Mitochondrial *cytochrome b* Sequence Variation and Phylogenetics of the Highly Specialized Schizothoracine Fishes (Teleostei: Cyprinidae) in the Qinghai-Tibet Plateau

Delin Qi,[1,2] **Taiping Li,**[3] **Xinquan Zhao,**[1] **Songchang Guo,**[1,2] **and Junxiang Li**[3]

The complete 1140 bp mitochondrial cytochrome b sequences were obtained from 39 individuals representing five species of all four genera of highly specialized schizothoracine fishes distributed in the Qinghai-Tibet plateau. Sequence variation of the cytochrome b gene was surveyed among the 39 individuals as well as three primitive schizothoracines and one outgroup. Phylogenetic analysis suggested that the group assignment based on 1140 bp of the cytochrome b sequence is obviously different from previous assignments, and the highly specialized schizothoracine fishes (Schizopygopsis pylzovi, Gymnocypris przewalskii, G. eckloni, Chuanchia labiosa, and Platypharodon extremus) *form a monophyletic group that is sister to the clade formed by the primitive schizothoracine fishes* (Schizothorax prenanti, S. pseudaksaiensis, and S. argentatus). *The haplotypes of Schizopygopsis pylzovi and G. przewalskii were paraphyletic based on cytochrome b data, which most likely reflected incomplete sorting of mitochondrial DNA lineages. The diploid chromosome numbers of Schizothoracinae were considered in phylogenetic analysis and provided a clear pattern of relationships. Molecular dating estimated for highly specialized schizothoracine fishes suggested that the highly specialized schizothoracine fishes diverged in the late Miocene Pliocene to Pleistocene $(4.5 \times 10^4$–4.05×10^6 years BP). The relationship between the*

[1]Northwest Plateau Institute of Biology, Chinese Academy of Science, Xining 810001 P.R. China.
[2]Graduate School, Chinese Academy of Science, Beijing 100039, P.R. China.
[3]College of Agriculture and Animal Science, Qinghai University, Xining 810003, P.R. China.
[4]To whom correspondence should be addressed; e-mail: xqzhao@nwipb.ac.cn.

cladogenesis of highly specialized schizothoracine fishes and geographical events of the Qinghai-Tibet plateau is discussed.

INTRODUCTION

Schizothoracine fishes, members of the family Cyprinidae, commonly known as snow trout, consist of 15 genera and over 100 species all over the world (Mirza, 1991). In China, about 70 species and 9 subspecies belonging to 11–12 extant or nominal genera account for over 80% of the world's schizothoracine species (Wu and Wu, 1992; Yue and Chen, 1998). Most are endemic to China and are mainly distributed in plateau lakes and rivers and in the upper reaches of the Yellow River on the Qinghai-Tibet plateau in western China. Based on modifications of scales, pharyngeal teeth, and barbells, the subfamily Schizothoracinae was divided into three grades of primitive, specialized, and highly specialized schizothoracine fishes, which represent three special stages during the evolutionary history of the Qinghai-Tibet plateau (Wu and Wu, 1992). Most highly specialized schizothoracine fishes tolerate high hypoxia and low temperatures, favoring slow currents and the surface level of clear and cold plateau lakes and tributaries in the upper reaches of the Yellow River, at an altitude of 3000 m. Their inherent biological features, such as short growth period and slow growth to maturity (which are typical of plateau schizothoracine fishes), are the main constraints hindering their resources and population increase. Many of the highly specialized schizothoracine species were listed as endangered in 1998 by the National Environmental Protection Agency and Endangered Species Scientific Commission (Yue and Chen, 1998).

Phylogenetic interrelationships of Cyprinid subfamilies have been extensively investigated from morphological, anatomical, and molecular perspectives (Cavender and Coburn, 1992; Chen *et al.*, 1984; Gilles *et al.*, 1998, 2001; Zardoya and Doadrio, 1999). The highly specialized category in the subfamily Schizothoracinae, however, has been poorly studied. Two studies mainly based on morphological and anatomical characters have investigated phylogenetic relationships among genera and species and explored the taxonomic status of these fishes (Wu, 1964; Wu and Wu, 1992), but the phylogenetic relationships based on mtDNA *cytochrome b* are somewhat understudied for the highly specialized schizothoracine fishes in the Qinghai-Tibet plateau.

Mitochondrial DNA is typically circular, and the molecule's high nucleotide substitution rate, lack of recombination, and maternal inheritance make it particularly useful for studies of intraspecific and interspecies variation (Avise, 1986; Avise and Saunders, 1984; Billington and Hebert, 1988). The availability of mtDNA data has provided new perspectives on taxonomically debatable taxa and confusing questions of phylogeny (Groves and Shields, 1996). Among many mitochondrial genes, the mitochondrial *cytochrome b* gene has been widely used to study genetic variation (McVeigh and Davidson, 1991), phylogenetic relationships (Cavender and Coburn, 1992; Groves and Shields, 1996; Gilles *et al.*, 1998; Perdices *et al.*, 2004; Xiao *et al.*, 2001), biogeographical patterns (Durand *et al.*, 2002; Gilles *et al.*, 2001; Xiao *et al.*, 2001; Zardoya and Doadrio, 1999), and taxonomy (Burridge, 1999; Xiao *et al.*, 2001) in many fishes and vertebrates. The rate of evolution of the *cytochrome b* gene (documented in Irwin *et al.*, 1991) is appropriate for investigating events that have occurred within the last 20 million years, such as the evolution of the Cyprinidae. In the present study, we use *cytochrome b* sequence data to investigate genetic variation and clarify relationships among five species of the highly specialized schizothoracine fishes and three species of the primitive schizothoracine fishes based on reconstructed phylogeny, and we discuss the relationship between cladogenetic events of the highly specialized schizothoracine fishes and geological tectonic events of the Qinghai-Tibet plateau through a tentative divergence time.

MATERIALS AND METHODS

Fish Sample Collection

A total of 39 samples representing five species of all four genera of the highly specialized schizothoracine fishes in the Qinghia-Tibet plateau were included in this study (Table I). Sequence analysis included an additional three species of primitive schizothoracine fishes as well as *Opsariichthys bidens* (Leuciscinae) as an outgroup because of its clear relationship to the subfamily Schizothoracinae (Greenwood, 1966). Muscle tissues were temporarily frozen with liquid nitrogen in the field and then taken to the lab and kept at −70°C until DNA extraction. Sequences generated for this study have been deposited in the GenBank database under accession nos. AY608049–AY608666.

DNA Extraction, PCR Amplification, and Sequencing

Total genomic DNA was extracted from about 20 mg of frozen tissue sample according to the standard procedure for proteinase K digestion

Table I. Characteristics of Samples Used in This Study

Taxonomic Designation	Collection Location[a]	No. Specimens	$2n$[b]	Haplotype
Gymnocypris				
G. eckloni	Tuosuo Lake	3	94	*G. eckloni* 1
G. eckloni	Tuosuo Lake	1	94	*G. eckloni* 2
G. eckloni	Zhalin Lake	4	94	*G. eckloni* 2
G. przewalskii	Qinghai Lake, Gonghe	3	92	*G. przewalskii* 1
G. przewalskii	Qinghai Lake, Gonghe	2	92	*G. przewalskii* 2
G. przewalskii	Qinghai Lake, Gonghe	1	92	*G. przewalskii* 3
Schizopygopsis				
Sp. pylzovi	Zhalin Lake	4	92	*Sp. pylzovi* 1
Sp. pylzovi	Zhalin Lake	1	92	*Sp. pylzovi* 2
Sp. pylzovi	Upper reach of Yellow River, Guide	1	92	*Sp. pylzovi* 3
Sp. pylzovi	Upper reach of Yellow River, Guide	2	92	*Sp. pylzovi* 1
Chuanchia				
Ch. labiosa	Zhalin Lake	2	92	*Ch. labiosa* 1
Ch. labiosa	Upper reach of Yellow River, Guide	1	92	*Ch. labiosa* 2
Ch. labiosa	Upper reach of Yellow River, Guide	1	92	*Ch. labiosa* 1
Platypharodon				
Pl. extremus	Tuosuo Lake	2	90	*Pl. extremus* 1
Pl. extremus	Tuosuo Lake	1	90	*Pl. extremus* 2
Pl. extremus	Tuosuo Lake	3	90	*Pl. extremus* 3
Pl. extremus	Zhalin Lake	1	90	*Pl. extremus* 4
Pl. extremus	Zhalin Lake	2	90	*Pl. extremus* 1
Pl. extremus	Zhalin Lake	1	90	*Pl. extremus* 5
Pl. extremus	Zhalin Lake	1	90	*Pl. extremus* 6
Pl. extremus	Zhalin Lake	2	90	*Pl. extremus* 7
Schizothorax				
S. prenanti	Banmake River	3	148	*S. prenanti*
S. argentatus	Alakol Lake, Kazakhstan[c]		98–150	*S. argentatus*
S. pseudaksaiensis	Chue River, Kazakhstan[c]			*S. pseudaksaiensis*
Opsariichthys bidens	Lishui, Hunan[d]			*Opsariichthys bidens*

[a]All locations in Guoluo, Qinghai, unless otherwise noted.
[b]Diploid chromosome number.
[c]Durand *et al.*, 2002; GenBank accession nos. AF180861, AF180827.
[d]Perdices *et al.*, 2004; GenBank accession no. AY245090.

followed by phenol/chloroform extraction. The complete sequence of the *cytochrome b* gene was amplified from all samples via the polymerase chain reaction (PCR) using a pair of primers: L14724, 5′ GAC-TTGAAAAACCACCGTTG 3′; and H15915, 5′ CTCCGATCTCCGG-ATTACAAGAC 3′ (Xiao *et al.*, 2001). L and H refer to light and heavy strands, respectively. Numbering is from the 3′-end oligonucleotide based on the published sequence of mammalian mtDNA (Anderson *et al.*, 1981). The sequence of the *cytochrome b* gene amplified by PCR was approximately 1140 base pairs (bp). PCR reactions consisted of the following: 0.1 μg of

total genomic DNA, 1.5 U of *Taq* DNA polymerase (Promega), 5.0 μL of 10× reaction buffer, 3.0 μL of 0.01 mmol/L MgCl$_2$, 4.0 μL of 0.5 mmol/L dNTPs, 1.0 μL of 0.5 μmol/L of each primer, and ddH$_2$O added for a final volume of 50 μL. Reactions were carried out for 35 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 90 s, and a final extension at 72°C for 300 s.

PCR products were electrophoresed on 1% agarose gels (containing about 0.1 μg of EB) and purified using the PCR Purification Mini Kit (Waston Corp.) according to the supplier's instructions. Purified DNA fragments were directly sequenced using an automated sequencer (Applied Biosystems 377) following the manufacturer's instructions. The primers used for sequencing (Xiao *et al.*, 2001) were L15138, 5′ ATGATGACCGC C1TCGTGGGCTA 3′; L15519, 5′ GGAGACCCAGAAAACTTTACCCC 3′; H15149, 5′ CCTCAGAAGGATATTTGTCCTC 3′; and H15560, 5′ GC GTAGGCAAATAGGAAGTATC 3′. To ensure accuracy, strands were sequenced in both directions for each individual. Both DNA strands were checked for ambiguous bases and edited manually.

Sequence Alignment and Phylogenetic Analysis

The DNA sequences were aligned using the Editseq 5.0 and Megalign 5.0 software of the DNA Star package (DNAStar Inc.) and checked by eye according to the published sequence of *Cyprinus carpio* (Chang *et al.*, 1994). Pairwise and base composition comparisons were performed using MEGA 2.1 (Kumar *et al.*, 1993). Phylogenetic analysis of sequences of 1140 bp of the *cytochrome b* gene was conducted using PAUP 3.1.1 (Swofford, 1993) for maximum-parsimony (MP) analysis and the DNAML program of Phylip 3.6b (Felsenstein, 1993) for maximum-likelihood analysis and MEGA 2.1 (Kimura, 1981) for distance analysis. The bootstrap analyses (1000 replicates) were performed in PAUP 3.1.1 and MEGA 2.1 to estimate relative support of clades.

RESULTS

In the present study, 1140 bp of *cytochrome b* sequences were obtained from 39 highly specialized schizothoracine fishes. Sequence alignment of 21 haplotypes identified among all taxa, including the outgroup, showed that 391 sites out of 1140 (32%) were variable without any insertions or deletions. Of the variable sites, 258 (66%) were parsimony informative polymorphic sites. Most variations (309 sites) occurred at the third codon position; 65 variable sites were in the first position, and only 17 were in the second position. Most of the mutation events were transitions (64%,

including 56% TC and 44% AG). Transversions were mostly AC (38%) and AT (33%), with TG (14%) and CG (14%) occurring less frequently. The number of haplotypes ranged from one (*Schizothorax prenanti*) to seven (*Platypharodon extremus*) within species. Seven haplotypes within *Pl. extremus* indicated high genetic diversity in this species (Table I). DNA of all taxa terminated with a GCC stop codon, except *Schizothorax argentatus*, S. *pseudaksaiensis*, and *Opsariichthys bidens*, which use GCT as the stop codon.

Base Composition

The base compositions of the three codon positions for all haplotypes in this study were similar to those described by Xiao *et al.* (2001) for the *cytochrome b* gene of xenocyprinine fishes (Table II), except that there was a bias in composition, especially in the third position of the codons.

Pairwise Comparisons

The sequence divergence between species based on all substitutions ranged from 0.09% (between *Gymnocypris przewalskii* and *Schizopygopsis pylzovi*) to 28% (between *Opsariichthys bidens* and *Chuanchia labiosa*). Sequence divergence within species ranged from 0.09% to 0.71% (Table III). These results indicate that the *cytochrome b* gene appears to be a good marker for speciation studies as well as for examination of the phylogenetic relationships at the species or higher taxonomic levels in schizothoracine fishes. The average transition:transversion ratio across all pairwise comparisons in the data set was 3.3, and it should decrease with increasing distance between taxa as transition sites gradually become saturated (Brown *et al.*, 1982). The highest ratios occurred between *Schizothorax pseudaksaiensis* and *S. argentatus*, both closely related species; the lowest ratios were between the divergent *Opsariichthys bidens* and other Schizothoracines (Table III).

Parsimony Analysis

Unweighted maximum parsimony (MP) analysis of the *cytochrome b* sequence data recovered a single most-parsimonious tree of 605 steps, with a consistency index of 0.772 and retention index of 0.864, when *Opsariichthys bidens* was designated as the outgroup (Fig. 1). In the tree topology, monophylies of *Schizopygopsis pylzovi* + *Gymnocypris przewalskii* + *G. eckloni*, *Sp. pylzovi* + *G. przewalskii* + *G. eckloni*, + *Chuanchia labiosa* + *Platypharodon extremus*, and *Schizothorax pseudaksaiensis* + *S. argentatus* + *S. prenanti* were well supported with high bootstrap values. The *Sp.*

Table II Percentage Base Composition by Codon Position for all Haplotypes of Highly Specialized Schizothoracine Fishes

	First Position (380 bp)				Second Position (380 bp)				Third Position (380 bp)			
	A	T	C	G	A	T	C	G	A	T	C	G
Range	23.7–24.2	23.2–24.5	25.0–26.3	26.6–26.8	19.5–20.3	41.6–43.2	24.2–25.0	13.2	33.2–37.9	18.9–27.1	27.4–33.9	8.2–13.7
Mean	23.8	23.8	25.7	26.7	19.6	42.8	24.5	13.2	35.1	25.4	29.4	10.1
A + T%	47.6				62.4				60.5			
Bias[a]	0.032				0.237				0.199			

[a]Bias in base composition is calculated as $C = (2/3)\Sigma (C_i - 0.25)$, where C is the compositional bias and C_i is the frequency of the ith base (Irwin et al., 1991).

Table III Percent Sequence Divergence and Transitions or Tranversions Based on the Complete Sequence of *Cytochrome b* Gene

	Cla 1	Cla 2	Gec 1	Gec 2	Spy 1	Spy 2	Spy 3	Pex 1	Pex 2	Pex 3	Pex 4	Pex 5	Pex 6	Pex 7	Gpr 1	Gpr 2	Gpr 3	Spr	Sps	Sar	Obi
Cla 1		0.44	6.41	6.41	6.12	6.51	6.41	6.29	6.49	6.68	6.69	6.59	6.79	6.79	6.41	6.31	6.21	21.91	20.19	19.55	27.52
Cla 2	4/1		6.71	6.41	6.41	6.80	6.71	6.39	6.59	6.78	6.78	6.68	6.88	6.88	6.50	6.61	6.31	22.29	20.05	19.66	28.21
Gec 1	62/7	64/8		0.18	0.62	0.80	0.71	7.08	7.28	7.28	7.48	7.38	7.59	7.59	0.71	0.80	0.71	21.22	20.14	20.41	27.72
Gec 2	61/8	63/9	6/1		0.62	0.80	0.71	6.89	7.09	7.08	7.29	7.19	7.39	7.39	0.71	0.80	0.71	21.08	20.13	20.40	27.60
Spy 1	59/7	61/8	4/0	1/1		0.35	0.26	7.38	7.59	7.58	7.79	7.69	7.89	7.89	0.26	0.18	0.09	21.09	19.77	20.03	27.72
Spy 2	63/7	65/8	5/0	7/0	4/0		0.44	7.59	7.79	7.78	7.99	7.89	8.09	8.09	0.62	0.53	0.44	21.48	20.14	20.41	28.15
Spy 3	62/7	64/8	5/0	9/0	3/0	5/0		7.48	7.48	7.48	7.69	7.59	7.79	7.79	0.53	0.26	0.35	21.35	19.77	20.03	28.00
Pex 1	57/11	57/12	63/11	64/12	67/12	69/12	66/12		0.18	0.53	0.35	0.26	0.44	0.44	7.48	7.38	7.48	21.06	19.99	19.60	26.64
Pex 2	59/11	59/12	65/11	66/12	69/12	71/12	68/12	2/0		0.71	0.53	0.44	0.62	0.62	7.68	7.59	7.69	21.32	20.24	19.85	26.92
Pex 3	60/12	60/13	64/12	65/13	68/13	70/13	67/13	5/1	7/1		0.53	0.44	0.62	0.62	7.68	7.58	7.68	21.18	20.35	19.84	26.76
Pex 4	61/11	61/12	67/11	68/12	71/12	73/12	70/12	4/0	6/0	5/1		0.09	0.26	0.26	7.88	7.79	7.89	21.06	20.24	19.85	27.05
Pex 5	60/11	60/12	66/11	67/12	70/12	72/12	69/12	5/0	5/0	4/1	1/0		0.18	0.18	7.78	7.69	7.79	20.04	20.11	19.73	26.92
Pex 6	62/11	62/12	68/11	69/12	72/12	74/12	71/12	5/0	7/0	6/1	3/0	2/0		0.35	7.99	7.89	7.99	21.19	20.03	19.98	27.19
Pex 7	62/11	62/12	68/11	69/12	72/12	74/12	71/12	5/0	7/0	6/1	3/0	2/0	4/0		7.99	7.89	7.99	21.19	20.03	19.98	27.19
Gpr 1	61/8	61/9	6/2	7/1	2/1	6/1	5/1	67/13	69/13	68/14	71/13	70/13	72/13	72/13		0.44	0.18	21.21	19.88	20.14	27.84
Gpr 2	61/7	63/8	8/1	9/0	2/0	6/0	3/0	67/12	69/12	68/13	71/12	70/12	72/12	72/12	4/1		0.26	21.09	19.52	19.78	27.86
Gpr 3	60/7	60/8	7/1	8/0	1/0	5/0	4/0	68/12	70/12	69/13	72/12	71/12	73/12	73/12	1/1	3/0		21.22	19.89	20.16	27.86
Spr	160/50	162/51	150/54	152/53	151/53	154/53	153/53	149/55	151/55	149/56	149/55	148/55	150/55	150/55	151/54	151/53	152/53		9.91	9.79	24.29
Sps	150/46	148/47	146/50	147/49	144/49	147/49	144/49	144/51	146/51	146/52	146/51	145/51	147/51	147/51	144/50	142/49	145/49	97/6		7.74	24.48
Sar	144/47	144/48	149/49	150/48	147/48	150/48	147/48	140/52	142/52	141/53	142/52	141/52	143/52	143/52	147/49	145/48	148/48	95/7	79/3		26.22
Obi	172/84	176/85	176/81	176/81	176/81	179/81	178/81	170/79	172/79	170/80	173/79	172/79	174/79	174/79	176/82	177/81	177/81	150/82	155/78	167/79	

Note: Values above the diagonal are pairwise sequence divergence based on all observed substitutions. Values below the diagonal are number of transitions or number of transversions. Species abbreviations: Cla, *Ch. labiosa*; Gec, *G. eckloni*; Spy, *Sp. pylzovi*; Pex, *Pl. extremus*; Gpr, *G. przewalskii*; Spr, *S. prenanti*; Sps, *S. pseudaksaiensis*; Sar, *S. argentatus*; and Obi, *Opsariichthys bidens*.

pylzovi + *G. przewalskii* and *Sp. pylzovi* + *G. przewalskii* + *G. eckloni* + *Ch. labiosa* groups were better supported, with 78 and 62 bootstrap values, respectively. The sequences from *Sp. pylzovi*, which is distributed in Zhalin Lake and the upper reaches of the Yellow River, were very similar to those from *G. przewalski*, distributed in Qinghai Lake, forming a single clade with a maximum of 0.62% sequence divergence (Table III). *Sp. pylzovi* and *G. przewalski* were paraphyletic based on the mitochondrial *cytochrome b* gene. It was difficult to separate them into two groups (Table I). *Chuanchia labiosa* was sister to *Sp. pylzovi*, *G. przewalskii*, and *G. eckloni*. The monophyly of the clade formed by *Sp. pylzovi*, *G. przewalskii*, *G. eckloni*, and *Ch. labiosa*, however, was relatively little supported by maximum parsimony, with a relatively low bootstrap value (62), and *Pl. extremus* was placed close to the clade of *Sp. pylzovi*, *G. przewalskii*, *G. eckloni*, and *Ch. labiosa*. Together they constituted a sister group of the clade formed by *S. prenanti*, *S. pseudaksaiensis*, and *S. argentatus*.

MP trees generated using 5:1, 10:1, or equal Tv/Ts weights produced similar and congruent topologies using unweighted parsimony analysis (not shown).

Distance Analysis

A neighbor-joining (NJ) tree (Fig. 1) was constructed using MEGA 2.1 (Kumar *et al.*, 1993). Distances were calculated using Kimura's two-parameter model (Kimura, 1981) with the observed ratio of transitions and transversions (3.3:1). The topologies of the NJ tree and the MP tree were identical. Bootstrap values of the *Sp. pylzovi* + *G. przewalskii* clade, the *Sp. pylzovi* + *G. przewalskii* + *G. eckloni* + *Ch. labiosa* clade, and the *S. pseudaksaiensis* + *S. argentatus* clade were higher in NJ (85, 73, and 64, respectively) than in MP (78, 62, and 55).

Maximum-likelihood Analysis

A maximum-likelihood tree was estimated with the DNAML program from Phylip 3.6b (Felsenstein, 1993) using the observed nucleotide frequencies and a transition:transversion ratio of 3.3:1, which was found to maximize the likelihood. This tree (Fig. 1) was different from the MP and NJ trees in the branching order of the primitive schizothoracine fishes.

DISCUSSION

Sequence Variation

In this research, the pattern of nucleotide composition among the *cytochrome b* of schizothoracine fishes is different from that reported in other
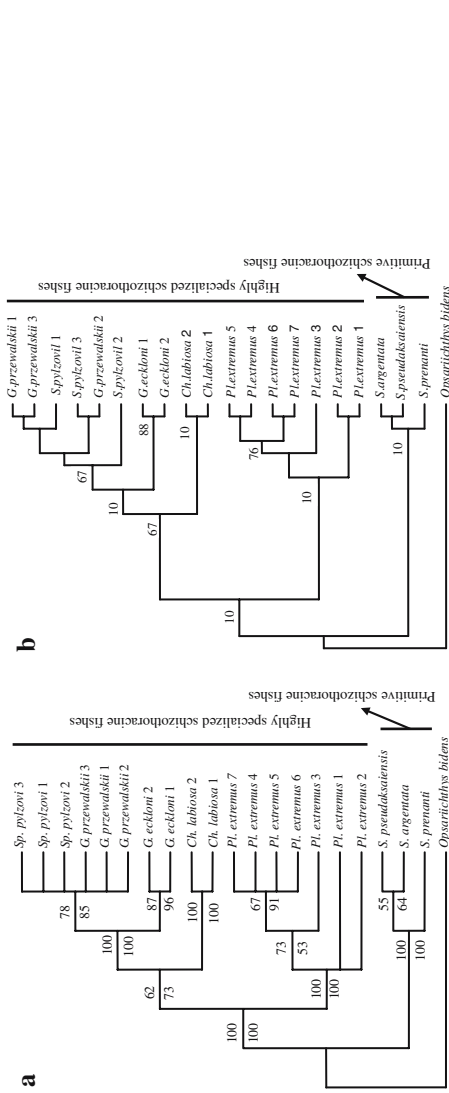
Fig. 1. Phylogenetic trees based on 1140 bp mitochondrial *cytochrome b* DNA sequences for Schizothoracinae in the Qinghai-Tibet plateau. (a) A single most-parsimonious tree constructed by PAUP 3.1.1 (length 605, CI 0.772, and RI 0.864) and neighbor-joining tree based on Kimura's two-parameter distance constructed using MEGA version 2.1. Numbers at the nodes represent bootstrap values (parsimony value above the branch, neighbor-joining value below the branch). (b) Maximum-likelihood tree (log likelihood −4261.1). Bootstrap values > 50 are displayed at the nodes.

fishes (Tang *et al.*, 2003; Xiao *et al.*, 2001), mammals (Irwin *et al.*, 1991), Perciformes (Cantarore *et al.*, 1994), sharks (Martin, 1995), Caprinae (Groves and Shields, 1996), and newts (Caccone *et al.*, 1997). At the third codon position, the nucleotide composition bias of schizothoracine fishes (average 0.199) is lower than that of Xenocyprinae (average 0.37; Xiao *et al.*, 2001), Caprinae (0.41), mammals (average 0.40), Perciformes (average 0.31), sharks (average 0.33), and birds (average 0.45) (cf. Cantarore *et al.*, 1994). Further studies are necessary to clarify the causes resulting in the relatively low nucleotide composition bias of schizothoracine fishes at the third codon position. At the first position, there is an almost equal frequency of the four nucleotides as reported in the study of Xenocyprinae (Xiao *et al.*, 2001). By contrast, at the third position, the frequencies are in the order A > C > T > > G. The highest bias against G content occurs at the third codon position. It has been suggested that selection might restrict nucleotide frequencies at the third codon position (Martin, 1995).

A high transition bias is well known in vertebrate mtDNA (Meyer, 1993). In this study, the high ratio of third-position to first- and second-position substitutions suggested that saturation of third-position substitutions has not occurred (Cook *et al.*, 1999).

Divergence Time and Biogeographical Consideration

One important aspect of molecular phylogenetic analysis is its potential for estimating branching times, in addition to branching order (Bermingham and Avise, 1986). Molecular clock estimates of schizothoracine fishes are based on a sequence divergence rate of approximately 2.0% per My (Brown *et al.*, 1979, 1982; Wilson *et al.*, 1985). Kimura's two-parameter sequence divergence of *cytochrome b* between *Sp. pylzovi* and *G. przewalskii* is 0.10%–0.60%. Applying the above-mentioned sequence divergence rate, the divergence between *G. przewalskii* (which is endemic to Qinghai Lake) and *Sp. pylzovi* (which is distributed in Zhalin Lake and the upper reaches of the Yellow River) occurred within 0.05–0.30 My. The data in this study do not conflict with the geological event that formed Qinghai Lake in the late Pliocene to middle Pleistocene (0.5 My BP) (Bian *et al.*, 2000; Yuan *et al.*, 1990) and are consistent with the view that Qinghai Lake was isolated from the upper reaches of the Yellow River during the Gonghe Movement (0.15 My BP) (Li and Fang, 1998). During that time, geographical barriers between Qinghai Lake and the upper reaches of the Yellow River separated the ancestors shared by *G. przewalskii* and *Sp. pylzovi* into two groups, thus preventing gene exchanges between them. The present distribution pattern and evolutionary process of the two species are direct results of their adaptation to environmental and climatic changes on the Qinghai-Tibet

plateau during the Gonghe Movement. Therefore, the calibration of the molecular clock (2.0% per My) is valid for estimating the separation time of the highly specialized schizothoracine fishes. Since 0.09–8.10% (Table III) sequence divergence in the *cytochrome b* sequence was observed between taxa of the highly specialized schizothoracine fishes, the approximate separation time is estimated to be about 4.5×10^4–4.05×10^6 years, suggesting that these fishes separated in the late Miocene Pliocene to Pleistocene (4.5×10^4–4.05×10^6 years BP). This result implies that a violent uplift of the Qinghai-Tibet plateau and marked environmental and climatic changes occurred at that time. Some other geographical events occurred around the same time. The intensive uplift of the Qinghai-Tibet plateau, for example, began at 3.4 My BP and comprised the Qinghai-Tibet Movement (3.6–1.7 My BP), the Kunlun–Huanghe Movement (1.1–0.6 My BP), and the Gonghe Movement (0.15 My BP) (Li and Fang, 1998). Over that time, large-scale extrusion, fault subsidence, and rapid and massive uplift of the Qinghai-Tibet plateau (Song *et al.*, 2003) caused damage to the drainage systems of the planation surfaces and brought about a division into different water bodies (Wu and Tan, 1991). Based on that information, we suggest that the Tuosuo and Zhalin lakes formed at this time.

The three species of primitive schizothoracine fishes (*S. prenanti, S. pseudaksaiensis*, and *S. argentatus*) diverged from a common ancestor approximately 3.85–4.75 My ago. The divergence time is in accordance with the geological age of fossil fishes collected from the Lunbola Basin of north Xizang (Wu and Chen, 1980).

Phylogenetic Analyses and Diploid Number Consideration

Based on the morphological characteristics of the shape of lower lip, position of ventral fin, shape of pharyngeal teeth, lower jaw, and dentary, Wu and Wu (1992) divided the highly specialized schizothoracine fishes into two groups: group 1 (*Gymnocypris*) and group 2 (*Schizopygopsis, Chuanchia*, and *Platypharodon*). The genus *Platypharodon* of group 2 was considered basal to the other genera. Phylogenetic analysis in the present study, however, indicates that the group assignment based on 1140 bp of the *cytochrome b* sequence is obviously different from previous contentions (Wu and Wu, 1992). Namely, all four genera of the highly specialized schizothoracine fishes divided into two groups with high bootstrap values using all three methods of analysis. Group 1 included the three genera of *Gymnocypris, Schizopygopsis*, and *Chuanchia*, while group 2 consists of only one genus, *Platypharodon*. The genus *Platypharodon* is still seen as basal to the other genera.

One surprising result of the present study is the inability of the *cytochrome b* sequence data to discriminate between the morphologically distinctive specimens of *G. przewalskii* and *Sp. pylzovi*. The result is not consistent with the traditional study (Wu and Wu, 1992) based on anatomical characters. Incongruencies between morphological and molecular phylogenies have been recognized and debated ever since molecular techniques have been in use (Patterson, 1987). A similar phenomenon has also been observed in some fishes and mammals (Avise, 1986; Avise and Saunders, 1984; Billington and Hebert, 1988; Burridge, 1999; Xiao *et al.*, 2001). In the two species of Schizothoracinae the discrepancy may be explained in terms of introgressive hybridization or incomplete lineage sorting. Although the spawning periods of *G. przewalskii* and *Sp. pylzovi* overlap (Wu and Wu, 1992), there are no records of sympatry for these two species or natural hybridization because of geographical barriers. Therefore, mtDNA exchange between *G. przewalskii* and *Sp. pylzovi* appears unlikely. Incomplete lineage sorting is the favored explanation, as reported in *Nemodactylus macropterus* and *Acantolatris monodactylus* by Burridge (1999). During speciation, ancestral mtDNA lineages may not sort congruently with respect to species boundaries, and individuals can possess haplotypes more similar to those of nonconspecific individuals of the same species (Avise, 1986; Burridge, 1999). That is, the gene tree for sister taxa may not be reciprocally monophyletic (Burridge, 1999). A simulation conducted by Neigel and Avise (1986) suggested that the probability of sister taxa possessing reciprocally monophyletic mtDNA lineages is high only after $4N$ generations of genetic isolation, where N is the number of females. According to the analysis of Burridge (1999) in *N. macropterus* and *A. monodactylus*, the maximum divergence time (0.05–0.30 My) suggested for *G. przewalskii* and *Sp. pylzovi* is too recent for lineage monophyly to have developed. The difficulty of distinguishing between homologous and analogous features can complicate the establishment of an accurate phylogeny based on morphological characteristics (Goodman *et al.*, 1987). Traditional phylogenetics have been based on the assumption that characters such as morphology change slowly over time, and that as species evolve, they select environments best suited to these characteristics, which are highly plastic and can change rapidly to suit environments (Groves and Shields, 1996). So, different morphological characteristics between the two species were rapidly established in order to reduce competition or increase food availability in different drainage systems. In addition, karyotype analysis (Yu *et al.*, 1990; Qi, 2004) suggests that the relationship of *G. przewalskii* and *Sp. pylzovi* is relatively close because both of them share the same diploid number ($2n = 92$).

Chromosomes may evolve at rates different from morphology and behavior, contributing confusion to proposed phylogenies (Groves and

Shields, 1996). Within the schizothoracine fishes included in this study, however, the diploid number (2n) ranges from 90 to 150 (Durand *et al.*, 2002; Qi, 2004; Yu *et al.*, 1990) and provides a clear pattern of relationships; that is, individuals sharing very close diploid chromosome numbers are very closely related in phylogenetic analysis. For example, the highly specialized schizothoracine fishes (*Sp. pylzovi*, *G. przewalskii*, *G. eckloni*, *Ch. labiosa*, and *Pl. extremus*) with diploid numbers between 90 and 94 have a very close relationship and cluster together, and the primitive schizothoracine fishes (*S. argentatus* and *S. prenanti*) with diploid numbers between 98 and 150 cluster together and are consistently more divergent from other taxa. Among all taxa, *Sp. pylzovi, G. przewalskii*, and *Ch. labiosa* share the same diploid chromosome number and have a very close relationship. Although there is no record of a diploid number for *S. pseudaksaiensis*, it is most likely that it is close to that of *S. argentatus* and *S. prenanti* because the three species cluster together and show a very close relationship. This study did not completely resolve the phylogenetic relationships among all the species of highly specialized schizothoracine fishes, but we have proposed some phylogenetic patterns that differ slightly from phylogenies based on morphological and anatomical characters. Further studies are required to investigate the phylogenetic relationships of all highly specialized schizothoracine fishes based on more mtDNA genes as well as some region of nuclear DNA.

ACKNOWLEDGMENTS

REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., De Bruijn, M. H L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* **290**:457–465.

Avise, J. C. (1986). Mitochondrial DNA and the evolutionary genetics of higher animals. *Phil. Trans. R. Soc. Lond.* **312**:325–342.

Avise, J. C., and Saunders, N. C. (1984). Hybridization and introgression among species of sunfish (*Lepomis*): Analysis by mitochondrial DNA and allozyme markers. *Genetics* **108**:237–255.

Bermingham, E., and Avise, J. C. (1986). Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* **113**(4):939–965.

Bian, Q. T., Liu, J. Q., Luo, X. Q., and Xiao, J. L. (2000). Geotectonic setting, formation, and evolution of the Qinghai Lake. *Seismology and Geology* **22**(1):20–26.

Billington, N., and Hebert, P. D. N. (1988). Mitochondrial DNA variation in Great Lakes walleye (*Stizostedion vitreum*) populations. *Can. J. Fish. Aquat. Sci.* **45**:643–654.

Brown, W. M., George, M. Jr., and Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **76**:1967–1971.

Brown, W. M., Prager, E. M., Wang, A., and Wilson, A. C. (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J. Mol. Evol.* **18**:225–239.

Burridge, C. P. (1999). Molecular phylogeny of *Nemadactylus* and *Acantholatris* (Perciformes: Cirrhitoides: Cheilodactylidae), with implications for taxonomy and biogeography. *Mol. Phyl. Evol.* **13**(1):93–109.

Caccone, A., Milinkovitch, M. C., Sbordoni, V., and Powell, J. R. (1997). Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). *Syst. Biol.* **46**:126–144.

Cantarore, P., Roberti, M., Pesole, G., Ludovico, A., Millella, F., Gadaleta, M. N., and Saccone, C. (1994). Evolutionary analysis of *cytochrome b* sequences in some Perciformes: Evidence for a slower rate of evolution than in mammals. *J. Mol. Evol.* **39**:589–597.

Cavender, T. M., and Coburn., M. M. (1992). Phylogenetic relationships of North American Cyprinidae. In Mayden, R. L. (ed.), *Systematics, Historical Ecology, and North American Freshwater Fishes*, Stanford Univ. Press, Stanford, pp. 293–327.

Chang, Y. S., Huang, F. L., and Lo, T. B. (1994). The complete nucleotide sequence and gene organization of carp (*Cyprinus caryio*) mitochondrial genome. *J. Mol. Evol.* **38**:138–155.

Chen, X. L., Yue, P. Q., and Lin, R. D. (1984). Major groups within the family Cyprinidae and their phylogenetic relationships. *Acta. Zootaxon. Sinica* **9**:424–440.

Cook, C. E., Wang, Y., and Sensabaugh, G. (1999). A mitochondrial control region and *cytochrome b* phylogeny of sika deer (*Cervus nippon*) and report of tandem repeats in the control region. *Mol. Phyl. Evol.* **12**(1):47–56.

Durand, J. D., Tsigenopoulos, C. S., Unlu, E., and Berrebi, P. (2002). Phylogeny and biogeography of the family Cyprinidae in the Middle East inferred from *cytochrome b* DNA: Evolutionary significance of this region. *Mol. Phyl. Evol.* **22**(1):91–100.

Felsenstein, J. (1993). *Phylip (Phylogeny inference package) version 3.5c*, University of Washington: Seattle.

Gilles, A., Lecointre, G., Faure, E., Chappaz, R., and Brun, G. (1998). Mitochondrial phylogeny of the European cyprinids: Implications for their systematics, reticulate evolution, and colonization time. *Mol. Phyl. Evol.* **10**:132–143.

Gilles, A., Lecointre, G., Miquelis, A., Chappaz, R., and Brun, G. (2001). Partial combination applied to phylogeny of European cyprinids using the mitochondrial control region. *Mol. Phyl. Evol.* **19**(1):22–33.

Goodman, M., Miyamoto, M., and Czelusniak, J. (1987). Pattern and process in vertebrate phylogeny revealed by coevolution of molecules and morphologies. In Patterson, C. (ed.), *Molecules and Morphology in Evolution: Conflict or Compromise?* Cambridge Univ. Press, Cambridge, pp. 141–176.

Greenwood, P. H. (1966). Phyletic studies of the teleostean fishes with provisional classification of living forms. *Bull. Am. Mus. Nat. Hist.* **131**(4):339–456.

Groves, P., and Shields, G. F. (1996). Phylogenetics of the Caprinae on *cytochrome b* sequence. *Mol. Phyl. Evol.* **5**(3):467–476.

Irwin, D. M., Kocher, T. D., and Wilson, A. C. (1991). Evolution of the *cytochrome b* gene in mammals. *J. Mol. Evol.* **32**:128–144.

Kimura, M. (1981). Estimation of evolutionary distances between homologous nucleotide sequences. *Proc. Natl. Acad. Sci. USA* **78**:454–458.

Kumar, S., Tamura, K., and Nei, M. (1993). *MEGA: Molecular Evolutionary Genetics Analysis, Version 1.0*, University Park: Pennsylvania State Univ.

Li, J. J., and Fang, X. M. (1998). Uplift of the Tibetan Plateau and environmental changes. *Chinese Sci. Bull.* **44**(23):2117–2125.

Martin, A. P. (1995). Mitochondrial DNA sequence evolution in sharks: Rates, patterns, and phylogenetic inferences. *Mol. Biol. Evol.* **12**:1114–1123.

McVeigh, H. P., and Davidson, W. S. (1991). A salmonid phylogeny inferred from mitochondrial *cytochrome b* gene sequences. *J. Fish Bio.* **39**(Supplement A):277–282.

Meyer, A. (1993). Evolution of mitochondrial DNA in fishes. In Hochachka, P. W. and Mommsen, T. P. (eds.), *The Biochemistry and Molecular Biology of Fishes, Vol. 2*, Elsevier, Amsterdam, pp. 1–38.

Mirza, M. R. (1991). A contribution to the systematics of the Schizothoracine fishes (Pisces: Cyprinidae) with the description of three new tribes. *Pakistan J. Zool.* **23**:339–341.

Neigel, J. E., and Avise, J. C. (1986). Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In Nevo, E. and Karlin, S. (eds.), *Evolutionary Processes and Theory*, Academic Press, New York, pp. 515–534.

Patterson, C. (1987). Introduction. In Patterson, C. (ed.), *Molecules and Morphology in Evolution: Conflict or Compromise?* Cambridge Univ. Press, Cambridge, pp. 1–22.

Perdices, A., Cunha, C., and Coelho, M. (2004). Phylogenetic structure of *Zacco platypus* (Teleostei, Cyprinidae) populations on the upper and middle Chang Jiang (Yangtze) drainage inferred from *cytochrome b* sequences. *Mol. Phyl. Evol.* **31**(1):192–203.

Qi, D. L. (2004). Preliminary studies on chromosome karyotype and polyploidy of Qinghai Lake naked carp. *J. Qinghai Univ.* **22**(2):44–47.

Song, C. H, Fang, X. M., Li, J. J., Gao, J. P., Sun, D., Nie, J. S., and Yan, M. D. (2003). Sedimentary evolution of the Guide Basin in the Cenozoic and the uplift of the Qinghai-Tibet plateau. *Geol. Rev.* **49**(4):337–346.

Swofford, D. (1993). *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1*, Champaign, 11: Illinois Natural History Survey.

Tang, Q. Y., Yang, X. P., and Liu, H. Z. (2003). Biogeographical process of *Spinibarbus caldwelli* revealed by sequence variations of mitochondrial *cytochrome b* gene. *Acta Hydrobio. Sinica* **27**(4):352–356.

Wilson, G. M., Thomas, W. K., and Beckenbach, A. T. (1985). Intra- and inter-specific mitochondrial DNA sequence in Salmo: Rainbow, steelhead, and cutthroat trouts. *Can. Zool.* **63**:2088–2094.

Wu, X. W. (1964). *The Fauna of Chinese Cyprinidea in China*, Shanghai: Shanghai Science and Technology Press.

Wu, Y. F., and Chen, Y. Y. (1980). Fossil Cyprinid fishes from the late tertiary of north Xizang, *China Verteb. Palasiatica* **18**(1):15–18.

Wu, Y. F., and Tan, Q. J. (1991). Characteristics of the fish-fauna of the Qinghai-Xizang plateau and its geological distribution and formation. *Acta Zool. Sinica* **37**(2):135–152.

Wu, Y. F., and Wu, C. Z. (1992). *The Fishes of the Qinghai-Xizang Plateau*, Chengdu: Sichuan Publishing House of Science and Technology.

Xiao, W., Zhang, Y., and Liu, H. (2001). Molecular systematics of Xenocyprinae (Teleostei: Cyprinidae): Taxonomy, biogeography, and coevolution of a special group restricted in East Asia. *Mol. Phyl. Evol.* **18**(2):163–173.

Yuan, B. Y., Chen, K. Z., Bowler, J. M., and Ye, S. J. (1990). The forming and evolution of the Qinghai Lake. *Quaternary Science* **3**:233–243.

Yue, P. Q., and Chen, Y. Y. (1998). *China Red Book of Endangered Animals, Pisces*, Beijing: Science Press.

Yu, X. Y., Li, Y. C., and Zhou, T. (1990). Study on chromosome karyotype of Chinese Cyprinidae: Chromosome karyotype of eight Schizothoracines. *J. Wuhan Univ.* **2**(2):97–103.

Zardoya, R., and Doadrio, I. (1999). Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *J. Mol. Evol.* **49**:227–23.