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Mitochondrial DNA phylogeny of two morphologically enigmatic fishes in the subfamily Schizothoracinae (Teleostei: Cyprinidae) in the Qinghai-Tibetan Plateau

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The complete mitochondrial DNA (mtDNA) cytochrome *b* gene (1140 bp) was sequenced in *Herzenstein macrocephalus* and *Gymnocypris namensis* and in 13 other species and sub-species ($n = 22$), representing four closely related genera in the subfamily Schizothoracinae. Conflicting taxonomies of *H. macrocephalus* and *G. namensis* have been proposed because of the character instability among individuals. Parsimony, maximum likelihood and Bayesian methods produced phylogenetic trees with the same topology and resolved several distinctive clades. Previous taxonomic treatments, which variously placed these two species of separate genera or as sub-species, are inconsistent with the mtDNA phylogeny. Both *H. macrocephalus* and *G. namensis* appear in a well-supported clade, which also includes nine species of *Schizopygopsis*, and hence should be transferred to the genus *Schizopygopsis*. Morphological changes are further illustrated, and their adaptive evolution in response to the local habitat shifts during the speciation process appears to be responsible for conflicting views on the systematics of these two species and hence the contrasting taxonomic treatments. These species are endemic to the Qinghai-Tibetan Plateau, a region with a history of geological activity and a rich diversity of habitats that may have result in the parallel and reversal evolution of some morphological characters used in their taxonomies. Our results further suggest that speciation and morphological evolution of fishes in this region may be more complex than those previously expected.

Key words: evolution; *Gymnocypris namensis*; *Herzenstein macrocephalus*; morphology; phylogeny; taxonomy.

INTRODUCTION

The Qinghai-Tibetan Plateau (QTP) is the World's largest high-elevation ecosystem, occupying nearly 2·5 million km^2, with an average elevation >4000 m a.s.l. (Zheng, 1996). This region, along with southeast China, the Himalayan biodiversity hotspot, has been designated as one of the World's 34 most important centres of biodiversity because of it high species richness and abundance of endemic

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species (Wilson, 1992; Myers *et al.*, 2000). An enduring question has been to understand the mechanisms leading to these high diversities and the processes producing endemics (Liu *et al.*, 2002, 2006), often with unusual morphologies (Wang *et al.*, 2005).

The subfamily Schizothoracinae has been a model group for testing hypotheses of speciation that explain these high levels of species richness, especially a possible correlation between diversification and geological uplift of the QTP (Cao *et al.*, 1981; Wu, 1984; Wu & Wu, 1991). This subfamily consists of 15 genera, including about 100 species (Mirza, 1991). More than 70 species of 12 of these genera are limited to the QTP (Wu, 1984; Wu & Wu, 1991). Although most of these endemics are easily assigned to well-circumscribed genera in Schizothoracinae, the systematic positions of two species, *Herzenstein microcephalus* (Herzenstein, 1891) and *Gymnocypris namensis* (Wu & Ren, 1982), have been disputed (Wu, 1964, 1984; Wu & Wu, 1991; Yue *et al.*, 2000). The taxonomies of these two species have been subjected to several contradictory revisions. *Herzenstein microcephalus* is distributed in the upper reaches of the Yangtze River and bears one unique trait in the Schizothoracinae, namely one row of pharyngeal teeth. This monotypic genus was established by Chu (1935), accepted by Wu (1964) but later rejected by Wu (1984) and Wu & Wu (1991). The latter authors re-assigned this species to the genus *Schizopygopsis* (Steindachner, 1866), as first described by Herzenstein (1891), because of similarities in the arrangement of pharyngeal teeth and the presence of outer horny sheaths on the lower jaw. However, Yue *et al.* (2000) resurrected the genus *Herzenstein* in a recent revision of Schizothoracinae and suggested that the unique morphology of this species indicated an early divergence from *Schizopygopsis*. *Gymnocypris namensis* is similar to *H. microcephalus* in having one row of pharyngeal teeth for most individuals (a few retaining two rows). This species, distributed in Nam Co Lake (Fig. 1), was first described by Ren & Wu (1982) under *Schizopygopsis* but later treated as a sister sub-species with *H. microcephalus* within *Schizopygopsis* (Wu & Wu, 1991). Yue *et al.* (2000), however, retained *G. namensis* within *Gymnocypris* (Günther, 1868). Although their distributions appear close, these two species are separated by the high Tanggula Mountains (*c.* 5200 m) and the habitats are different (lacustrine lake for *G. namensis v.* fluvial river for *H. microcephalus*). These two species show a considerable amount of morphological distinctness in diagnostic characters (Wu & Wu, 1991; Yue *et al.*, 2002). For example, *G. namensis* has both inner and outer horny sheaths, while all examined individuals of *H. microcephalus* have only outer types (Wu & Wu, 1991). Also, the mouth positions of *G. namensis* are subterminal or terminal, while all individuals of *H. microcephalus* have inferior mouths.

Some of the characters used for taxonomic assignments of these two species, as well as species of *Schizopygopsis*, including absence of scales, arrangement of pharyngeal teeth, mouth positions, sheaths on the lower jaw and abundances of mucus pores on the cheek and chin, were suggested to result from early adaptive evolution to the high altitude of the Schizothoracinae when this group diverged with its low altitude sister group (Cao *et al.*, 1981; Wu, 1984, Wu & Wu, 1991). However, this lineage must have experienced the Quaternary glacial and interglacial cycles, during which the lakes or rivers shrunk or expanded repeatedly with the developments and recessions of ice sheets (Shi *et al.*, 1998). In addition, the local habitats of Schizothoracinae fishes are also diverse in this

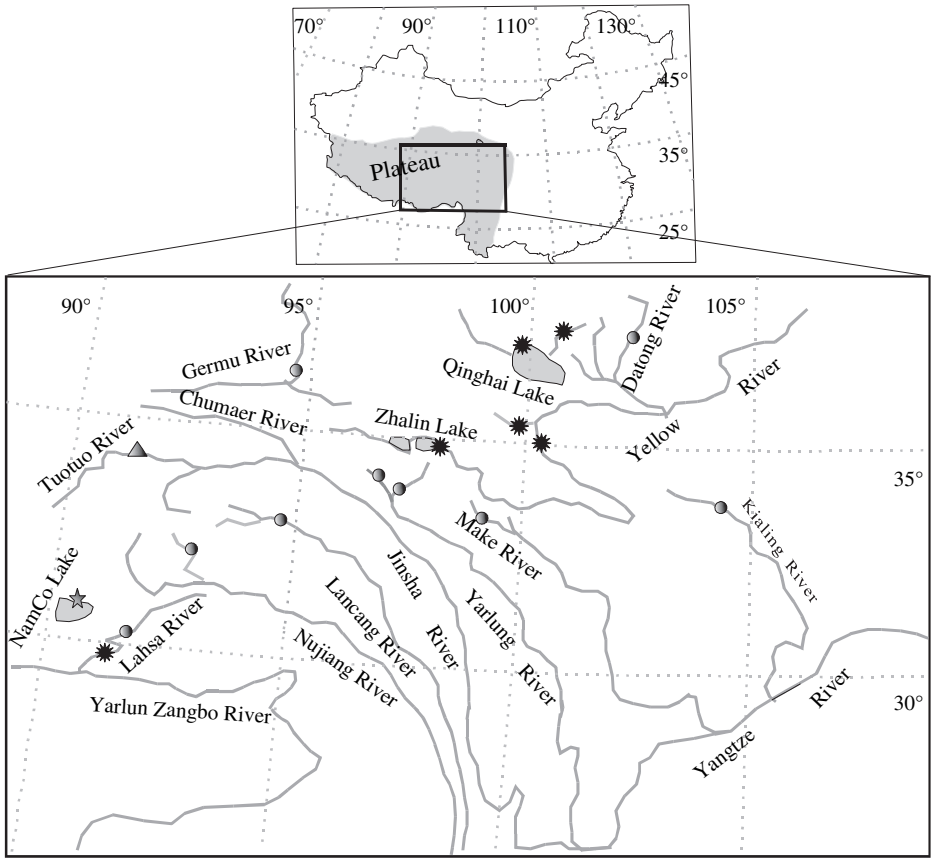

FIG. 1. Sample locations of taxa in the Schizothoracinae. The shaded area in the upper map indicates the Qinghai-Tibetan Plateau. ★, *G. namensis*; ▲, *H. microcephalus*; ●, *Schizopygopsis*; ✸, species from the other genera.

region, for example, fluvial drainages *v.* lacustrine lakes. All these historical and habitat shifts might have caused repeated loss and gain of some adaptive morphological traits during speciations within this subfamily. However, this possible scenario has received little attention.

The analysis of molecular genetic variation can potentially help resolve these taxonomic problems and illustrate morphological evolution because molecular variants are considered to be largely neutral to the selective forces, producing morphological convergence. DNA sequences have been useful for reconstructing phylogenies, understanding the course of evolution and providing a basis for taxonomic assignment (Groves & Shields, 1996; Peng *et al.*, 2004; Guo *et al.*, 2005). Molecular phylogenies are especially useful for untangling the taxonomies of species, for which morphological data are lacking or ambiguous, or of species experiencing parallel and reversal evolution of the same adaptive morphology (Xiao *et al.*, 2001; Bernard & Michael, 2004).

The goal of this study is to construct a molecular phylogeny for *H. microcephalus*, *G. namensis* and several closely related species from complete sequences

of the mitochondrial DNA (mtDNA) cytochrome *b* gene (cyt *b*). A recent study of molecular variation in Schizothoracinae fishes showed that *G. namensis* was nested within *Schizopygopsis* (He *et al.*, 2004). However, this study did not include *H. microcephalus*, so the relationship with *G. namensis* remains unresolved. A more complete molecular phylogeny will be important in illustrating morphological evolutions in the Schizothoracinae and in resolving the taxonomic standings of *H. microcephalus* and *G. namensis*. In addition, such a phylogeny and evolutionary paths of morphological may highlight the speciation processes of fishes occurring in this region.

MATERIALS AND METHODS

SAMPLE COLLECTION

Wild individuals of *H. microcephalus* and *G. namensis* were collected in the field at locations in the QTP (Fig. 1). An additional 13 species and sub-species representing four closely related genera in the subfamily Schizothoracinae were included in the present study. These species dwell in two typical habitats: lacustrine lake and fluvial river. Except for resolving both taxonomic conflicts of the two species, the constructed phylogeny was further designed to map morphological characters to these two special habitats. *Gymnodiptychus pachycheilus* (Herzenstein, 1892) within the subfamily Schizothoracinae was chosen as an out-group. Previous studies suggested that this genus is the sister group to the sampled genera (Wu & Wu, 1991; He *et al.*, 2004).

Muscle tissue was dissected from field-caught fish, immediately frozen in liquid nitrogen and stored at −70° C until DNA extraction. Voucher specimens were deposited in the Fishery Environmental Monitoring Station of Qinghai Province.

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

Thawed tissues were digested with proteinase K, and total DNA was extracted using a standard phenol–chloroform protocol. The complete cyt *b* gene was amplified by the polymerase chain reaction (PCR) using the primer pairs LI4724 (5′-GACTTGAAAACCACCGTTG-3′) and H15915 (5′-CTCCGATCTCCGGATT-ACAAGAC-3′) (Xiao *et al.*, 2001). PCR amplifications were performed in total reaction volumes of 30 μl, containing 1·0 U TaKaRa Ex Taq (TaKaRa Corp., Dalien, China), 1·0 μl dNTP mix (2·5 nM each), 3·0 μl 10×Taq buffer (TaKaRa), 0·5 μl of each primer (10 nM) and 0·1 μg of total genomic DNA. The PCR cycle consisted of an initial denaturation at 95° C for 4 min, followed by 35 cycles at 94° C for 1 min, 50° C for 1 min and 72° C for 1 min, and a final extension at 72° C for 5 min. PCR products were purified using a CASpure PCR purification kit following the manufacturer's protocol (Casarray, Shanghai, China). The sequencing reactions were carried out in a Biometra thermocycler using a DYEnamic dye terminator cycle sequencing kit (Amersham Biosciences Corp., Piscataway, NJ, USA) following the manufacturer's protocol. Purified DNA fragments were directly sequenced using a MegaBACE 500 DNA Analysis System following the manufacturer's instructions. To ensure accuracy, strands were sequenced in both directions for each individual. Both DNA strands were checked for ambiguous base assignments.

SEQUENCE ALIGNMENT AND DATA ANALYSIS

Sequences were aligned by ClustalX (Thompson *et al.*, 1997) with default settings and adjusted by eye. The extent of the cyt *b* gene was determined by comparison with sequences of other genera of Schizothoracinae in GenBank. New sequences were

deposited in GenBank under the accession numbers shown in Table I. Nucleotide composition and Kimura's two-parameter sequence divergences (Kimura, 1980) between haplotypes were calculated by MEGA 3.·1 (Kumar *et al.*, 2004).

Phylogenetic trees were constructed from sequences with maximum parsimony (MP), maximum likelihood (ML) and Bayesian methods. MP trees were constructed with a heuristic search, 100 replicates of random additions of sequences, equally weighted characters and nucleotide transformations, ACCTRAN character optimization, MULPARS+TBR branch swapping and STEEPEST DESCENT options (Maddison, 1991) with PAUP* 4.0b10 (Swofford, 2000). The ML analysis was performed under the best-fit model TrN+G selected by MODELTEST 3.06 (Posada & Crandall, 1998) based on the likelihood ratio tests. Settings for this model were as follows: Base = (0·265, 0·284, 0·155), Nat = 6, Rmat = (1·000, 25·751, 1·000, 1·000, 8·194), Rates = gamma, Shape = 0·160 and Pinvar = 0. A heuristic search with simple addition of sequences and TBR branch swapping, MULTREES and COLLAPSE were used to produce ML trees. The robustness of the phylogenetic hypotheses was tested by 1000 and 100 bootstrap replicates for MP and ML, respectively. For Bayesian trees (Huelsenbeck & Ronquist, 2001), four simultaneous Monte-Carlo Markov Chains of 5 000 000 steps were used, with a tree saved every 1000 steps. Posterior probabilities (PP, shown as percentages) indicated branch support (Huelsenbeck & Ronquist, 2001). The first 5000 trees were discarded and 45 001 trees (whose log-likelihoods converged to stable values) were used to construct a 50% majority rule consensus tree with PP. The number of burn-in steps was determined by visual inspection of log-likelihood values.

A hypothesis of *Schizopygopsis* monophyly was explicitly tested by constrained (monophyletic) and unconstrained ML trees. Similarly, a hypothesis of *Gymnocypris* monophyly was also tested. Alternative topologies were statistically compared using the Kishino–Hasegawa and Shimodaira–Hasegawa tests implemented in PAUP* (Kishino & Hasegawa, 1989; Shimodaira & Hasegawa, 1999). To illustrate the morphological evolution in this group, three diagnostic characters, *i.e.* lower jaw morphology, mouth position and arrangement of pharyngeal teeth, were mapped onto the ML tree using MacClade 3.04 (Maddison & Maddison, 1992).

To estimate divergence times, a likelihood ratio test with and without molecular clock constraints was conducted using PUZZLE 4.0.1 (Strimmer & von Haeseler, 1996). The likelihood ratio test could not reject the null hypothesis of molecular clock rate consistency when including all sequences ($-\ln L$ non-clock = 3711·84, $-\ln L$ clock = 3724·25, $\delta = 24{\cdot}81$, d.f. = 18; $P = 0{\cdot}13$). Subsequently, a molecular clock-constrained ML tree was constructed using PAUP* 4.0b10, and the branch lengths were used to estimate tentative divergence date.

RESULTS

SEQUENCE VARIATION

Twenty haplotypes were identified among 22 individuals based on complete sequences of cyt *b* gene. A total of 278 (24·4%) of the 1140 sites were variable. Of these, 147 (52·9%) were parsimony informative. No indels were observed among species. Most of the nucleotide polymorphisms (229 sites) occurred in the third codon position, 40 polymorphisms occurred in the first position and 9 in the second position. The base composition was on average A, 25·9%; T, 30·7%; C, 26·7% and G, 16·7% and showed an anti-G bias, which is characteristic for the mitochondrial genome (Cantatore *et al.*, 1994). Kimura's two-parameter sequence divergences between *G. namensis* and three other species of *Gymnocypris* ranged from 6·79% (*G. namensis v. Gymnocypris eckloni* Herzenstein, 1981) to 6·89% (*G. namensis v. Gymnocypris przewalskii* Kessler, 1876). However, sequence divergence between *G. namensis* and *Schizopygopsis*

TABLE I. Species, sampling locality, sample size, drainages, altitude, number of haplotypes and GenBank accession numbers

Species	Collection location (sample size)	Drainages	Altitude (m)	Number of haplotypes	GenBank accession number
Gymnocypris eckloni	Madoi, Qinghai (1)	Zhalin Lake (Yellow River)	4100	1	DQ309350
G. przewalskii przewalskii	Gonghe, Qinghai (1)	Qinghai Lake	3200	1	DQ309362
G. p. ganzihonensis	Haiyan, Qinghai (1)	Ganzi River (Qinghai Lake)	3340	1	DQ309363
G. namensis	Naqu, Tibet (1)	Nam Co Lake	4700	1	DQ309353
Herzenstein microcephalus	Tuotuohe, Qinghai (6)	Tuotuo River (Jinsha River)	4570	4	DQ309354–57
Oxygymnocypris stewartii	Lhasa, Tibet (1)	Lhasa River (Yarlung Zangbo River)	3700	1	DQ309358
Platypharodon extremus	Xinghai, Qinghai (1)	Yellow River	3400	1	DQ309349
Gymnodiptychus pachycheilus	Xinghai, Qinghai (1)	Yellow River	3400	1	DQ309348
Schizopygopsis kialingensis	Langmusi, Sichuan (1)	Kialing River (Yangtze River)	2400	1	DQ309364
S. kessleri	Geermu, Qinghai (1)	Geermu River	3200	1	DQ309366
S. chengi	Banma, Qinghai (1)	Make River (Yangtze River)	4100	1	DQ309351
S. malacanthus	Chengdu, Qinghai (1)	Yarlung River (Yangtze River)	4380	1	DQ309360
	Chengdu, Qinghai (1)	Qingshui River (Yarlung River)	4400	1	DQ309361
S. pylzovi	Datong, Qinghai (1)	Datong River (Yellow R.)	2800	1	DQ309359
S. anteroventris	Zadoi, Qinghai (1)	Jiqu River (Lancang River)	4250	1	DQ309365
S. younghusbandi	Lhasa, Tibet (1)	Lhasa River (Yarlung Zangbo River)	3700	1	DQ309352
Schizopygopsis thermalis	Tanggula, Qinghai (1)	Tanggula, Nujiang River	47 500	1	DQ309367

younghusbandi (Regan, 1905) was only 0·62%. Four haplotypes (*H. microcephalus* T1, *H. microcephalus* T2, *H. microcephalus* T3, *H. microcephalus* T4) were observed in *H. microcephalus*, with sequence divergences between these haplotypes and haplotypes in other species of *Schizopygopsis* ranging from 1·69% (*H. microcephalus* T2 *v. Schizopygopsis pylzovi* Kessler, 1876) to 5·04% (*H. microcephalus* T1 *v. S. younghusbandi*) (Table II).

PHYLOGENETIC ANALYSIS

A single MP tree (length = 445 steps, consistency index = 0·721, retention index = 0·757) was found. This tree had the same topology as the ML tree (−lnL = 3709·23) and the 50% majority rule consensus Bayesian tree (Fig. 2). In this topology, *G. namensis* clustered with *S. younghusbandi* with high statistical support (100% BP (Bootstrap value) for both the ML and the MP trees, and 100% PP for the Bayesian tree). The four haplotypes found in *H. microcephalus* appeared within a larger monophyletic clade with high statistical support (99% BP for the ML tree, 98% BP for the MP tree and 100% PP for the Bayesian tree), which also contained six species of *Schizopygopsis*. Within this clade, *H. microcephalus* was a sister taxon to a group consisting of *Schizopygopsis chengi* Fang, 1936, *S. pylzovi*, *Schizopygopsis kessleri* Herzenstein, 1891, *Schizopygopsis kialingensis*, *Schizopygopsis malacanthus* and *Schizopygopsis anteroventris* Wu, Tsao, Zhu & Chen, 1979. The clade consisting of *G. namensis* and *S. younghusbandi* was sister to the group formed by the six species of *Schizopygopsis* and *H. microcephalus*. These three clades included the well circumscribed *Schizopygopsis* with high statistical support (95% BP for the ML tree, 90% BP for the MP tree and 100% PP for the Bayesian tree). Three taxa of *Gymnocypris* clustered as a monophyletic clade (100% BP for both the ML tree and the MP tree and 100% PP for the Bayesian tree) and, together with *Platypharodon extremus* (Herzenstein, 1891), formed a sister group to the *Schizopygopsis* species and *H. microcephalus* and *G. namensis*.

When the monophyly of *Schizopygopsis* species was forced using the CONSTRAINTS function in PAUP*, the likelihood score (−lnL = 4069·94) was significantly larger than the unconstrained ML tree (−lnL = 3899·23) based on the Kishino–Hasegawa ($P < 0{\cdot}01$) and Shimodaira–Hasegawa ($P < 0{\cdot}01$) tests. When the monophyly of *Gymnocypris* was similarly forced, the likelihood score (−lnL = 3968·77) was also significantly larger than the unconstrained topology for Kishino–Hasegawa test ($P < 0{\cdot}01$) and for Shimodaira–Hasegawa test ($P < 0{\cdot}05$). All these tests did not support the monophyletic assumptions of these two genera.

CHARACTER MAPPING

Absence of sharp horny sheath was inferred to be the ancestral condition, followed by the appearance of the outer horny sheath. The inner horny sheath then arose from the outer horny sheath, as in *G. namensis* [Fig. 3(a)]. The evolution of mouth position is highly complex in this group [Fig. 3(b)]. The ancestral state in this group is equivocal, and the subterminal or terminal positions

TABLE II. Per cent sequence divergence (Kimura's two-parameter distances) based on the complete sequence of cytochrome *b* gene

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Gd. pachycheilus*																			
2	*P. extremus*	16·17																		
3	*G. eckloni*	15·21	6·12																	
4	*G. p. przewalskii*	15·54	6·41	0·26																
5	*G. p. ganzihonensis*	15·20	6·41	0·26	0·35															
6	*O. stewartii*	16·59	11·16	11·16	11·47	11·47														
7	*S. chengi*	15·19	6·49	7·59	7·68	7·78	0·82													
8	*S. younghusbandi*	15·44	6·91	6·61	6·70	6·70	10·53	4·38												
9	*G. namensis*	15·76	7·10	6·79	6·89	6·89	10·62	4·28	0·62											
10	*H. microcephalus* T1	15·38	6·67	7·66	7·76	7·86	11·11	2·05	5·04	5·13										
11	*H. microcephalus* T2	14·97	6·30	7·29	7·38	7·48	10·93	1·69	4·48	4·57	0·88									
12	*H. microcephalus* T3	15·06	6·39	7·37	7·47	7·57	10·81	1·87	4·85	4·94	0·53	0·71								
13	*H. microcephalus* T4	15·07	6·50	7·28	7·37	7·47	10·39	2·15	4·57	4·66	0·97	0·97	0·44							
14	*S. pylzovi*	15·19	6·49	7·38	7·48	7·58	11·03	0·35	4·38	4·47	2·05	1·69	1·87	2·15						
15	*S. malacanthus* Z1	15·09	6·59	7·18	7·28	7·38	10·61	1·87	4·38	4·28	2·51	2·15	2·33	2·24	2·06					
16	*S. malacanthus* Q3	15·09	6·59	7·18	7·28	7·38	10·61	1·87	4·38	4·28	2·51	2·15	2·33	2·24	2·06	0·01				
17	*S. kialingensis*	15·29	6·78	8·09	8·18	8·29	10·71	1·33	5·15	5·24	2·60	2·24	2·42	2·69	1·33	2·60	2·60			
18	*S. anteroventris*	14·74	7·11	7·20	7·29	7·50	10·10	4·38	4·68	4·58	5·04	4·77	4·85	4·57	4·76	4·38	4·38	5·53		
19	*S. kessleri*	15·07	6·39	7·28	7·38	7·48	10·93	0·44	4·29	4·38	2·14	1·78	1·96	2·06	0·26	1·96	1·96	1·42	4·67	
20	*S. thermalis*	16·03	7·19	7·19	7·28	7·38	11·69	2·79	5·16	5·05	3·24	2·70	2·88	2·97	2·97	1·06	1·06	3·34	5·25	2·88

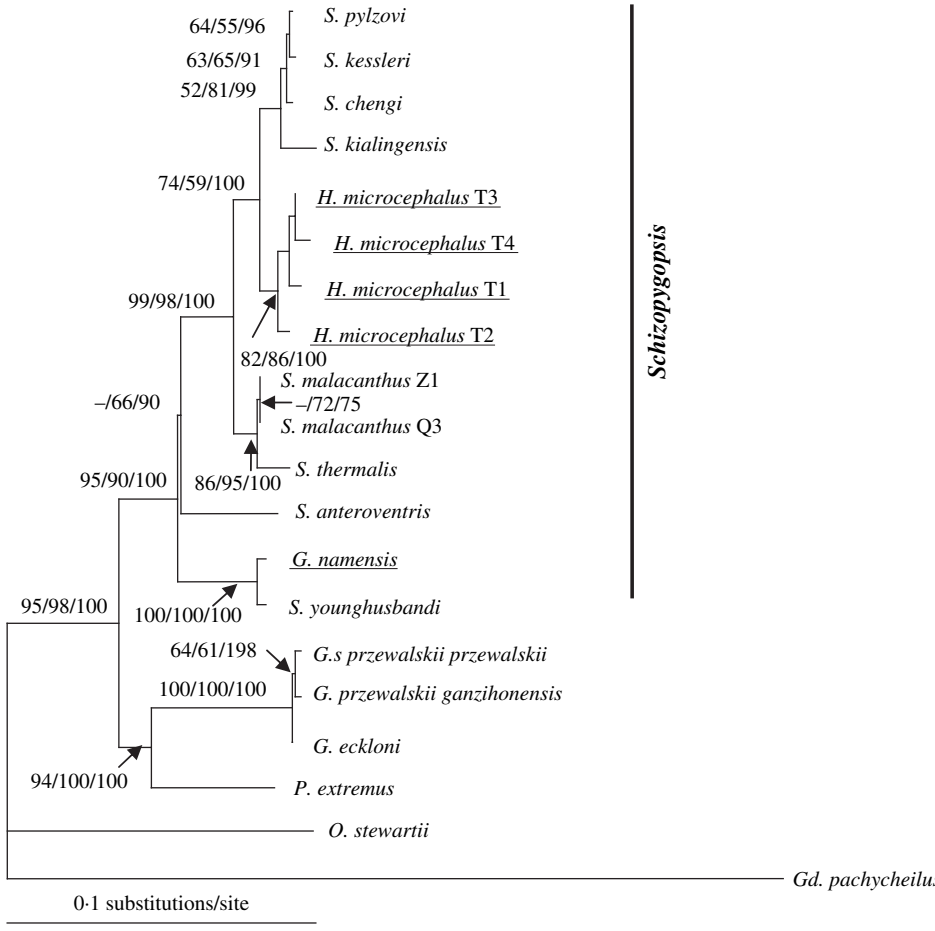

FIG. 2. The single-most parsimonious phylogenetic tree based on 1140 bp mitochondrial cytochrome *b* DNA sequences for two problematic and other species in the Schizothoracinae in the Qinghai-Tibetan plateau (length = 445, consistency index (CI) = 0·721 and retention index (RI) = 0·757). The maximum likelihood (ML) tree (−lnL = 3709·23) has the same topology with this tree. Bootstrap values from the ML and parsimony analyses and Bayesian posterior probabilities appear at branch nodes.

were independently derived in two groups. One group includes *G. eckloni, G. przewalskii przewalskii*, *Gymnocypris przewalskii ganzihonensis and P. extremus*. The other group comprises only *G. namensis*, whose terminal positions were apparently derived from inferior positions through a character-state reversal. It seems probably that terminal positions were lost and appeared again in the Schizothoracinae more than once. The arrangement of pharyngeal teeth in two rows was inferred to be ancestral, and one row of teeth in *G. namensis* and *H. microcephalus* was independently derived from a double row of teeth [Fig. 3(c)]. Mouth position shows a distinct correlation with ecological habitat. Fluvial fishes, *i.e. Schizopygopsis* and *H. microcephalus*, have inferior mouths, while the lacustrine types, *i.e. Gymnocypris* and *G. namensis*, bear subterminal or terminal mouths.

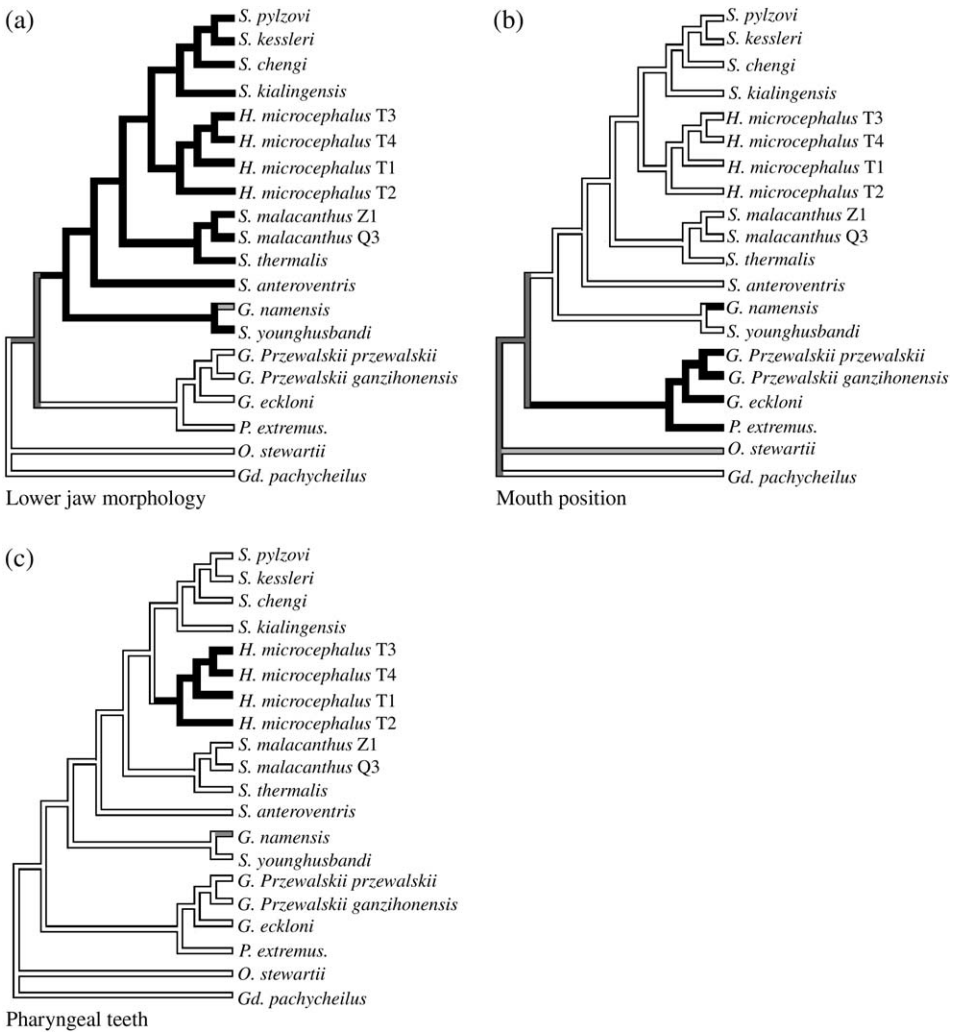

FIG. 3. Mapping of changes in lower jaw morphology, mouth position and arrangement of pharyngeal teeth onto the ML tree. (a) Lower jaw morphology. There is one optimal reconstruction of four steps (RI = 0·73, CI = 0·43). ▭, Without horny sheath; ▬, with inner or outer horny sheath; ▬, with sharp outer horny sheath; ▬, equivocal. (b) Mouth position. There is one optimal reconstruction of five steps (RI = 0·58, CI = 0·33). ▭, Inferior; ▬, terminal; ▬, subterminal or terminal; ▬, equivocal. (c) Arrangement of pharyngeal teeth. There is one optimal reconstruction of four steps (RI = 0·81, CI = 0·58). ▭, Two rows; ▬, one or two rows; ▬, one row.

DIVERGENCE TIME ESTIMATE

Since there is no fossil record of species in the subfamily Schizothoracinae, the time of separation of two species G. *eckloni* and G. *przewalskii* appears to be in the late Pleistocene (0·15 MYA) (Li *et al.*, 1996). This date was used to estimate the tentative divergence times of major clades, as suggested by He *et al.* (2004). According to this calibration, the genus *Schizopygopsis* originated *c.* 2·66 MYA

(middle Pliocene) at a time when intensive uplifts occurred in the QTP (3·4–1·1 MYA) (Li *et al.*, 1996; Li & Fang, 1998). Divergence between *H. microcephalus* and its sister clade (*S. kialingensis*, *S. chengi*, *S. kessleri* and *S. pylzovi*) was estimated to be *c.* 0·98 MYA and that of *G. namensis* and *S. younghusbandi c.* 0·30 MYA. These two estimations correspond with the Kunlun-Huanghe movement between 1·10 and 0·15 MYA, inferred from geological evidence (Li *et al.*, 1996; Li & Fang, 1998).

DISCUSSION

SYSTEMATICS AND TAXONOMY

The results of the present study help to resolve the longstanding debate over the systematic positions and taxonomies of *H. microcephalus* and *G. namensis* within the subfamily Schizothoracinae. Several contrasting taxonomic treatments have variously placed these two taxa as a single species or as species of different genera because of their unusual morphologies (Chu, 1935; Ren & Wu, 1982; Wu & Wu, 1991; Yue *et al.*, 2000). However, the phylogeny reconstructed from the complete mtDNA cyt *b* gene clearly shows that *H. microcephalus* is embedded in a clade, which also includes *S. chengi*, *S. pylzovi*, *S. kessleri* and *S. kialingensis*, and that *G. namensis* is closely related to *S. younghusbandi*. Alternative hypotheses that *Schizopygopsis* and *Gymnocypris* species are each monophyletic were rejected by the Kishino–Hasegawa and Shimodaira–Hasegawa tests. These phylogenetic relationships also contrast sharply with the treatment of *H. microcephalus* and *G. namensis* as sub-specific entities within a single species. Instead, these taxa appear to be derived from different lineages within the genus *Schizopygopsis*.

The molecular phylogeny presented here calls into question the value of some morphological characters to assign taxonomic rank. The assignment to sub-specific rank was based on the arrangement of pharyngeal teeth, the position and sheath morphology of the lower jaw and mouth and the presence of mucus pores on the cheek (Ren & Wu, 1982; Wu & Wu, 1991). The level of difference in these characters between the two taxa was thought to be commensurate with infraspecific variation because they show a large amount of variation among individuals within species. *Herzenstein microcephalus* has only one row of pharyngeal teeth and retains only a few scales, and these traits were used to establish a monotypic genus (Chu, 1935). However, scale abundance varies considerably among individuals. Although scales are generally absent in species of *Schizopygopsis*, *Gymnodiptychus*, *Platypharodon*, *Gymnocypris* and *Oxygymnocypris* (Lloyd, 1908), Wu & Wu (1991) found scales on both sides of the body for a few individuals of *H. microcephalus*.

From a broader perspective, several other characters are typical of species of the genus *Schizopygopsis* and support the results of the molecular phylogeny. These characters include an inferior mouth, small mucus pores on the cheek and chin, a moderately sized skull and sharp ax-shaped pharyngeal teeth. In addition, a sequence divergence of 1·69–5·04% from the other species of *Schizopygopsis*, as estimated in the present study, provides a strong justification to treat *H. microcephalus* as a distinct species of this genus.

In a similar fashion, the large amount of morphological variation among individuals of *G. namensis* has also led to conflicting taxonomic views (Wu, 1984; Wu & Wu, 1991; Yue *et al.*, 2000). In a study by Wu & Wu (1991), 77 of 123 individuals sampled had outer horny sheaths, while 46 fish in this sample had inner horny sheaths. The arrangements of pharyngeal teeth varied greatly in 2 of 225 fish of this species that were sampled, in having two or three rows instead of just one. However, the results here indicate that this species is a closely related sister taxon to *S. younghusbandi* (Fig. 1). The sequence divergence between these two species of 0·62% is lower than the divergences between other pairs of species (Table II). These taxa are best treated as separate species because of their distinct morphology and allopatric distributions, which precludes the possibility of gene flow between them.

EVOLUTION OF MORPHOLOGY AND SPECIATION

Three genetic mechanisms, including hybridization and introgression, incomplete lineage sorting and convergent evolution, may explain the apparent morphological similarity in *H. microcephalus* and *G. namensis*. Examples invoking each of these mechanisms have been described for fishes (Avise & Saunders, 1984; Avise, 1986; Billington & Hebert, 1988; Burridge, 1999; Xiao *et al.*, 2001; Bernard & Michael, 2004). The character-state mappings outlined here support a hypothesis of the convergent evolution of one row of teeth in these two species [Fig. 3(c)]. This trait was the major diagnostic character used to combine these two species together as one species (Wu & Wu, 1991). The arrangement of pharyngeal teeth in one row was independently derived from different ancestral lineages with two rows of teeth. This parallel evolution might result from further adaptations to the high altitudes and cold habitats because their distributions are higher than those of the other species (Fig. 1). However, the adaptive value of reduced numbers of pharyngeal teeth remains largely unknown. The evolutionary process of mouth position is closely correlated with habitat. The reversal of subterminal or terminal mouths from inferior mouths in *G. namensis* may have been accompanied by a habitat shift from fluvial rivers to lacustrine lakes. Other lake species of *Gymnocypris* similarly have terminal mouths. In the QTP, rivers are universally abundant in benthic algae but short of zooplankton and phytoplankton, while lakes are rich in zooplankton and phytoplankton (Wu & Wu, 1991). Under this hypothesis, fluvial fishes, which feed mainly on benthic algae, may have evolved inferior mouths, while the lacustrine fishes may have developed subterminal and terminal mouths better to exploit zooplankton and phytoplankton populations (Wu & Wu, 1991). Adaptive evolution to the local habitats during speciation may also account for similar changes in lower jaw morphology in *G. namensis* and the reversal gain of a few scales in *H. microcephalus*, after diverging from ancestral species at the lower altitude.

The low sequence divergences between *G. namensis* and its sister species indicate that it is a recently derived species. This hypothesis is also supported by geological evidence. The major drainage systems in the QTP were developed in the middle and the late Pleistocene (1·1–0·15 MYA) because of the

Kunlun-Huanghe and Gonghe surface deformations (Li & Fang, 1998; Li *et al.*, 2000). Molecular clock estimates indicate that *G. namensis* originated *c.* 0·30 MYA, earlier than the geological evidence for the formation of Nam Co Lake *c.* 0·12 MYA (Zhu *et al.*, 2003; Zhu *et al.*, 2004). It is possible that this species started to diverge from its sister species before Nam Co Lake was completely isolated. Another possibility is that the time of divergence between G. *eckloni* and G. *przewalskii* estimated from geological data is earlier than the actual time of separation between them.

Molecular data further indicate that the appearance of *H. microcephalus* may also have been associated with the Kunlun-Huanghe surface displacement *c.* 0·98 MYA. The evolutionary scenarios outlined here for *H. microcephalus* and *G. namensis* indicate that geological shifts over the past three million years and allopatric separations may have played a central role in the diversification of species in the Schizothoracinae in the QTP (He *et al.*, 2004). However, these evolving species may also have been subjected to the Quaternary habitat changes driven by the climatic oscillations after the uplifts. During repeated climatic shifts that led to the shrinkage and expansion of lakes, some adaptive morphologies may have exhibited repeated losses and gains within a lineage. Therefore, the evolution of these adaptive traits may be more complex than previously thought, and they do not suggest a single adaptation to the high altitude during the early divergence (Cao *et al.*, 1981; Wu, 1984; Wu & Wu, 1991). Our results highlight the importance of molecular methods for inferring the course of morphological evolutions and for resolving taxonomic conflicts in the Schizothoracinae in the QTP. Additional molecular and morphological comparisons are needed to understand the diversification process of organisms distributed over the plateau.

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