



Convergent evolution misled taxonomy in schizothoracine fishes (Cypriniformes: Cyprinidae)

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ABSTRACT

Highly specialized grade (HSG; genera *Gymnocypris*, *Oxygymnocypris*, *Schizopygopsis*, *Platypharodon* and *Chuanchia*) of the Schizothoracinae (Cypriniformes: Cyprinidae) are endemic to the Qinghai-Tibet Plateau (QTP). Previously, two distinct ecomorphs were recognized according to trophic traits. One was a limnetic omnivore with normal lower jaw morphology, terminal mouth, and moderate or dense gill rakers, mostly inhabiting in open water of lakes, including *Gymnocypris* and *Oxygymnocypris*. Another was a benthic feeder with inferior mouth, sparse gill rakers and sharp horny sheath on the lower jaw for scraping of attached prey off hard substrates, including *Schizopygopsis*, *Platypharodon* and *Chuanchia*. However, traditional taxonomy of HSG based on these trophic traits presented extensive conflicts with the molecular studies in recent years. The possible cause could be convergent evolution in morphology, retention of ancestral polymorphisms or mitochondrial introgression, but these hypotheses could not be assessed due to incomplete taxon sampling and only mitochondrial data employed in previous works. Here, we conducted the most comprehensive molecular analysis on HSG fishes to date, using four mitochondrial loci and 152,464 genome-wide SNPs, and including 21 of 24 putative species and one undescribed *Schizopygopsis* species. Both SNP and mtDNA trees confirmed extensive paraphyly of genera *Gymnocypris* and *Schizopygopsis*, where species often were clustered together by watershed instead of by genus. Basal split into the north clade B and the south clade C (ca. 3.03 Ma) approximately by the Tanggula-Tanitawen Mountains in SLAF tree coincided with a violent uplift of the QTP during the phase A of 'Qingzang movement' (ca. 3.6 Ma). Ancestral state reconstruction of the trophic ecomorph showed that the limnetic omnivore ecomorph had evolved repeatedly in clade B and C. Furthermore, we presented a striking case of convergent evolution between two 'subspecies' *Gymnocypris chui chui* and *G. chui longimandibularis*, which had diverged as early as two million years ago (ca. 2.42 Ma). Ecological analyses revealed that similar food utilization, particularly in zooplankton, was the main underlying driving force. This work showed an example of taxonomy with the most extensive errors at the genus/species levels due to convergent evolution and suggested that trophic traits could be misleading in fish taxonomy. Therefore, we propose a major generic revision for HSG species.

1. Introduction

Species description and classification usually were based on morphological characters, but morphological taxonomies were not always consistent with molecular studies (Fleischer et al., 2008; Aliabadian

et al., 2012; Ukuwela et al., 2013). For example, under adaptive radiation, morphologically distinct species were not necessarily genetically diverged (Samonte et al., 2007). On the contrary, morphologically similar species could be actually from lineages that diverged millions of years ago (Wada et al., 2013). Rapid radiation coupled with adaptation

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to similar environments may result in repeated evolution of similar phenotypic traits independently (Meyer et al., 1990; Losos et al., 1998; Ruber et al., 1999; Bossuyt and Milinkovitch, 2000; Cassens et al., 2000; Rundle et al., 2000), which could mislead taxonomy based on those traits. Nevertheless, large scale misclassifications of genera and species were rarely seen in the literature even with those confounding factors. Unexpectedly, phylogenetic studies based on mitochondrial data revealed many potential misclassifications in the traditional morphology-based taxonomy in the schizothoracine fishes at the genus/species level (He and Chen, 2007; Duan et al., 2009; Yonezawa et al., 2014; Zhang et al., 2016).

The schizothoracine fishes (Cypriniformes: Cyprinidae), meaning ‘cloven-breast,’ derived their name from a groove between two rows of large specialized anal scales located between the pelvic fin and the anal fin (Wu and Wu, 1992; Chen and Cao, 2000). They are the predominant species of the Qinghai-Tibet Plateau (QTP) ichthyofauna, with their main occurrence in the mid-upper reaches of drainages of the QTP, as well as the Tien Shan region to the north of QTP (Wu and Wu, 1992; Chen and Cao, 2000). These endemic freshwater fishes provide wonderful opportunities to investigate patterns of biogeography and paleo-drainage changes (He et al., 2004; He and Chen, 2006, 2007; Li et al., 2015a, 2016b; Qi et al., 2015). It has been an excellent model for studying ecological radiation and genomic adaptation to the extreme conditions of the QTP (Zhao et al., 2009; Qi et al., 2012; Zhang et al., 2013; Guan et al., 2014; Tong et al., 2017a,b). Based on specialization in scales, pharyngeal teeth (PT) and barbels, Cao et al. (1981) divided schizothoracine fishes into three grades: (1) primitive grade (PG), with tiny scales on whole body or except for the venter, three rows of PT, two pairs of barbels for all except *Aspiorhynchus laticeps*; (2) specialized grade (SG), more or less with tiny scales, two rows of PT, one pair of barbels; (3) highly specialized grade (HSG), almost entirely naked body, one or two rows of PT, no barbels. This interesting phenotypic specialization is supposed to be correlated to stepwise uplift of the QTP (Cao et al., 1981).

As the most specialized and youngest group, HSG species are predominant in most of lakes and rivers in the center of the QTP, with the highest-altitude habitat among all three grades (Fig. 1). A variety of trophic phenotypes were observed for HSG species, including variation in mouth position, lower jaw morphology, shape of the pharyngeal bones and number of gill rakers, which were widely used to establish intergeneric and infrageneric classification. Generally, five genera, *Gymnocypris*, *Oxygymnocypris*, *Schizopygopsis*, *Platypharodon* and *Chuanchia*, were recognized, with 24 or so species-level taxa (Supplementary Table S1) (Wu, 1984; Wu and Wu, 1992; Chen and Cao, 2000; Qi et al., 2007; Zhang et al., 2015; Tang et al., 2016). Phylogeny inferred from osteological and superficial traits recovered two clades or ecomorphs (Fig. 2a) (Wu, 1984). One included a species-rich genus *Gymnocypris* and a monotypic genus *Oxygymnocypris*, which have normal lower jaw, terminal mouth, and moderate or dense gill rakers, and mostly inhabit lakes and rarely deep water area of rivers, and are considered as limnetic omnivores (Fig. 2b). Another group consisted of one species-rich genus *Schizopygopsis* and two monotypic genera, *Platypharodon* and *Chuanchia*, which possess inferior mouths, as well as short and sparse gill rakers. Compared to the first group, they are benthic feeder mostly inhabiting rivers and rarely along lakeshores, and are characterized by sharp anterior edge of lower jaw for scraping attached prey off hard substrates (Fig. 2b).

Several molecular studies have been conducted to investigate evolutionary and biogeographic processes of HSG species, and to assess the taxonomic validity of species and subspecies (He and Chen, 2007; Duan et al., 2009; Qi et al., 2009, 2015; Zhao et al., 2011; Zhang et al., 2013, 2015; Yonezawa et al., 2014; He et al., 2016a; Tang et al., 2016). Surprisingly, incongruence between molecular phylogenies and the morphological taxonomy was found everywhere, such that extensive paraphyly for *Gymnocypris* and *Schizopygopsis* was consistently recovered. Interestingly, species of distinct genera in the same or

neighboring water systems were always closely related. Some underlying reasons suggested for these incongruences were (1) characters used for systematics are unreliable and undergoing convergent evolution, (2) retention of ancestral polymorphism when species are reproductively isolated but too young for lineage sorting to complete, (3) mitochondrial introgression if reproductive isolation is incomplete (Wendel and Doyle, 1998; Fleischer et al., 2008; Edwards, 2009; Aliabadian et al., 2012; Ukuwela et al., 2013). Nonetheless, these competing hypotheses are difficult to test due to limited taxon sampling and only mitochondrial data being used in previous studies (Qi et al., 2006).

Herein, we reconstructed a robust phylogeny of HSG species based on four mitochondrial loci and 152,464 genome-wide SNPs discovered using specific-locus amplified fragments sequencing (SLAF-seq) (Sun et al., 2013), a new sequencing technology like RAD-seq, with the most comprehensive sampling of the HSG to date, including 21 of the 24 species. In addition, ancestral state reconstruction of trophic traits revealed that convergent evolution prevailed in the diversification of HSG fishes. Morphometric and diet analysis suggested an important role of natural selection in shaping shared phenotypes during their adaptations to similar habitats and resources. These provided suggestions for the taxonomic work in Schizothoracinae.

2. Materials and methods

2.1. Taxon sampling

We sampled 21 of 24 species (except *Gymnocypris firmispinatus*, *G. pengquensis*, *G. scolostomus*) including all five genera of HSG species, plus one undescribed *Schizopygopsis* species from Lake Namtso and five subspecies (sample numbers per species or subspecies ranged from two to three), making a total of 79 individuals for ingroups (Supplementary Table S2). Seven SG species, *Ptychobarbus dipogon*, *Pt. conirostris*, *Pt. kaznakovi*, *Diptychus maculatus*, *Gymnodiptychus pachycheilus*, *Gy. dybowskii* and *Gy. integrigymnatus* were also obtained as outgroups which were considered as sister groups to the HSG species (He and Chen, 2007) (one individual per species) (Supplementary Table S2). All individuals were collected from the QTP and adjacent regions except *Gy. dybowskii* from the Tianshan region in central Asia during 2007–2014 (Supplementary Table S2, Fig. 1). Whole specimens were caught via seining and additional samples were obtained through tissue exchange. All captured specimens were stored directly in 95% ethanol during field trips and archived in Qinghai-Tibet Plateau Museum of Biology, Northwest Institute of Plateau Biology, Chinese Academy of Sciences. To compare food preferences of two subspecies, *Gymnocypris chui chui* and *G. chui longimandibularis*, the intestines (for stomach and gut content analyses, SGCA) and a piece of white dorsal muscle tissue (for stable isotope analysis, SIA) were preserved in 10% formalin solution and 95% ethanol, respectively. For comparison, the other two sympatric species, *G. scleracanthus* and *S. stoliczkai maphamumensis*, were also included in SIA.

2.2. Amplification, sequencing and data preparation of mitochondrial loci

Total genomic DNA was extracted from fin clips or muscle tissue. The tissue samples were lysed by proteinase K digestion followed by standard three-step phenol-choroform DNA extraction method (Green and Sambrook, 1989). Four mitochondrial gene fragments were amplified for all individuals using PCR: (i) the complete cytochrome *b* gene (Cyt *b*) (1140 bp); (ii) the entire NADH dehydrogenase subunit 2 gene (ND2) (1047 bp); (iii) a 1097-bp fragment of 16S rRNA; (iv) a 702-bp fragment of control region (D-loop). Detailed primer information is given in Supplementary Table S3. All primers were synthesized at Beijing Tianyi Huiyuan Bioscience and Technology Incorporation (Beijing, China). PCR was performed in 35 µL reactions containing 50–200 ng of DNA, 3 µL of dNTP mix (2.5 mM each), 1 U TaKaRa rTaq

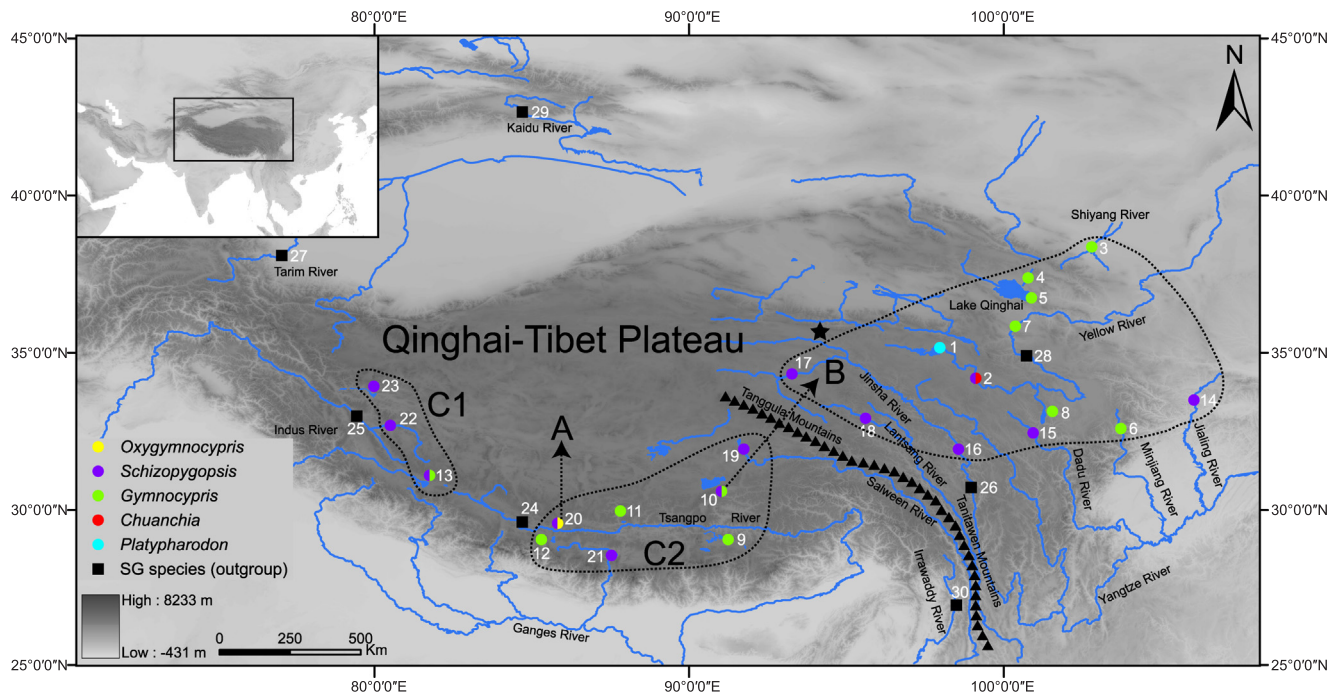


Fig. 1. Sampling localities of the schizothoracine fishes of the specialized grade (SG) and highly specialized grade (HSG) used in this study. Black square indicates SG species, and circle indicates HSG species. Colors correspond to genus identities (yellow: *Oxygymnocypris*; purple: *Schizopygopsis*; green: *Gymnocypris*; red: *Chuanchia*; cyan: *Platypharodon*). Locality numbers correspond to Locality ID in Supplementary Table S2. The capital letters (A, B, C1 and C2) and closed dashed lines correspond to major clades identified in the phylogeny of specific-locus amplified fragments sequencing (SLAF-seq) data, where one dotted arrow indicates *Oxygymnocypris stewartii* belonging to clade A (sample site 20), and another dotted arrow indicates *Gymnocypris namensis* belonging to clade B (sample site 10) (Fig. 4). Black triangles show the approximate positions of the Tanggulaand-Tanitawen Mountains. Major rivers and lakes are shown. The black five-pointed star indicate the place of 'Kunlun-Huanghe movement.' (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

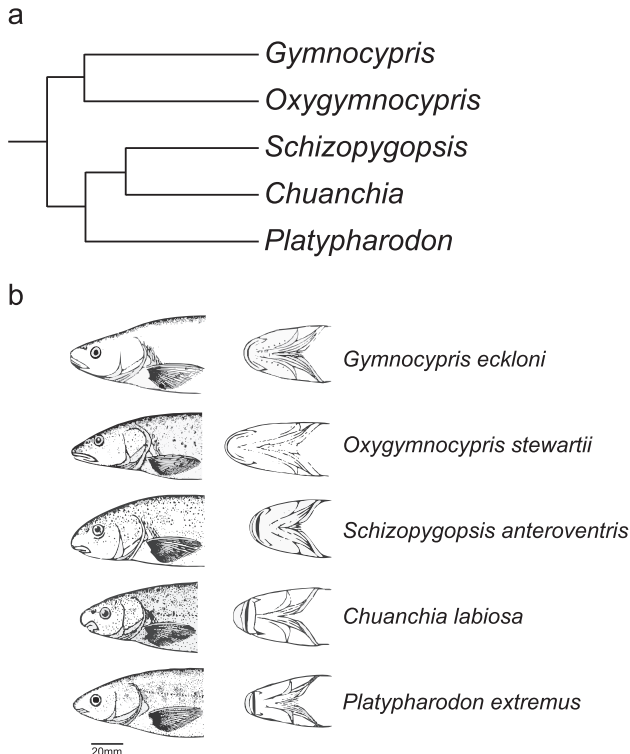


Fig. 2. Morphological phylogeny and diagrams of five genera of HSG species. (a) Phylogeny inferred from osteological and morphological traits of five genera (*Gymnocypris*, *Oxygymnocypris*, *Schizopygopsis*, *Chuanchia* and *Platypharodon*) of HSG species by Wu (1984); (b) lateral and ventral diagrams of head of typical species for five genera (Wu and Wu, 1992).

(TaKaRa Corp., Dalian, China), 3.5 μ L 10 $\times$ reaction buffer, 0.7 μ L of each primer (10 mM). All reactions were performed under the following thermal cycler conditions: denaturation at 95 $^{\circ}$ C for 5 min followed by 35 cycles of 95 $^{\circ}$ C for 30 s, annealing at 48–52 $^{\circ}$ C for 30 s (Supplementary Table S3) and extension at 72 $^{\circ}$ C, 90 s for Cyt b, ND2, 16S, and 45 s for D-loop, respectively, and with a final extension at 72 $^{\circ}$ C for 10 min. After visualization of the fragments using 1% agarose gel, the PCR products were sequenced from both ends using an ABI PRISM 3700 sequencing system with the same primers used for PCR.

The DNA sequences from resulting chromatograms were proofread and assembled with Seqman and EditSeq (DNASTAR, Lasergene v7.1). Cyt b and D-loop of two individuals of *G. scoliosomus* were downloaded from GenBank (Supplementary Table S2). Edited sequences were aligned with Clustal X v1.83 (Thompson et al., 1997). Protein coding genes were translated into amino acid residues in MEGA 5.0 (Tamura et al., 2011) to test the absence of premature stop codons or 'indels', and subsequently adjusted manually to maximize positional homology.

2.3. Preparation of SLAF sample for sequencing, data processing and SNP calling

Because some individual DNA quality could not satisfy the demand of library construction, a subset of samples (59 individuals, Supplementary Table S2) were picked to generate genome-wide SNP data using SLAF-seq (Sun et al., 2013), a new sequencing technology like RAD-seq which targeted a reduced representation of the genome. Firstly, high-quality genomic DNA was isolated from fin and muscle by the same method described above. DNA quality and concentration were assessed by a NanoDrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA) and electrophoretic separation through a 0.8% agarose gel. As there was no reference genome sequence for the schizothoracine fishes, the genome of a closely relative species, *Sinocyclocheilus graham* (<https://www.ncbi.nlm.nih.gov/genome/?term=>

Sinocyclocheilus%20grahami; (Yang et al., 2016)), was selected to perform *in silico* digestion prediction aiming to obtain different restriction enzyme combinations. Based on uniqueness and uniformity of simulated fragment alignments, two restriction enzymes (*RsaI* and *HaeIII*) were selected. Genomic DNA (about 500 ng) was digested to completion with *RsaI* (New England Biolabs, NEB, Ipswich, MA, USA), T4 DNA ligase (NEB), ATP (NEB) and *RsaI* adapter at 37 °C. Restriction-ligation reactions were heat inactivated at 65 °C, and then digested with the second restriction enzyme *HaeIII* at 37 °C. The Duplex Tag-labeled sequencing adapters (PAGE purified, Life Technologies) were ligated to the A-tailed DNA with T4 DNA ligase. PCR reactions were performed using diluted restriction-ligation samples, dNTP, Q5® High-Fidelity DNA Polymerase and PCR primers: AATGATACGGCGACCACCGA and CAAGCAGAAGACGGCATACG (PAGE purified, Life Technologies). The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK) and pooled. The pooled sample was separated by electrophoresis in a 2% agarose gel. Fragments with 350–450 bp (with indexes and adaptors) in size were excised and purified using QIAquick Gel Extraction Kit (QIAGEN). The gel-purified product was sequenced on the Illumina HiSeq 2500 system (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's recommendations.

The raw data were parsed by barcodes. Reads with quality scores < 30 were discarded. SLAF pair-end reads with clear index information were clustered and detected using BLAT (Kent, 2002). Sequences with over 80% identity were considered as one SLAF locus. Due to the lack of schizothoracine fish reference sequences, the highest depth tag in each SLAF was taken as reference sequence tag. Both GATK (McKenna et al., 2010) and SAMTOOLS (Li et al., 2009) were used to call SNPs. SNPs with minor allele frequency (MAF) < 5% were discarded.

2.4. Phylogenetic analysis and estimation of divergence time

Four aligned mitochondrial fragments were concatenated to construct phylogenetic trees. The data matrix was partitioned based on each gene and by codon position (for Cyt *b* and ND2). We implemented PartitionFinder 1.1 (Lanfear et al., 2012) to select appropriate partition schemes and substitution models for these partitions using the corrected Akaike's Information Criterion (AICc), unlinked branch lengths, and the 'greedy' heuristic algorithm. We ran two separate analyses with models restrained to those implemented in MrBayes and RAxML, respectively. ML analyses were performed using the graphical front-end RAxML GUI v1.3.1 (Silvestro and Michalak, 2012) with a GTRGAMMAI model for each partition identified by PartitionFinder (Supplementary Table S4). Searches for the most likely ML tree and calculation of nodal bootstrap support (BS) were carried out using the 'ML + rapid bootstrap' option with 1000 nonparametric bootstrap replicates. Bayesian inference (BI) was conducted in MrBayes v.3.2.6 (Ronquist et al., 2012) with unlinked branch lengths and a partitioning scheme suggested by PartitionFinder

(Supplementary Table S4). Two independent runs of one million generations, each comprising four Markov chain Monte Carlo (MCMC) chains, were sampled every 1000 generations with a 25% burn-in. We checked the average standard deviation of split frequencies for assessing convergence by < 0.01. Additionally, convergence was evaluated by comparing posterior distributions and effective samples sizes of all parameters (> 200) of two independent runs with TRACER v1.5 (Rambaut and Drummond, 2007). The posterior probabilities (PP) indicating support values for each branch were also estimated.

A total of 152,464 SNPs of SLAF-seq data were used to infer phylogenetic relationships among HSG species through both ML and BI analyses with *D. maculatus*, *Gy. pachycheilus* and *Gy. dybowskii* as outgroups. ML analyses were conducted using RAxML-HP2 on XSEDE via CIPRES Science Gateway (Miller et al., 2010) with GTRGAMMA model of sequence evolution and 1000 bootstrap replicates. Bayesian analyses were also performed via CIPRES Science Gateway using MrBayes 3.2.6, setting GTR + gamma model, one million generations, sampling every 1000 generations with a burn-in fraction of 0.25.

We used an approximate likelihood method to estimate divergence time as implemented with MCMCTREE in PAML (Yang, 2007) with an independent rates clock model (clock = 2 in MCMCTREE). The best SLAF ML tree was used, and the ML branch lengths were estimated using the BASEML program in PAML under the GTR substitution model. As no fossil record of the schizothoracine fishes was available, we used two secondary calibrations estimated from a previous study (He et al., 2004) to calibrate divergence time: (1) constraining the most recent common ancestor (MRCA) of *D. maculatus*, *Gy. pachycheilus* and *Gy. dybowskii* to 7.61–7.93 Ma, (2) constraining the MRCA of *Gy. pachycheilus* and *Gy. dybowskii* to 3.38–3.70 Ma. An accurately dated geological event, 'Kunlun-Huanghe movement,' occurred at ca. 1.1–0.7 Ma, which caused the separation of the schizothoracine fishes distributed in the upper Yangtze River and Yellow River (Cui et al., 1998; He and Chen, 2007). Therefore we constrained the MRCA of *S. microcephalus* and *S. pylzovi* to 1.10–0.70 Ma. Two independent MCMC runs of ten million cycles were conducted with every 1000 cycles sampled, and the first one million cycles were discarded as burn-in. The distributions of the parameter values from the MCMC samples were assessed using Tracer v1.5 (ESS > 200).

2.5. Hypotheses testing

In order to compare the ML trees based on the mitochondrial data and SLAF data with traditional morphological circumscriptions as monophyletic groups, the likelihoods of alternative hypotheses (Table 1) were assessed against that of the unconstrained ML tree using the approximately unbiased (AU) test (Shimodaira, 2002) and the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999), as implemented in CONSEL v 0.1j (Shimodaira and Hasegawa, 2001). We adopted the same data partitions and models as those described previously (see ML method) for mitochondrial data. Hypotheses having *p*-

Table 1

Monophyletic hypotheses test results. Hypothesis: A, No constraint; B, *Schizopygopsis* monophyly constraint; C, *Gymnocypris* monophyly constraint; D, both *Gymnocypris* and *Schizopygopsis* monophyly constraint respectively; E, *Gymnocypris chui* monophyly constraint; F, HSG species monophyly constraint. Rejected monophyly: *p* < 0.05. lnL: likelihood of the hypothesis; ΔlnL: difference in likelihood between the alternative hypothesis and the Maximum Likelihood (ML) tree as best explanation of the data. Mt indicates concatenated mitochondrial data. – indicates that the value is lacked.

Hypothesis	lnL		ΔlnL		AU test <i>P</i> -value		SH test <i>P</i> -value	
	Mt	SLAF	Mt	SLAF	Mt	SLAF	Mt	SLAF
A	–20385.8	–2085436.7	–	–	–	–	–	–
B	–21541.7	–2173220.1	1155.9	87783.4	1e–74	2e–64	< 0.01	0
C	–21257.8	–2164472.1	872.0	79035.4	2e–11	2e–41	< 0.01	0
D	–21660.8	–2227286.1	1275.0	141849.4	1e–64	6e–45	< 0.01	0
E	–20824.7	–2108341.9	438.9	22905.2	8e–7	1e–39	< 0.01	0
F	–20404.1	–	18.3	–	0.005	–	0.515	–

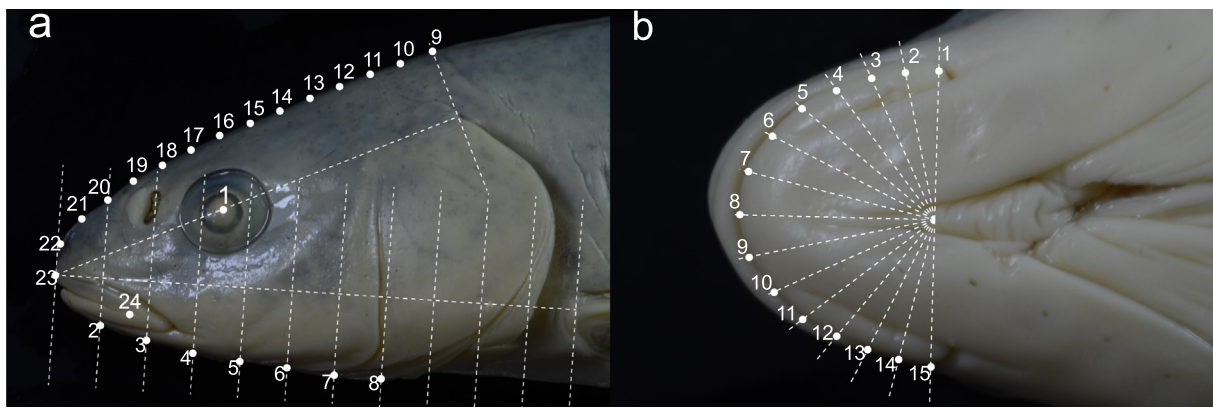


Fig. 3. Distribution of landmarks for the geometric morphometric analyses of head shape (a) and lower jaw shape (b).

values significant at the alpha-level of 0.05 were rejected.

2.6. Geometric morphometric and morphological analyses

We assessed the shape variation of head and lower jaw from 78 individuals of the HSG species (Supplementary Table S2) using landmark-based geometric morphometric methods (Adams et al., 2004; Zelditch et al., 2012). The left lateral heads and ventral lower jaw of fishes were photographed in a standardized way using Nikon D7000 digital camera. Coordinates (xy) of all landmarks distributed across the head and lower jaw were recorded using TPSDIG2 (Rolhf, 2006), and MakeFan8 Tool (Sheet, 2003) was used to draw fans or guidelines for digitizing landmarks on images. After comparison with corresponding studies (Chakrabarty et al., 2010; Wanek and Sturmbauer, 2015; Abaad et al., 2016), head shape (Fig. 3a) was assessed using a set of 24 landmarks (LM). LM1, LM23 and LM24 were set as the center of the eye, the anterior most point of upper lip and the corner of the mouth, respectively. Twelve equidistant lines perpendicular to the line between LM23 and the origin of pectoral fin were made. Eleven intersection points of these lines with the lower outline of the head were generated, of which seven were selected as landmarks 2–8 from left to right. A perpendicular line to the line between LM23 and the upper origin of operculum was made, and the intersection with the upper outline of the head was set as LM9. Thirteen landmarks (LM10–22) averaged the length of the outline between LM9 and LM23. For assessment of lower jaw shape (Fig. 3b), fifteen landmarks distributed on the outline of lower lip were employed. Firstly, a circle was produced with a diameter of the connecting line between the right (LM1) and left (LM15) end point of lower lip, with the center at the mid-point of LM1 and LM15. The left part of the circle was divided into 14 equal parts by thirteen lines radiating from the center. Thirteen intersection points of these lines with the outline of the lower lip were treated as LM2–14. Raw landmark coordinates were Procrustes aligned and the resulting Procrustes coordinates were used for a pooled-within-species regression of shape against centroid size in MorphoJ 1.05f software (Klingenberg, 2011). The resulting residuals were averaged for each species and subspecies and used for principal component analysis (PCA). The shape changes of four genera were visualized relative to the average shape of all individuals.

2.7. Ancestral state reconstruction

The evolutionary history of the foraging ecology and diet was reconstructed based on MP and ML approaches implemented in Mesquite 2.7 (Maddison and Maddison, 2009), using SLAF ML trees as input trees excluding SG species. Based on data of trophic morphology retrieved from the literature (Wu and Wu, 1992; Chen and Cao, 2000) and examining the voucher specimens personally, HSG species were assigned

into three main trophic ecomorphs to reconstruct the ancestral state. First, we assigned three genera (*Schizopygopsis*, *Platypharodon* and *Chuanchia*) with straight, sharp anterior edge or wedged, rectangle or ladder-shaped lower jaw morphology for scraping prey attached on hard substrates to the benthic feeder ecomorph; second, we assigned species of *Gymnocypris* and *Oxygymnocypris* except *G. chui chui* and *G. chui longimandibularis* to the limnetic omnivore ecomorph according to their normal lower jaw morphology and omnivorous dietary style; finally, we assigned *G. chui chui* and *G. chui longimandibularis* to the planktonic specialist ecomorph based on their unique trophic morphological adaptation to planktonic prey, such as nearly superior mouth position and the extremely dense gill rakers. The Markov K-state one parameter model (Lewis, 2001) was used for ML optimizations. Character transitions were considered to be reversible and unordered.

2.8. Diet analysis

To evaluate the diet similarity of the two subspecies of *G. chui*, we inspected stomach and gut contents of 20 individuals for *G. chui chui*, and 5 individuals for *G. chui longimandibularis*. These stomachs were dissected under a dissecting stereomicroscope, and all items were separated into five categories: plant scraps, periphytic algae, insects, zoobenthos and zooplankton. The other gelatinous substance, something like gastric juice, was excluded. The wet weight of each category in each individual was estimated and used to calculate Schoener's index of proportional diet overlap, where values range between 0 (no diet overlap) and 1 (complete diet overlap). Values greater than 0.6 generally indicate biologically significant overlap (Schoener, 1970).

There were some drawbacks of SGCA, which just simply presented food intake in the last few hours or days before the capture. Therefore, we also conducted a more reliable method, carbon stable isotope analysis ($\delta^{13}\text{C}$), which could indicate individual long-term food resource use (Maruyama et al., 2001; Dromard et al., 2015). In lakes, $\delta^{13}\text{C}$ has proven beneficial for differentiating between two major sources of carbon production, because the production from the nearshore littoral food web tends to be enriched in $\delta^{13}\text{C}$ relative to that from the open water, pelagic food web (France, 1995). By comparison with the stable isotope of nitrogen ($\delta^{15}\text{N}$), another beneficial tool to estimate trophic position, $\delta^{13}\text{C}$ signatures of consumers should provide insight into both feeding history and the primary habitat occupied when assimilating the consumed prey material. In total, including *G. scleracanthus* and *S. stoliczkai maphamyumensis*, 67 specimens were used for SIA (*G. chui chui*, $n = 20$; *G. chui longimandibularis*, $n = 20$; *G. scleracanthus*, $n = 20$; *S. stoliczkai maphamyumensis*, $n = 7$). All individuals were adults and had been sampled during a single field expedition (June 2014). Each pair of sympatric species of Lake Langtso (*G. chui chui* and *G. scleracanthus*) and Lake Manasarovar (*G. chui longimandibularis* and *S. stoliczkai maphamyumensis*) was collected from the same location which

was 10 to 20 m away from the shore and less than two meter in depth. White dorsal muscle tissues were over-dried to constant mass at 55 °C for 48 h, and then ground into a fine powder. A small amount (0.6–1.0 mg) of powder was wrapped into tin capsules and analyzed on an elemental analyzer (Thermo Flash EA2000) coupled to a MAT 253 isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), with standard setup for CO₂ analysis. We expressed the ratio of stable isotopes in the conventional delta (δ) notation as the parts per thousand (‰) deviations from standard IAEA-600 caffeine (Coplen et al., 2006; Letourneur et al., 2013).

3. Results

3.1. Phylogenetic analyses and divergence time estimation using SNP data

In total, 59 individuals including all five genera of HSG species were successfully used to develop genome-wide SNPs through SLAF-seq (Supplementary Table S2). The SLAF sequencing generated a mean of 359,984,512 paired-end reads per sample, with the average quality score of 90.75%. The mean number of SLAFs per entry was 270,340, equivalent to a sequencing depth of $14.14 \times$ (Supplementary Table S5). From the total number of 8,690,958 SNPs, 152,464 informative ones were identified with an MAF ≥ 0.05 and integrity $\geq 80\%$ (Supplementary Table S6). All SLAF-seq raw data are available on the NCBI SRA database (Bioproject ID PRJNA483516).

The phylogenetic relationships among HSG species were first investigated based on genome-wide SNPs through SLAF-seq (Fig. 4). Because of unavailability of four SG species, particularly lacking *Gy. integrigymnatus* which was embedded in HSG species based on previous mtDNA data (He and Chen, 2007), the monophyly of HSG species could not be confirmed under SLAF data. Overall, in our SLAF tree, three major clades in HSG species were recovered with strong support (BS = 100 for clades A and C; BS = 92 for clade B; PP = 1.00 for all nodes). Both *Gymnocypris* and *Schizopygopsis* were not monophyletic, and close relationships were observed between species of different genera rather than in the same genus in some clades, such as clade B2-4, C1 and C2b. The monotypic genus *Oxygymnocypris* (*O. stewartii*, clade A) was sister to the other HSG species. A subsequent split occurred between clade B and C. Most species and subspecies were monophyletic and highly supported units throughout the tree except *G. scleracanthus* and *S. thermalis* (BS = 100 for all except BS = 91 for *S. stoliczkai maphamyumensis*).

The clade B was the most heterogeneous taxonomically, consisting of two monotypic genera *Platypharodon* (*P. extremus*) and *Chuanchia* (*C. labiosa*), as well as about half of the *Gymnocypris* and *Schizopygopsis* species. In the clade B, four well resolved subclades B1–B4 were recovered (BS ranges from 82 to 100). *Schizopygopsis anteroventris* together with *S. malacanthus* formed clade B1, which located at the basal position. *Schizopygopsis microcephalus* and *G. namensis* formed a well-defined clade B2, and was a sister lineage to clade B3 plus B4. In the subclade B3, three *C. labiosa* individuals formed a basal lineage, followed by *S. pylzovi* as well as the group of *S. kialingensis* and *G. chilianensis*. In the subclade B4, *G. potanini* was separated first, and *S. chengi* together with *P. extremus* formed the sister lineage to a group of *G. eckloni* and two subspecies of *G. przewalskii*. However, *G. przewalskii* was not monophyletic because *G. przewalskii przewalskii* was more closely related to *G. eckloni* than to *G. przewalskii ganzihonensis*.

Clade C contained two well supported subclades C1 and C2, and was composed of only *Gymnocypris* and *Schizopygopsis* species. Two subspecies, *Gymnocypris chui chui* and *G. chui longimandibularis* did not cluster together and showed close relationships with their sympatric species, *G. scleracanthus* and *S. stoliczkai maphamyumensis*, respectively. *Schizopygopsis stoliczkai* was paraphyletic because *G. chui longimandibularis* nested within it. *Schizopygopsis thermalis* located the base of clade C2b, and it was not monophyletic because one individual (T151) did not cluster with the other two (T150 and T152). Two

subspecies of *S. younghusbandi* were separated into two clades, one clustered with an undescribed *Schizopygopsis* species from Lake Namtso and the other grouped with a clade of *G. dobula* and *G. waddellii*.

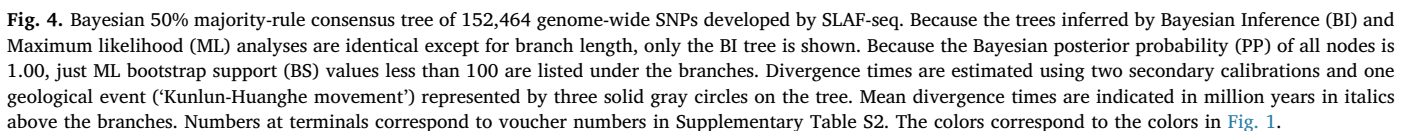
According to the results of divergence time estimation (Fig. 4 and Supplementary Fig. S1), the MRCA of HSG species was dated back to 5.35 Ma, and diverged from SG species in the Late Miocene (ca. 8.53 Ma). The major splits between and within clade B and clade C dated to 3.03–0.97 Ma. The divergence time of the two subspecies of *G. chui* was ca. 2.42 Ma.

3.2. Mitochondrial analysis

The ND2 could not be successfully amplified in three samples due to technical issues or low quality of the genomic DNA. In addition to four downloaded sequences of *G. scolostomus*, 337 sequences were obtained from 85 specimens in this study, and were deposited in GenBank and accession numbers were listed in Supplementary Table S2. The length of aligned Cyt b, ND2, 16S and D-loop were 1140 bp, 1047 bp, 1097 bp and 702 bp respectively, and totaled 3986 bp when concatenated.

The ML and BI phylogenies based on the mtDNA were almost identical, and were similar to previous studies (Fig. 5) (He and Chen, 2007; Duan et al., 2009; Zhang et al., 2016). MtDNA topology recovered the same clades A, B4, C1 and C2a as those in the SLAF tree. We found that HSG species were not monophyletic with respect to one embedded SG species *Gymnoditychus integrigymnatus*, as previously reported (He and Chen, 2007). Monophyly of *Platypharodon* was supported with high statistical support (PP = 1.00, BS = 100). Monophyly of traditionally defined genera *Gymnocypris*, *Schizopygopsis* and *Chuanchia* was not supported, where they were nested in clades B3-4 and C1-2. Monophyly of 16 species or subspecies, *Platypharodon extremus*, *S. chengi*, *S. stoliczkai stoliczkai*, *S. stoliczkai bangongensis*, *S. anteroventris*, *S. malacanthus*, *S. kialingensis*, *S. microcephalus*, *S. younghusbandi himalayensis*, *S. thermalis*, *G. potanini*, *G. scolostomus*, *G. chilianensis*, *G. scleracanthus*, *G. waddellii* and *G. dobula*, was recovered by strong support (PP ≥ 0.93 , BS $\geq 84\%$, except for *G. scolostomus*, BS = 58% in ML analysis). The other species were not monophyletic and failed to be clearly separated from sympatric or parapatric species on the tree, including *S. sp.*, *G. namensis*, *S. younghusbandi younghusbandi*, *G. chui chui*, *S. pylzovi*, *C. labiosa*, *S. stoliczkai maphamyumensis*, *G. chui longimandibularis*, *G. eckloni*, *G. przewalskii przewalskii* and *G. przewalskii ganzihonensis*. As observed in SLAF-based tree, two subspecies of *G. chui* were also not closest relatives. Compared to He and Chen (2007), one of the most obvious inconsistencies was the interchanged position of *Platypharodon extremus* and *Chuanchia labiosa*. Two runs of standard nucleotide BLAST in the National Center of Biotechnology Information (NCBI) using our Cyt b sequences of *Platypharodon extremus* (KY461375) and *Chuanchia labiosa* (KY461371) as the query sequence indicated that our sequences showed very high identity with those from the same species respectively (Duan et al., 2009; Su et al., 2015; Chen et al., 2016; He et al., 2016b; Li et al., 2016a). However, the two sequences (AY608650, AY608651) of *Chuanchia labiosa* originally submitted by Qi et al. (2006) and subsequently used by He and Chen (2007) were more similar with our sequences of *Platypharodon extremus*. A similar situation was also observed for the seven sequences (AY608657–AY608663) of *Platypharodon extremus* in Qi et al. (2006). In addition, Wang et al. (2016) presented similar phylogenetic positions of *Platypharodon extremus* and *Chuanchia labiosa* with our study. Together, we think a mistake in species identification of *Platypharodon extremus* as *Chuanchia labiosa* accounts for this inconsistency and vice versa.

Significant incongruence between the topologies inferred from SLAF versus mtDNA data was observed (Figs. 4 and 5). For example, clade B1-4 clustered together in SLAF data, whereas the mtDNA data supported clade B4 as a basal lineage separately, and then placed the remaining taxa of clade B between subclades C1 and C2. In clade B4 of mtDNA data, *G. potanini* grouped together with four *Gymnocypris*



3.3. Hypotheses tests

Four alternative hypotheses (B-E) were rejected by all tests using both datasets (mitochondrial and SLAF data) ($p < 0.01$; [Table 1](#)), suggesting that the genera *Gymnocypris* and *Schizopygopsis* are polyphyletic, as well as that *G. chui chui* and *G. chui longimandibularis* are two distinct species instead of two subspecies. The monophyly of HSG species was supported by the AU test ($p = 0.005$), but was not rejected by the SH test for mitochondrial data ($p = 0.515$; [Table 1](#)). Due to the

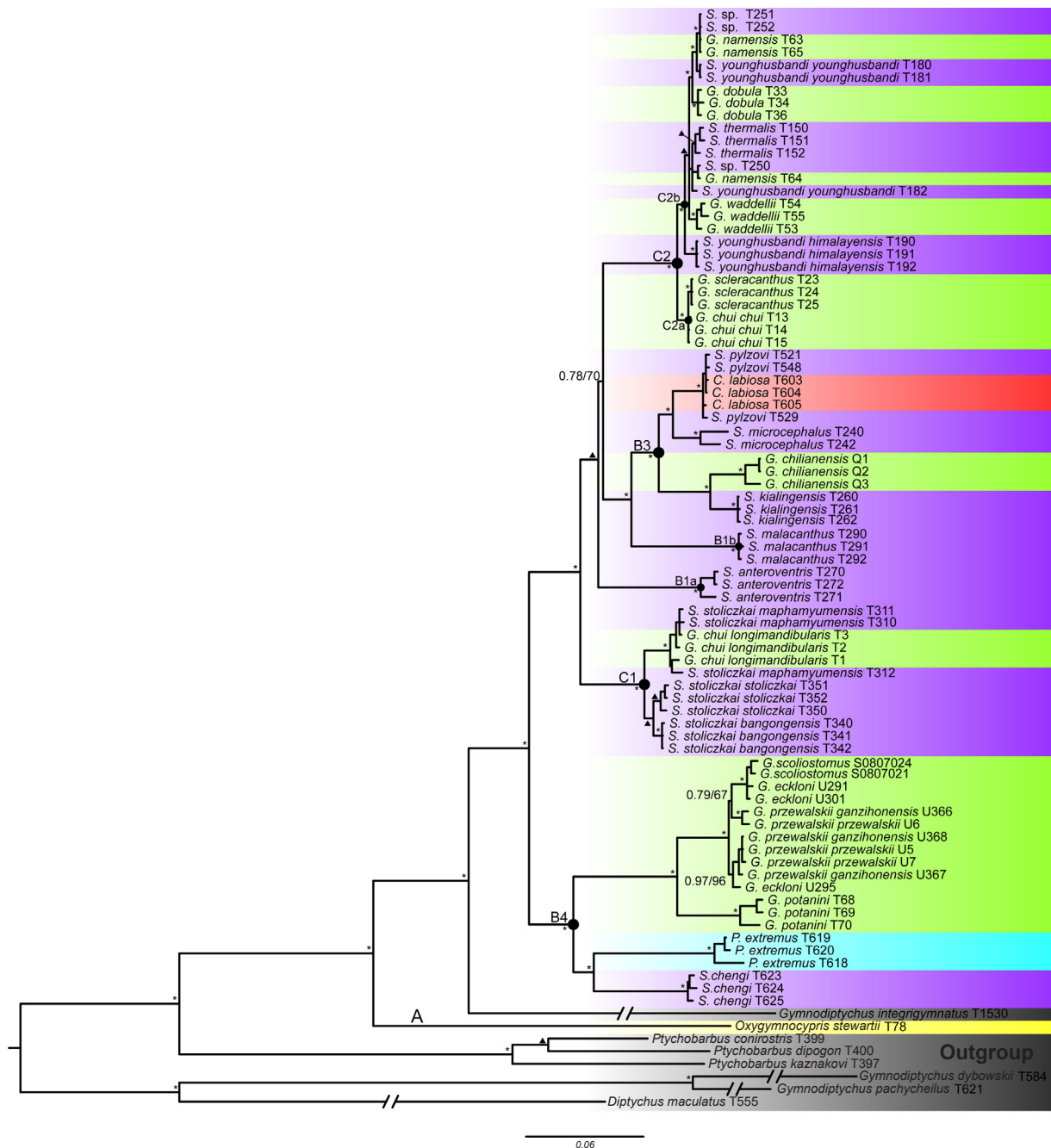


Fig. 5. Bayesian 50% majority-rule consensus tree off our concatenated mitochondrial DNA markers. Because the trees inferred by BI and ML analyses are almost identical except for several non-dichotomous branches at terminals, only the BI tree is shown. Numbers along the branches are PP and BS values. Asterisks denote PP ≥ 0.99 and BS $\geq 90\%$. Black triangles denote $0.99 > PP \geq 0.95$ and $90\% > BS \geq 70\%$. The capital letters (A–C) next to the nodes correspond to the clades in Fig. 4. Numbers at terminals correspond to voucher numbers in Supplementary Table S2. The colors correspond to the colors in Fig. 1.

absence of SLAF data for some SG species, monophyly of HSG species could not be tested with SLAF data.

3.4. Morphological differentiation

In principal component analyses of the head and lower jaw, we discovered four groups corresponding well to four genera defined by traditional classification. Three benthic-feeding genera (*Schizopygopsis*, *Platypharodon* and *Chuanchia*) had similar morphology of the head and lower jaw (Fig. 6c and d). Head shape changes along the PC1 axis showed short and nearly straight oral fissure as well as inferior mouth for *Chuanchia* and *Platypharodon*, long and oblique oral fissure and terminal mouth for *Gymnocypris*, and then intermediate phenotype for

Schizopygopsis (Fig. 6a). Furthermore, *Gymnocypris*'s lower jaw was the narrowest among the four genera (Fig. 6b). It was noteworthy that *G. chui chui* and *G. chui longimandibularis* had a very close relationship along the PC1 axis (Fig. 6c and d).

3.5. Ancestral state reconstruction

The result of the ancestral state reconstruction is presented in Fig. 7. The ancestral state of the MRCA of HSG species (N1) remained unresolved under both MP and ML methods. However, the most likely ancestral state of other basal nodes (i.e., N2, N3, and N4) was benthic feeder ecomorph under both methods. Both limnetic omnivore and planktonic specialist ecomorphs were inferred to have experienced

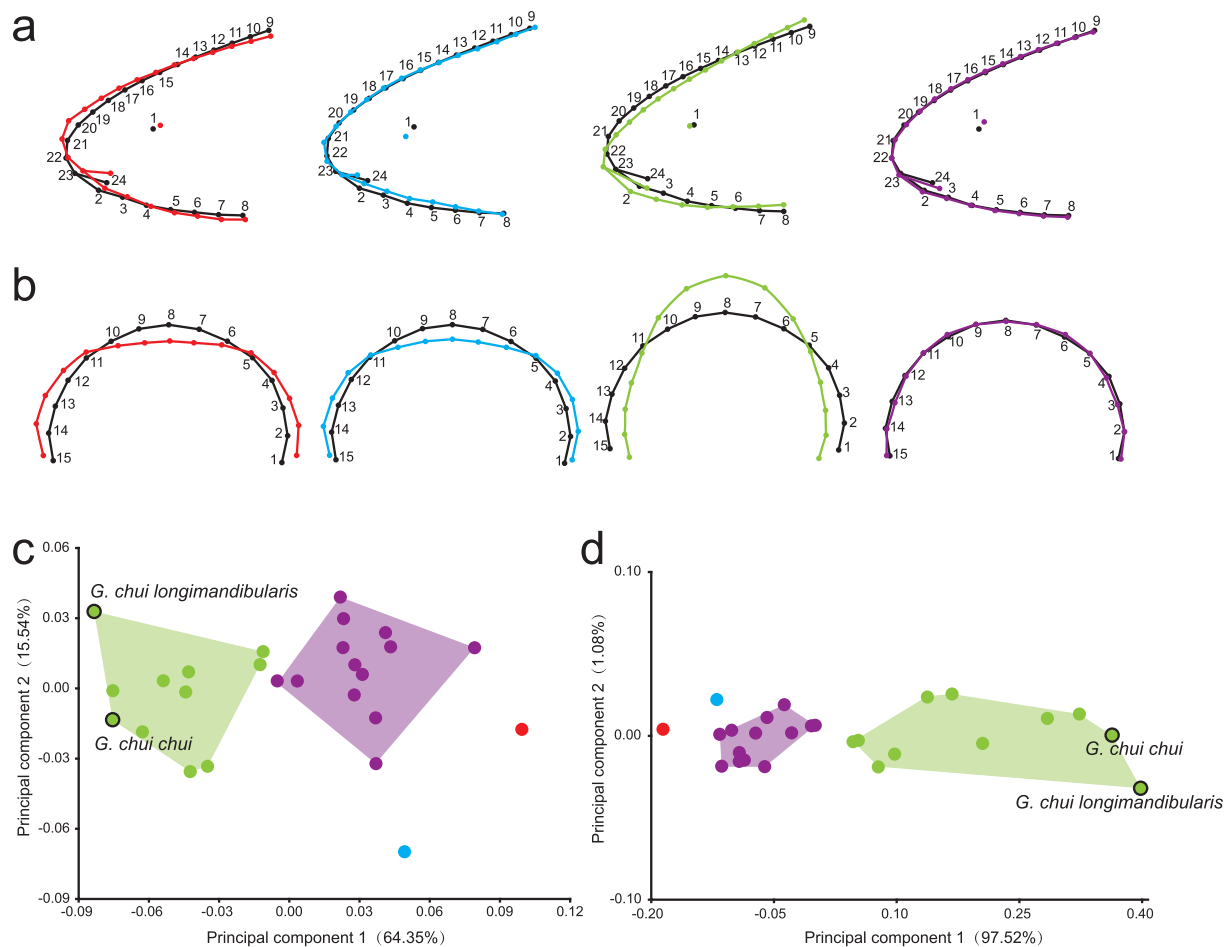


Fig. 6. Morphological differentiation among four genera. Shape changes along PC1 axis for (a) head shape and (b) lower jaw shape relative to the average shape. Scatter plots of first and second principal component scores of Principal Components Analysis for (c) head shape and (d) lower jaw shape. The colors correspond to the colors in Fig. 1.

convergent evolution. Without consideration of estimated equivocal states for some nodes, five independent origins of the limnetic omnivore ecomorph were observed. The planktonic specialist ecomorph, which was only seen in *G. chui chui* and *G. chui longimandibularis*, was independently derived from two different ancestral states, of which one was the benthic feeder ecomorph, and another was the limnetic omnivore ecomorph.

3.6. Diet comparison between *G. chui chui* and *G. chui longimandibularis*

Diet compositions for two pairs of sympatric species within Lake Langtso and Lake Manasarovar were quantified from both SGCA and SIA (Fig. 8). For two morphologically convergent species, *G. chui chui* and *G. chui longimandibularis*, SGCA indicated significant dietary similarity (Schoener's index = 0.68), with a high proportion of zooplankton, plant scraps and insects (i.e., chironomids at the water surface), and a very low proportion of zoobenthos (Fig. 8c). In line with gut content analysis, SIA showed similar divergence across two lakes (Fig. 8d). The smaller stable C isotope signatures ($\delta^{13}\text{C}$) of *G. chui chui* and *G. chui longimandibularis* indicated that they tended to forage in open water and pelagic areas compared to *G. scleracanthus* and *S. stoliczkae maphamyumensis*.

4. Discussion

According to all of our analyses in this study, *Gymnocypris* is polyphyletic and the taxonomic revision is necessary. For clarification and

convenience, we use “*Gymnocypris*” or “*G.*” to refer to putative genus in the discussion.

4.1. SLAF vs. MtDNA based phylogenies

Multiple contradictions between SLAF- and mtDNA-based phylogenies were recovered in basal and terminal branch order in clades B and C (Figs. 4 and 5). The phylogeny reconstructed from SLAF data was more convincing than that of mtDNA because it was not only based on whole genome information, but also supported by the following morphological and geographical evidence. First, regardless of generic limits, all species of clade C were morphologically similar in the position of the origin of ventral fin which corresponded to or was behind the fourth branched dorsal ray; whereas in clade B, the position of ventral fin corresponded to or was in the front of the third branched dorsal ray for all except three basal species, *S. microcephalus*, *S. malacanthus*, “*G.*” *namensis*, and one recently derived species, *S. chengi*, whose situations were similar to clade C (Wu and Wu, 1992; Chen and Cao, 2000). Second, because of limited dispersal ability across major water systems, the biogeographical patterns of freshwater fishes are often closely related to the physical geography (Slatkin, 1987; Unmack, 2001; Li et al., 2015a, 2016b). In our study, the northern clade B and southern clade C were separated by the Tanggula and Tanitawen Mountains ca. 3.03 Ma, coinciding with a violent uplift of the QTP in phase A of the ‘Qingzang movement’ (ca. 3.6 Ma) (Figs. 1 and 4) (Li et al., 2001, 2015b). Clade B contained all species distributed north of the Tanggula-Tanitawen Mountains and “*G.*” *namensis* in Lake Namtso.

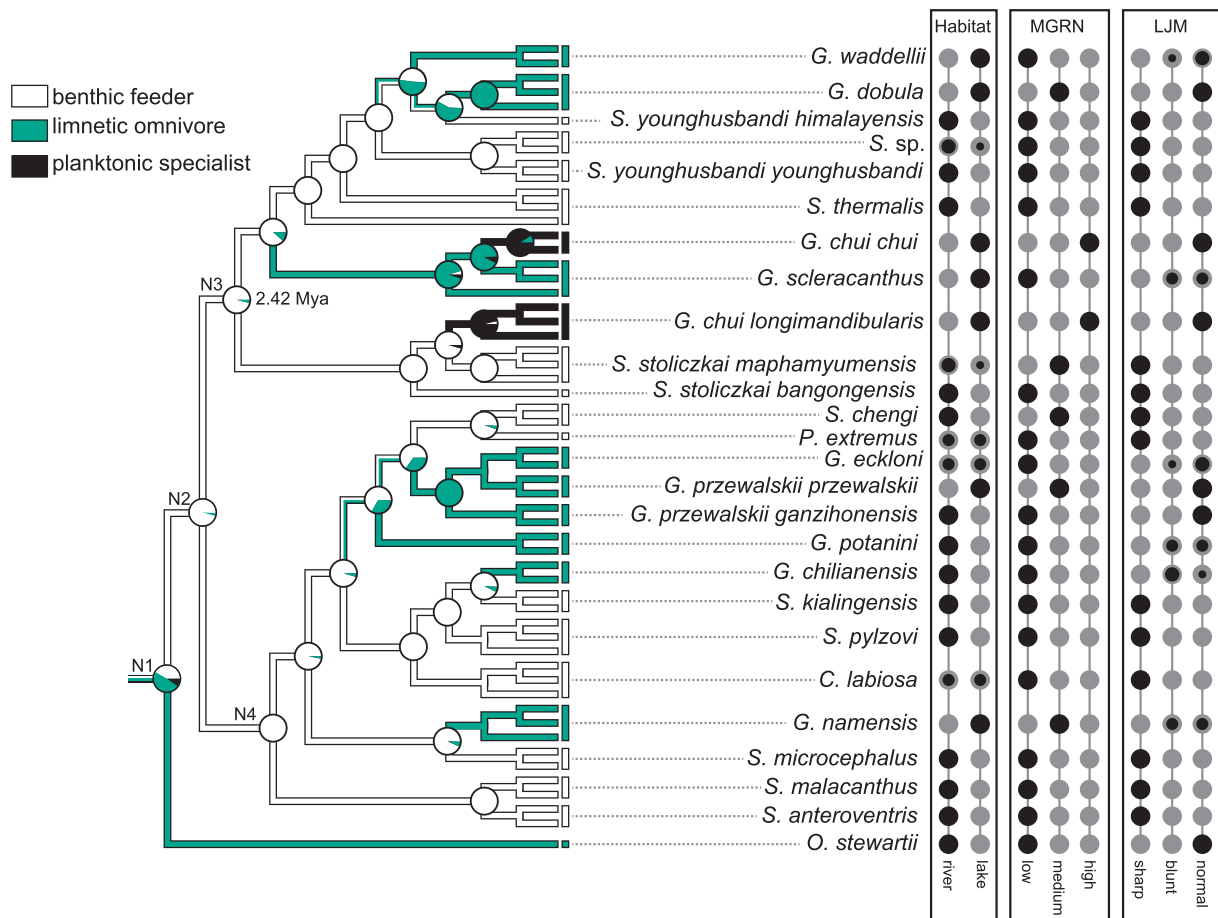


Fig. 7. Ancestral state reconstruction of trophic ecomorphs based on MP and ML methods in Mesquite 2.7 using SLAF ML trees (outgroup not shown). Pie charts indicate relative ML support at ancestral nodes for the corresponding character state. Colors in legend correspond to three ecomorphs. Three genera with sharp anterior edge on the lower jaw, *Schizopygopsis*, *Platypharodon* and *Chuanchia*, are assigned to the benthic feeder ecomorph. Two subspecies of *Gymnocypris chui* are considered as the planktonic specialist ecomorph. *Oxygymnocypris stewartii* and other *Gymnocypris* species are included in the limnetic omnivore ecomorph. Four basal nodes in the HSG species clade are defined as N1–4. The right part shows ecological and morphological characteristics of HSG species. Data are retrieved from previous literature (Wu and Wu 1992; Chen and Cao 2000). Habitat: river (black), lake (white) and both (gray). Mean outside gill raker number of first gill arch, MGRN: low < 20 (black), 20 ≤ medium ≤ 30 (gray) and high ≥ 30 (white). Lower jaw morphology, LJM: sharp anterior edge (black), normal or with blunt horny sheath (gray) and normal with no horny sheath (white).

Clade C included the remaining species from south of the Tanggula-Tanitawen Mountains except *O. stewartii*. The Tanggula-Tanitawen Mountains, also called the ‘Ward Line–Mekong–Salween Divide’ (Ward, 1921), was considered as a classic geographical boundary playing an important role in shaping the biogeographical structures of fauna and flora endemic to the Himalaya and Hengduan Mountains (Gao et al., 2007; Geissmann et al., 2011; Luo et al., 2017). This biogeographical pattern was discovered for the first time in schizothoracine fishes. Surprisingly, one northern clade species, “*G.*” *namensis* was distributed in Lake Namtso, south of the Tanggula Mountain (Fig. 1). The divergence between *S. microcephalus* and “*G.*” *namensis* occurred at ca. 0.84 Ma, corresponding well with the ‘Kunlun-Huanghe movement.’ We suggest that the river reversal event associated with this movement probably caused some individuals of *S. microcephalus* to be isolated from the Tuotuohe River population followed by migration to Lake Namtso, and subsequent speciation by local adaptation to lacustrine conditions. Additionally, it is also possible that the meltwater of the Pleistocene glaciations created several temporary channels between both sides of the Tanggula Mountain and provided ephemeral migration routes for fishes during the warm and wet interglacial periods (Shi, 1995; He et al., 2016a).

However, the mtDNA tree failed to show both patterns of corresponding positions of the ventral-dorsal fins and these geographical clades recovered by the SLAF tree, concerning the positions of

subclades within clades B and C (Figs. 4 and 5). In the mtDNA tree, subclade C2 had a close relationship with subclades B1–3 instead of subclade C1. Interestingly, a previous study (Shu et al., 2018) investigating the phylogeny of Schizothoracinae based on the complete mitochondrion genome supported a sister relationship of species in subclades C1 and C2, just like the cladistic relationship in our SLAF tree (Fig. 4). Accordingly, insufficient mutational information of our four mtDNA fragments may explain in part the SLAF-mt conflicts, which also could be reflected by the short branches and moderate supports (PP = 0.78, BS = 70) for the relationships of subclades C2 and B1–3 (Fig. 5). The closer relationship between subclades B1–3 and clade C shown in the mitochondrial-genome phylogeny of Shu et al. (2018) was more likely owing to an ancient hybridization event considering their adjacent geographical range (Fig. 1), although we could not fully exclude the possibility of incomplete lineage sorting. As the QTP experienced frequent tectonic events during the Late Pliocene and Pleistocene, the resulting river captures and rearrangements could promote secondary contact of previously divergent forms, leading to hybridization (Clark et al., 2004; Li et al., 2014). Additionally, mtDNA only resolves the history of a cytoplasmic genome and can often be misleading when relying on these sequences alone (Ballard and Whitlock, 2004; Moyer et al., 2009; Schmidt et al., 2017). Therefore, multiple nuclear markers are now considered as prerequisites for confident inference of phylogenetic relationships of species (Wallis et al., 2017).

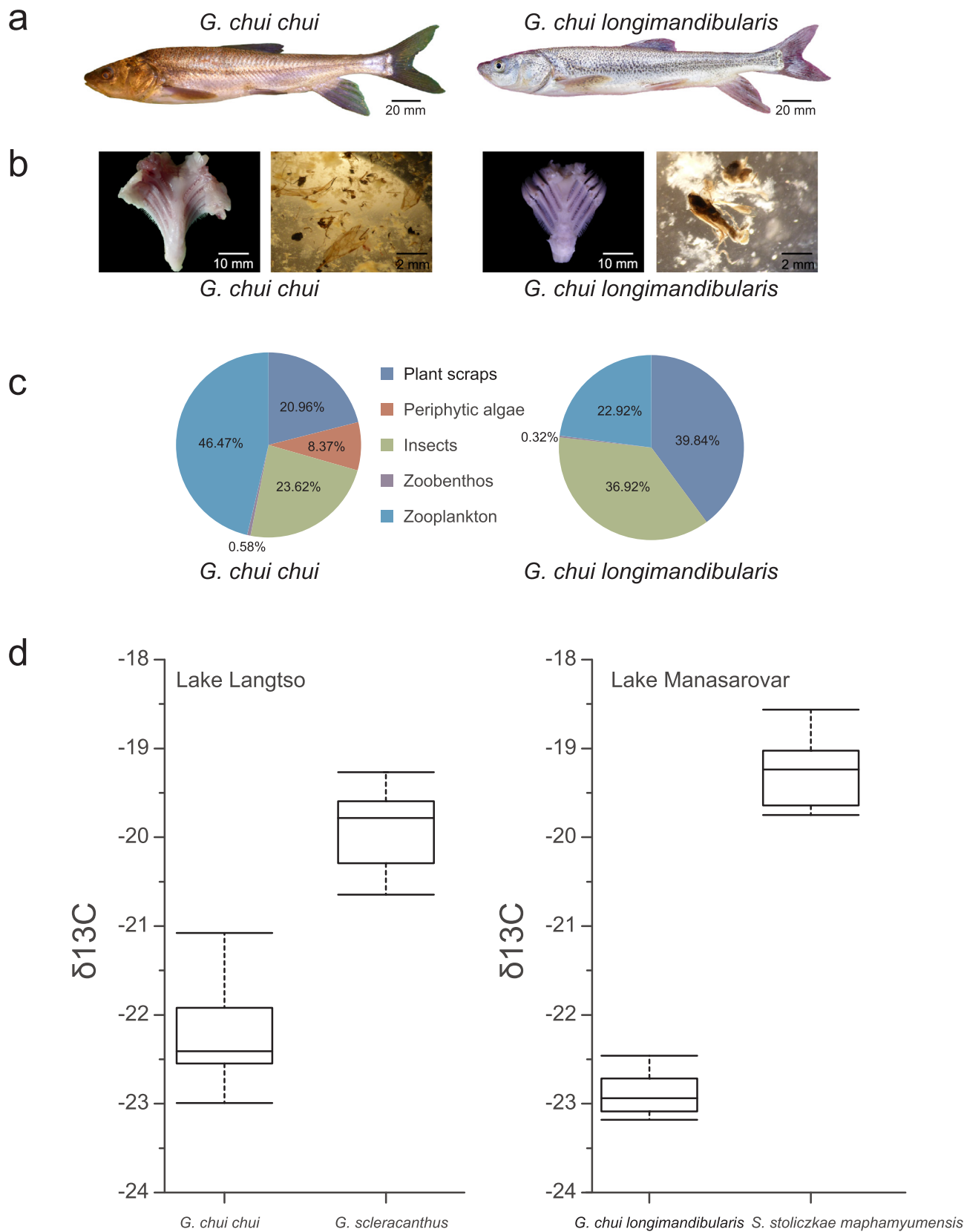


Fig. 8. The extreme case of convergent evolution between *Gymnocypris chui chui* and *G. chui longimandibularis*. (a) Photograph of *G. chui chui* and *G. chui longimandibularis*; (b) gill rakers and chironomids found in the gut contents; (c) pie chart of food composition for the two convergent species; (d) Carbon stable isotope analysis of two pairs of sympatric species (*G. chui chui* vs. *G. scleracanthus*, *G. chui longimandibularis* vs. *S. stoliczkae maphamyumensis*).

Species delimitation and relationships among HSG species have long been disputed, and primary debates are focused on the validity of several species or subspecies within “*Gymnocypris*” and *Schizopygopsis*, which mainly exist between two conflicting taxonomic systems advocated by Wu and Wu (1992) and Chen and Cao (2000). In the taxonomy of Wu and Wu (1992), “*G.*” *namensis* was placed in the genus *Schizopygopsis* and considered as a subspecies of *S. microcephalus*, based on the fact that they were the only two fishes possessing one row of pharyngeal teeth. However, it was reclassified as a separate “*Gymnocypris*” species by Chen and Cao (2000) because it shared similarities with “*Gymnocypris*,” such as blunt lower jaw morphology and well-developed canalis preoperculomandibularis. In our study, the SLAF-based tree confirmed a close relationship between *S. microcephalus* and “*G.*” *namensis*, which agrees with the classification by Wu and Wu (1992). Whereas in the mtDNA-based tree, “*Gymnocypris*” *namensis* grouped with the sympatric *Schizopygopsis* sp. in Lake Namtso, which likely resulted from mitochondrial introgression. “*Gymnocypris*” *eckloni* *scoliotomus* and “*G.*” *eckloni* *chilianensis* were classified as subspecies of “*Gymnocypris*” *eckloni* by Chen and Cao (2000), but were regarded as separate species by Wu and Wu (1992). Although the mtDNA tree showed the same position with previous study (Zhao et al., 2009), we did not discuss the subspecies status of “*G.*” *scoliotomus* further because of the deficiency of nuclear genes. However, given its unique distribution endemic to Lake Sumtso and that it was used as a model to study sympatric speciation (Zhao et al., 2009), clarifying the taxonomic status of “*G.*” *eckloni* is necessary. The separate species status of “*G.*” *chilianensis* supported by a recent molecular analysis (Zhang et al., 2015) was also confirmed in this study. Existence of a deep split between “*G.*” *eckloni* and “*G.*” *chilianensis* was observed; they had diverged from each other ca. 0.97 Ma (Fig. 4). A well-supported sister relationship between “*G.*” *chilianensis* and *S. kialingensis* was recovered in both SLAF and mtDNA trees. Based on morphological examinations of Li and Zhang (1974) and Wu and Wu (1992), “*G.*” *chilianensis* had fewer gill rakers and anal scales, and adult individuals of “*G.*” *chilianensis* were smaller than those of “*G.*” *eckloni*. In addition, “*G.*” *chilianensis* is endemic to three major rivers of the Hexi Corridor. In view of the above evidence, we suggest that “*G.*” *chilianensis* should be classified as an independent species. Individuals of “*G.*” *scleracanthus*, *S. anteroventris* and “*G.*” *chui longimandibularis* recognized by Wu and Wu (1992) also formed separate lineages in both trees. Furthermore, topology tests rejected subspecies status for “*G.*” *chui longimandibularis* (Table 1, $p < 0.01$). *Schizopygopsis* *younghusbandi* *himalayensis* was not the sister taxon to *S. younghusbandi* *younghusbandi*, but was grouped with two “*Gymnocypris*” species with high support in the SLAF tree and forms a single lineage in mtDNA tree. Moreover, *Schizopygopsis* *y. himalayensis* is endemic to the tributaries of the Ganges River, south of the Himalayas (nominant subspecies distributed in the Tsangpo River, north of the Himalayas). We recognize it as a separate species, *Schizopygopsis* *himalayensis*. A similar situation was observed for three subspecies of *Schizopygopsis* *stoliczkai*. Each has an endemic distribution, with *S. s. stoliczkai* in the upper Indus River, *S. s. maphamyumensis* in the endorheic Lakes Manasarovar and Langa, and *S. s. bangongensis* in the endorheic Lake Pangotso respectively. They were genetically and morphologically distinguished from each other by previous studies concerning the phylogeography and morphology of *S. stoliczkai* (Wanghe et al., 2017a, 2017b). Therefore, we recognize each subspecies of *S. stoliczkai* as a separate species. An undescribed *Schizopygopsis* sp. has the sister relationship with *S. younghusbandi* *younghusbandi* in the SLAF-based tree, and the species needs to be described in the future, following a more detailed morphological investigation.

Our mtDNA tree indicated that the monophyly of HSG fishes was broken by the insertion of *Gy. integrigymnatus*, distributed in Irrawaddy River (Figs. 1 and 5), which was also supported by the AU test ($p = 0.005$). The close relationship of *Gy. integrigymnatus* with HSG species could be understood from the following aspects. (1) Except for nearly degenerate barbels, it shares more traits with HSG fishes than

congeneric species such as almost entirely absent scales and poorly developed lips (distinguished from the other two thick-lipped *Gymnodiptychus* species). (2) This intermediate morphology of *Gy. integrigymnatus* and its basal position in HSG species implies it could be the transitional species from SG to HSG fishes (He et al., 2004). The residual barbels were extremely short and possibly presented a state of evolutionary stasis of morphology and function during the course of phyletic divergence (Winemiller, 1991). (3) In this study, an obviously positive correlation between drainage and species relationships was observed. Its exclusive habitat, the headwaters of the Irrawaddy River, is far from that of congeneric species, *Gy. pachycheilus* and *Gy. dybowskii* (Fig. 1). Its close affinity with *O. stewartii* could be inferred from geological evidence which indicated that the ancestral Tsangpo possibly flowed into the upper Irrawaddy River and was likely truncated by the Brahmaputra in the Late Miocene (Brookfield, 1998; Clark et al., 2004). Meanwhile, other fishes also show a close affinity between these two rivers, such as Badidae, Chinese sisorid catfishes, and genus *Schizothorax* (Ruber et al., 2004; Guo et al., 2005; He and Chen, 2006).

4.2. Convergent evolution was the cause of inconsistent taxonomy

“*Gymnocypris*” and *Schizopygopsis* were not recovered as monophyletic groups by either SLAF or mtDNA data (Figs. 4 and 5). Alternative topologies enforcing the respective monophyly of “*Gymnocypris*” and *Schizopygopsis* were also extremely unlikely (Table 1). Our general results found three major clades, namely clade A (*Oxygymnocypris stewartii*) and two geographical clades B–C, in which limnetic omnivore and benthic feeder ecomorphs repeatedly occurred in sympatric or adjacent water systems and often were each other’s closest relatives (Figs. 1 and 4). Three hypotheses might account for the discordance between the molecular phylogeny and traditional classification. First, some taxonomic characters are highly homoplastic and do not have systematic significance. They were repeatedly obtained in distinct lineages when facing similar environments. Conversely, closely related species are morphologically distinct due to divergent selection in different environments (e.g., riverine vs. lacustrine habitats). For example, trophic characters like the position of mouth, the shape of lip, the lower jaw morphology and the number of gill rakers are dependent on fishes’ feeding behavior, food type, and habitat. A similar phenomenon was observed in the primitive grade fishes of *Schizothorax*, in which subgeneric divisions based on mouth and lip characters were not supported by molecular analysis (He and Chen, 2006). Second, rapid diversification of species did not allow enough time to complete lineage sorting and thus resulted in retention of ancestral polymorphisms. According to our date estimation, HSG species originated in the Pliocene (ca. 5.35 Ma) and underwent a rapid radiation in the late Early Pleistocene (ca. 1.12 Ma) (Fig. 4). This corresponds with the ‘Kunlun-Huanghe movement’ which occurred at ca. 1.1–0.7 Ma. However, this explanation does not seem plausible because ancestral state reconstruction suggested the benthic feeder ecomorph as an ancestral state for N2–4 and “*Gymnocypris*” species having evolved more recently under both MP and ML methods (Fig. 7). In contrast, it is more credible that “*Gymnocypris*” species independently originated from ancestral *Schizopygopsis* species after the split of clades B and C (3.03 Ma) (Figs. 4 and 7). Third, SLAF and mtDNA trees consistently rejected the monophyly of the taxonomic genera “*Gymnocypris*” and *Schizopygopsis*, therefore introgression as an explanation for such extensive paraphyly is not as likely as convergent evolution of traits, even though some hybridization events possibly occurred among HSG species. Accordingly, we suggested that convergent evolution, but not introgression or incomplete lineage sorting was the cause of errors in traditional taxonomy.

4.3. Convergent evolution of trophic traits

Ecologically alike but spatially separated environments can exert similar selection pressures on diverging populations, which may lead to

repeated occurrences of specific ecotypes associated with specific niches (Schluter, 2000; Hudson et al., 2011). Importantly, we found that species inhabiting the same environment tend to be morphologically similar, irrespective of their genetic background (Figs. 4–6). For instance, “*Gymnocypris*” species primarily live in lakes (Figs. 1 and 7), and independently evolved lake-adapted foraging characters. Their terminal mouth position and normal lower jaw morphology (Fig. 6a) indicated an extremely wide foraging scope, ranging across the entire water column. The deeply oblique oral fissure and moderate to dense gill rakers allow them to consume a wide spectrum of food items including plankton, zoobenthos, algae, and small fishes, and be considered as a generalist predator (Tsao and Wu, 1962; Wu and Chen, 1979). In contrast, benefiting from the trophic traits of inferior mouth position, sharp outer horny sheath on the lower jaw and sparse gill rakers, *Schizopygopsis* species, *Chuanchia labiosa* and *Platypharodon extremus*, would be more efficient than “*Gymnocypris*” species in feeding on benthos like zoobenthos or periphytic algae attached to the substrate, the major food items in rivers (Tsao and Wu, 1962). This close correlation between habitat and trophic traits suggested a habitat-driven ecomorphological adaptation. This finding was not surprising given the oligotrophic nature of the lakes and rivers on the QTP and the distinct food items they offered, where rivers mainly offer benthos at the substrate and lakes offer relatively rich prey scattered in the water body (Ren and Sun, 1982; Jiang et al., 1983; Wu and Wu, 1992; Wan and Zhang, 2004; Yuan et al., 2007). A functional trade-off between speed of movement of the jaw and the force at the jaw tip is presented by the ratio of in-lever (articular) to out-lever (dentary), where a lower ratio will speed the jaw velocity and reduce the potential bite force (Wainwright and Richard, 1995). Particularly in this study, “*Gymnocypris*” species had a narrow and long lower jaw with a slender dentary, which favored a rapid suction and relatively weak bite to capture prey (Fig. 6) (Wu and Wu, 1992). In contrast, the wide and short lower jaw with sharp outer horny sheath, together with a robust dentary, provided a strong bite force for *Schizopygopsis* species, *Chuanchia labiosa* and *Platypharodon extremus* to graze algae and grip zoobenthos (Fig. 6) (Wu and Wu, 1992).

Perhaps the most amazing case of convergent evolution within HSG fishes occurred between two ‘subspecies,’ “*G.*” *chui chui* and “*G.*” *chui longimandibularis*, across two isolated lakes (Fig. 8). Our molecular data and time estimation showed that they were not closest relatives, but had diverged ca. 2.42 Ma (Fig. 4). Ancestral state reconstruction significantly favored planktonic specialist as a more recently derived ecomorph originating from their sympatric species by both MP and ML methods (Fig. 7). The alternative scenario, that they previously had a wide geographical distribution and later became extinct in all other locations was far less parsimonious. Considering all the evidence, we concluded that they were two distinct species and selection pressures related to similar diet probably explained the convergence of trophic morphology. “*Gymnocypris*” *chui chui* and “*G.*” *chui longimandibularis* were unique among “*Gymnocypris*” species with nearly superior mouth positions, the densest gill rakers, and a preference to feed almost exclusively on zooplankton, chironomids and plant scraps at the water surface as shown by similar stomach contents (Schoener's index = 0.68) and stable C isotope signatures (Fig. 8) (Wu and Wu, 1992). In line with taxonomy, geometric morphometric analyses also grouped them together (Fig. 6c and d). In our field observations, these two species often leaped up to capture chironomids at the water surface, which was also verified by SGCA (Fig. 8a). This is the first documented example of convergent evolution in Tibetan fishes, in which the two species were ecomorphologically similar to a degree that even taxonomists considered them as one. Meanwhile, they offer a potential model to study how ecological speciation acts on the different populations or lineages through ecologically-based divergent natural selection when facing similar niches (Rundle and Nosil, 2012).

4.4. Taxonomic implications

Based on our findings that the generic limits are strongly incongruent with the molecular phylogenies (Figs. 2, 4 and 5), we propose a major reclassification as the following:

1. Extensive paraphyly of *Gymnocypris* Günther, 1868 and *Schizopygopsis* Steindachner, 1866 rejects the validity of both genera. Moreover, the type species (*S. stoliczkai* and *G. dobula*) of both genera are found in the clade C from the SLAF tree (the nominate subspecies, *S. stoliczkai stoliczkai*, was not included in the SLAF data but in mtDNA data). To keep monophyletic assemblages and consider the established priority, we formally place *Gymnocypris* Günther, 1868 in the synonymy of *Schizopygopsis* Steindachner, 1866.
2. Two monotypic genera, *Chuanchia* Herzenstein, 1891 and *Platypharodon* Herzenstein, 1891, nested within clade B, are considered as the junior synonym of *Schizopygopsis* Steindachner, 1866. Although obvious morphologically distinctive trophic traits are observed for *Chuanchia labiosa* and *Platypharodon extremus*, seven additional genera at least need to be established to assure their monotypy. Therefore, we treat *Chuanchia labiosa* and *Platypharodon extremus* as *Schizopygopsis labiosa* Herzenstein, 1891 and *Schizopygopsis extrema* Herzenstein, 1891, respectively.
3. We elevate “*Gymnocypris*” *chui longimandibularis*, *Schizopygopsis younghusbandi himalayensis*, *Schizopygopsis stoliczkai maphamyensis* and *Schizopygopsis stoliczkai bangongensis* to separate species status as *Schizopygopsis longimandibularis* Tsao, Chen, Wu & Zhu, 1983, *Schizopygopsis himalayensis* Tsao, 1974, *Schizopygopsis maphamyensis* Wu & Zhu, 1979 and *Schizopygopsis bangongensis* Wu & Zhu, 1979.

5. Conclusions

According to our results, extensive paraphyly of “*Gymnocypris*” and *Schizopygopsis* suggests a paucity of phylogenetically informative morphological characters in HSG under the present taxonomic system. Convergent morphological traits, particularly those associated with feeding behavior, like mouth position, lower jaw morphology and number of gill rakers, are highly ecologically relevant and frequently confuse taxonomists. These homoplastic characters that were generally used to differentiate schizothoracine fishes at intergeneric and infrageneric levels are clearly obstacles to exploring their actual evolutionary history. Thus, more novel synapomorphies that accurately reflect evolutionary relationships should be identified for revising the taxonomy of the HSG fishes.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2019.01.008>.

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