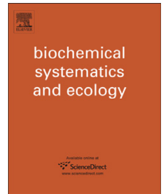
Biochemical Systematics and Ecology 64 (2016) 6–13

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco



Molecular phylogeny of the subfamily Schizothoracinae (Teleostei: Cypriniformes: Cyprinidae) inferred from complete mitochondrial genomes

Jie Zhang [a, b], Zhuo Chen [a], Chuanjiang Zhou [b], Xianghui Kong [b, *]

[a] College of Life Science, Henan Normal University, Xinxiang 453007, PR China
[b] College of Fisheries, Henan Normal University, Xinxiang 453007, PR China

A R T I C L E I N F O

Article history:
Received 16 June 2015
Received in revised form 19 October 2015
Accepted 14 November 2015
Available online 26 November 2015

Keywords:
Mitochondrial genome
Phylogeny
Schizothoracinae
Schizopygopsis malacanthus

A B S T R A C T

The schizothoracine fishes, members of the Teleost order Cypriniformes, are one of the most diverse group of cyprinids in the Qinghai–Tibetan Plateau and surrounding regions. However, taxonomy and phylogeny of these species remain unclear. In this study, we determined the complete mitochondrial genome of *Schizopygopsis malacanthus*. We also used the newly obtained sequence, together with 31 published schizothoracine mitochondrial genomes that represent eight schizothoracine genera and six outgroup taxa to reconstruct the phylogenetic relationships of the subfamily Schizothoracinae by different partitioned maximum likelihood and partitioned Bayesian inference at nucleotide and amino acid levels. The schizothoracine fishes sampled form a strongly supported monophyletic group that is the sister taxon to *Barbus barbus*. A sister group relationship between the primitive schizothoracine group and the specialized schizothoracine group + the highly specialized schizothoracine group was supported. Moreover, members of the specialized schizothoracine group and the genera *Schizothorax*, *Schizopygopsis*, and *Gymnocypris* were found to be paraphyletic.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The schizothoracine fishes (Teleostei: Cyprinidae), which are composed of approximately 100 species in 11–12 genera, are endemic to the Qinghai–Tibetan Plateau and surrounding regions (Mirza, 1991). They are distinguished from other cyprinids by two lines of enlarged scales along both sides of the urogenital opening and anus (Cao et al., 1981). The subfamily Schizothoracinae is traditionally divided into three groups based on morphological data: the primitive group (including the genera *Schizothorax*, *Schizocypris*, and *Aspiorhynchus*), the specialized group (including the genera *Diptychus*, *Gymnodiptychus*, and *Ptychobarbus*), and the highly specialized group (including the genera *Chuanchia*, *Gymnocypris*, *Herzensteinia*, *Oxygymnocypris*, *Platypharodon*, and *Schizopygopsis*) (Cao et al., 1981). The taxonomy and phylogeny of Schizothoracinae have been investigated by a series of morphological and molecular data over the past three decades (Cao et al., 1981; He et al., 2004; Qi et al., 2012; Yonezawa et al., 2014). Great advances have been made in elucidating the phylogenetics and taxonomy of Schizothoracinae, but these studies have also created some new issues. For example, one controversy was the

* Corresponding author.
E-mail address: xhkong@htu.cn (X. Kong).

http://dx.doi.org/10.1016/j.bse.2015.11.004
0305-1978/© 2015 Elsevier Ltd. All rights reserved.

monophyly of Schizothoracinae. Analyses of datasets consisting of morphological characteristics, single nuclear genes, and mitochondrial gene sequences all supported the monophyly of Schizothoracinae (He et al., 2004; Qi et al., 2012). However, recent molecular analyses based on their complete mitogenomes disproved their monophyly and suggested polyphyletic origins of Schizothoracinae (Li et al., 2013; Yonezawa et al., 2014). Another contentious issue is the taxonomy of Schizothoracinae. Only two distinct groups, i.e., the morphologically primitive and morphologically specialized groups, were identified with the analyses of molecular data (He et al., 2004; Qi et al., 2012; Yonezawa et al., 2014). In addition to these controversies, previous phylogenetic analyses had different conclusions regarding the interrelationships within Schizothoracinae (He et al., 2004; Qi et al., 2012; Yonezawa et al., 2014).

Mitochondrial DNA (mtDNA) has been proposed as a useful marker for studies of systematics, phylogenetics, evolution, and population biology because of its relative lack of recombination and maternal mode of inheritance. mtDNA has also been successfully applied to recover robust phylogenies for various groups at different taxonomic ranks (He et al., 2008). In addition, substantial amounts of amino acid and DNA data involved with mtDNA can be provided for phylogenetic analyses. Yonezawa et al. (2014) pioneered the use of complete mitochondrial genomes to investigate the phylogenetic relationships among schizothoracine fishes. However, only 21 species of schizothoracine fish were involved, and only single maximum likelihood analyses were conducted using the concatenated data of the 12 protein-coding genes and two rRNAs. A separate partition for the concatenated tRNAs, each rRNA, and each codon position of each protein-coding gene is suggested for mitogenome analyses (Mueller et al., 2004). Therefore, increasing the number of mitochondrial genome sequences of Schizothoracinae and reconstructing the Schizothoracinae phylogenetic tree based on a more comprehensive dataset are necessary to address the remaining problematic clades.

In this study, we determined and analyzed the complete nucleotide sequence of *Schizopygopsis malacanthus* mtDNA. Phylogenetic analyses were also conducted based on the nucleotide and the deduced amino acid sequences of the 13 mitochondrial protein genes, as well as the entire mitochondrial genome sequences using the maximum likelihood and Bayesian inference methods to reveal the phylogenetic relationships among 32 species of 8 genera in Schizothoracinae.

2. Materials and methods

2.1. Sample collection and DNA extraction

S. malacanthus (Voucher: HNNU20060805036) collected from Litang County, Sichuan Province, China in August 2006 was used in the experiments. Total DNA was extracted from the ethanol-preserved muscle tissues by using a standard phenol/chloroform procedure followed by ethanol precipitation (Sambrook and Russell, 2006).

2.2. Primer design, PCR amplification, and sequencing

Normal PCR and long-and-accurate PCR (LA-PCR) were employed to amplify 19 DNA overlapping fragments by using 10 pairs of highly conserved primers (Zhou et al., 2014) and 9 pairs of newly designed primers to determine the complete mitogenomic sequence of *S. malacanthus*. The primer information is given in Table 1. All PCR amplifications were conducted using a BioRAD PTC-200 (BioRad Laboratories, Hercules, CA) with 2 × EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China) or LA-Taq kit (Takara, Dalian, China). Normal PCR was carried out in a 25 μl reaction mixture containing 12.5 μl of

Table 1
Primers used in this study.

Fragment	Region	Forward primer sequences (5′-3′)	Reverse primer sequences (5′-3′)	Source
1	*tRNA^{Phe}-12S rRNA*	GTAGCTTAAHRYAAAGCATRRCACTG	GGCGGTGTGTACGCGYCTYAGAGCC	Zhou et al. (2014)
2	*12S rRNA -16S rRNA*	AAGGAGGATTAGTAGTAAARRRGGAA	CAACCAGCTATYACCARGTTCGRTA	Zhou et al. (2014)
3	*16S rRNA-16S rRNA*	AACCCKTCTCTGGTGGCAAAGAGTGG	AGCTCCAWAGGGTCTTCTCGTCTTGT	Present study
4	*16S rRNA-16S rRNA*	TGACCGTGCAAAGGTAGCGYAATCACT	TACAGATAGAAACTGACCTGGATT	Zhou et al. (2014)
5	*16S rRNA- tRNA^{Gln}*	TGGTGCAGCCGCTATTAAGGGTTCGTT	TAGRRAGTGGTGTADAGGARGCAC	Zhou et al. (2014)
6	*tRNA^{Gln} - tRNA^{Asn}*	TAGAAAGAAGGGRRTYGAACCCAT	TTGRGCRCTTAGCTGTTAACTAAGA	Zhou et al. (2014)
7	*ND2-COII*	ACATCCTCATACTCACCACCTAG	GATAGGAGTCAAAGCCCAGAT	Present study
8	*COII- tRNA^{Lys}*	GGMTTYCAAGACGCRGCRTCACCYGT	CACCAWWCTTTGGCTTAAAGGCCRA	Zhou et al. (2014)
9	*tRNA^{Lys} -COIII*	AGCRTYGGCCTTTTAAGCCRAAG	GGTCAKGGGCKDGGRTCRACYAT	Zhou et al. (2014)
10	*COIII- tRNA^{Gly}*	TAATGRCHCACCAAGCACAYGC	TCCYTGGRRTTYAACCAAGACT	Zhou et al. (2014)
11	*COIII-ND4L*	AGACGGGGTATACGGCTC	AGCTAGCCTCACAAGCAGAA	Present study
12	*tRNA^{Arg}-ND4*	AAGAYCYCTGATTTCGGCT	TCVGTVGCVCCRAADGCYA	Zhou et al. (2014)
13	*ND4-ND4*	GCCTCTTGATTGCTTTCATG	ATGTAGCGTTACTARTAAGTGTTCC	Present study
14	*ND4-ND5*	TGCAGTRTGATGATTCRTCG	ATAATGCTCCGAGGCAGAG	Present study
15	*ND5-ND5*	AGCTTCGACATAACGATTCC	TGTCCGAGGGTAAGTTTGG	Present study
16	*ND5- tRNA^{Glu}*	ARCGRGCYTGRGGRAGHAT	GTTTTYCGWAGGCTTGYCAT	Zhou et al. (2014)
17	*ND6- Cytb*	TGCCGAATAAGCAAAGACTAC	GCGGAATGTTAGTCCTCGT	Present study
18	*Cytb- D-loop*	CCTTCCACCCATACTTYACAT	TCGTTGGTGGTTTCTTACTACAT	Present study
19	*D-loop-12S rRNA*	CACCCYTRRCTCCCAAAGCYA	GGTGCGGRKACTTGCATGTRTAA	Present study

Notes: R = A/G; Y = C/T; W = A/T; M = A/C; K = G/T; H = A/T/C; D = G/A/T; V = G/A/C.

2 × EasyTaq PCR SuperMix, 9.5 μl of sterile distilled water, 1 μl of each primer (10 μM), and 1 μl of template DNA with the following thermal profile: initial denaturation for 5 min at 94 °C, followed by 34 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 53–59 °C, and extension for 30 s at 72 °C, and then a final extension for 10 min at 72 °C. LA-PCR was performed in a 25 μl reaction mixture containing 2.5 μl of $MgCl_2$ (25 mM), 2.5 μl of 10× reaction buffer, 1 μl of each primer (20 μM), 4 μl of dNTPs (2.5 mM), 12.75 μl of sterile distilled water, 0.25 μl of Ex-Taq™ polymerase, and 1 μl of template DNA with the following thermal profile: initial denaturation for 4 min at 94 °C, followed by 35 cycles of denaturation at for 30 s at 98 °C, annealing for 1 min at 55–60 °C, and extension for 14 min at 68 °C, and then a final extension for 20 min at 68 °C.

The amplified PCR products were purified through gel excision (1.5% agarose in Tris-borate-EDTA buffer) using AxyPrep™ DNA gel extraction kit (Axygen Scientific, Inc. USA). The products were then sequenced in both directions using an ABI 3730 automated genetic analyzer. For some large PCR fragments, specific primers were designed based on the newly obtained sequences to fulfill primer walking.

2.3. Sequence editing and analysis

Sequences were carefully checked and assembled using the program SeqMan in Lasergene version 5.0 (Burland, 2000). The locations of 2 rRNA genes and 13 protein-coding genes were determined by comparison of the homologous sequences between other schizothoracine fishes using ClustalW (Thompson et al., 1997). All the tRNA genes except for the tRNA-Ser (AGY) gene were determined by their cloverleaf secondary structure with tRNA-scan SE 1.21 (Lowe and Eddy, 1997). The tRNA-Ser (AGY) gene was identified by comparison with other schizothoracine homologous sequences. The complete mitogenome sequence of *S. malacanthus* obtained in the present study has been deposited in GenBank with accession number KR527479.

2.4. Phylogenetic analyses

In addition to the newly obtained sequence of *S. malacanthus*, we obtained another 31 complete mitogenomic sequences of 8 genera in Schizothoracinae to elucidate the phylogenetic relationships among members of the subfamily Schizothoracinae. Complete mitogenomic sequences of four species in the subfamily Barbinae and three species in Cryprininae were obtained from GenBank to serve as outgroups in the subsequent phylogenetic analyses. All species and the respective GenBank numbers used in the present study are listed in Table 2.

All the L-strand-encoding gene sequences (eight tRNA genes and ND6) were converted into their complementary strand sequences. Twenty-two tRNA and two rRNA sequences were separately aligned by their secondary structures. All 22 tRNA alignments were combined, and a concatenated alignment was then generated. Thirteen protein-coding gene sequences were translated into their corresponding amino acids and aligned using ClustalX (Thompson et al., 1997) with default settings. The amino acids were then used as a backbone for the alignment of the corresponding nucleotide sequences. All gap-containing sites and unalignable sites analyzed using Gblocks (Castresana, 2000) were deleted from the initial alignments. Finally, three datasets (i.e., the concatenated amino acid sequences of the 13 protein-coding genes, the concatenated nucleotide sequences of the 13 protein-coding genes, and the concatenated nucleotide sequences of the 22 tRNA, 2 rRNA, and 13 protein genes) were generated for the subsequent phylogenetic analyses. A partition strategy that defined 42 partitions for every codon position in each protein-coding gene, the concatenated tRNAs, and each rRNA was selected for the two concatenated nucleotide sequences according to the recommendation of Mueller et al. (2004).

Phylogenies were reconstructed using maximum-likelihood algorithms in MetaPIGA 2.0 (Helaers and Milinkovitch, 2010) and Bayesian inference in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) for the three datasets mentioned above. The optimal models for each partition were determined with Modeltest 3.7 (Posada and Crandall, 1998) according to the Akaike Information Criterion, and the best-fit substitution models selected for the protein dataset and the two DNA datasets were mtREV + I+Γ and GTR + I+Γ, respectively. Maximum likelihood analyses were conducted using MetaPIGA 2.0 (Helaers and Milinkovitch, 2010) with 1000 metaGA replicate searches. Twenty million generations were conducted, and every 1000 generations were sampled with four Markov chains. Tracer 1.4 (Rambaut and Drummond, 2007) was employed to determine the stationarity of the likelihood scores of sampled trees. The first 10% of the trees were deleted as the "burn-in" stage, and the 50% majority-rule consensus trees of the post "burn-in" sampled at stationarity.

3. Results

3.1. General features of S. malacanthus mtDNA achieved in this study

The mitogenomic organization of *S. malacanthus* is shown in Fig. 1. It is a circular molecule of 16,672 bp in length, including 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and 17 non-coding regions (including the control region) (Table 3). The orientation and order of the protein-coding, tRNA, and rRNA genes are identical to those of other vertebrates. As in other schizothoracine fishes, most of the genes were encoded on the H-strand except for *ND6* and 8 tRNA genes (Table 3 and Fig. 1).

The 13 protein-coding genes found in the mitogenome of *S. malacanthus* are similar in length to those of the other schizothoracine fishes, and no frameshift mutations or premature stop codons were found. As shown in Table 3, 12 out of the 13 protein-coding genes begin with ATG as start codon, whereas COI starts with GTG. Five genes (i.e., COI, ATP6, ND4L, ND5, and ND6) are terminated with TAA as stop codon, ND1 and ATP8 end with TAG, and the six other protein-coding genes end

Table 2
List of species and sequences used in this study.

Family	Subfamily	Genus	Species	GenBank no.
Ingroup				
Cryrinidae	schizothoracinae	*Aspiorhynchus*	*Aspiorhynchus laticeps*	KF564793
		Diptychus	*Diptychus maculatus*	KM659026
		Gymnocypris	*Gymnocypris dobula*	KC558497
			Gymnocypris eckloni	JQ004279
			Gymnocypris namensis	KC558498
			Gymnocypris przewalskii	AB239595
			Gymnocypris przewalskii ganzihonensis	JQ004278
		Gymnodiptychus	*Gymnodiptychus pachycheilus*	KF976395
		Oxygymnocypris	*Oxygymnocypris stewartii*	KF528985
		Ptychobarbus	*Ptychobarbus dipogon*	KF597526
			Ptychobarbus kaznakovi	KM268050
		Schizopygopsis	*Schizopygopsis malacanthus*	KR527479
			Schizopygopsis malacanthus baoxingensis	KM593242
			Schizopygopsis thermalis	KC558499
			Schizopygopsis younghusbandi	KC351895
			Schizopygopsis younghusbandi 2	JX232379
			Schizopygopsis pylzovi	KP316067
		Schizothorax	*Schizothorax biddulphi*	JQ844133
			Schizothorax chongi	KJ718889
			Schizothorax dolichonema	KJ184546
			Schizothorax esocinus	KF600713
			Schizothorax labiatus	KF739398
			Schizothorax macropogon	KC020113
			Schizothorax oconnori	KC513575
			Schizothorax plagiostomus	KF928796
			Schizothorax prenanti	KJ126773
			Schizothorax progastus	KF739399
			Schizothorax pseudoaksaiensis	KM079630
			Schizothorax richardsonii	KC790369
			Schizothorax waltoni 2	JX202592
			Schizothorax waltoni	KC513574
			Schizothorax wangchiachii	KC292197
Outgroup				
Cyprinidae	Barbinae	*Barbus*	*Barbus barbus*	AB238965
			Barbus trimaculatus	AB239600
		Puntius	*Puntius chalakkudiensis*	JX311437
			Puntius denisonii	KF019637
	Cyprininae	*Carassius*	*Carassius auratus*	KJ476998
			Carassius gibelio	GU170401
		Cyprinus	*Cyprinus carpio*	AP009047

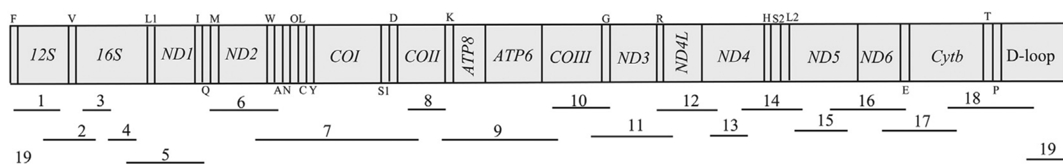


Fig. 1. Gene organization of the mitogenome of the *Schizopygopsis malacanthus* and schematic of the amplification strategy employed. Three letter codes represent the rRNA and protein-coding genes, and single uppercase letter amino acid codes represent the tRNA genes. Two distinct $tRNA^{Leu}$ genes are labeled L1 and L2. S1 and S2 denote $tRNA^{Ser(UCN)}$ and $tRNA^{Ser(AGY)}$. Gene sizes are roughly approximated. Lines below the map show the amplifying region, and the primer pair numbers (detailed in Table 1) are shown above the line.

with an incomplete stop codon (i.e., a single stop nucleotide T). A common feature was found in the metazoan mitogenome; these incomplete termination codons can be recovered into a complete stop codon TAA with post-transcriptional poly-adenylation. The 22 tRNA genes range from 66 to 73 nucleotides in length (Table 3), and the typical cloverleaf secondary structure can be folded except for the tRNA-Ser (AGN) gene. Similar to other metazoan mtDNAs, the DHU arm was absent in the tRNA-Ser (AGN) gene and was folded manually.

The non-coding regions of *S. malacanthus* include a few intergenic spacers and the control region (*D-loop*) (Table 3). The *D-loop* is 932 bp in length and located between the tRNA-Pro and tRNA-Phe genes. The A + T content of this region is 63.9% (A, 31.4%; T, 32.5%; C, 21%; G, 15%). Significant repeat regions were not observed in the *D-loop*. Without considering the *D-loop*, the mitochondrial genes of *S. malacanthus* are separated by 150 bp of intergenic spacer sequences, which are spread over 16 regions and range from 1 bp to 78 bp (Table 3). The largest spacer (78 bp) is present between the tRNA-Thr and tRNA-Pro

Table 3
Location of features in the mtDNA of *Schizopygopsis malacanthus*.

Gene/region	Position		Spacer (+), Overlap (−)	Length (bp)	Codon		Strand
	From	To			Start	Stop	
*tRNA^Phe^*	1	69		69			H
12S rRNA	70	1028		959			H
*tRNA^Val^*	1029	1100		72			H
16S rRNA	1101	2781		1681			H
*tRNA^Leu^*	2782	2857	+1	76			H
ND1	2859	3833	+3	975	ATG	TAG	H
*tRNA^Ile^*	3837	3908	−2	72			H
*tRNA^Gln^*	3907	3978	+2	72			L
*tRNA^Met^*	3980	4049		69			H
ND2	4050	5094		1045	ATG	T	H
*tRNA^Trp^*	5095	5165	+1	71			H
*tRNA^Ala^*	5166	5235	+1	69			L
*tRNA^Asn^*	5237	5309	+33	73			L
*tRNA^Cys^*	5343	5408	−1	66			L
*tRNA^Tyr^*	5408	5478	+1	71			L
COI	5480	7030		1551	GTG	TAA	H
*tRNA^Ser^*	7031	7101	+3	71			L
*tRNA^Asp^*	7105	7176	+13	72			H
COII	7190	7880		691	ATG	T	H
*tRNA^Lys^*	7881	7956	+1	76			H
ATP8	7958	8122	−7	165	ATG	TAG	H
ATP6	8116	8799	−1	684	ATG	TAA	H
COIII	8799	9582	+1	784	ATG	T	H
*tRNA^Gly^*	9584	9655		72			H
ND3	9656	10,014		349	ATG	T	H
*tRNA^Arg^*	10,015	10,074		70			H
ND4L	10,075	10,371	−7	297	ATG	TAA	H
ND4	10,365	11,745		1381	ATG	TAG	H
*tRNA^His^*	11,746	11,814		69			H
*tRNA^Ser^*	11,815	11,883	+1	69			H
*tRNA^Leu^*	11,885	11,957	+3	73			H
ND5	11,961	13,784	−4	1824	ATG	TAA	H
ND6	13,781	14,302		522	ATG	TAA	L
*tRNA^Glu^*	14,303	14,371	+4	69			L
Cytb	14,376	15,516		1141	ATG	T	H
*tRNA^Thr^*	15,517	15,588	+78	72			H
*tRNA^Pro^*	15,667	15,736	+4	70			L
D-loop	15,741	16,672		932			H

genes. Most of the spacer positions are conserved among the mitogenomes of schizothoracine fishes. Furthermore, the origin of light strand replication was 33 bp in length and located between the tRNA-Asn and tRNA-Cys genes.

3.2. Mitogenomic dataset characteristics

The mitochondrial genomic DNA dataset combining the 13 protein-coding genes, the concatenated tRNAs, and two rRNA alignments includes 15,577 characters. A total of 9103 out of 15,577 sites are constant and 6474 are variable, with 5025 being parsimony-informative. Within the subfamily Schizothoracinae, the number of parsimony-informative sites is 3820. Another DNA dataset combining the concatenated nucleotide sequences of the 13 protein-coding genes contains 11,376 characters. Of these, 6191 are constant, 5185 are variable, and 4251 are parsimony-informative. The protein dataset combining the deduced amino acid sequences of all 13 mitochondrial protein-coding genes includes 3794 characters (2775 constant, 1019 variable, and 557 parsimony-informative), and the number of parsimony-informative sites within Schizothoracinae is 405.

3.3. Phylogenetic relationships

The topologies of the maximum likelihood and Bayesian inference trees obtained from the partitioned analysis of the two DNA datasets were nearly identical, and the Bayesian posterior probability (PP) and maximum likelihood bootstrap support (BP) are represented on the Bayesian inference tree (Fig. 2 and Appendix 1). The phylogenies reconstructed both by the protein-coding gene DNA dataset and mitogenomic DNA dataset (37 genes) are well-resolved with support 1.0 (PP) and greater than 90% (BP) for most branches (Fig. 2 and Appendix 1). The topology strongly supported the sister group relationship of *Barbus barbus* and a clade that contains all of the included schizothoracine species (PP = 1.0, BP = 100) (Fig. 2 and Appendix 1). Two major clades within Schizothoracinae were identified and denoted as Clades A and B (Fig. 2 and Appendix 1). A clade (Clade B) of the primitive group (*Schizothorax* and *Aspiorhynchus*) is strongly supported (PP = 1.0, BP = 100) and is

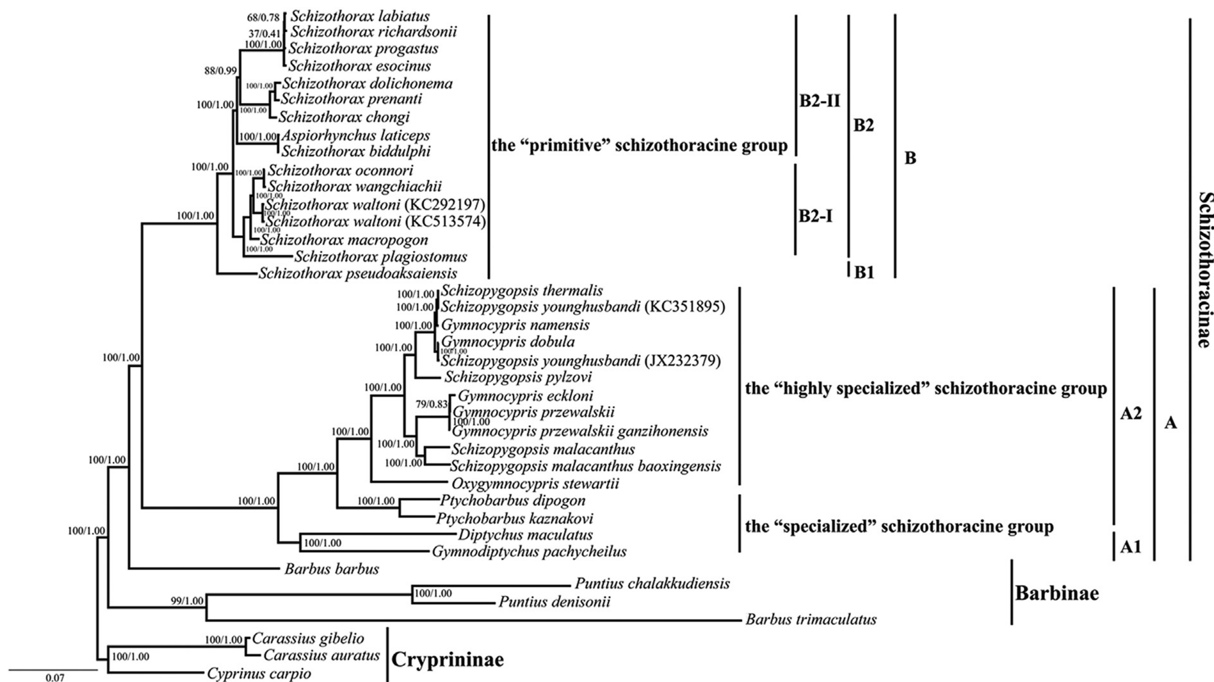


Fig. 2. Phylogenetic trees reconstructed using partitioned maximum likelihood and partitioned Bayesian inference methods using the concatenated dataset combining 13 protein-coding genes, two rRNAs, and the concatenated tRNAs for species of Schizothoracinae and related species. Bootstrap support values for maximum likelihood inference and Bayesian posterior probability are labeled with integers and values of 1 or less associated with branches, respectively. Numbers in parentheses correspond to the GenBank accession numbers for the mitochondrial genome of the related species.

sister to another well-supported clade (Clade A) that includes all other schizothoracine fishes included in the present study (*Diptychus*, *Gymnodiptychus*, *Ptychobarbus*, *Gymnocypris*, *Oxygymnocypris*, and *Schizopygopsis*) (PP = 1.0, BP = 100).

In Clade A, Subclades A1 and A2 were recovered with strong supports (PP = 1.0, BP = 100). In Subclade A1, the sister relationship between *Diptychus maculatus* and *Gymnodiptychus pachycheilus* was well-resolved with strong support (PP = 1.0, BP = 100). Subclade A2 consisted of the two species of the genus *Ptychobarbus* and a highly supported monophyletic group comprising the 12 representatives of the highly specialized group (*Gymnocypris*, *Oxygymnocypris*, and *Schizopygopsis*). The first phylogenetic split within the highly specialized group separates *Oxygymnocypris stewartii* from a clade involving the species of the genus *Gymnocypris* and *Schizopygopsis* (PP = 1.0, BP = 100). Interspecific relationships among the nine species of the two genera *Gymnocypris* and *Schizopygopsis* appear to be well-resolved except for one node comprising *Gymnocypris eckloni*, *Gymnocypris przewalskii*, and *Gymnocypris przewalskii ganzihonensis*. *G. eckloni* and *G. przewalskii* are sister taxa (PP = 0.83, BP = 79) that have a close affinity with *G. przewalskii ganzihonensis* (PP = 1.0, BP = 100) based on the analyses of the mitogenomic DNA dataset (37 genes). However, the relationship between *G. eckloni* and *G. przewalskii* + *G. przewalskii ganzihonensis* (PP = 0.84, BP = 78) was well-resolved (PP = 1.0, BP = 100) based on the analyses of the protein-coding gene DNA dataset. Previous studies reported that species of *Gymnocypris* and *Schizopygopsis* did not form respective monophyletic groups, suggesting the paraphyly of both genera. Moreover, the monophyly of two individuals of *Schizopygopsis younghusbandi* was not supported. *S. younghusbandi* (GenBank No. KC351895) and *Schizopygopsis thermalis*, as well as *S. younghusbandi* (GenBank No. JX232379), and *Gymnocypris dobula* were grouped as sister species (PP = 1.0, BP = 100).

Clade B consisted of *Aspiorhynchus laticeps* and 15 species of the genus *Schizothorax* (Fig. 2 and Appendix 1). The basal split within Clade B separated *Schizothorax pseudoaksaiensis* from a well-supported clade (Subclade B2 in Fig. 2 and Appendix 1) consisting of *A. laticeps* and the other 14 species of *Schizothorax* (PP = 1.0, BP = 100). Two well-supported monophyletic groups were identified in Subclade B2 (Clades B2-I and B2-II in Fig. 2 and Appendix 1). In clade B2-I, *Schizothorax plagiostomus* is the sister taxon to a highly supported group comprising *Schizothorax macropogon*, *Schizothorax waltoni*, *Schizothorax oconnori*, and *Schizothorax wangchiachii* (PP = 1.0, BP = 100); among the latter three species, the basal divergence separates *S. waltoni* from *S. oconnori* and *S. wangchiachii* (PP = 1.0, BP = 100). Within Clade B2-II, *A. laticeps* and *Schizothorax biddulphi* are grouped (PP = 1.0, BP = 100) as the sister taxon to a clade consisting of the remaining seven species of *Schizothorax* included in the present study. *Schizothorax dolichonema* and *Schizothorax prenanti* are sister taxa (PP = 1.0, BP = 100) that have a close affinity with *Schizothorax chongi*. *Schizothorax esocinus*, *Schizothorax progastus*, *Schizothorax richardsonii*, and *Schizothorax labiatus* formed a well-supported monophyletic group (PP = 1.0, BP = 100), within which interspecific relationships are not well-resolved.

The phylogenetic relationships among schizothoracine fishes inferred by the protein dataset show slight differences from the DNA phylogeny. (1) the protein results favor a sister group relationship of *B. barbus* and a clade comprising the specialized

schizothoracine group and the highly specialized schizothoracine group (Fig. 3, node a), whereas the DNA tree supports the sister group relationship of *B. barbus* and a monophyletic group containing all of the included schizothoracine species. (2) Within the primitive schizothoracine group, the protein tree places *S. plagiostomus* as the sister taxon to a clade comprising *A. laticeps* and 14 species of the genus *Schizothorax* included (Fig. 3, node b) instead of grouping it with a clade containing *S. macropogon*, *S. waltoni*, *S. wangchiachii*, and *S. oconnori*, as in the DNA tree. (3) A clade including *A. laticeps* and *S. biddulphi* is a sister taxon to a well-supported monophyletic group (*S. macropogon*, *S. waltoni*, *S. wangchiachii*, and *S. oconnori*) (Fig. 3, node c) in the protein tree, whereas it was grouped with seven other species of *Schizothorax* (Clade B2-II in Fig. 2 and Appendix 1). (4) A relationship of (((*S. labiatus*, *S. richardsonii*), *S. esocinus*), *S. progastus*) is supported in the protein tree (Fig. 3, nodes d and e), but the DNA results favor (((*S. labiatus*, *S. richardsonii*), *S. progastus*), *S. esocinus*). (5) In the protein tree, *Gymnocypris namensis* is placed as the sister taxon to a clade ((*G. dobula*, *S. younghusbandi* JX232379), (*S. thermalis*, *S. younghusbandi* KC351895)) (Fig. 3, node f), whereas different relationship of (((*S. thermalis*, *S. younghusbandi* KC351895), *G. namensis*), (*G. dobula*, *S. younghusbandi* JX232379)) is supported in the DNA trees. However, the supports for these conflicting nodes in the protein tree are weak (Fig. 3), and the DNA phylogeny with strong support appears to be more reliable.

4. Discussion

A more robust schizothoracine phylogeny was reconstructed and evaluated in this study by increasing the amount of mitogenome sequences and using different phylogenetic analytical methods (i.e., bootstrapping partitioned maximum likelihood and partitioned Bayesian inference). The monophyly of Schizothoracinae obtained in our DNA phylogeny was compatible with previous molecular analyses (He et al., 2004; Qi et al., 2012) and supported by morphological evidence (Cao et al., 1981; Qi et al., 2012), but contradicted with the results of Li et al. (2013) and Yonezawa et al. (2014). In agreement with the findings of previous molecular analyses (He et al., 2004; Qi et al., 2012), two major clades, i.e., the primitive schizothoracine group and the specialized schizothoracine group + the highly specialized schizothoracine group were recovered in Schizothoracinae, which contradicts prior morphological results (Cao et al., 1981). In addition, the specialized schizothoracine group does not seem to be a monophyletic group, a finding consistent with several previous molecular studies (He et al., 2004; Yonezawa et al., 2014).

The monophyly and status of the genera within Schizothoracinae have been disputed by morphologists and molecular systematists (He et al., 2004; Qi et al., 2012; Yonezawa et al., 2014). The sister group relationship of *Diptychus* and *Gymnodiptychus* and the basal position of this group in the morphologically specialized Schizothoracinae (the specialized schizothoracine group + the highly specialized schizothoracine group) agreed with many previous molecular hypotheses (He et al.,

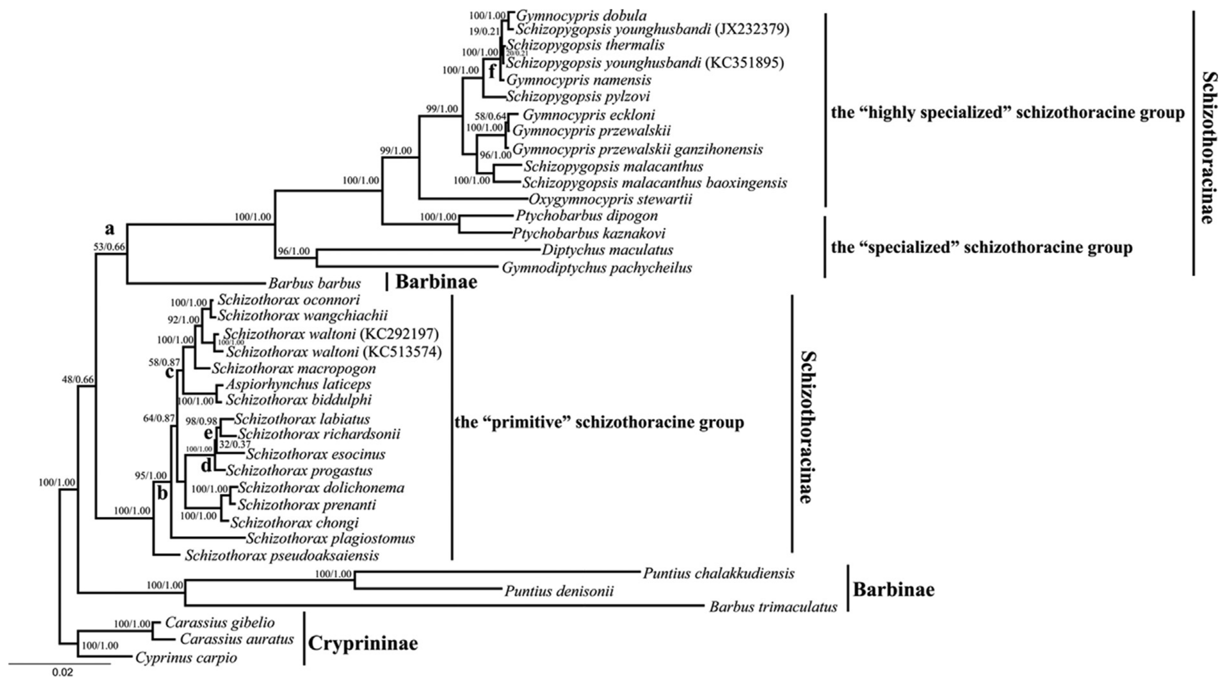


Fig. 3. Phylogenetic trees reconstructed using partitioned maximum likelihood and partitioned Bayesian inference methods using the concatenated dataset of the amino acid sequences of the 13 protein-coding genes for species of Schizothoracinae and related species. Bootstrap support values for maximum likelihood inference and Bayesian posterior probability are labeled with integers and values of 1 or less associated with branches, respectively. Numbers in parentheses correspond to the GenBank accession numbers for the mitochondrial genome of the related species. Branches with letters (a–f) represent the differences between the protein tree and the DNA phylogeny.

2004; Qi et al., 2012; Yonezawa et al., 2014), but contradicted with the relationship of ((*Ptychobarbus*, *Gymnodiptychus*), *Diptychus*) based on morphological data (Cao et al., 1981). In addition, the present finding that species of *Ptychobarbus* formed a monophyletic group is concordant with prior molecular studies (He et al., 2004; Yonezawa et al., 2014). Evidently, the mitogenomic data discounts the monophyly of genera *Gymnocypris*, *Schizopygopsis*, and *Schizothorax*, a finding well concordant with previous molecular analyses (He et al., 2004; Li et al., 2013; Yonezawa et al., 2014).

Of the highly specialized schizothoracine species examined, the close affinity of *S. younghusbandi* and *Gymnodiptychus namensi*, *G. eckloni*, and *G. przewalskii* was congruent with the studies based on mitochondrial cytochrome *b* data (He et al., 2004; Li et al., 2013). Furthermore, the affinity strongly supported the alliance of *S. malacanthus* with *S. younghusbandi* and *Schizopygopsis pylzovi*. This finding is in contrast with the suggestion of Qi et al. (2012) who reported the differentiation between *S. younghusbandi* and *S. malacanthus* + *S. pylzovi*. In agreement with the findings of Qi et al. (2012), the relationship of ((*S. oconnori*, *S. waltoni*), *S. macropogon*) was confirmed in our DNA phylogeny with high support (Fig. 2 and Appendix 1). In addition, the grouping of *A. laticeps* with *S. prenanti*, *S. chongi*, and *S. dolichonema* was concordant with Qi et al. (2012), but contradicted with Yonezawa et al. (2014), whose findings are similar to our protein tree and have weak support (Fig. 3, node c).

In summary, our phylogenetic results based on mitochondrial genomes are in agreement with previous morphological and molecular analyses. The schizothoracine fishes sampled form a strongly supported monophyletic group that is the sister taxon to *B. barbus*, and a sister group relationship between the primitive schizothoracine group and the specialized schizothoracine group + the highly specialized schizothoracine group is supported. The present study is the first comprehensive analysis of the phylogenetic relationships of the subfamily Schizothoracinae and the phylogeny presented here could be regarded as a further step towards reliably resolving relationships among Schizothoracinae.

Acknowledgments

Financial support was provided by the Program for Innovative Research Team in Science and Technology in the University of Henan Province (Project No. 15IRTSTHN018) and the National Natural Science Foundation of China (NSFC) (Grant No. U1404306 to ZC).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2015.11.004.

References

Burland, T.G., 2000. DNASTAR's Lasergene sequence analysis software. Methods. Mol. Biol. 132, 71–91.
Cao, W., Chen, Y., Wu, Y., Zhu, S., 1981. Origin and evolution of schizothoracine fishes in relation to the upheaval of the Xizang Plateau. Tibetan Expedition Team of the Chinese Academy of Science. In: Studies on the Period, Amplitude and Type of the Uplift of the Qinghai-xizang Plateau. Science Press, Beijing, pp. 118–130.
Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.
He, D., Chen, Y., Chen, Y., Chen, Z., 2004. Molecular phylogeny of the specialized schizothoracine fishes (Teleostei: Cyprinidae), with their implications for the uplift of the Qinghai-Tibetan Plateau. Chin. Sci. Bull. 49, 39–48.
He, S., Gu, X., Mayden, R.L., Chen, W., Conway, K.W., Chen, Y., 2008. Phylogenetic position of the enigmatic genus *Psilorhynchus* (Ostariophysi: Cypriniformes): evidence from the mitochondrial genome. Mol. Phylogenet. Evol. 47, 419–425.
Helaers, R., Milinkovitch, M.C., 2010. MetaPIGA v2.0: maximum likelihood large phylogeny estimation using the metapopulation genetic algorithm and other stochastic heuristics. BMC Bioinforma. 11, 379.
Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.
Li, Y., Ren, Z., Shedlock, A.M., Wu, J., Sang, L., Tersing, T., Hasegawa, M., Yonezawa, T., Zhong, Y., 2013. High altitude adaptation of the schizothoracine fishes (Cyprinidae) revealed by the mitochondrial genome analyses. Gene 517, 169–178.
Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic. Acids. Res. 25, 955–964.
Mirza, M.R., 1991. A contribution to the systematics of the schizothoracine fishes (Pisces: Cyprinidae) with the description of three new tribes. Pak. J. Zool. 23, 339–341.
Mueller, R.L., Macey, J.R., Jaekel, M., Wake, D.B., Boore, J.L., 2004. Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders inferred from complete mitochondrial genomes. Proc. Natl. Acad. Sci. U. S. A. 101, 13820–13825.
Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.
Qi, D., Chao, Y., Guo, S., Zhao, L., Li, T., Wei, F., Zhao, X., 2012. Convergent parallel and correlated evolution of trophic morphologies in the subfamily Schizothoracinae from the Qinghai-Tibetan Plateau. PLoS. One 7, e34070.
Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Available from: http://beast.bio.ed.ac.uk/Trace.
Sambrook, J., Russell, D.W., 2006. Molecular Cloning, fourth ed. Cold Spring Harbor Laboratory Press, New York.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acids. Res. 24, 4876–4882.
Yonezawa, T., Hasegawa, M., Zhong, Y., 2014. Polyphyletic origins of schizothoracine fish (Cryprinidae, Osteichthyes) and adaptive evolution in their mitochondrial genomes. Genes. Genet. Syst. 89, 187–191.
Zhou, C., Li, B., Ma, L., Zhao, Y., Kong, X., 2014. The complete mitogenome of natural triploid Carassius auratus in Qihe River. Mitochondrial DNA. http://dx.doi.org/10.3109/19401736.2014.908367 [Epub ahead of print].