

Molecular phylogeny and biogeography of the highly specialized grade schizothoracine fishes (Teleostei: Cyprinidae) inferred from cytochrome *b* sequences

HE DeKui & CHEN YiFeng[†]

Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

We recovered the phylogenetic relationships among 23 species and subspecies of the highly specialized grade schizothoracine fishes distributing at 36 geographical sites in the Tibetan Plateau and its surrounding regions by analyzing sequences of cytochrome *b* genes. Furthermore, we estimated the possible divergent times among lineages based on a historical geological isolation event in the Tibetan Plateau. The molecular data revealed that the highly specialized grade schizothoracine fishes were not a monophyletic group, but were the same as genera *Gymnocypris* and *Schizogopsis*. Our results indicated that the molecular phylogenetic relationships apparently reflected their geographical and historical associations with drainages, namely species from the same and adjacent drainages clustered together and had close relationships. The divergence times of different lineages were well consistent with the rapid uplift phases of the Tibetan Plateau in the late Cenozoic, suggesting that the origin and evolution of schizothoracine fishes were strongly influenced by environment changes resulting from the upheaval of the Tibetan Plateau.

highly specialized grade schizothoracine fishes, Tibetan Plateau, molecular phylogeny, biogeography, cytochrome *b* gene

The theories of modern historical biogeography are based on the assumption that biological evolution and geographical changes are synchronous^[1]. Geographical and ecological changes result in spatial distribution segregation and species speciation. Deciphering the current distribution of species may help us to explore and trace the geological and geographical histories of the region. Schizothoracine is a good experimental subject to understand the biological and geological evolution process of the Tibetan Plateau. As the primary freshwater and cold water-adapting species, these fishes are not only restricted to drainage systems, but are also confined to high altitude habitat, showing little capacity for trans-watershed dispersal. Therefore, the evolution and distribution patterns of schizothoracine reflect the paleogeographical history of the Tibetan Plateau, especially the development of hydrographic basins and their isolation and interconnection processes.

According to the differences in scales, pharyngeal teeth and barbels of schizothoracine fishes, Cao et al.^[2] divided the subfamily Schizothoracinae into three evolutionary grades: primitive, specialized and highly specialized fishes. Each grade is related with specific periods of geological evolution of the Tibetan Plateau. The highly specialized grade schizothoracine fishes are characterized by the following features: the absence of barbelst, one or two rows of pharyngeal teeth and degenerated entirely scales, including six genera, *Gymnocypris*, *Oxygymnocypris*, *Schizopygosis*, *Platypharodon*, *Chuanchia* and *Herzensteinia*, and 26 species and subspecies^[3]. The fishes distribute in the middle and upper

Received September 27, 2005; accepted January 16, 2007

doi: 10.1007/s11434-007-0123-2

[†]Corresponding author (email: chenyf@ihb.ac.cn)

Supported by the National Natural Science Foundation of China (Grant Nos. 30670287, 30471345 and 30200029) and the Chinese Academy of Sciences (Grant No. KSCX2-SW-125)

reaches of rivers in the Tibetan Plateau and its adjacent areas with an elevation range from 1500 to 5000 m. Except the genera *Gymnocypris* and *Schizopygosis*, the other four genera are monotypic and endemic to China.

In the present study, we assessed the interspecific relationships among 23 species and subspecies of the highly specialized grade schizothoracine fishes collected from the main drainage systems in the Tibetan Plateau with sequences from the mitochondrial cytochrome *b* gene (*cyt b*). The *Cyt b* gene, a well-known protein-coding gene, is considered a good phylogenetic marker for recovering the true phylogenetic relationships among taxa^[4]. It has been used in phylogenetic studies of various Cyprinidae groups^[5–10]. The purposes of this study are: (1) to provide a phylogenetic hypothesis for the highly specialized grade schizothoracine fishes; (2) to explore the biogeographical implications of the molecular phylogeny; (3) to explore the evolutionary processes of the major river systems across the Tibetan Plateau.

1 Materials and methods

1.1 Sample collection

A total of 161 individuals (excluding GenBank Data) collected from 16 different drainage systems and 36

geographical locations (Figure 1 and Table 1) were examined. These samples were assigned to 21 species and subspecies. In addition, the *cyt b* sequences of *P. extremus* and *C. labiosa* were downloaded from GenBank and analyzed in this study. Samples were collected by gill net or casting net during 1997–2005. Muscle tissues were preserved in 95% ethanol in the field. All specimens were fixed in formalin and preserved in the Museum of Freshwater Fishes, Institute of Hydrobiology, the Chinese Academy of Sciences (CAS). Species names, drainages, voucher, collecting locations and GenBank Accession numbers are given in Table 1.

1.2 DNA extractions, amplification and sequencing

Total genomic DNA was isolated by standard phenol-chloroform extraction protocol^[11]. The *cyt b* gene was amplified using primers L14724 (5'-GACTTGAAA-ACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3'). PCR amplifications were performed in a programmable thermal cycler using the following profile: one preliminary denaturation at 94°C for 3 min, followed by strand denaturation at 94°C (0.5 min), annealing at 56°C (1 min), and primer extension at 72°C (1.5 min) for 35 cycles. Amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen,

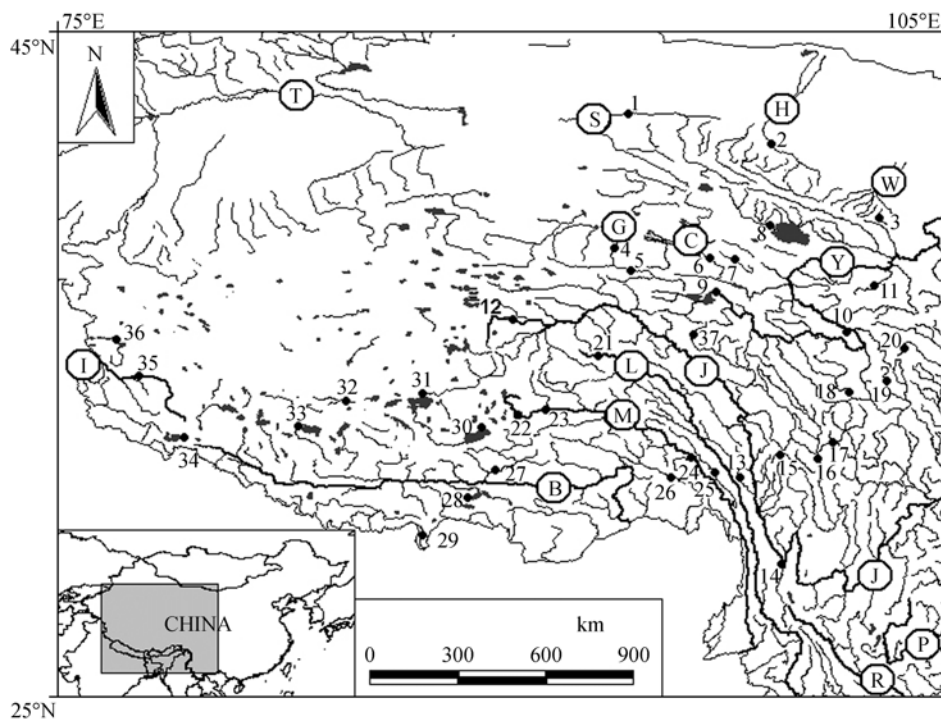


Figure 1 Map of western China showing the sampling localities of the highly specialized schizothoracine fishes used in this study. The numbers refer to the localities listed in Table 1. Major rivers mentioned in the text are indicated by circles: B, Tsangpo R.; C, Tsaidam R.; G, Golmud R.; H, Heihe R.; I, Indus; J, Jinshajiang R.; L, Mekong R.; N, Salween R.; P, Pear R.; R, Red R.; S, Shulehe R.; T, Tarim R.; Y, Yellow R. and W, Shiyanghe R.

Table 1 Species, drainages, collection sites, number of specimens and haplotype, and GenBank Accession No. of specimens for this study^{a)}

Genus	Species	Collection sites (number is in Figure 1)	Number of specimens	Drainage	Haplotype	GenBank Accession No.
<i>Oxygymnocypris</i>	<i>O. stewartii</i>	Lhasa, Tibet (27)	7	Tsangpo	LS1-3	
<i>Gymnocypris</i>	<i>G. waddelli</i>	Yamzho Yumco, Tibet (28)	2	Yamzho Yumco	YZYC	
	<i>G. potanini potanini</i>	Heishui, Sichuan (19)	3	Yangtze R.	HS	
		Marlkang, Sichuan (18)	2	Yangtze R.	DD	
	<i>G. p. firmispinatus</i>	Shigu, Yunnan (14)	2	Yangtze R.	SG1-2	
	<i>G. chui</i>	Nyogze, Tibet (34)	4	Mapam Yumco	GZC1-2	
	<i>G. eckloni</i>	Maduo, Qinghai (9)	9	Yellow R.	MD1-5	
		Ma Qu, Gansu (10)		Yellow R.	MQ1-3	*AY463494-5, 463522
		Golmud, Qinghai (4)	6	Golmud R.	WQ	
		Wenquan, Qinghai (5)	2	Golmud R.	WQ	
	<i>G. chilienensis</i>	Zhangye, Gansu (2)	7	Heihe R.	XM1-3	
		Anxi, Gansu (1)	2	Shulehe R.	AX	
	<i>G. namensis</i>	Nam Co, Tibet (30)	2	Nam Co	NMC1-2	*AY463500,
		Siling Co, Tibet (31)	7	Siling Co	ZJZB1-2	
	<i>G. p. prezwaslskii</i>	Lake Qinghai, Qinghai (8)		Lake Qinghai	QHH1-3	*AY463496-7, 463523
<i>Schizopygosis</i>	<i>S. pylzovi</i>	Qagan US, Qinghai (7)	4	Tsaidam R.	DL	
		Xiangride, Qinghai (6)	6	Tsaidam R.	XRD	
		Wenquan, Qinghai, (5)	6	Golmud R.	WQ	
		Golmud, Qinghai (4)	3	Golmud R.	WQ	
		Maduo, Qinghai (9)	12	Yellow R.	MD1-2	
		Ma Qu, Gansu (10)	3	Yellow R.	DL	
		Linxia, Qansu (11)		Yellow R.	LX	*AY463503
		Xiama, Gansu (3)	6	Shiyanghe R.	XM1-3	
	<i>S. stliczkai stliczkai</i>	Shiquanhe, Tibet (35)	5	Indus	SQH1-3	
	<i>S. s. bangongensis</i>	Bangong Co, Tibet (36)	3	Bangong Co	BGC	
	<i>S. malacanthus malacanthus</i>	Mangkang, Tibet (13)	2	Yangtze R.	MK	
		Litang, Sichan (15)	2	Yangtze R.	LT	
		Yajiang, Sichuan (16)	3	Yangtze R.	YJ1-2	
		Xinduqiao, Sichuan (17)	3	Yangtze R.	XDQ1-2	
		Qingshuihe, Qinghai (37)	6	Yangtze R.	QSH1-3	
	<i>S. thermalis</i>	Nagqu, Tibet (22)	4	Salween	NQ1-2	
		Chalong, Tibet (23)	3	Salween	CL	
		Baima, Tibet(24)	2	Salween	BM2	
	<i>S. younghusbandi younghusbandi</i>	Lhasa, Tibet (27)	3	Tsangpo	LS1-2	*AY463501
		Po-Mi, Tibet (26)	4	Tsangpo	BM1-2	
	<i>S. y. himalayensis</i>	Xarsingma, Tibet (29)	4	Brahmaputra	YD1-2	
	<i>S. kialingensis</i>	Jiuzhaigou, Sichuan (20)	4	Yangtze R.		
	<i>S. anteroventris</i>	Zaduo, Qinghai (21)	4	Mekong	ZD1-3	*AY463499,
		Wangda, Tibet (25)	2	Salween	ZG1-2	
	<i>S. undescribe I</i>	Nyma, Tibet (31)	2	Bocang Tsangpo	NM1-2	*AY463502
	<i>S. undescribe II</i>	Zhari Namco, Tibet (32)	3	Zhari Namco	ZRNMC	
<i>Herzensteinia</i>	<i>H. microcephalus</i>	Tuotuohe, Qinghai (12)	7	YangtzeR.	TTH1-3	
<i>Platypharodon</i>	<i>P. extremus</i>					*AY608657-8, *AY608659-60
<i>Chuanchia</i>	<i>C. labiosa</i>					*AY608650-1
<i>Ptychobarbus</i>	<i>Pb. dipogon</i>	Lhasa, Tibet (27)		Tsangpo		*AY463510
	<i>Pb. conirostris</i>	Shiquanhe, Tibet (35)		Indus		*AY463509
	<i>Pb. c. chungtienensis</i>	Zaduo, Qinghai (21)		Mekong		*AY463508
<i>Gymnodiptychus</i>	<i>Gd. integrigymnatus</i>	Ruidian, Yunnan		Irrawaddy		*AY463526

a) *, Sequences were obtained from the GenBank site.

Hilden, Germany). The amplification primers were used for sequencing. All samples were sequenced with Big-dye sequencing chemistry with an ABI 377 Automated

Sequencer (Applied Biosystems, Inc., Foster City, California). All sequences have been deposited in GenBank under Accession Nos.

1.3 Data analyses

Sequences were initially aligned using the CLUSTAL X 1.8^[12], followed by manual correction by eye. Base composition homogeneity was tested with chi-square (χ^2) test for equal base frequencies across taxa. Nucleotide saturation was analyzed by plotting absolute number of transitions (Ts) and transversions (Tv) against absolute distance values. Phylogenetic analyses of maximum parsimony (MP) and maximum likelihood (ML) were implemented with PAUP* versions 4.0b10^[13]. According to the analysis results of morphological and molecular phylogenies, 4 species of the specialized grade schizothoracine fishes were designated as outgroup^[10,14,15]. The hierarchical likelihood ratio test (hLRT) implemented in MODELTEST 3.06^[16] was used to determine the appropriate model of molecular evolution for ML analysis. The selected model was general time-reversible^[17] (GTR + I + Γ , I = 0.5089, Γ = 0.8401, A = 0.3021, C = 0.3272, G = 0.1017, T = 0.2690). MP tree searches were performed by heuristic searches (TBR branch swapping, MULTREES option effective, and 100 random stepwise additions of taxa). Tv was given higher weights than Ts (6:1) based on empirical Ts:Tv ratios (6.41:1) estimated from the ML trees. Support for resolved clades was estimated using non-parametric bootstrapping with 1000 pseudoreplicates for MP trees^[18]. The Bayesian inference (BI) was implemented using MrBayes 3.1.1b^[19]. We assumed GTR + I + Γ model of evolution to run 2000000 generations of 4 simultaneous Monte Carlo Markov chains (MCMC), sampling every 100 generations. Trees with likelihood scores lower than those at saturation (burn-in = 600) were discarded from the analysis. Posterior probabilities (BBP) of nodes were estimated based on the 50% majority rule consensus of the trees. To determine whether a particular tree topology corresponded to a significantly better or worse interpretation of the data than an alternative tree, we used the Shimodaira-Hasegawa test (RELL, 1000 bootstrap replicates)^[20], as implemented in PAUP*.

A likelihood ratio test was performed with ML trees with and without a molecular clock constraint^[21]. In order to date major cladogenetic events, a linearized tree based on the ML tree was constructed using the non-parametric rate smoothing (NPRS) method as implemented in TreeEdit v1.0^[22]. In order to estimate the magnitude of error from substitutional noise, confidence

intervals were obtained by parametric bootstrapping. Seq-Gen v1.2.2^[23] was used to simulate 100 parametric bootstrap data sets (1140 bp long) along the original ML tree under the GTR+I+ Γ model. For each parametric bootstrap data set the ML tree was reconstructed in PAUP* v4.0b10 and converted into a linearized tree in TreeEdit v1.0 as described above. Mean and 95% confidence interval for each node depth was then calculated.

He et al.^[10] estimated the divergence times using molecular clock calibrated by geological segregation event between the upper Yellow River and Lake Qinghai. However, this segregation event occurred at a late time of Pleistocene (0.15 Ma), and either the species *G. eckloni* or *G. przewalskii* did not form a monophyly^[24]. Thus, we calibrated the age of the most recent common ancestor of the *H. microcephalus* and *S. pylzovi*, using a reliable geological event, the “Kunlun-Huanghe Movement”^[25]. The large-scale uplift commenced 1.1 Ma ago in the Kunlun Mountains Pass area. We suppose that the uplift event isolated the upper Yangtze River from Yellow River and drainages of the Tsaidam Basin, which stops genetic exchanges of fish distributing in these two areas.

2 Results

2.1 Sequence variation

A total of 1140 positions were analyzed, of which 348 sites were variable and 269 sites were parsimony informative. 79.60% variable sites were at the third codon positions and 16.95% were at the first codon positions. Mean base composition of cyt *b* sequences of all examined schizothoracine fishes had a low G content (10.17%) and almost equal A, T, and C content (30.21, 26.90, and 32.72%, respectively). Strong bias in base composition is a typical feature of the cyt *b* gene and other mitochondrial protein-coding genes^[26]. Significant compositional biases exist at the second and especially the third codon positions, where there is a marked underrepresentation of guanine, while base composition at the first codon positions is almost equal. No base compositional biases were observed ($\chi^2 = 50.0336$, $df = 243$, $P = 1.00$).

Sixty-six unique haplotypes were identified among the 161 individuals examined (Table 1). Except for five species, *G. eckloni*, *S. pylzovi*, *G. namensis*, *S. thermalis* and *S. younghusbandi younghusbandi*, the shared haplotypes occurred only among the species from the same geographical sites. There are shared haplotypes between

G. eckloni and *S. pylzovi* from the upper Yellow River and Tsaidam Basin (Tables 2 and 3). The species *S. pylzovi* and *P. extremus* (AY608658) also share one haplotype. Furthermore, shared haplotypes were detected between *G. namensis* and *S. thermalis*, as well as *S. thermalis* and *S. younghusbandi younghusbandi* (Tables 2 and 3).

Table 2 The number of individuals shared haplotypes between interspecies and intraspecies of the high-specialized schizothoracine fishes

Haplotype	Geographical Population					
	Golmud	Wenquan	Xiangride	Qagan US	Maduo	Ma Qu
<i>G. eckloni</i> WQ	4	2			4	
<i>S. pylzovi</i> WQ	3	3				
<i>S. pylzovi</i> DL		3	2	4	4	3

The maximum sequence divergence within examined schizothoracine exists between *Ptychobarbus dipogon* and *Gymnodiptychus integrigymnatus*, which is 14.30%. The levels of sequence divergence between outgroup and ingroup lineages range from 10.09% (between *G. eckloni* and *Gd. integrigymnatus*) to 13.51% (*C. labiosa* and *Pb. chungtienensis chungtienensis*). The maximum value of pairwise distance within the highly specialized grade schizothoracine fishes is 10.61% (between *G. eckloni* and *O. stewartii*).

Plots of transitions and transversions against uncorrected pairwise distance indicate an absence of nucleotide saturation (not shown). All plots are linear whether the data were plotted by position, or by the full sequence. Thus, all nucleotide positions were employed in the following phylogenetic analysis.

2.2 Phylogeny of the highly specialized schizothoracine fishes

The phylogenetic tree recovered with BI is shown in Figure 2. Seven major clades are identified. ML tree shows identical topologies to the BI tree. The MP phylogenetic analysis with a 6:1 (Tv:Ts) weighting scheme yielded 80 equally short trees (not shown) with a length of 1323 steps, a consistency index (CI) excluding uninformative characters of 0.6424, and a retention index (RI) of 0.9041. Different Tv:Ts weighting schemes (e.g.

1:1, 2:1) arrived at similar topologies of the strict consensus tree. The relative bootstrap supports (BP) for MP (Tv:Ts = 6:1), posterior probabilities (BPP) for BI are also shown in Figure 2. The monophyly of the highly specialized grade schizothoracine fishes is not supported by all analytical methods when designating *Ptychobarbus* and *Gd. integrigymnatus* as outgroup. The *Gd. integrigymnatus* is embedded in the lineages of the highly specialized grade schizothoracine fishes, and is placed as the sister group to the other highly specialized grade schizothoracine fishes except *O. stewartii*, with strong supports (BP = 100, BBP = 1.00). On the contrary, species *O. stewartii* from the Tsangp forms a clade with a high support (BP = 95, BBP = 1.00). Nevertheless, SH tests do not reject the hypothesis that the highly specialized grade schizothoracine fishes are a monophyletic group ($P = 0.66$). All other species of the highly specialized grade schizothoracine fishes, except for *O. stewartii*, might diverge into two lineages. One of the lineages includes *G. eckloni*, *G. przewasliskii przewasliskii*, *G. potanini potanini* and *C. labiosa*, with high supports (BP = 95, BBP = 1.00). The other lineage includes the rest species of the highly specialized grade schizothoracine fishes, with high supports (BP = 98, BBP = 1.00). Of the species examined in this study, the genera *Gymnocypris* or *Schizopygosis* does not form a monophyly. Furthermore, alternative hypotheses enforcing the monophyly of two genera were strongly rejected by SH tests ($P = 0.00$).

The phylogenetic relationships recovered from the *cyt b* data are not consistent with those of traditional taxonomy. In general, phylogenetic relationships exhibit a significant geographical distribution pattern; that is, species from the same drainage systems or adjacent regions often cluster together (Figure 3). For example, clade 3 contains species distributing in the Tsaidam Basin, Lake Qinghai, the upper Yellow and Minjiang rivers. Clade 4 consists of species collected from the drainages of western Tibetan Plateau. Clade 5 is composed of species from the Salween and Mekong rivers. Clade 6 includes

Table 3 The shared haplotypes between interspecies and intraspecies of the highly specialized schizothoracine fishes

Haplotype	Species and Geographical Population					
	Nam Co <i>G. namensis</i>	Siling CO <i>G. namensis</i>	Nagqu <i>S. thermalis</i>	Chalong <i>S. thermalis</i>	Baima <i>S. thermalis</i>	Po-Mi <i>S. y. younghusbandi</i>
<i>G. namensis</i> NMC1	1	2				
<i>S. thermalis</i> NQ1		3	1	1		
<i>S. y. younghusbandi</i> BM2				1	2	3

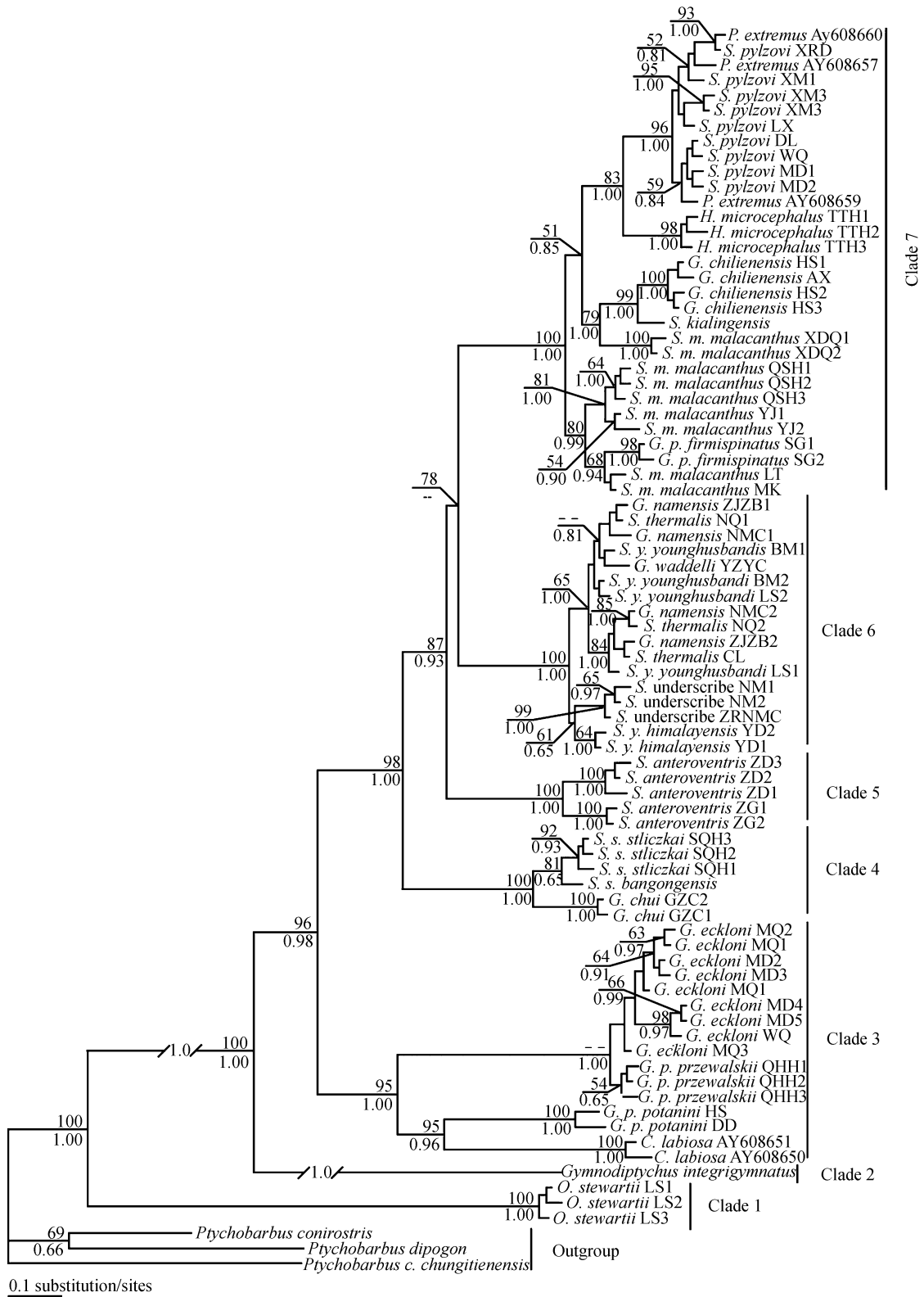


Figure 2 The 50% majority-rule consensus tree of the highly specialized schizothoracine fishes recovered by Bayesian approach based on cytochrome *b* sequence data. The numbers above each branch refer to the >50% Bayesian posterior probability (GTR + I + Γ , 2000000 generations, Burnin = 600). Bootstrap values (>50%) for 6:1 (Tv:Ts) weighting scheme MP are shown below branches. The haplotype names are shown after taxa.

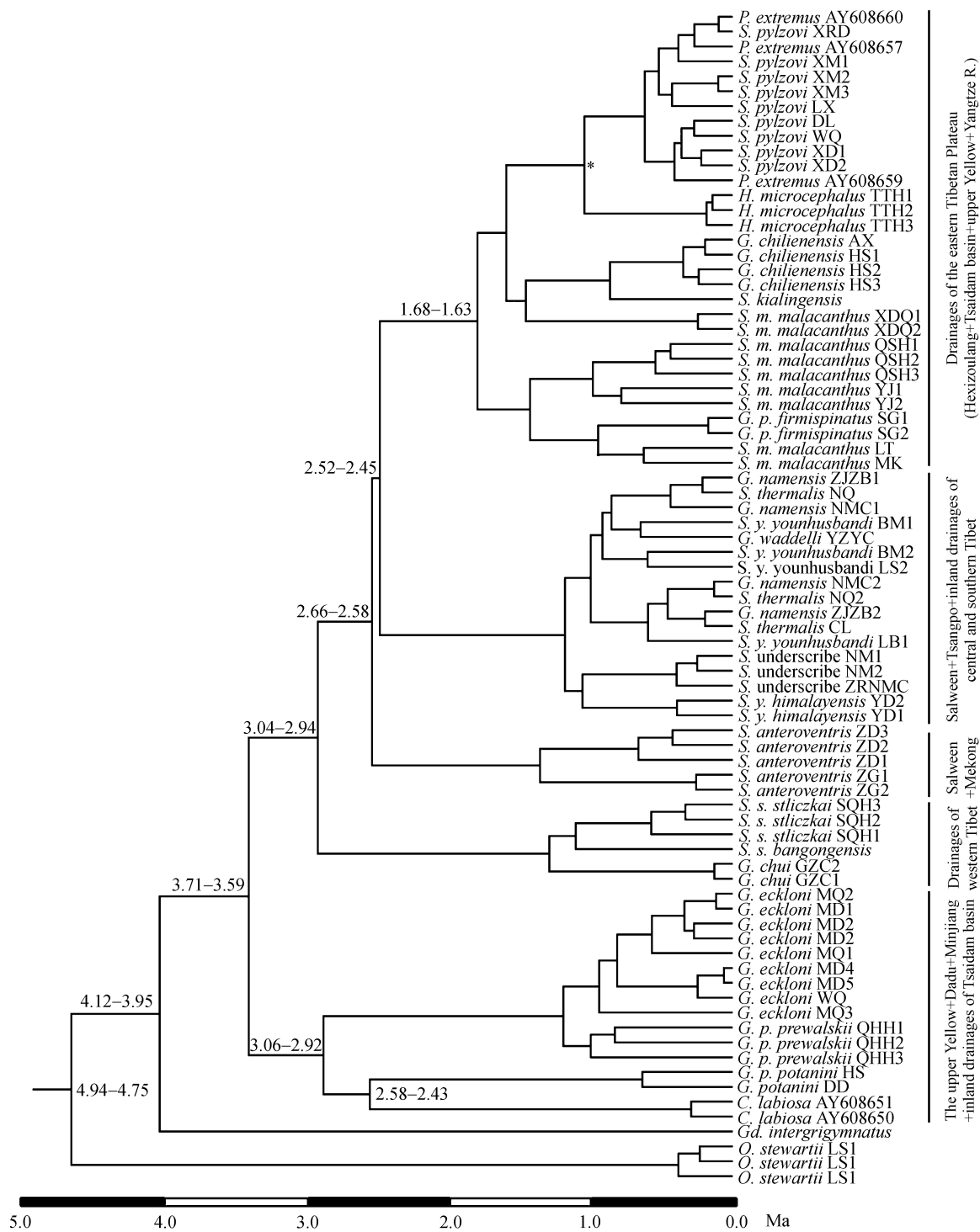


Figure 3 Ultrametric ML tree of the highly specialized schizothoracine fishes based on the NPRS transformation using the *cyt b* data. The scale bar below the tree shows the time scale resulting from molecular estimated divergence using a calibration point based on the node highlighted with a star. The 95% confidence intervals of divergence times for major nodes are given on above branches. The drainages distributions of each terminal taxon are also given on the right side of the tree.

species distributing in central and southern Tibetan Plateau as well as the Salween, Tsangpo rivers. Clade 7

comprises species inhabiting in eastern Tibetan Plateau drainages (the upper Yangtze River, the Yellow River,

Tsaidam Basin and Hexi Corridor).

2.3 Divergence times among lineages

A log-likelihood ratio test rejected the null hypothesis of rate constancy of evolution among schizothoracine fishes ($-\ln L_{\text{nonclock}} = 5681.3985$, $-\ln L = 5788.2195$, $\chi^2 = 213.6420$, $df = 80$, $P = 0.00$). The lineage corresponding to *Gd. integrigymnatus* has a somewhat longer branch than those of other schizothoracine fishes. Instead, divergence times on tree were estimated using the non-parametric rate smoothing (NPRS) method. The calibration point was corrected using the dated geological event (1.1 Ma), which separated *H. microcephalus* from *S. pylzovi*. Tentative divergence dates for major cladogenetic events were as follows: the highly specialized grade schizothoracine fishes and *Gd. integrigymnatus* may have originated in the early Pliocene, and *O. stewartii* diverged from *Gd. integrigymnatus* + *Gymnocypris* + *Schizopygopsis* + *Platypharodon* + *Chuanchia* + *Herzensteinia* in 4.94–4.75 Ma (95% confidence limited). The divergence between clade 3 and the remaining highly specialized grade schizothoracine fishes occurred in 4.12–3.95 Ma. Two major lineages were split in 3.71–3.59 Ma. Major speciation event mainly occurred during the Pleiocene (Figure 3). The frequencies of cladogenetic events prior to 0.7 Ma are shown in Figure 4, indicating that the cladogenetic events occurred in the following periods: 3.6–3.5, 3.0–2.9, 2.7–2.4, 1.7–1.6 and 1.3–0.9 Ma.

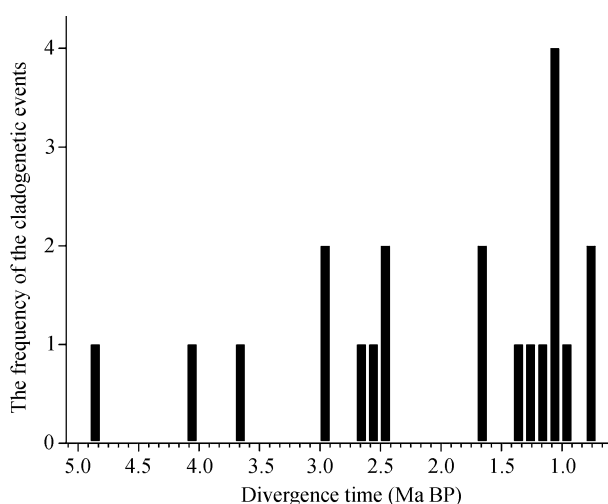


Figure 4 The frequencies of the cladogenetic events prior to 0.7 Ma based on molecular estimated divergence times for the highly specialized schizothoracine fishes.

3 Discussion

3.1 Phylogenetic relationships

Nearly all previous researches [2,4,15] on estimate relationships among genera of the subfamily Schizothoracinae, based on morphological characters, have attained similar conclusions. That is, (1) the specialized and highly specialized grade schizothoracine fishes formed a monophyletic group, respectively. (2) The genera *Gymnocypris* and *Oxygymnocypris* formed monophyly, respectively, and they were the sister group relationship. The phylogenetic relationships based on *cyt b* sequences are not fully consistent with the results deduced from morphological comparisons. *Gd. integrigymnatus* is clustered with the lineage of the high-specialized grade schizothoracine fishes. In addition, neither genera *Gymnocypris* nor *Schizopygopsis* is monophyly.

Four hypotheses (but not mutually exclusive) might help to explain the inconsistency between traditional taxonomy based on morphology and molecular phylogeny. (1) Traditional taxonomy might use some morphological characters that do not trace the evolutionary history of the groups but are shaped by adaptations to environmental conditions. As already noted by several researchers [8,27], the morphological characters used to infer phylogenetic relationships within cyprinids are often not informative, and this is especially true at and below the species level. (2) The low level of sequence divergence and ancestral polymorphism might have caused confusion to discriminate species. Species level phylogenies are often difficult to reconstruct due to the low level of genetic divergence, hence sufficient synapomorphies for establishing reliable sister group relationships are lacking. It is the case in this study, expect for the *O. stewartii*, sequence divergence levels of the other species in the highly specialized grade schizothoracine fishes are less than 7.72%. Simulation studies have shown that the accuracy of the tree reconstruction declines fast with low substitution rates [28]. (3) The highly specialized grade schizothoracine fishes might undergo a rapid evolution, expansion and diversification process, which occurred over a shorter time period than is required for the appearance and fixation of a novel mutation. The geological studies have indicated that the Tibetan Plateau entered into a rapidly changing period 3.6 Ma ago [29,30], which promoted the differentiation of schizothoracine fishes. Moreover, fishes in plateau usually have a rela-

tively “loose” niche, which facilitates species speciation. However, few of these rapid morphological differentiations have been reflected in their molecular variation. (4) The mitochondrial introgressive hybridization between different lineages might have played an important role in species evolution, given that this phenomenon is particularly frequent in cyprinids^[5,31]. The schizothoracine is notorious for hybridization, both interspecific and intergeneric. Furthermore, the schizothoracine might have suffered several complex introgression events due to connections of river systems. Similar phenomena are also found in other freshwater fishes^[32–34]. It has long been recognized that the river systems of the Tibetan Plateau had undergone large reorganization and capture events associated with the initiation of Miocene uplifts in Tibet^[35,36]. The temporary connections between river systems had resulted in migration and genetic exchanges of fishes between lineages. In the Yalongjiang, Yellow and Golmud rivers, phylogenetic analysis revealed that introgressive hybridization caused by river capture and temporary connection, e.g. *S. malacanthus malacanthus* from Mangkang, was closely related to those from Mangkang, while the specimens from Xinduqiao, had an affinity to those from the Minjiang River. Similar phenomena are detected in the genera *Gymnocypris* and *Schizopygopsis* between the upper Yellow River and Tsaidam Basin.

Chen^[37] attributed a new species collected from the upper Longchuanjiang River in Yunnan Province to genus *Gymnodiptychus* due to its one pair of barbels. However, our results obtained from mtDNA revealed that this specie is more closely related to the highly specialized grade schizothoracine fishes than the other two species of *Gymnodiptychus*. Moreover, with another two DNA sequences, D-loop and S-7 genes (our unpublished data), similar results were obtained. Although we do not exclude here the possibility that *Gd. integrigymnatus* has a closer relationship with *Gymnodiptychus*, at least, the present molecular data revealed that it should not be assigned to genus *Gymnodiptychus*. The results suggest that *Gd. integrigymnatus* might be an intergrade species from specialized to highly specialized grade in the evolutionary process of schizothoracine fishes. This hypothesis might be further supported by the morphological characters and current distribution pattern of *Gymnodiptychus*. The characters of *Gd. integrigymnatus* distinctly distinguishes from other species of genus *Gym-*

nodiptychus in having small size, minimus barbels, narrower folds of lower lip, gray peritoneum and less gill rakers of the first arch. Furthermore, *Gd. integrigymnatus* only distributes in the Longchuanjiang River, a tributary of the upper Irrawaddy River in border of southern Tibetan Plateau, while the other species distribute in the drainages of eastern Tibetan Plateau and Central Asia.

Chu^[38] erected the genus *Herzensteinia* by type-species *S. microcephalus* (Herzenstein, 1891). Considering the broad variations of the species in major diagnostic characters, e.g. row number of pharyngeal teeth, horny edge of lower jaw, Wu and Wu^[39] pointed out that it is not reasonable to erect genus *Herzensteinia* only by limited specimens from local region and limited characters. Thus, they proposed to attribute *H. microcephalus* to genus *Schizopygopsis*. In addition, they further suggested that *S. microcephalus* should be classified as a polytype including two subspecies *S. microcephalus microcephalus* and *S. m. namensis*. The phylogenetic analysis of the cyt *b* data did not support the validity of genus *Herzensteinia*. However, our data also showed that *H. microcephalus* is more closely related to *S. pylzovi*, and *S. m. namensis* (*G. namensis*), and *S. microcephalus* (*H. microcephalus*) should be treated as separate species.

3.2 Biogeography and river systems evolution on the Tibetan Plateau

For mtDNA protein coding genes of teleostean, studies using a fossil-based or other reliable calibration indeed suggest divergence rates of 0.5%–1.3% per million years^[6,8,40–48]. Substitution rates in cyprinid fishes are usually adopted as 0.76%^[6], 0.66%^[42], 0.91%^[10] or standard substitution rate 1.0% per million years^[8]. The substitute rates were obtained using various substitution models, taxa and molecular markers; thus they may not all be exactly comparable. In this study, we used the geological event of Kunlun Mountains Pass to calibrate the divergent time of *H. microcephalus* and *S. pylzovi*^[25]. In the Kunlun Mountains Pass area, a violent tectonic movement occurred between 1.1–0.7 Ma. This movement led to a large-scale uplifting first, and then fault-block rising and fault depression occurred suddenly in the northern Plateau. We proposed that the uplift of the Kunlun Mountains Pass area might have separated the species *H. microcephalus* from *S. pylzovi*. The average levels of sequence divergence between *H. mi-*

crocephalus and *S. pylzovi* is 1.85%, corresponding to a divergence rate of 1.68% per million years. The divergence rate is well consistent with 1.64% per million years of European cyprinids that have been widely used in recent studies on freshwater fishes^[6,44–47].

The major cladogenetic events of the highly specialized schizothoracine fishes well coincided with the late Cenozoic uplift phases in the Tibetan Plateau. The geological studies indicated that Tibetan Plateau has entered a rapid uplift period since 3.6 Ma ago, including three major phases. The early stage, the ‘Qingzang (Tibet) Movement’, occurred between 3.6–1.7 Ma and included A, B and C phases commencing at 3.6, 2.5, and 1.7 Ma, respectively^[48]. The middle stage is the ‘Kunlun-Huanghe Movement’, which occurred between 1.1–0.6 Ma and included three phases starting at 1.1, 0.8 and 0.6 Ma, respectively^[25]. The late stage, the ‘Gonghe Movement’ took place 0.15 Ma ago^[48]. Molecular estimates of divergence times revealed that the major cladogenetic events of the highly specialized schizothoracine fishes occurred at these phases: 3.6–3.5, 3.0–2.9, 2.7–2.4, 1.7–1.6, 1.3–0.9 Ma (Figure 4), as is well consistent with geological assumptions of the rapid uplift phases of Tibetan Plateau. Our data supported the assumption that the origin and evolution of schizothoracine were in close correlation with marked environmental changes caused by violent upheaval of the Plateau^[2]. In short, the ultimate factors that give rise to the current distribution pattern of the extant schizothoracine were the environment changes and river systems transitions due to the uplift of plateau.

The tentative divergence date for two main clades of the highly specialized grade schizothoracine fishes occurred in 3.71–3.59 Ma, coinciding with the splitting of *Gd. pachycheilus* and *Gd. dybowskii* (3.54±0.39 Ma)^[10]. The data is also consistent with the lineages divergence of genus *Triplophysa* occurring in Xinjiang vs. the eastern Tibetan Plateau^[49], as well as the divergence between the “alpine shrub” vs. “coniferous forest” (3.4 Ma) within *Nannoglottis*, a genus endemic to the Tibetan Plateau^[50]. The coincidence of divergence times estimated from different taxa may be a shared response to a common geological event. The Tibetan Plateau has entered a large-scale uplifting phase since 3.6 Ma ago, which ended the continuous plain topography formed in the early Pliocene and resulted in huge environmental changes in the central Tibetan Plateau.

Differing from the cladogenetic event that occurred in 3.6 Ma, the divergences that occurred in 3.0–2.4 Ma might reflect the separation between river systems in the Tibetan Plateau, indicative of the affiliation and evolution of different drainages in history. At that period, the planation surface in the middle-late Pliocene epoch was disintegrated. The separation of major drainages in and surrounding Tibet ensued the tectonic uplift, including the western drainages (Bangong Co, Indus and Mapam Yumco Co) (3.04–2.94 Ma), the Salween-Mekong rivers (2.66–2.58 Ma), the central drainages (Inland lakes in the northern Tibet, the Tsangpo, Salween and southern Tibet) (2.52–2.45 Ma) and the drainages of eastern Tibetan Plateau (Hexi Corridor, Tsaidam basin, the upper Yellow and Yangtze rivers). The isolations between lineages in different drainages implied that the Tibetan Plateau further rose as a whole, but the heights at different parts of the Plateau are not equal at all, which have caused further evolution of fishes. However, within four areas (the Salween-Mekong, western, central and eastern Tibet), fishes might still exchange between different drainages till the “Kunlun-Huanghe Movement” in 1.1–0.7 Ma due to temporary river connection in the ice age and interglacial period in the Pleistocene and the difference in sequence and time between rivers headward erosion. In the western Tibetan Plateau, the Banggan Co, La’nga Co, Mapam Yumco and Nyogze were lakes with out-flow to Indus^[51]. In the central Tibetan Plateau, the precipitation increased due to the strengthening monsoon^[52]. As a result, high lake level emerged widely, most of which had out-flow to the Tsangpo or Salween rivers.

The Salween and Mekong rivers (2.03–1.97 Ma) were the last to separate among major drainages on the Tibetan Plateau, indicating that the two rivers might continued exchanging till the early or middle Pleistocene. The zoogeographical studies of the Hengduan Mountains region, based on analysis of the similarities of ichthyofauna between rivers, showed that there is an affinity between Salween and Mekong rivers^[37,53]. Moreover, there was an affinity for the drainages in the eastern Tibetan Plateau, including the upper Yangtze, Yellow and inland rivers in the Tsaidam Basin. The development of the Yellow River commenced 1.6 Ma ago. Thereafter, the Yellow River went through the present headwaters area by a series of tectonic events and river captures^[48]. We concluded that the ancestral headwater of the Yel-

low River possibly flowed along the present Zoigê Basin and outflowed into the tributary of the Yangtze River in Sichuan Basin before the Yellow River cut through the Zoigê Basin, while the headwaters of the Yellow River were headward captured by the inland rivers of the Tsaidam Basin.

It is noticeable that current data show that *G. chilienensis* distribution in Hexi Corridor is closely related to *S. kialingensis* distributing in the Jialingjiang River. Molecular estimates of the divergence times allowed us to date the splitting between these two species back to 0.75–0.72 Ma ago. Chen^[37] revealed similar results when analyzing the similarity of ichthyofauna in 11 river systems in the Hengduan Mountains region. That is, the Jialingjiang River has an affinity to the Yellow River

according to the analysis of all 237 species or genus *Triplophysa* fishes. We suggested that the glaciers in the major mountains probably played an important role in linking different river systems in the eastern Tibetan Plateau during the largest ice age in the middle Pleistocene. The headwaters of rivers connected temporarily when ice melted during inter-glacial period, which resulted in the dispersal of fishes to adjacent drainages. This conclusion is supported further by the current distribution patterns of genus *Triplophysa* and schizothoracine fishes, e.g. *Gd. pachycheilus* distributes in the upper Yellow and Yalongjiang rivers, a tributary of the Yangtze River, and *T. pseudoscleroptera* distributes in the Tsaidam Basin, the upper Yellow River and tributaries of the Yangtze River in western of Sichuan.

- Nelson G, Platnick N. Biogeography. Carolina Bio Readers, 1984, 119: 1–20
- Cao W X, Chen Y Y, Wu Y F, et al. Origin and evolution of schizothoracine fishes in relation to the upheaval of the Xizang Plateau. In: The Comprehensive Scientific Expedition to the Qinghai-Xizang Plateau, Chinese Academy of Sciences, ed. Collection in Studies on the Period, Amplitude and Type of the Uplift of the Qinghai-Xizang Plateau (in Chinese). Beijing: Science Press, 1981. 118–130
- Chen Y F, Cao W Y. Schizothoracinae. In: Yue P, ed. Fauna Sinica, Osteichthyes, Cypriniformes III (in Chinese). Beijing: Science Press, 2000. 273–335
- Zardoya R, Meyer A. Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. *Mol Biol Evol*, 1996, 13: 933–942
- Briolay J, Galtier N, Brito R M, et al. Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. *Mol Phylogenet Evol*, 1998, 9: 100–108[DOI]
- Zardoya R, Doadrio I. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *J Mol Evol*, 1999, 49: 227–237[DOI]
- Tsigenopoulos C S, Berrebi P. Molecular phylogeny of North Mediterranean freshwater barbs (Genus *Barbus*: Cyprinidae) inferred from cytochrome *b* sequences: Biogeographic and systematic implications. *Mol Phylogenet Evol*, 2000, 14(2): 165–179[DOI]
- Durand J, Tsigenopoulos C, Ünlü E, et al. Phylogeny and biogeography of the family Cyprinidae in the Middle East inferred from cytochrome *b* DNA -evolutionary significance of this region. *Mol Phylogenet Evol*, 2002, 22: 91–100[DOI]
- He S P, Liu H Z, Chen Y Y, et al. Molecular phylogenetic relationships of Eastern Asian Cyprinidae (Pisces: Cypriniformes) inferred from cytochrome *b* sequences. *Sci China Ser C-Life Sci*, 2004, 47(2): 130–138
- He D K, Chen Y F, Chen Y Y, et al., Molecular phylogeny of the specialized grade schizothoracine fishes (Teleostei: Cyprinidae), with their implications for the uplift of the Qinghai-Tibetan Plateau. *Chin Sci Bull*, 2004, 49(1), 39–48
- Sambrook J. Molecular Cloning: A Laboratory Manual, 3rd ed. (Translated by Huang P T, et al.). Beijing: Science Press, 2002. 463–618
- Thompson J D, Gibson T J, Plewniak F, et al. The Clustal X windows interface: Flexible strategies for multiple sequences alignment aided by quality analysis tools. *Nucleic Acids Res*, 1997, 25: 4876–4882 [DOI]
- Swofford D L. PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4. Massachusetts: Sinauer Associates, 2002
- Wu Y F. Studies on phylogeny of subfamily Schizothoracinae (Pisces, Cypriniformes, Cyprinidae) in China. *Acta Biol Plat Sin* (in Chinese), 1984, 3: 119–140
- Chen Y F. Phylogeny and distributional patterns of subfamily Schizothoracinae (Pisces, Cypriniformes, Cyprinidae) I. Phylogenetic Relationships. *Acta Zootaxon Sin* (in Chinese), 1998, 23 (Spec J Zoogeogr): 17–25
- Posada D, Crandall K A. Modeltest: Testing the model of DNA substitution. *Bioinformatics*, 1998, 14: 817–818[DOI]
- Yang Z H. Estimating the pattern of nucleotide substitution. *J Mol Evol*, 1994, 39(1): 105–111
- Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 1985, 39: 783–791[DOI]
- Huelsenbeck J P, Ronquist F. MrBayes: Bayesian inference of phylogeny. *Bioinformatics*, 2001, 17(8): 754–755[DOI]
- Shimodaira H, Hasegawa M. Multiple comparisons of loglikelihoods with applications to phylogenetic inference. *Mol Biol Evol*, 1999, 16(8): 1114–1116
- Huelsenbeck J P, Rannala B. Phylogenetic methods come of age: Testing hypotheses in an evolutionary context. *Science*, 1997, 276(11):

- 227–232[DOI]
- 22 Sanderson M. A nonparametric approach to estimate divergence times in the absence of rate constancy. *Mol Biol Evol*, 1997, 14: 1218–1231
- 23 Rambaut A, Charleston M. TreeEdit. Version 1.0. London: University of Oxford, 2001
- 24 Zhao K, Li J, Yang G, et al. Molecular phylogenetics of *Gymnocypris* (Teleostei: Cyprinidae) in Lake Qinghai and adjacent drainages. *Chin Sci Bull*, 2005, 50(13): 1325–1333
- 25 Cui Z, Wu Y, Liu G, et al. On Kunlun–Yellow River tectonic movement. *Sci China Ser D–Earth Sci*, 1998, 41(2): 592–600
- 26 Iwin D M, Kocher T D, Wilson A C. Evolution of the Cytochrome *b* gene of mammals. *J Mol Evol*, 1991, 32: 128–144[DOI]
- 27 Ketmaier V, Bianco P G, Cobolli M, et al. Molecular phylogeny of two lineages of Leuciscinae cyprinids (*Telestes* and *Scardinius*) from the peri-Mediterranean area based on cytochrome *b* data. *Mol Phylogenet Evol*, 2004, 32: 1061–1071[DOI]
- 28 Yang Z. On the best evolutionary rate for phylogenetic analysis. *Syst Biol*, 1998, 47: 125–133[DOI]
- 29 Li J J, Fang X M. Uplift of the Tibetan Plateau and environmental changes. *Chin Sci Bull*, 1998, 44(23): 2117–2125
- 30 Li J J, Fang X M, Pan B T. Late Cenozoic intensive uplift of Qinghai-Xijiang Plateau and its impacts on environments in surrounding area. *Quaternary Sci (in Chinese)*, 2001(5): 381–391
- 31 Berrebi P. Speciation of the genus *Barbus* in the north Mediterranean basin: Recent advances from biochemical genetics. *Biol Conserv*, 1995, 72: 237–249[DOI]
- 32 Brito R M, Briolay J, Galtier N, et al. Phylogenetic relationships within genus *Leuciscus* (Pisces, Cyprinidae) in Portuguese freshwaters, based on mitochondrial DNA cytochrome *b* sequences. *Mol Phylogenet Evol*, 1997, 8: 435–442[DOI]
- 33 Kotlík P, Berrebi P. Phylogeography of the barbell (*Barbus barbus*) assessed by mitochondrial DNA variation. *Mol Ecol*, 2001, 10: 2177–2185[DOI]
- 34 Mateos M, Sanjur O I, Vrijenhoek R C. Historical biogeography of the livebearing fish genus *Poeciliopsis* (Poeciliidae: Cyprinodontiformes). *Evolution*, 2002, 56(5): 972–984[DOI]
- 35 Brookfield M E. The evolution of the great river systems of southern Asia during the Cenozoic India-Asia collision: rivers draining southwards. *Geomorphology*, 1998, 22: 285–312[DOI]
- 36 Clark M K, Schoenbohm L M, Royden L H, et al. Surface uplift, tectonics, and erosion of eastern Tibet from largescale drainage patterns. *Tectonics*, 2004, 23, TC106, doi:10.1029/2002TC001402
- 37 Chen Y Y. The Fishes of the Hengduan Mountains Region (in Chinese). Beijing: Science Press, 1998
- 38 Chu Y T. Comparative studies on the scales and on the pharyngeals and their teeth in Chinese Cyprinids, with particular reference to taxonomy and evolution. *Biol Bull St John's Univ*, 1935, 2: 1–255
- 39 Wu Y F, Wu C Z. The Fishes of the Qinghai-Xizang Plateau (in Chinese). Chendu: Sichuan Publishing House of Science & Technology, 1992
- 40 Martin A P, Palumbi S R. Body size, metabolic rate, generation time and the molecular clock. *Proc Natl Acad Sci USA*, 1993, 90: 4087–4091[DOI]
- 41 Bermingham E, McCafferty S S, Martin A P. Fish biogeography and molecular clocks: Perspectives from the Panamanian Isthmus. In: Kocher T D, Stepien C A, eds. *Molecular Systematics of Fishes*. San Diego: Academic Press, 1997. 113–128
- 42 Machordom A, Doadrio I. Evidence of a Cenozoic Betic-Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). *Mol Phylogenet Evol*, 2001, 18: 252–263[DOI]
- 43 Perdices A, Doadrio I. The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. *Mol Phylogenet Evol*, 2001, 19: 468–478[DOI]
- 44 Sanjur O I, Carmona J A, Doadrio I. Evolutionary and biogeographical patterns within Iberian populations of the genus *Squalius* inferred from molecular data. *Mol Phylogenet Evol*, 2003, 29: 20–30[DOI]
- 45 Perdices A, Bermingham E, Montilla A, et al. Evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America. *Mol Phylogenet Evol*, 2002, 25: 172–189[DOI]
- 46 Perdices A, Doadrio I, Economidis P S, et al. Pleistocene effects on the European freshwater fish fauna: Double origin of the cobitid genus *Sabanejewia* in the Danube basin (Osteichthyes: Cobitidae). *Mol Phylogenet Evol*, 2003, 26: 289–299[DOI]
- 47 Hrbek T, Stölting K N, Bardakci F, et al. Molecular Plate tectonics and biogeographical patterns of the *Pseudophoxinus* (Pisces: Cypriniformes) species complex of central Anatolia, Turkey. *Mol Phylogenet Evol*, 2004, 32: 297–308[DOI]
- 48 Li J J, Fang X M, Ma H Z, et al. Geomorphological and environment evolution in upper reaches of Yellow River during the late Cenozoic. *Sci China Ser D–Earth Sci*, 1996, 39(4): 380–390
- 49 He D K, Chen Y X, Chen Y F. Molecular phylogeny and biogeography of the genus *Triplophysa* (Osteichthyes: Nemacheilinae) in the Tibetan Plateau inferred from cytochrome *b* DNA sequences. *Prog Nat Sci (in Chinese)*, 2006, 16(11): 1395–1404
- 50 Liu J Q, Gao T G, Chen Z D, et al. Molecular phylogeny and biogeography of the Qinghai-Tibet Plateau endemic *Nannoglottis* (Asteraceae). *Mol Phylogenet Evol*, 2002, 23: 307–325[DOI]
- 51 Chen Z. Approaches to the changes of ecological environment of lakes in Xizang based on the upheaval of the Qinghai-Xizang Plateau. *Oceanol Limnol Sin (in Chinese)*, 1981, 12(5): 402–411
- 52 Shen J, LÜ H, Wang S, et al. A 2.8 Ma record of environmental evolution and tectonic events inferred from the Cuoe core in the middle of Tibetan Plateau. *Sci China Ser D–Earth Sci*, 2004, 47(11): 1025–1034
- 53 Chu X L, Chen Y R. The Ichthyofauna of Yunnan (II) (in Chinese). Beijing: Science Press, 1989