

# The biogeography and phylogeny of schizothoracine fishes (*Schizopygopsis*) in the Qinghai-Tibetan Plateau

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Freshwater fish belonging to the genus *Schizopygopsis* are widespread in drainages throughout the Qinghai-Tibetan Plateau and, thus, a model group with which to investigate how paleo-drainage changes linked to historical uplifting within the Qinghai-Tibetan Plateau influence speciation. To date, the phylogenetic and taxonomic relationships within *Schizopygopsis* remain controversial. In this study, we constructed a comprehensive molecular phylogeny of *Schizopygopsis* based on six mitochondrial gene sequences. We compared the taxonomic relationships revealed by this phylogeny with those obtained from morphological data. We also used this phylogeny to assess the extent to which the evolution of *Schizopygopsis* has been driven by paleo-drainage changes linked to uplifting of the Qinghai-Tibetan Plateau. Results indicated that all *Schizopygopsis* taxa formed a monophyletic group comprising five major clades, which were inconsistent with the taxonomic relationships based on morphology for this group. Our results also strongly supported the validity of *S. anteroventris* and *S. microcephalus* as distinct species within *Schizopygopsis*. Molecular calibrations showed that species within the middle Yangtze species diverged earlier (~4.5 Mya) than species within the Indus River (~3.0 Mya), the Mekong River (~2.8 Mya) and the Tsangpo + Salween rivers (~2.5 Mya). The most recent evolutionary splits occurred among species from the upper and lower Yangtze River, the Yellow River and the Qiadam Basin at about 1.8 to 0.3 Mya. Our molecular evidence and use of the molecular clock calibration have allowed us to associate speciation events within the genus *Schizopygopsis* to the formation and separation of paleo-drainage connections caused by tectonic events during the uplifting of the Qinghai-Tibetan Plateau (~4.5 Mya). This work underlines the dominant role of vicariance in shaping the evolutionary history of the genus *Schizopygopsis*. Further research using multiple loci and more extensive sampling will reveal a more complete picture of the phylogenetic relationships and biogeography of *Schizopygopsis* fishes.

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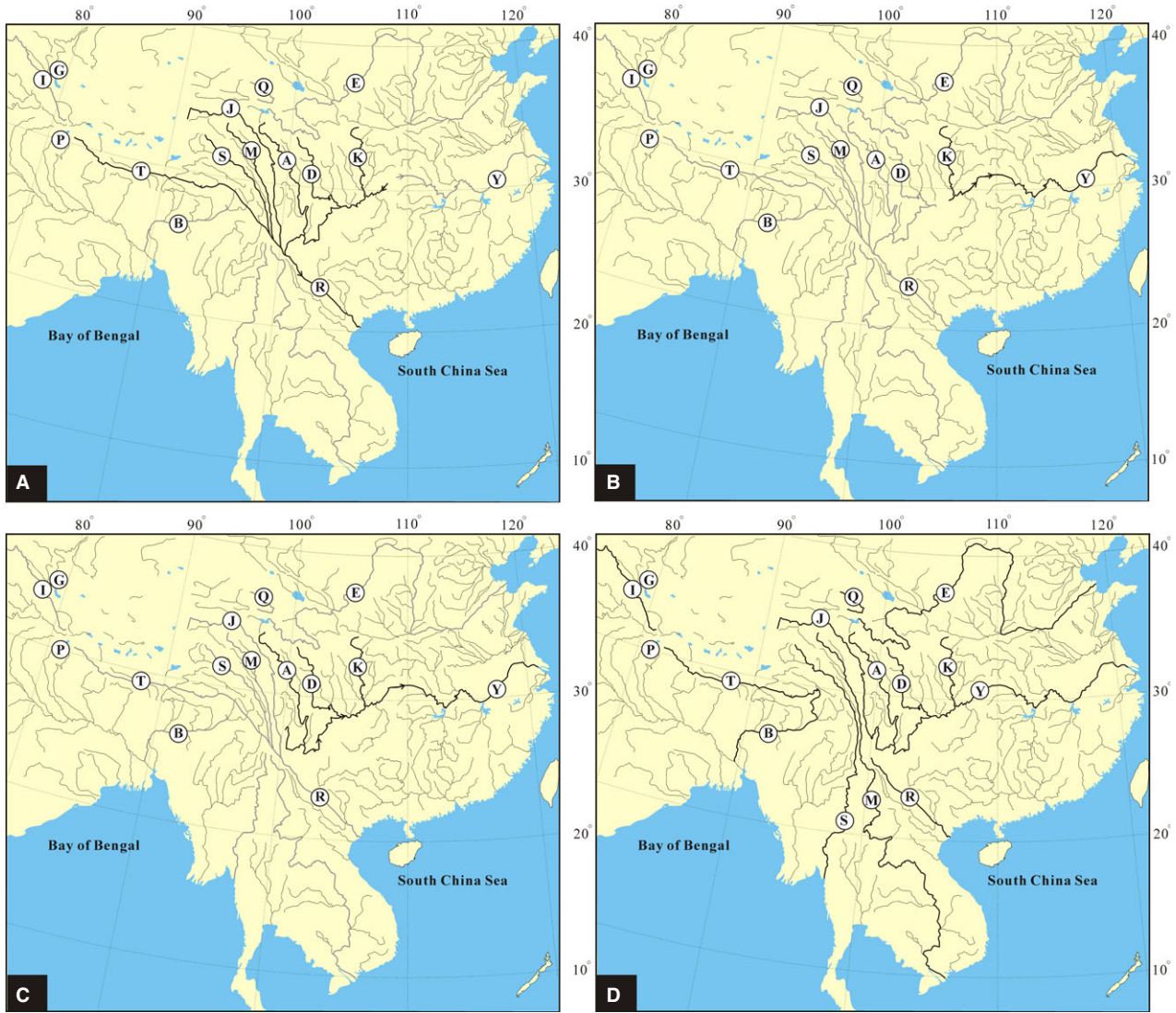
## Introduction

Phylogeographical studies play an important role in understanding the evolutionary history of the biota of a geographical area in relation to paleo-environmental changes (Hewitt 1996; Bermingham & Martin 1998; Pavlova *et al.* 2005; Lessios & Robertson 2013). Freshwater fishes, which are constrained by drainage systems, have provided key insights into how historical paleo-drainage changes shape current species distributions (Murphy & Collier 1996; Berendzen *et al.* 2003; Hewitt 2004). China's Qinghai-Tibetan Plateau is home to between nine and 12 species of schizothoracine fishes (genus *Schizopygopsis* Steindachner, 1866), which are distributed throughout the majority of the plateau's main drainages (including the Indus River and the isolated lakes of western Tibet; see Supporting Information Table S1 and Fig. 1). The wide distributional range of these species and their limited ability to migrate make this genus of obvious biogeographical interest. Additionally, being confined to high altitudes and high latitudes, these species have evolved unique morphological and physiological adaptations to their plateau environment and play a significant role in the trophic web of freshwater communities within the Qinghai-Tibetan Plateau (Wu & Wu 1992; Qi *et al.* 2012). *Schizopygopsis*, therefore, is a good model group with which to investigate how paleo-drainage changes linked to historical uplifting within the Qinghai-Tibetan Plateau can influence speciation.

Wu (1964) first described the taxonomy of the genus *Schizopygopsis* based on morphological and anatomical characteristics, recognizing six species: *S. stoliczkae* Steindachner, 1866; *S. kessleri* Herzenstein, 1891; *S. pylzovi* Kessler, 1876; *S. malacanthus* Herzenstein, 1891; *S. thermalis* Herzenstein, 1891; *S. kialingensis* Tsao & Tun, 1962; and a subspecies of *S. malacanthus* (*S. malacanthus chengi* Fang, 1936). Later, Wu & Wu (1992) proposed a new morphological phylogeny containing nine species in which they (i) assigned the species *Herzenstein microcephalus* Herzenstein, 1891, and *S. youngbusbandi* Regan, 1905, to the genus *Schizopygopsis*, (ii) elevated *S. malacanthus chengi* to species status, (iii) subsumed *S. kessleri* as synonymous to *S. pylzovi* and (iv) included a new species, *S. anteroventris* Wu, Tsao, Zhu & Chen, 1979. Based on gross morphology, Wu & Wu (1992) showed there were four major clades within the genus *Schizopygopsis*: (i) a primitive clade consisting solely of *S. kialingensis* and characterized by a narrow, slender horny sheath on the lower jaw and slender pharyngeal bone, which are distributed in rivers from 1300 to 3000 m

above sea level; (ii) a clade consisting of *S. pylzovi*, *S. chengi* and *S. malacanthus*, characterized by a broad and flat horny sheath on the lower jaw, broad pharyngeal bone and more than 16 gill rakers on the outer row of the first gill arch, which are distributed in rivers from 2200 to 4400 m above sea level; (iii) a clade consisting of *S. anteroventris*, *S. youngbusbandi* and *S. thermalis*, characterized by a broad and flat horny sheath on the lower jaw, broad pharyngeal bone and less than 14 gill rakers on the outer row of the first gill arch, which are distributed in rivers from 3000 to 4500 m above sea level; and (iv) a clade consisting of *S. stoliczkae* and *S. microcephalus* that possess highly specialized examples of morphology, such as a broad horny sheath on the lower jaw, markedly broad pharyngeal bone and a mixed number of gill rakers on the outer row of the first gill arch, which are distributed in rivers from 3500 to 5000 m above sea level. Wu & Wu (1992) proposed morphological trends in the evolution of some traits, such as the broadening of the horny sheath on the lower jaw and the broadening of the pharyngeal bone, and noted an increase in the altitudinal habitat preferences of the clades (the most basal group in this genus occurs at lowest altitude, while the most highly specialized group is restricted to the highest altitude). More recently, Chen & Cao (2000) again revised the classification of *Schizopygopsis* based on major morphological and anatomical characteristics, while Qi *et al.* (2007) proposed a taxonomic revision of this clade based on a new molecular phylogeny. While these previous studies have extended our understanding of the phylogenetic relationships of *Schizopygopsis*, the small number of morphological characteristics used and the absence of species from the analyses limit their ability to resolve the taxonomic conflicts, evolutionary history, phylogenetic relationships and biogeography within this group. Phylogeographical analyses that include more species and are based on robust molecular data are, thus, necessary to reconstruct *Schizopygopsis* more accurately and determine the biogeographical patterns of this genus within the Qinghai-Tibetan Plateau.

It has long been recognized that the paleo-drainage patterns of the major continental east Asian rivers that drain the south-eastern margin of the Tibetan Plateau differed markedly from their current patterns (Brookfield 1998; Hallet & Molnar 2001; Clark *et al.* 2004; Rüber *et al.* 2004; He & Chen 2007). The major rivers of the Qinghai-Tibetan Plateau were once tributaries of a single southward-flowing river system that drained into the South China Sea through the paleo-Red River (Fig. 1a; Clark



**Fig. 1** Summary of major changes to the paleo-drainages of the Qinghai-Tibetan Plateau [adapted from Clark *et al.* (2004) and Rüber *et al.* (2004)]. —A. Drainage patterns prior to major captures of rivers, where the upper Yangtze, middle Yangtze, upper Mekong, upper Salween and the Tsangpo rivers drained together to the South China Sea through the paleo-Red River. —B. The capture of the Kialing River to the east, into the lower Yangtze River (changes shown in bold black). —C. The reversal/capture of the middle Yangtze River into the lower Yangtze River. —D. The capture of the upper Yangtze River by the middle Yangtze, and of the upper Mekong and upper Salween rivers into their modern drainage positions. Also shown is the capture of the Tsangpo River through the Brahmaputra River into its modern course. The letters in circles refer to the following: A, Yalung River; B, Brahmaputra River; D, Dadu River; E, Yellow River; G, Bangongco Lake; I, Indus River; J, Jinsha River; K, Kialing River; M, Mekong River; P, Mapangyongco Lake; Q, Qiadam Basin; R, Red River; S, Salween River; T, Tsangpo River; Y, Yangtze River.

*et al.* 2004). The subsequent reorganization of this river system into the current major river drainages was caused primarily by river capture and reversal events that are linked with the uplifting of the Qinghai-Tibetan Plateau. Of these events, the development of the Yangtze River exhibits particularly great paleo-geographical complexity as a result of the progressive capture and reversal events that occurred in the lower, middle and upper reaches of the

Yangtze River (Fig. 1b–d). Previous studies have shown that the development of rivers in the Qinghai-Tibetan Plateau, along with the processes leading to their isolation or interconnection during uplifting of the plateau, have played a dominant role in determining the evolution and current distributions of the primary freshwater fishes in this region (Rüber *et al.* 2004; Guo *et al.* 2005; He & Chen 2006). However, a thorough phylogeographical analysis of the



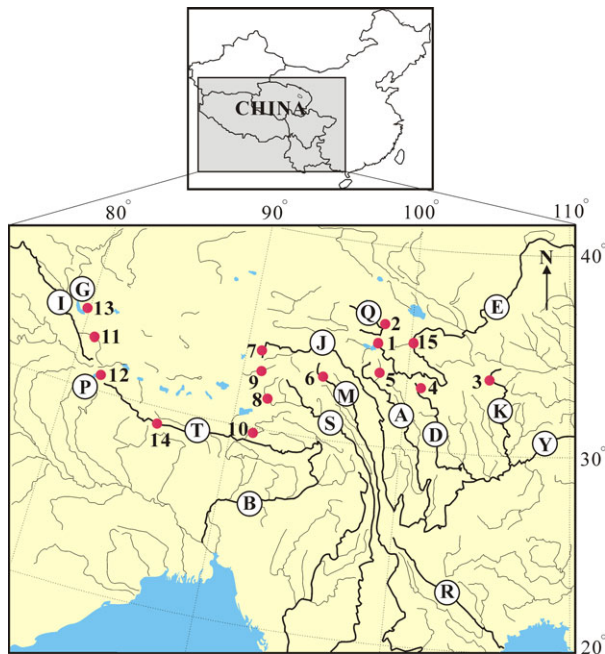
genus *Schizopygopsis* will help to elucidate the evolution of drainages in the Qinghai-Tibetan Plateau, especially the evolution of drainages within the western and central Tibetan Plateau and the relationships among the Yangtze River, Yellow River and Qiadam Basin.

In this study, we assessed the deep phylogenetic relationships of the genus *Schizopygopsis* using sequences from six mitochondrial genes: cytochrome b gene (*Cyt b*), NADH dehydrogenase subunit 1 and 2 (ND1, ND2), cytochrome c oxidase subunit I, II and III (CO I, COII and COIII). We aimed to (i) clarify the phylogeny of *Schizopygopsis* and test whether the molecular phylogenetic relationships reflect taxonomic relationships based on morphology, and (ii) use our phylogeny and a molecular clock to assess the extent to which the evolution of *Schizopygopsis* has been driven by paleo-drainage changes linked to uplifting of the Qinghai-Tibetan Plateau.

## Material and methods

### Sample collection

Fish were collected from the field using gill nets over a 10-year period (2003–2013). Whole fish were preserved in 90% ethanol in the field and subsequently transferred into 95% ethanol before being deposited in the ichthyology collection of the Animal Science Department of the Agriculture and Animal Husbandry College of Qinghai University, China. The specimens used in this study include 12 species



**Fig. 2** Location of sites where fish were sampled for use in this study. The numbers refer to the localities listed in Table S1. The letters in circles are the same localities as in Fig. 1.

and subspecies representing almost all the species of the genus *Schizopygopsis* known to be present in the Qinghai-Tibetan Plateau. These species were collected from all of the main drainages of the Qinghai-Tibetan Plateau. In addition, five species of primitive and specialized schizothoracine fishes were chosen as the outgroup (see Fig. 2 and Supporting Information Table S1).

All research involving animals in this study followed the guidelines of, and was conducted under the approval of, the Animal Care and Use Committee, Qinghai University, China.

### DNA extraction, PCR amplification and sequencing

Total genomic DNA was isolated from ethanol-fixed muscle tissue or fin clips by proteinase K digestion followed by standard phenol–chloroform extraction. Complete sequences of six protein-coding mitochondrial genes (*Cyt b*, ND 1, ND 2, CO I, CO II and CO III) were amplified using PCR with primers (Supporting Information Table S2). All the primers were synthesized by Sangon Biotech (Shanghai, China) Co. Ltd. PCR amplifications were performed in total reaction volumes of 30  $\mu$ L, containing 1.0 U TaKaRa EX Taq (TaKaRa, Dalian, China), 1  $\mu$ L of dNTP mix (2.5 mM each), 3.0  $\mu$ L of 10 $\times$  Taq buffer (TaKaRa), 0.5  $\mu$ L of each primer (10 mM) and 0.1  $\mu$ g of total genomic DNA. Reactions were carried out for initial denaturation at 94  $^{\circ}$ C for 4 min, followed by 35 cycles of denaturation at 94  $^{\circ}$ C for 1 min, annealing at 50–55  $^{\circ}$ C for 1 min and extension at 72  $^{\circ}$ C for 1 min, with a final extension at 72  $^{\circ}$ C for 5 min. PCR products were visualized via electrophoresis on a 1.0% agarose gel. Successful amplifications were excised from the gel and purified with a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa) following the manufacture's protocol. The purified fragments were then sequenced by Sangon Biotech Co., Ltd. New sequences have been deposited in GenBank (Accession numbers given in Table S1).

### Data analyses

The nucleotide sequences of the six protein-coding mitochondrial genes were subjected to concatenated alignment using CLUSTAL X (Thompson *et al.* 1997) with default settings and adjusted by eye. Statistics on nucleotide composition were conducted using MEGA version 3.0 (Kumar *et al.* 2004). We first conducted maximum-likelihood (ML) analysis under a one single model of molecular evolution for the whole concatenate data set in PAUP\* 4.0b10 (Swofford 2000). The best-fitting models of sequence evolution were determined with MODELTEST 3.7 (Posada & Crandall 1998), where the heuristic search parameters were the simple addition sequences of the taxa with TBR branch-swapping and 10 random sequence additions. Branch support

(BS) for the ML tree was assessed using nonparametric bootstrapping (Felsenstein 1985) with 100 heuristic replicates and with single random addition replicates.

Subsequent phylogenetic analyses were conducted under an optimal partitioning scheme using the maximum-likelihood (ML) approach in RAXML GUI v. 1.3.1 (Silvestro & Michalak 2012) and Bayesian inference (BI) in MRBAYES 3.0 (Huelsenbeck & Ronquist 2001). The best-fit partitioning scheme and the best nucleotide evolution model for each partition were evaluated using the PARTITIONFINDER program (Lanfear *et al.* 2012) based on Akaike information criterion (AIC). For the ML analysis, RAXML implements the rapid hill-climbing algorithm of heuristic searches with bootstrap pseudo-replicates (RBS). Bootstrap (BS) resampling (Felsenstein 1985) was applied to assess support for individual nodes using 1000 replicates. Random starting trees were used for each independent ML tree search, and all other parameters were left as the default values. The ML analyses were conducted under different models for each partition of the matrix as evaluated in PARTITIONFINDER. The Bayesian analysis was similarly conducted under the optimal partitioning scheme evaluated in by the PARTITIONFINDER program with the 'set partition' and 'unlink' options chosen. We used four simultaneous Monte Carlo Markov chains of 5 000 000 steps each, saving a tree every 100 steps. Posterior probabilities (PP, shown as percentages) indicated branch support (Huelsenbeck & Ronquist 2001). The first 5000 trees were discarded, and 45 000 trees (whose log-likelihoods converged to stable values) were used to construct a 50% majority rule consensus tree with the PP. Acceptable sampling from the posterior and convergence to the stationary distribution were checked using the diagnostic software TRACER v1.5 (Rambaut & Drummond 2007a).

The Shimodaira–Hasegawa test (SH; Burrige 1999) was used to evaluate the alternative phylogenetic trees we obtained by constraining the topologies of interest based on unpartitioned PAUP tree. These were tested using the RELL and 1000 bootstrap replicates in PAUP\*.

Species divergence dates were estimated using the uncorrelated relaxed molecular clock model (lognormal) under a Bayesian framework implemented in the program BEAST v2.1.2 (Drummond & Rambaut 2007). All clade-age estimates were presented as means and their 95% highest posterior density (HPD) values, which are the upper and lower bounds of the HPD interval. As no fossil records of the schizothoracine fish were available, we used two important and accurately dated geological events, as well as published substitution rate of Cyt *b* (0.65–1.86% per Mya; Machordom & Doadrio 2001; Duan *et al.* 2009) to calibrate the molecular clock. First, to calibrate the external node we used the opening of the Gibraltar Strait after the

Messinian salinity crisis, which separated North Africa from the Iberian Peninsula and prompted the separation of the genus *Barbus* (~5.0 Mya). In this method, the tentative divergence dates were estimated using Cyt *b* gene sequences based on six additional species of the genus *Barbus* (*Barbus sclateri*, AF0459720; *Barbus guiraonis*, AF045972; *Barbus callensis*, AF045974); *Barbus bocagei*, AF045969; *Barbus graellsii*, AF045973; and *Barbus microcephalus*, AF045971; Zardoya & Doadrio 1999). Second, to calibrate the internal node we used the 'Kunlun–Huanghe Movement' that occurred ~1.1 Mya during the uplifting of the Qinghai–Tibetan Plateau, which prompted the evolutionary separation of the schizothoracine fishes distributed in the upper Yangtze River, Yellow River and Qiadam Basin (He & Chen 2007). In this method, the tentative divergence dates were estimated using a combined data set of all six genes. The first calibration point was implemented as a normally distributed prior, with a mean of 5.0 Mya and a standard deviation of 0.1 Mya. The second calibration point used the same normally distributed prior, with a mean of 1.1 Mya and a standard deviation of 0.1 Mya. This analysis was conducted under the optimal partitioning scheme evaluated by the PartitionFinder program. We combined previously published substitution rates of Cyt *b* and the geological calibration points to infer species divergence dates. The analyses were run for 100 000 000 generations and sampled every 1000th generation. Stationarity and sufficient mixing of parameters (ESS.200) were verified using TRACER v1.5 (Rambaut & Drummond 2007a). A consensus tree was built using TREE ANNOTATOR v1.7.5 (Rambaut & Drummond 2007b).

## Results

### *Sequences characteristics*

The complete sequences of the Cyt *b*, ND 1, ND 2, CO I, CO II and CO III genes were 1140, 975, 1046, 1551, 691 and 786 bp in length, respectively. The six genes were concatenated to form a combined data set of 6189 aligned sites. In the analysed nucleotide sites of the ingroup taxa (the 12 *Schizopygopsis* species, see Supporting Information Table S3), 805 characters were variable and 500 were parsimony informative. The third codon positions were the most informative (456 parsimony informative characters), followed by the first codon positions (39 parsimony informative characters) and then the second codon positions (5 parsimony informative characters). Nucleotide base composition showed there was a low proportion of G among the six genes, which is characteristic for the mitochondrial genome (Cantatore *et al.* 1994; see Supporting Information Table S3).

Levels of sequence divergence (Kimura 2-parameter) between the outgroup and the ingroup taxa based on all

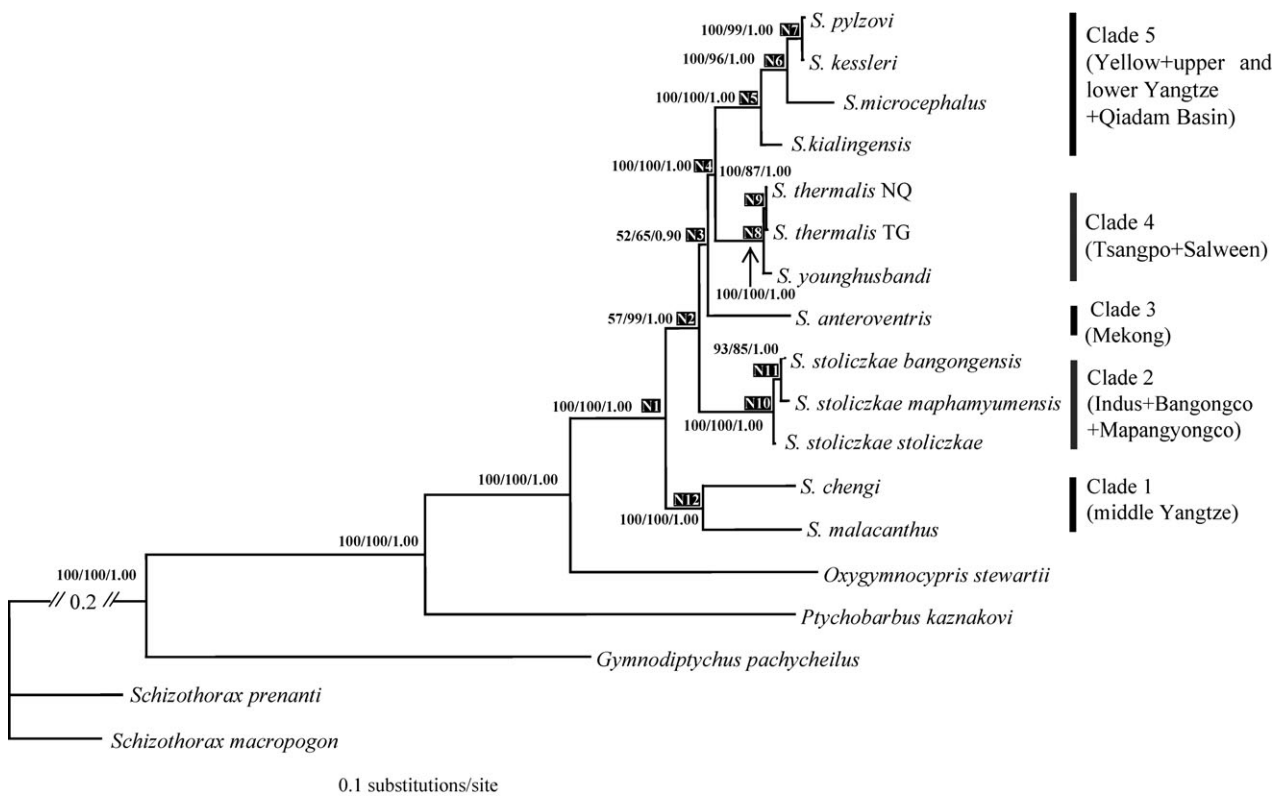
substitutions ranged from 9.43% (between *Oxygymnocypris stewartii* and *S. anteroventris* or *S. kessleri*) to 18.67% (between *Schizothorax prenanti* and *S. chengi*). Sequence divergence within the ingroup taxa ranged from 0.05% (between *S. thermalis* TG and *S. thermalis* NQ) to 6.72% (between *S. kialingensis* and *S. malacanthus*; Supporting Information Table S4).

Plots of transitions and transversions versus uncorrected pairwise distance indicated an absence of nucleotide saturation: all plots were linear regardless of whether the data were plotted by the codon position or by the full sequence (data not shown). Thus, all nucleotide positions were employed in the ensuing phylogenetic analysis of *Schizopygopsis*.

**Phylogenetic analyses**

The best-fit model of molecular evolution for the entire data set obtained from MODELTEST 3.06 (Posada & Crandall 1998) based on the likelihood ratio tests was the TrN + I + G model, which had the following settings:

Base = (0.2768 0.2866 0.1592), Nat = 6, Rmat = (2.1230 76.4483 1.7907 2.5788 20.2813), Rates = gamma Shape = 1.2886 and Pinvar = 0.5976. The parameters obtained from this analysis were used for the construction of the ML phylogeny in PAUP v4.0b10. The heuristic search found a single ML phylogenetic tree with a log-likelihood (–lnL) score of 24389.27 (Fig. 3), in which the genus *Schizopygopsis* is comprised of five major clades (clusters), each with strong support: clade 1 includes two species (*S. malacanthus* and *S. chengi*) from the middle Yangtze River; clade 2 contains three species or subspecies (*S. stoliczkae stoliczkae*, *S. stoliczkae bangongensis* and *S. stoliczkae maphamyumensis*) from the Indus River system; clade 3 includes just one species (*S. anteroventris*) from the Mekong River; clade 4 includes two species (*S. younghusbandi* and *S. thermalis*) from the Tsangpo River and the Salween River; and clade 5 includes four species (*S. kialingensis*, *S. microcephalus*, *S. kessleri* and *S. pylzovi*) from the upper and lower Yangtze River, the Yellow River and the Qiadam Basin.



**Fig. 3** Maximum-likelihood (ML) tree (–lnL (unconstrained) = 24389.27) of the phylogenetic relationships of the genus *Schizopygopsis* based on six mitochondrial genes, using ML settings from the best-fit model (TrN + I + G) selected by hLRT in MODELTEST 3.7. Numbers at the nodes denote the bootstrap values (BS, %) for ML tree (at right) obtained from the analysis in PAUP v4.0b10 under one best-fit model, for ML tree (at middle) and the posterior probabilities (PP, %) for the Bayesian inference (BI) tree (at left) obtained from the analyses in RAXML and MRBAYES under an optimal partitioning scheme. The capital letters behind *S. thermalis* refer to sampling sites: TG: Tanggula, Tibet; NQ: Naqu, Tibet.

The best-fit partitioning scheme and the best nucleotide evolution model selected by the software PARTITIONFINDER were as follows: a cluster for Cyt *b*-ND1 (GTR + G); a second cluster of CO I-CO II-CO III (GTR + I + G); and the ND2 gene, which was given its own partition (TrN + I + G). The topologies of phylogenetic trees obtained using Bayesian inference and ML methods (both based on the optimal partitioning scheme) were identical to that of the ML tree obtained from the entire data set in PAUP v4.0b10. Five clades were recovered as described above, each with high posterior probabilities (only BS values shown in Fig. 3).

To verify the classification of *S. microcephalus*, we tested the hypothesis that *Schizopygopsis* is a monophyletic group by removing *S. microcephalus* from the genus *Schizopygopsis*, and using both constrained (monophyletic) and unconstrained ML trees based on the Shimodaira–Hasegawa test (SH; Burridge 1999). The SH test strongly rejected the removal of *S. microcephalus* from the monophyletic group formed by the genus *Schizopygopsis* ( $-\ln L$  unconstrained = 26571.16;  $-\ln L$  constrained = 33386.02;  $P < 0.0001$ ).

#### Divergence times among lineages

Based on the two geological calibration points, the evolutionary rates were estimated to be 1.16% per Mya for the Cyt *b* sequences, 1.08% per Mya for the Cyt *b* – ND1 sequences, 0.64% per Mya CO I-CO II-CO III sequences and 1.82% per Mya for the ND2 sequences. The age of the most recent common ancestor of the genus *Schizopygopsis* was estimated at approximately 4.49–3.48 Mya. The main cladogenic events identified were as follows: (i) clade 2 splits from clades 3, 4, and 5 approximately 2.98–2.55 Mya, (ii) clade 3 splits from clades 4 and 5 approximately 2.73–2.42 Mya, (iii) clade 4 splits from clade 5 approximately 2.46–2.20 Mya and (iv) the schizothoracine fishes which are distributed in the upper Yangtze River, the Yellow River and the Qidam Basin separate approximately 1.77–0.27 Mya (see Supporting Information Table S5).

## Discussion

### Phylogenetic relationships within *Schizopygopsis*

The traditional taxonomy of the schizothoracine fishes was based primarily on the use of trophic morphologies, such as the arrangement of pharyngeal teeth, position of the mouth, lower jaw morphology, the shape of pharyngeal bone and the number of gill rakers. Indeed, trophic morphologies in the schizothoracine fishes are associated with foraging habits and trade-offs for resource use and are a consequence of adaptive evolution (Qi et al. 2012). In this study, however, we show that the results of our molecular phylogenetic analyses are inconsistent with those of the standard taxonomy for this group of fishes that is based on

morphological characteristics (Wu & Wu 1992). In particular, our more robust molecular phylogeny reveals that species possessing a narrow, slender horny sheath on their lower jaw and a slender pharyngeal bone are not basal in the phylogenetic trees, while those with broad, flat horny sheath on their lower jaws and a broad pharyngeal bone are not more evolutionary distal. Moreover, we found that species that share a similar pharyngeal bone structure, such as *S. youngbusbandi* and *S. malacanthus*, do not form a monophyletic group, but cluster among the other clades.

Incongruence between morphological and molecular phylogenies is a well-known phenomenon (Patterson 1987; Burridge 1999; Xiao et al. 2005; He & Chen 2006) that can be explained in terms of convergent evolution in morphological characters among species. For example, the single row of pharyngeal teeth exhibited by *S. microcephalus* was initially used to establish a monotypic genus *Herzenstein* (Chu 1935). Later, however, Wu & Wu (1992) re-assigned the species, *Herzenstein microcephalus*, to the genus *Schizopygopsis* on the basis of that it shared other characters with this group (an inferior mouth, small mucus pores on the cheeks and chin, a moderately sized skull, sharp ax-shaped pharyngeal teeth and a horny sheath on its lower jaw). Morphological variation between conspecific populations within the genus *Schizopygopsis* is common for species with large ranges (e.g. *S. microcephalus*, *S. pylzovi*, *S. stoliczkae* and *S. malacanthus*). This type of within-species variation has also been noted in other genera of the schizothoracine fishes endemic to the Qinghai-Tibetan Plateau (He & Chen 2006; Qi et al. 2007, 2012). Here, we have shown that the results of molecular phylogenetic analyses, along with the sequence divergence and SH test in the present study, strongly support the classification of Wu & Wu (1992), and suggest that *S. microcephalus* should be a species of *Schizopygopsis*.

Wu & Wu (1992) first described the species *S. anteroventris* noting that the new species inhabited a limited geographical range, including the upper reaches of Mekong River. However, the validity of *S. anteroventris* was not supported in a recent classification (Chen & Cao 2000) that did not sample the upper reaches of Mekong River. Using molecular data, we show that *S. anteroventris* forms a distinct clade that is genetically distant from the other species of genus *Schizopygopsis* (4.22–6.29%). Interestingly, our samples from the upper reaches of Mekong River possessed both characters typical of the genus *Schizopygopsis* and unique characters. For example, unlike other members of *Schizopygopsis*, in *S. anteroventris* the ventral fin originates before the first branched dorsal ray rather than behind the fourth branched dorsal ray.

The nature of mitochondrial evolution decreases its applicability for studies of species delimitation and taxon-



omy, particularly for closely related species (Will *et al.* 2005; de Carvalho *et al.* 2008; Dupuis *et al.* 2012). Low levels of sequence divergence and ancestral polymorphism might limit the ability to discriminate species. In this study, the sequence divergence levels of several species (e.g. *S. pylzovi* vs. *S. microcephalus*; *S. younbusbanid* vs. *S. thermalis*) were <1.3%. The relatively low interspecific sequence divergence may be explained in terms of introgressive hybridization, recent divergence or incomplete lineage sorting. Previous studies showed that the schizothoracine group of fishes is notorious for hybridization, both interspecific and intergeneric. Migration and genetic exchanges during historical temporary connections between the upper Yangtze River, the Yellow River and the Qiadam Basin river systems have been detected in *Gymnocypris* and *Schizopygopsis* (He & Chen 2007). In addition, incomplete lineage sorting resulted in incongruence between morphological and molecular phylogenies, as well as low interspecific sequence divergence has been reported in the schizothoracine fishes (He & Chen 2006; Qi *et al.* 2006). Nevertheless, the limited sampling per species in this study would not allow us to infer the favoured explanation in terms of introgression and/or incomplete lineage sorting. Studies that combine morphological and molecular data will help to resolve these questions.

#### **Divergence time estimates and historical biogeography**

Geological studies suggest that the evolution of drainage systems in the Qinghai-Tibetan Plateau occurred in four stages (Clark *et al.* 2004; see Fig. 1). Initially, the major rivers of the Qinghai-Tibetan Plateau (the Yangtze, the Mekong, the Salween and the Tsangpo) were tributaries of a single southward-flowing river system that drained into the South China Sea through the paleo-Red River. The Kialing River was then captured into the lower Yangtze River, following which the reversal/capture of the middle Yangtze River redirected drainage away from the palaeo-Red River and into the China Sea through the lower Yangtze River. Finally, the capture of the upper Yangtze River by the middle Yangtze, and of the upper Mekong and upper Salween rivers into their modern drainage positions occurred, along with the capture of the Tsangpo River through the Brahmaputra River into its modern course. Given the topology of the phylogenetic tree obtained from this study, we observed that the geographical splits within clades were associated with changes to the Yangtze, Mekong, Salween and the Tsangpo drainages.

Previous studies of cyprinid fishes have obtained molecular clock calibration rates of mitochondrial protein-coding genes using both fossil and geological data, and report substitution rates of 0.73%/Mya (Zardoya & Doadrio 1999), 0.65%/Mya (Machordom & Doadrio 2001), 1.05%/Mya

(Dowling *et al.* 2002), 0.91%/Mya (He *et al.* 2004) and 1.86%/Mya (Duan *et al.* 2009) for cytochrome *b*, and between 0.5 and 1.69%/Mya for combined data (Martin & Palumbi 1993; Zhao *et al.* 2009). In the present study, we used two important geological events whose dates are well resolved, along with the previously published substitution rate of Cyt *b*, to estimate that species residing in the middle Yangtze species diverged first (*c.* 4.5 Mya), followed by those in the Indus River at ~3.0 Mya, those in the Mekong River species at ~2.8 Mya and finally those in the Tsangpo Salween River species at ~2.5 Mya. The most recent splitting events occurred in species from the upper and lower Yangtze river, the Yellow river and the Qiadam Basin at about 1.8 to 0.3 Mya. Our estimated date of the origin for the genus *Schizopygopsis* is broadly consistent with that found by He & Chen (2007) and suggests that the evolutionary events linked to the origin and main speciation of the genus *Schizopygopsis* are correlated with the large-scale alterations of paleo-drainages resulting from uplifting of the Qinghai-Tibetan Plateau since the mid-Pliocene (*c.* 4.0 Mya). Hence, our study supports the results of previous studies reporting the importance of the uplifting of the Qinghai-Tibetan Plateau for altering drainage patterns and the geographical environment in the plateau, and thus promoting speciation of the fishes endemic to this region (Li & Yin 1998; Li & Fang 1999; Li *et al.* 2000; He *et al.* 2001; Peng *et al.* 2004; Guo *et al.* 2005).

A key finding of this study was that the species from the Yangtze River did not cluster as a single monophyletic group, suggesting that the Yangtze River possibly arose from a series of complicated events. The species from the middle Yangtze (*S. chengi* and *S. malacanthus*) form a monophyletic isolated group. However, the species from the upper (*S. microcephalus*) and lower Yangtze (*S. kialingensis*) are phylogenetically closer to those from the Yellow River and the Qiadam basin, with which they form a monophyletic group that is the result of a relatively recent splitting event. Tentatively, we proposed that the Yangtze River *Schizopygopsis* lineages had a dual origin as suggested in the genus *Schizothorax* (He & Chen 2006). An old lineage (Clade 1) is the result of the separation of the middle Yangtze from the palaeo-Red River. Another lineage might then have arisen from later dispersal from adjacent drainages (the upper Salween and upper Yellow rivers) as a result of headwater capture or temporary connections during the Late Pliocene to Pleistocene. The phylogenetic relationships among species in Clade 5 revealed that there was affinity for the drainages in the eastern Tibetan Plateau, including the upper Yangtze, upper Yellow, lower Yangtze and the Qiadam Basin. Geographical data show that the Yellow River emerged ~1.2 Mya at the edge of plateau and subsequently went through the present head-



waters area via a series of tectonic events and river captures. We suggest that there were temporary connections among the upper Yangtze, upper Yellow and Qiadam drainage systems that occurred through headwater captures of the two rivers, which resulted in the dispersal and exchange of fishes to adjacent drainages. This pattern is also found in other lineages of the schizothoracine fishes and within the subfamily *Nemacheilinae* in the Qinghai-Tibetan Plateau (He & Chen 2006, 2007; He *et al.* 2006). We estimated that the lineages residing in the upper Yangtze and the upper Yellow-Qiadam diverged between 1.11 and 0.56 Mya. This is consistent with the tectonic events that occurred during the Kunlun-Huanghe Movement resulting in a large eastward outflow drainage system in the Qiadam Basin, which connected the Gemu River in the Qiadam Basin with Zhaling Lake and Yellow River. Additionally, our data support the hypothesis suggested by He & Chen (2007) that the ancestral headwater of the Yellow River flowed along the present Zoigê Basin and into the tributary of the lower Yangtze River in the Sichuan Basin, promoting the dispersal of *Schizopygopsis* fishes and their exchange between the Yellow River and the lower Yangtze River (e.g. the Kialing River).

The phylogenetic relationships among species residing in the western Tibetan Plateau (Clade 2) we observed reveal a geographical split between lineages of the Indus River and lineages of the Bangongco and Mapangyongco lakes, estimated to have occurred ~0.30–0.24 Mya. In fact, tectonic events that occurred during that time resulted in an outflow drainage system in the western Tibetan Plateau that connected the Bangongco and Mapangyongco lakes with Indus River, supporting our findings. Meanwhile, our phylogenetic analyses support a geographical split of the lineages residing in the Tsangpo and the Salween rivers (Clade 4) at ~0.20–0.18 Mya. In the central Tibetan Plateau, precipitation is high and variable due to the monsoon, resulting in great variation in the water level within lakes and increased outflow to the Tsangpo and Salween rivers.

In this study, the relatively short internal branch lengths and longer tips of the mitochondrial DNA-based phylogenetic trees support the hypothesis of a recent, rapid radiation in the evolutionary history of these taxa. The dates estimated for the divergence between lineages also suggest that a number of speciation events have taken place in a short time. Rapid diversification leading to incongruence between the gene history of the mitochondrion and the evolutionary history of the recently diverged species is a common phenomenon (Hoelzer & Melnick 1994; Crespi & Fulton 2004; Kingston *et al.* 2009). The mitochondrion bears the evolutionary history of a single molecule nested within a species, which while often producing a powerful signal must be interpreted with caution (Kingston *et al.* 2009). It is possible

that a single gene may not be able to fully resolve the phylogenetics and biogeography of this genus of fish as they diverged within a narrow time period. Further studies based on multiple loci and more extensive sampling will reveal a more complete picture of the phylogenetic relationships and biogeography of *Schizopygopsis* fishes.

## Conclusions

Despite the morphological and ecological diversity among species of the genus *Schizopygopsis*, our study resolves some of the controversies of the taxonomic classification of this group of organisms and provides the first robust molecular phylogenetic relationships based on six mitochondrial genes. We have shown that the genetic relationships revealed by this molecular phylogeny are not consistent with those based on morphological characters. Our molecular evidence and use of the molecular clock calibration have allowed us to associate speciation events within the genus *Schizopygopsis* to the formation and separation of paleo-drainage connections caused by tectonic events during the uplifting of the Qinghai-Tibetan Plateau (~4.5 Mya). This work underlines the dominant role of vicariance in shaping the evolutionary history of the genus *Schizopygopsis*.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1:** Species used in this study, along with the locality, catchment and drainage system from which they were sampled.

**Table S2:** List of primers used in this study.

**Table S3:** Molecular characteristics of the mitochondrial genes for the ingroup taxa (the 12 *Schizopygopsis* species, see Table S1).

**Table S4:** Sequence divergence among the 12 *Schizopygopsis* species and the outgroup species.

**Table S5:** Divergence dates estimated from the molecular data using two geological events (the opening of the Gibraltar Strait after the Messinian salinity crisis at ~5.0 Mya, and the isolation of the upper Yangtze River from the Yellow River and the drainages of the Qidam Basin at 1.1 Ma) along with the published substitution rate of the Cyt *b* gene (0.65–1.86%).