs (Figure 3C; Table 7). Therein, *cox3* (0.0076) and *nd4l* (0.0087) were evolving under a strong purifying selection, whereas *nd4* (0.0549), *nd5* (0.0782), and *nd2* (0.0784) were evolving under comparatively relaxed mutational constraints. Currently, selective pressure in mitochondrial PCGs has been studied poorly on other

Cobitidae species [13,18,38,39,55–57]. While the same pattern of widespread purifying selection has been discovered in several other decapod crustaceans [58].

The comparison of the mitochondrial genome sequences between *L. elongata* and 6 Leptobotia species showed a significant sequence homology within the Leptobotia genus (Figure 4; Figure 5). *L. elongata* showed a higher identity with the other five species, except for *L. microphthalma*, which lacked the *tRNA-Arg* and a shorter *tRNA-Asp*, indicating that the arrangement of genes of Leptobotia species is comparatively conserved.

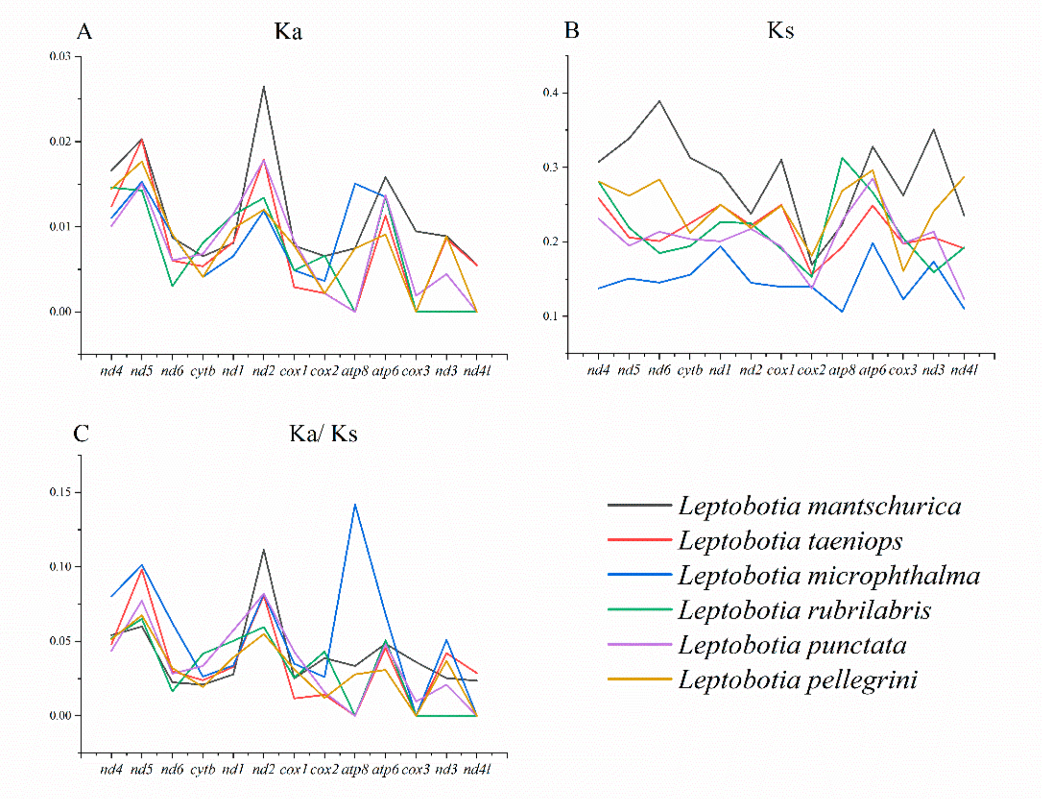

Figure 3. Non-ynonymous (A) and synonymous (B) substitutional rates and the ratios of KaKs (C) of protein coding genes of *L. elongata*.

Table 5. Non-synonymous substitution rate of mitochondrial genes in Leptobotia species.

Species	*Leptobotia mantschurica*	*Leptobotia taeniops*	*Leptobotia microphthalma*	*Leptobotia rubrilabris*	*Leptobotia punctata*	*Leptobotia pellegrini*	Average
nd4	0.0166	0.0124	0.0110	0.0146	0.0101	0.0145	0.0132
nd5	0.0203	0.0202	0.0153	0.0143	0.0150	0.0177	0.0171
nd6	0.0087	0.0060	0.0091	0.0030	0.0061	0.0091	0.0070
cytb	0.0065	0.0054	0.0041	0.0081	0.0068	0.0041	0.0058
nd1	0.0081	0.0082	0.0066	0.0114	0.0115	0.0098	0.0093
nd2	0.0265	0.0179	0.0119	0.0134	0.0178	0.0120	0.0166
cox1	0.0078	0.0029	0.0049	0.0049	0.0083	0.0078	0.0061
cox2	0.0066	0.0022	0.0036	0.0066	0.0022	0.0022	0.0039
atp8	0.0075	NA	0.0151	NA	NA	0.0075	0.0100
atp6	0.0158	0.0113	0.0135	0.0135	0.0137	0.0091	0.0129
cox3	0.0095	NA	NA	NA	0.0019	NA	0.0057
nd3	0.0089	0.0086	0.0089	NA	0.0045	0.0089	0.0080
nd4l	0.0055	0.0055	NA	NA	NA	NA	0.0055
Average	0.0114	0.0092	0.0094	0.0100	0.0089	0.0093	

Table 6. Synonymous substitution rate of mitochondrial genes in Leptobotia species.

Species	***Leptobotia mantschurica***	***Leptobotia taeniops***	***Leptobotia microphthalma***	***Leptobotia rubrilabris***	***Leptobotia punctata***	***Leptobotia pellegrini***	**Average**

nd4	0.3072	0.2587	0.1378	0.2807	0.2312	0.281	0.2494
nd5	0.339	0.2061	0.1508	0.2192	0.1948	0.262	0.2286
nd6	0.3892	0.2009	0.1454	0.1851	0.2136	0.2839	0.2363
cytb	0.313	0.2249	0.1558	0.1944	0.2038	0.2119	0.2173
nd1	0.2916	0.2499	0.1942	0.2269	0.2008	0.2503	0.2356
nd2	0.2375	0.2222	0.1452	0.2249	0.2176	0.2187	0.211
cox1	0.3102	0.2498	0.1397	0.1906	0.1935	0.2488	0.2221
cox2	0.1696	0.1562	0.1393	0.153	0.1376	0.1815	0.1562
atp8	0.2238	0.193	0.1062	0.3132	0.2284	0.2686	0.2222
atp6	0.3279	0.2489	0.1982	0.2665	0.2852	0.2965	0.2705
cox3	0.2624	0.1976	0.123	0.205	0.1981	0.1609	0.1912
nd3	0.351	0.2059	0.1736	0.1592	0.2136	0.241	0.2241
nd4l	0.2358	0.1912	0.1103	0.1924	0.1234	0.2873	0.1901
Avergae	0.2891	0.2158	0.1476	0.2162	0.2032	0.2456	

Table 7. The ratio of the number of nonsynonymous substitutions per nonsynonymous site (Ka) to the number of synonymous substitutions per synonymous site (Ks) of mitochondrial genes in Leptobotia species.

Species	***Leptobotia mantschurica***	***Leptobotia taeniops***	***Leptobotia microphthalma***	***Leptobotia rubrilabris***	***Leptobotia punctata***	***Leptobotia pellegrini***	**Average**
nd4	0.0541	0.0479	0.0802	0.0521	0.0437	0.0514	0.0549
nd5	0.0599	0.0982	0.1014	0.0651	0.0773	0.0675	0.0782
nd6	0.0224	0.0300	0.0623	0.0163	0.0283	0.0321	0.0319
cytb	0.0209	0.0239	0.0264	0.0417	0.0335	0.0193	0.0276
nd1	0.0278	0.0329	0.0338	0.0503	0.0573	0.0391	0.0402
nd2	0.1114	0.0807	0.0818	0.0596	0.0820	0.0550	0.0784
cox1	0.0251	0.0117	0.0350	0.0255	0.0430	0.0312	0.0286
cox2	0.0387	0.0140	0.0261	0.0431	0.0158	0.0121	0.0250
atp8	0.0335	0.0000	0.1420	0.0000	0.0000	0.0278	0.0339
atp6	0.0483	0.0455	0.0682	0.0508	0.0481	0.0308	0.0486
cox3	0.0361	0.0000	0.0000	0.0000	0.0096	0.0000	0.0076
nd3	0.0253	0.0420	0.0512	0.0000	0.0209	0.0369	0.0294
nd4l	0.0235	0.0286	0.0000	0.0000	0.0000	0.0000	0.0087
Average	0.0405	0.0350	0.0545	0.0311	0.0353	0.0310	

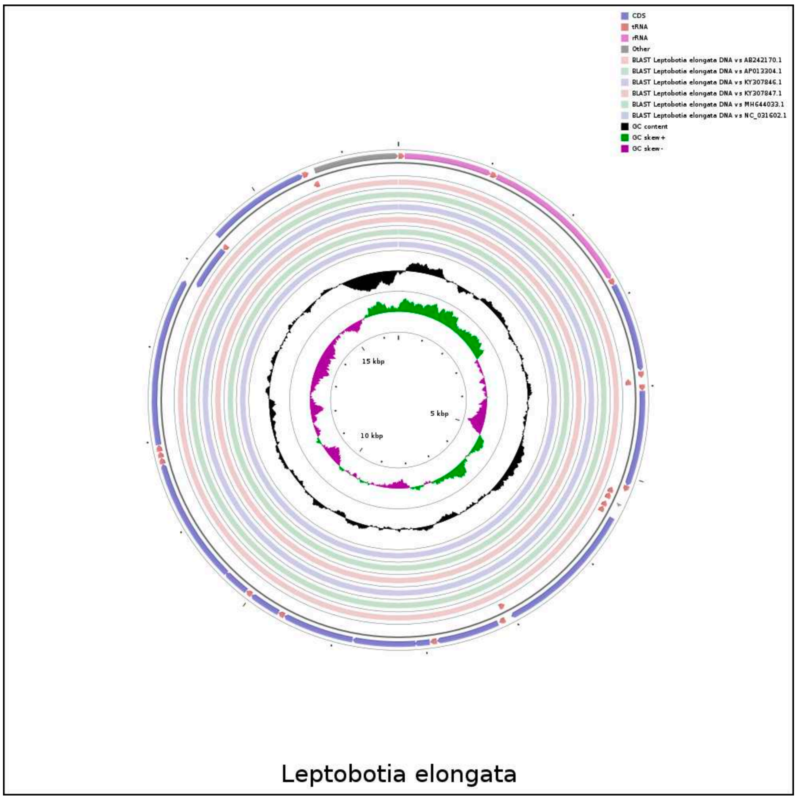

Figure 4. The comparative circle diagram of the genomestructure of Leptobotia species.

Figure 5. The visualized results of the genome comparison of *L.elongata*.

3.4. Ribosomal RNA and Transfer RNA Genes

The total length of rRNAs was 2638bp, with an AT skew value of 0.272 and a GC skew value of -0.095. The lengths of 12SrRNA and 16SrRNA were 955bp and 1683bp, respectively (Table 3). These rRNAs were located between *tRNA-Phe* and *tRNA-Leu* and are separated by *tRNA-Val*, which is consistent with the most reported teleost [59].

There were 22 tRNAs in the mitochondrial genome of *L. elongata*, with a total length of 1558bp. The AT content was 53.89% and AT skew value was 0.044. Each tRNA has a length of 66-76bp, with 14 encoded in the H chain and 8 encoded in the L chain. Most of the secondary structure of tRNA genes (Figure 6) in *L. elongata* were standard clover-shaped, except for *trnS1*, which lacked the DHU stem. It was very common to defect DHU stem in metazoan [42]. Additionally, there were 18 false GU pairings in the tRNA sequences of *L. elongata*. GU mismatch was frequently observed in teleost fishes and allowed for an expanded chemical and conformational diversity of double-stranded RNA. This diversity provided unique sites that were recognized by amino acids, contributing to a higher genetic diversity for *L. elongate* [60]. The base mismatch was essential for the secondary structure of tRNA and played a crucial role in the accurate translation of the genetic code. It also helped minimize errors during mRNA transcription [61].

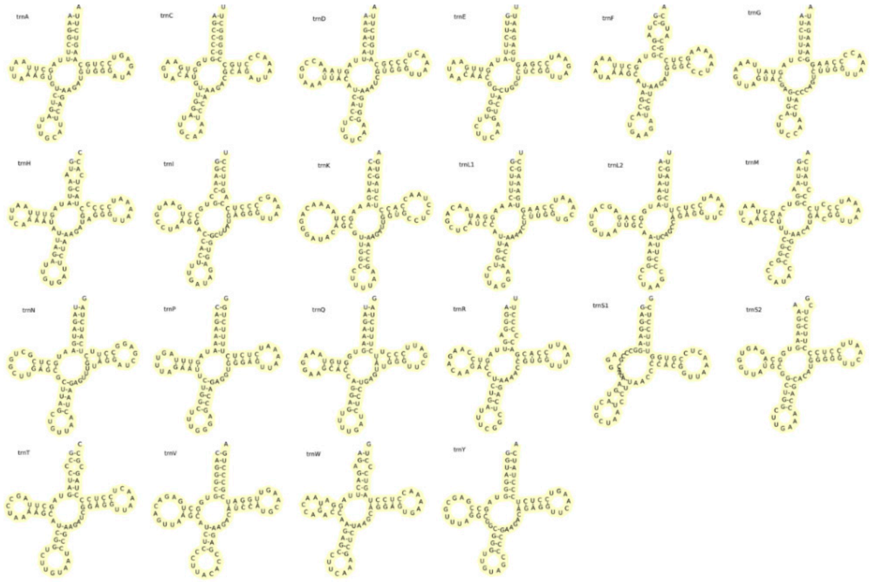

Figure 6. Putative secondary structure of *L. elongata* tRNA.

3.5. Non-Coding Regions

Two common non-coding regions (OL and CR) were identified in the *L. elongata* mitogenome, the OL region was 31 bp in length and was located between *tRNA-Asn* and *tRNA-Cys*. The CR region was located between *tRNA-Pro* and *tRNA-Phe*, which is the longest no-coding region in the entire mitochondrial genome with a span distance of 926 bp. It plays a key role in the replication and transcription [62]. Similar to other vertebrates [21,63], the CR of *L. elongata* exhibited the highest AT content (67.39%) among all regions of the mitochondrial genome. The palindromic sequence motifs 'tacat' and 'atgta' were related to the termination of H strand replication found in the CR of *L. elongata* (Figure 6), which might complete the termination by forming a stable hairpin structure [64].

ACCAGCATTATGGTTTAGTACATAATATGCATAATATTACATCATGTGTTAGTACATAC
TATGTATTATCACCAATAATTTATCTTAACCACAAAGCAAGTACTAAAATATAAGGTA
TGCATAAGACATACATTTAAACTCAACATGAACTCATTTTAAAATAAAGAAATAGGT
CATCTCCCATATAAATCGTCCTCACAATTTACTTTGGAGGAATCAACTGACATCTAT
ATTAACAAATATTAATGTAGTAAGAAACCACCAACCAGTTTATATAAAGGTAAATTA
TTAATGATAGAATCAGGGACAATAATTGTGGGGGTCGCACAAAATGAACTATTACT
GGCATTTGGTTCCTATTTCAGGTACATAACTGTAAGACTCCACCCTCGGATAATTAT
ACTGGCATTTGATTAATGGTGTAGTACATATGTCTCGTTACCCACCATGCCGAGCAT
TCTTTTATATGCATAACGTATCTTTTTTCTGGTTTCCTTTCAATTGGCATCTCACAGT
GCAAGCTCAAATAACTGTTAAGGTGGATCATTTATACCTGCTTTAATTAATATAGGTT
AATGATTGAAAGACATAACATAAGAGTTACATTAGTTTAATTCAAGTGCATAAGATA
TATTTACTCAACACATCCTTATACTATATGCCCCCTTTTGGTTTTTGCGCGACAAACC
CCCTTACCCCCTACGCTCAGAAAATCCTGTTATCCTTGTCAAACCCCGAAACCAAG
GAAGGCTCAACTAAACGTTCAAAGTTAACAAGTTGTAGTATGGGTCAACTTATGCC
ACCACATATTATATATAACATATATAAATATAACACCATAAATTATTCACTGCCGAAAG
CCTAAAAATTAGGATTAAAATTTTATGAAACGGGCCTCAATACTAAAATTTCCAAGT
TTTAAATT

Figure 7. Compositional features of the control region of the *L. elongata* mitochondrial genome. Palindromic motif sequence "TACAT' and 'ATGTA' are marked in yellow and purple respectively.

3.6. Phylogenetic Relationships

Based on the complete mitochondrial genome sequences of the *L. elongata* and 36 Cyprinidaes, the phylogenetic tree was constructed. It was shown that the entire phylogenetic tree was grouped into two major clades (Figure 7). The genus Cobitis, Pangio, Triplophysa, and Acanthocobitis formed one clade and matched the subfamily Cobitinae. The Cobitis and the Pangio were sister-lineage, the Triplophysa and the Acanthocobitis were sister-lineage, and the two sister-lineages were sister-lineages to each other. The other clade consisted of Yasuhikotakia, Sinibotia, Chromobotia, Botia, Parabotia, and Leptobotia, corresponding to the subfamily Botiinae. In the subfamily Botiinae, *L. elongata* was more closely related to *L. microphthalma* than other species.

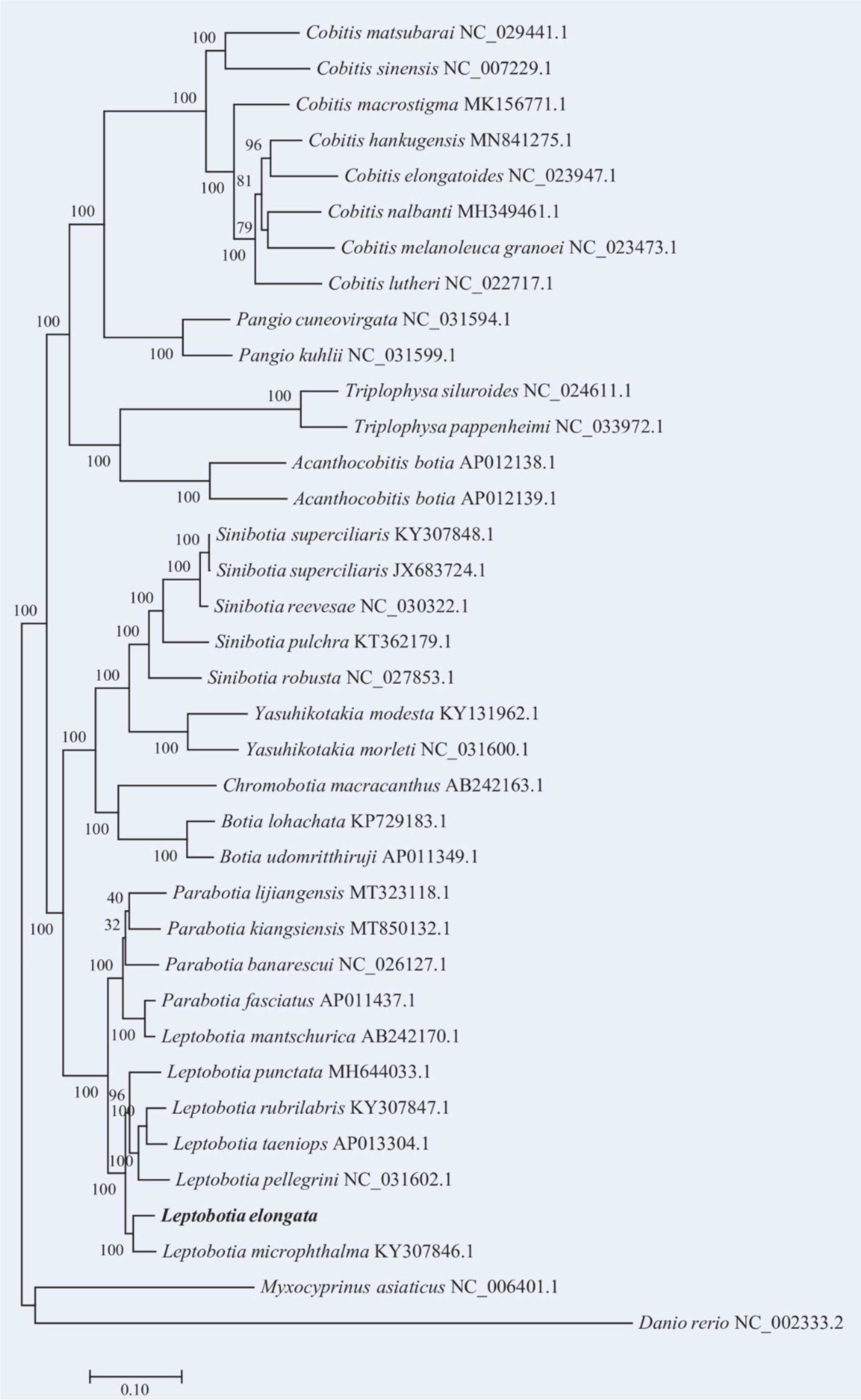

Figure 8. ML tree with boostrap values on the nodes constructed by using complete mitogenome sequences of *L. elongata*.

As a diverse population, there was a controversy in the taxonomic relationship of the subfamily Cobitinae. This study exhibited a monophyly of the subfamily CobitinaeN, which consists of four clades. However, according to Liu et al [11], there were sisterhoods in many branches. Therefore, the species in Cobitinae can’t form a monophyletic group, and the classification of Cobitinae in our study is incomplete, and more taxon should be used in future studies.

It is generally considered that the subfamily Botiinae is a group with relatively clear taxonomic relationship. In this study, according to their respective genera separately, all individuals except *L. mantschurica* of the subfamily Botiinae were clustered into a common branch, which could be confirmed the monophyly of the subfamily Botiinae. In the previous study, the genera Botia was separated into a separate genus [7], and the genera Botia was divided into three subgenera: Sinibotia, Botia, and Hymenophysa [65]. Others did not further categorize these subgenra, but instead grouped them under the genus Botia [12,66]. In this study, subgenera Botia and subgenra sinibotia species were clustered separately and formed parallel branches with the species of other genera. Thus, the results supported that subgenera Botia and subgenra sinibotia should be raised to genus. Additionally, the phylogenetic tree showed that *L. elongata* and *L. microphthalma* formed a sister group, which together formed a sister group of other Leptobotia species. Acorrding to Li et al. [18], the *L. elongata* and *L. mantschurica* were classified as sister lineages using protein genome sequence to construct the phylogenetic tree, however, this study was analysed based on limited taxon sampling and mitochondrial genes, lacking of sufficient information of phylogenetic the *L. elongata*.

Slechtova et al. [67] suggested that the Leptobotia and Parabotia genera were monophyletic using only the *Cytb* and *12S*. However, the phylogenetic tree in our study clearly showed that *L. mantschurica* was nested with Parabotia, indicating that Leptobotia and Parabotia genera were an unnatural group and not reciprocally monophyletic groups as previously hypothesized [13,66,68]. Additionally, there was obvious structural variation among the mitochondrial genome of *L. mantschurica*, compared with other Leptobotia species, proving that *L. mantschurica* formed a sister relationship with *Parabotia fasciatus* instead of Leptobotia species is reliable, which shared the same results with Tang et al. [13]. In the Parabotia species, part of the support vaule in the branch was low, suggestting that the phylogenetic relationships of these species haven‘t been solved well. Futher investigations should be performed to solve this problem.

4. Conclusion

In this study, we reported the complete mitogenome of *L. elongata*, the structural characteristics of the mitogenome of *L. elongata* were analyzed in detail, and the phylogenetic analyses of *L. elongata* were inferred using the complete mitogenome. The full length of the genome sequence was 16591 bp, and the arrangement of the *L. elongata* mitochondrial genome is similar to most teleost fishes. Almost all 13 PCGs showed the regular start codon ATG except gene *cox1*, which started with GTG. 6 PCGs have incomplete stop codons T--. 13PCGs were evolving under purifying selection, and the mitogenome shared the high identity with Leptobotia species. All the tRNA genes were standard clover-shaped except the lacking of DHU stem in *trnS1*. The phylogenetic analysis showed that *L. elongata* was more closely related to *L. microphthalma* than other species. *L. mantschurica* formed a sister relationship with *Parabotia fasciatus*, and the Leptobotia and Parabotia genera was polyphyletic. In this study, we first studied the selection pressure of complete PCGs in the *L. elongata*. Overall, we have a deeper understanding of the mitochondrial genome structure and phylogenetic analysis of *L. elongata*. However, exact information of about many Cobitidae fishes is still unkown. Extra taxon should be used for the phylogenetic research of Cobitidae in the future.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Animal Care and Use Committee of University of Southwest (Approval ID was 20190922, and the approval date was on 22 September 2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets presented in this study were submitted to The National Center for Biotechnology Information (NCBI) database.

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Conflicts of Interest: The authors declare no conflict of interest.

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