

## APPLIED ISSUES

# Genetic diversity and historical population structure of *Schizopygopsis pylzovi* (Teleostei: Cyprinidae) in the Qinghai–Tibetan Plateau

DELIN QI,<sup>\*,†</sup> SONGCHANG GUO,<sup>\*,†</sup> XINQUAN ZHAO,<sup>\*</sup> JIE YANG,<sup>\*,†</sup> AND WENJIA TANG<sup>‡</sup><sup>\*</sup>Key Laboratory of Qinghai–Tibetan Plateau Biological Evolution and Adaptation, Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, Qinghai, China<sup>†</sup>Graduate School of Chinese Academy of Science, Beijing, China<sup>‡</sup>Fishery Environmental Monitoring Station of Qinghai Province, Xining, Qinghai, China

## SUMMARY

1. Complete sequences of 1140 base pair of the cytochrome *b* gene from 133 specimens were obtained from nine localities including the inflow drainage system, isolated lakes and outflow drainage system in Qinghai–Tibetan Plateau to assess genetic diversity and to infer population histories of the freshwater fish *Schizopygopsis pylzovi*.
2. Nucleotide diversities ( $\pi$ ) were moderate (0.0024–0.0045) in populations from the outflow drainage system and Tuosuo Lake, but low (0.0018–0.0021) in populations from Qiadam Basin. It is probable that the low intra-population variability is related with the paleoenvironmental fluctuation in Qiadam Basin, suggesting that the populations from Qiadam Basin have experienced severe bottleneck events in history.
3. Phylogenetic tree topologies indicate that the individuals from different populations did not form reciprocal monophyly, but the populations from the adjacent drainages cluster geographically. Most population pairwise  $F_{ST}$  tests were significant, with non-significant pairwise tests between Tuosuo Lake and Tuosuo Lake in the north-west of the Qinghai–Tibetan Plateau. Analysis of molecular variance (AMOVA) indicates that the significant genetic variation was explained at the levels of catchments within and among, not among specific boundaries or inflow and outflow drainage systems.
4. The nested clade phylogeographical analysis indicates that historical processes are very important in the observed geographical structuring of *S. pylzovi*, and the contemporary population structure and differentiation of *S. pylzovi* may be consistent with the historical tectonic events occurred in the course of uplifts of the Qinghai–Tibetan Plateau. Fluctuations of the ecogeographical environment and major hydrographic formation might have promoted contiguous range expansion of freshwater fish populations, whereas the geological barriers among drainages have resulted in the fragmentation of population and restricted the gene flow among populations.
5. The significantly large negative  $F_s$ -value ( $-24.91$ ,  $P < 0.01$ ) of Fu's  $F_s$ -test and the unimodal mismatch distribution indicate that the species *S. pylzovi* underwent a sudden population expansion after the historical tectonic event of the Gonghe Movement.
6. The results of this study indicate that each population from the Qinghai–Tibetan Plateau should be managed and conserved separately and that efforts should be directed towards preserving the genetic integrity of each group.

Correspondence: Xinquan Zhao, Northwest Plateau Institute of Biology, Chinese Academy of Sciences, 59 Xiguan Street, Xining 810001, Qinghai, China. E-mail: xqzhao@nwipb.ac.cn

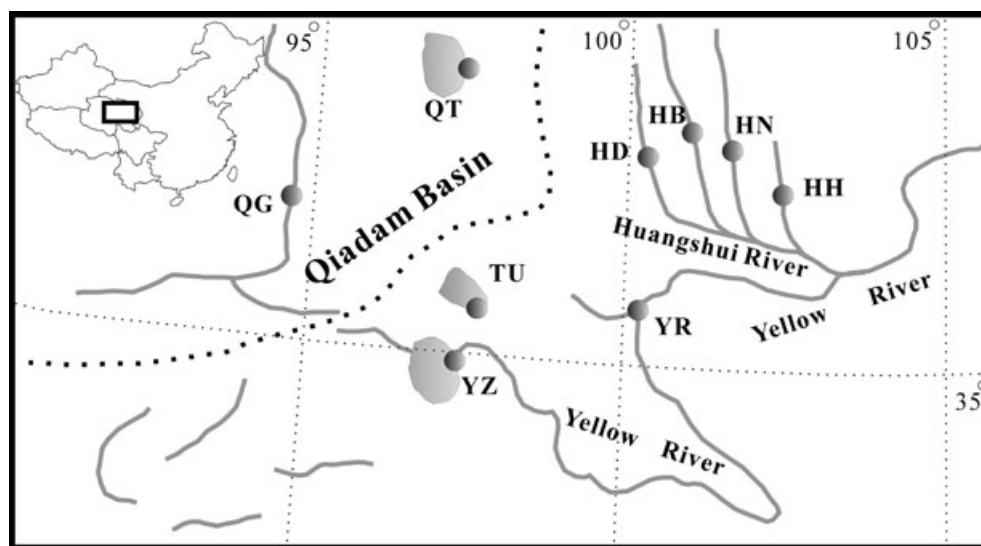
**Keywords:** conservation, genetic structure, mtDNA cytochrome *b*, Qinghai–Tibetan Plateau, *Schizopygopsis pylzovi*

## Introduction

Phylogeographic studies play very important roles in understanding the evolutionary history of species in the context of paleoenvironmental changes (Hewitt, 1996, 2004; Pavlova, Zink & Rohwer, 2005; Zaccara, Stefani & Crosa, 2005). Freshwater fishes, strictly constrained by drainage systems, have provided key insights into the relationships between the contemporary genetic structure and the historical changes in the environment (Murphy & Collier, 1996; Xiao, Zhang & Liu, 2001; Hewitt, 2004). The intensive uplifts of the Qinghai–Tibetan Plateau, especially strong tectonic movements that have occurred since the middle Pleistocene, are generally accepted to have had significant impact on the evolution and development of the contemporary drainage systems (Li & Ying, 1998; Li, Ying & Yu, 2000; Zhu *et al.*, 2003), which led to considerable habitat alterations that impacted taxa, including freshwater fishes, in the region (Cao *et al.*, 1981; Wu & Wu, 1991). The modern arrangement of drainages in the region was mostly established in the Gonghe Movement around 150 000 years ago (Li *et al.*, 2000; Zhu *et al.*, 2003). This arrangement includes the presence of the barriers between the inflow (Germu River) in the Qiadam

Basin and outflow drainage systems (Yellow River) and many isolated lakes such as Tuosu Lake and Tuosuo Lake (Fig. 1). Coupled with the markedly arid conditions in the Pleistocene epochs, the populations of freshwater fishes restricted to these drainage systems may well have been isolated since this time. Under this hypothesis, even contemporary fishes still display the intraspecific genetic structure formed in the past (Avice, 1994) or a deeper differentiation may result from paleoenvironmental changes. *Schizopygopsis pylzovi* (Kessler, 1876), a freshwater fish, is a natural group that offers an excellent opportunity to demonstrate the evolutionary history of species in response to historical changes of drainage systems and the geographical environment in the Qinghai–Tibetan Plateau.

*Schizopygopsis pylzovi*, one species of the endemic schizothoracine fishes (Teleostei: Cyprinidae) of freshwater ecosystem in the Qinghai–Tibetan Plateau, is omnivorous and plays significant roles in the trophic web of plateau freshwater communities. As an important natural fishery resource, it has a discrete geographic distribution, which is restricted to the inflow drainage system (i.e. Germu River) in the Qiadam Basin, the outflow drainage systems (i.e. the upper reaches of Yellow River and the Huangshui



**Fig. 1** Sampling sites for *Schizopygopsis pylzovi* from the Qinghai–Tibetan Plateau. The dashed-line represents the boundary between inflow drainage system in Qiadam Basin and outflow drainage system in the Qinghai–Tibetan Plateau (see Table 1 for population codes).

River) and isolated lakes (i.e. Tuosu Lake and Tuosuo Lake) in the Qinghai–Tibetan Plateau. Although the native freshwater fish *S. pylzovi* may be abundant in some restricted areas, the population size has declined dramatically in recent years and its distribution has been drastically reduced (Wu & Wu, 1991). Its decline has been attributed to a number of factors including habitat destruction and alteration, changes in reproductive habitat, introduction of exotic competitors and diseases.

In view of the ecological importance and the present status of *S. pylzovi*, several action plans for the restoration of native freshwater fishes have been established by the government in Qinghai Province. However, the absence of detailed population genetic information has led conservation agencies to employ management programmes based on the only available data from morphological studies. Wu (1964), based on the morphological characteristic, recognised the *S. pylzovi* distributed in the Germu River and Tuosu Lake in the Qiadam Basin as a new species *Schizopygopsis kessleri*. The validity of this classification was rejected in a subsequent phylogenetic analyses (Wu, 1984; Wu & Wu, 1991) based on 12 mainly morphological and anatomical characteristics. In this classification, Wu & Wu (1991) incorporated *S. kessleri* as a synonym of *S. pylzovi*. A recent revision, however, by Yue *et al.* (2000) resurrected the species *S. kessleri* and suggested that the considerable variation in morphological characteristic of this species indicated an early divergence from *S. pylzovi*. Conservation strategies based on specific categories which are not well defined, and which may not reflect the underlying genetic diversity, are at best inefficient and at worst

might reduce the long-term evolutionary potential of the species (Alpers *et al.*, 2004).

In the present study, we analysed the complete sequences of the mtDNA cytochrome *b* (*cyt-b*) gene among *S. pylzovi* populations from the outflow (Yellow River, Huangshui River) and the inflow (Germu River and the isolated Tuosu Lake) drainage systems in Qiadam Basin, as well as from the isolated lake Tuosuo to: (i) estimate the genetic population structure; (ii) interpret the population genetic differentiation in response to historical changes of drainage systems and the geographical environment and (iii) provide a genetic basis for conservation management of this endemic species.

## Methods

### Samples

A total of 133 individuals of *S. pylzovi* were collected from nine sites. Sampling sites covered the main isolated lakes and inflow and outflow drainage systems in Qinghai–Tibetan Plateau, which included Germu River (sample site labelled QG) and Tuosu Lake (QT) in Qiadam Basin and the upper Yellow River (YR), Huangshui River (HD, HB, HH and HN), Zhaling Lake (YZ) and Tuosuo Lake (TU) (Fig. 1 and Table 1). Fin clips were taken from field caught fish, and immediately stored in 90% ethanol for DNA extraction.

### DNA extraction, PCR amplification and sequencing

Total DNA was isolated from ethanol-fixed tissue by proteinase K digestion followed by standard phenol-

**Table 1** Sampling sites for *Schizopygopsis pylzovi*: collection location (specimen numbers in parentheses), catchment, number of haplotypes and drainage system

Population code	Collection location	Catchment	No. of haplotypes	Drainage system
QG	Qiadam Basin, Qinghai (13)	Germu River	7	Qiadam Basin
QT	Qiadam Basin, Qinghai (13)	Tuosuo Lake	6	Qiadam Basin
HB	Datong, Qinghai (21)	Beichuan River	15	Huangshui River
HD	Haiyan, Qinghai (23)	Dongdatan River	14	Huangshui River
HH	Ledu, Qinghai (16)	Heishuiutan River	8	Huangshui River
HN	Huzhu, Qinghai (18)	Nanmenxia River	10	Huangshui River
YR	Xinghai, Qinghai (4)	Yellow River	3	Yellow River
YZ	Madoi, Qinghai (12)	Zhaling Lake	6	Yellow River
TU	Madoi, Qinghai (13)	Tuosuo Lake	8	Tuosuo Lake
Outgroup	∑133		∑77	
<i>Oxygymnocypris stewartii</i>	Lhasa, Tibet (1)	Lhasa River	1	
<i>Gymnodiptychus pachycheilus</i>	Xinghai, Qinghai (1)	Yellow River	1	

chloroform extraction. The complete sequence of *cyt-b* gene was amplified via the polymerase chain reaction (PCR) using the primers pair L14724 (5'-GAC-TTGAAAACCACCGTTG-3') and H15915 (5'-CTCCGATCTCCGGATTACAAGAC-3') (Xiao *et al.*, 2001). PCR amplifications were performed in total reaction volumes of 30  $\mu$ L, containing 1.0 U of TaKaRa EX Taq (TaKaRa, Dalian, China), 0.15 pmol of dNTP mix, 3.0  $\mu$ L 10xTaq buffer (TaKaRa), 10 pmol of each primer and 20 ng of total genomic DNA. The PCR cycle consisted of an initial denaturation at 95 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were purified using a CASure PCR Purification Kit following the manufacturer's protocol (Casarray, Shanghai, China). The sequencing reactions were carried out in a Biometra thermocycler using a DYEnamic Dye Terminator Cycle Sequencing Kit (Amersham Biosciences Corporation, Shanghai, China) following the manufacturer's protocol. Purified DNA fragments were directly sequenced using a MegaBACE 500 DNA Analysis System Shanghai, China following the manufacturer's instructions. To ensure accuracy, strands were sequenced in both directions for each individual. Both DNA strands were checked for ambiguous base assignments.

### Analysis

Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) with default settings and adjusted by eye. The extent of the *cyt-b* gene was determined by comparison with sequences of other genera of *Schizothoracinae* in GenBank. New sequences were deposited in GenBank under the accession numbers DQ491113–DQ491191.

The hypothesis of neutral evolution was tested by Tajima's D (Tajima, 1989) test and Fu's  $F_s$ -test (Fu, 1997) with 10 000 permutations using Arlequin 2.0 software (Schneider, Roessli & Excoffier, 2000). The pairwise mismatch distribution was generated using the DnaSP 3.0 (Rozas & Rozas, 1999) program to test for populations that have undergone a sudden population expansion (Rogers & Harpending, 1992). Time since population expansion was calculated using the formula  $\tau = 2ut$ ; where  $u$  is the mutational rate per sequence per generation and  $t$  is time in generations (Rogers & Harpending, 1992). Estimates

of genetic variation were obtained in the form of haplotype diversity  $h$  (Nei, 1987), nucleotide diversity  $\pi$  (Nei & Jin, 1989) and an average pairwise distance between populations using Arlequin 2.0 software (Schneider *et al.*, 2000).

Phylogenetic analysis was performed with PAUP\* 4.0b10 software (Swofford, 2000), using maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML). ModelTest 3.06 software (Posada & Crandall, 1998) was used to determine the best-fit model of molecular evolution, which was used for the NJ and ML analysis. MP trees were constructed with a heuristic search, 100 replicates of random additions of sequences, equally weighted characters and nucleotide transformations, ACC-TRAN character optimisation, MULTPARS + TBR branch-swapping and STEEPEST DESCENT options with PAUP\* 4.0b10 (Swofford, 2000). A heuristic search with simple addition of sequences, and TBR branch swapping, MULTREES and COLLAPSE were used to produce ML trees in PAUP 4.0b10 (Swofford, 2000). Evaluation of statistical confidence in nodes was based on 1000, 1000 and 100 bootstrap replicates in MP, NJ and ML respectively (Felsenstein, 1985). *Oxygymnocypris stewartii* (Lloyd, 1908) and *Gymnodiptychus pachycheilus* (Kessler, 1876) were used as outgroups for all trees because they are very closely related to the species of the genus *Schizopygopsis* (Wu, 1984; Wu & Wu, 1991).

Hierarchical structure in the data was tested by partitioning variance components among and within drainage systems using analysis of molecular variance (AMOVA) in ARELQUIN 2.0 (Schneider *et al.*, 2000). Two hierarchies were analysed. First, four groups were distinguished based on the sample drainage systems. Groups were as follows: (i) Qiadam Basin, populations from the Germu River (QG) and the Tuosu Lake (QT); (ii) Huangshui River, the rivers Beichuan (HB), Dongdatan (HD), Heishuitan (HH) and Nanmenxia (HN); (iii) Yellow River, populations from the Zhaling Lake (YZ) and the Yellow River (YR) and (iv) Tuosuo Lake, population TU. Second, two groups were distinguished based on the hypothesis that populations from the inflow drainage and outflow drainage systems are two distinct species: (i) the inflow drainage system, populations from the Germu River (QG) and the Tuosu Lake (QT) and (ii) the outflow drainage systems, populations from the remaining sites (HB, HD, HH, HN, YZ, YR and TU). Permutation tests (1000) of significance were used to

test genetic structure by comparisons to null distributions with 10 000 random permutations of the original data matrix. Pairwise  $F_{ST}$  between populations were also computed in ARLEQUIN 2.0 package (Schneider *et al.*, 2000).

A nested clade phylogeographical analysis (NCPA, Templeton, 1998) was used to evaluate the contemporary and historical processes that could be responsible for the observed patterns of mtDNA variation. We used TCS version 1.13 (Clement, Posada & Crandall, 2000) to create a network showing relationships among haplotypes. This method uses MP to obtain the 95% plausible set of alternative networks. Nested clades were determined following the rules set out in Templeton, Crandall & Sing (1992) and Templeton (1998). All tip haplotypes were related to the closest internal haplotype forming one-step clade. When all haplotypes were connected in one-step clades, they served as the units for second-step clades, until the last step-level represented by the complete haplotype tree. GEODIS version 2.2 (Posada, Crandall & Templeton, 2000) was used first to test for geographical association of haplotypes and clades at each nesting level, using contingency tests. Second, we calculated the clade distance ( $D_c$ ) which determines the geographical range of a given clade, and nested clade distance ( $D_n$ ) which measures how this clade is geographically distributed relative to its closest evolutionary sister clades (Templeton, Routman & Phillips, 1995; Templeton, 1998). Based on these distances, two other measures can be calculated, the difference between the clade distance of tip and interior clades of a nesting clade (I-TD $c$ ) and the difference between nested clade distance of interior and tip clades (I-TD $n$ ). Each of these  $D_c$  and  $D_n$ -values was tested for significance using 1000 permutations. Clades with statistically significant values ( $P < 0.05$ ) for the measures of geographical distance ( $D_c$  and  $D_n$ ) were taken through the updated inference key (Templeton, 2004) to infer the processes leading to the significant geographical structure.

## Results

### *Polymorphic sites and genetic diversity*

The complete *cyt-b* sequence (1140 bp) was obtained for all 133 specimens of *S. pylzovi*, yielding 77 distinct haplotypes. For the 1140 bp of sequences, 83 charac-

ters were variable (no transversions) and 37 were phylogenetically informative for parsimony analysis. Third codon positions were the most informative (24 parsimony informative characters), followed by first codon positions (eight) and second codon positions (five). The base composition was on average A, 25.5%; T, 30.8%; C, 26.7%; G, 17.0% and showed an anti-G bias which is characteristic for the mitochondrial genome (Cantatore *et al.*, 1994). All populations showed high levels of haplotype diversity ( $h$ ) ranging from 0.83 to 0.98 and low or moderate of nucleotide diversities ( $\pi$ ) ranging from 0.0018 to 0.0045. Average sequence distance between populations ranged from 0.40% to 0.91% (Table 2).

### *Phylogenetic tree construction*

The best-fit model of molecular evolution obtained from ModelTest 3.06 (Posada & Crandall, 1998) based on the likelihood ratio tests was the TrN + I + G model. Settings for this model were as follows: base frequencies (A = 0.2513, C = 0.2697, G = 0.1754 and T = 0.3036); transition/transversion ratio 29.8245; proportion of invariable sites 0.7577, and the shape parameter of the gamma distribution 1.0573. Parameters obtained from this analysis were used for the construction of the NJ and ML phylogeny.

The phylogenetic analyses (MP, NJ and ML) resulted in largely congruent tree topologies, and the phylogenetic relationships among 77 mtDNA haplotypes of *S. pylzovi* are shown in a strict consensus tree of 200 equally maximum parsimony trees (tree length = 347, consistency index = 0.8530, homoplasy index = 0.1470, retention index = 0.8223 and rescaled consistency index = 0.7014) rooted with two species of sub-family *Schizothoracinae*: *O. stewartii* and *G. pachycheilus* (Fig. 2). As revealed, all haplotypes from *S. pylzovi* were defined as four major clades in all the three phylogenetic analyses, although most clades had relatively low-bootstrap values. Clade I consisted of all haplotypes from Tuosuo Lake (TU), five haplotypes from the Huangshui River (HD and HH), two haplotypes from Zhaling Lake (YZ) and one haplotype from Tuosu Lake (QT). Clade II was comprised of all haplotypes from the Germu River (QG), all haplotypes from the Yellow River (YR), five haplotypes from Tuosu Lake and four haplotypes from Zhaling Lake. Clades III and IV included haplotypes only from the Huangshui River (HB, HD, HH and HN).

**Table 2** Frequency of cytochrome *b* gene (*cyt-b*) haplotype of *Schizopygopsis pylzovi* detected at nine sample sites. Site abbreviations as in Table 1

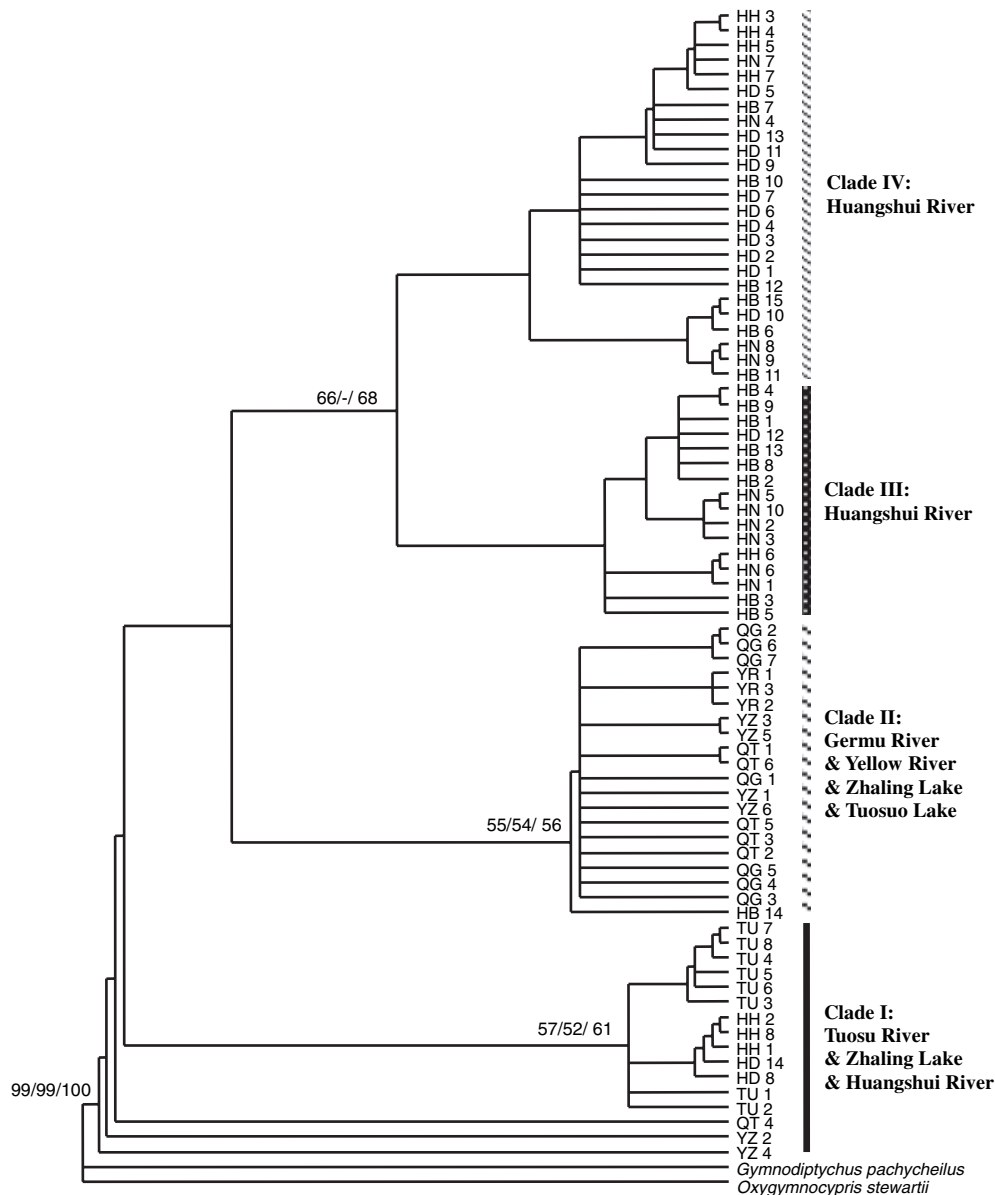
Haplotype	QG	QT	HB	HD	HH	HN	YR	YZ	TU
1	1								
2	1								
3	5								
4	3	4						4	
5	1								
6	1								
7	1								
8		1							
9		2							
10		2							
11		1							
12		2							
13		1							
14			2						
15			1						
16			1						
17			3	3		2			
18			1						
19			1						
20			1						
21			1						
22			4	4					
23			1						
24			1						
25			1						
26			1						
27			1						
28			1						
29				1					
30				1					
31				1					
32				1					
33				1					
34				1					
35				1					
36				1					
37				2					
38				1					
39				2					
40				1					
41				1					
42				1					
43					4				
44					3				
45					2				
46					1				
47					1				
48					2				
49					2				
50					1				
51						5			
52						2			
53						1			
54						1			

**Table 2** (Continued)

Haplotype	QG	QT	HB	HD	HH	HN	YR	YZ	TU
55							1		
56							2		
57							1		
58							1		
59							1		
60							1		
61								1	
62								1	
63								2	
64									3
65									1
66									1
67									1
68									1
69									1
70									5
71									1
72									2
73									1
74									1
75									1
76									1
77									1
Total	13	13	21	23	16	18	4	12	13

### Population structure

Most population pairwise  $F_{ST}$  tests were significant. Non-significant pairwise  $F_{ST}$  tests occurred between the populations from Tuosu Lake and Zhaling Lake. A global AMOVA estimated that 61.5% ( $P < 0.001$ ) of the variation was within populations and only 38.5% ( $P < 0.001$ ) was among populations (Table 3). When all individuals of *S. pylzovi* were grouped into four drainage systems (Qiadam Basin versus Huangshui River versus Yellow River versus Tuosuo Lake), 58.4% of the total variation resulted from differences within populations ( $\Phi_{ST} = 0.4185$ ,  $P < 0.001$ ). Significant differences were also attributed to variation among populations within drainage systems (24.6%) with  $\Phi_{SC} = 0.2986$  ( $P < 0.001$ ) and among drainage systems (17.0%) with  $\Phi_{CT} = 0.1709$  ( $P < 0.05$ ) (Table 4). Results of AMOVA in which populations were divided into two species or two drainage systems (inflow versus outflow drainage systems) indicated that little of the variation (5.2%,  $P = 0.172$ ) can be attributed to species (Table 4). Again, most of the variation was due to differences among populations ( $\Phi_{SC} = 0.3727$ ,  $P < 0.001$ ) and within populations ( $\Phi_{ST} = 0.4051$ ,  $P < 0.001$ ), indicating that the significant genetic



**Fig. 2** The strict consensus tree of 200 equally maximum parsimony (MP) trees (tree length = 347, consistency index = 0.8530, homoplasy index = 0.1470, retention index = 0.8223 and rescaled consistency index = 0.7014). *Gymnodiptychus pachycheilus* and *O. stewartii* are used as outgroup. The tree topology is largely congruent with the neighbour-joining (NJ) and maximum-likelihood (ML) analyses. Bootstrap confidence limits for MP, NJ and ML trees respectively, appear at branch nodes (>50%). Names of haplotypes follow population codes and are numbered sequentially.

variation was explained at the levels of catchments within and among, not among specific boundaries (i.e. inflow and outflow drainage systems).

#### *Nested clade analysis*

The 77 haplotypes were fitted into 42 1-step clades, 17 2-step clades and 5 3-step clades (Fig. 3), which are largely congruent with the phylogenetic tree (Fig. 2).

The contingency analyses revealed that significant associations of clades and locations, one 1-step clade, four 2-step clades, four 3-step clades and the total cladogram, showed significant association with geographical location allowing rejection of the panmixia hypothesis (Table 5). For each of the clades, by following the inference key (Templeton, 2004), the main evolutionary patterns identified from permutational analysis included restricted gene flow with

**Table 3** Nucleotide diversity within population [ $\pi \pm$  standard deviation (SD), along diagonal], average pairwise distance between populations (percentage, above diagonal), pairwise  $F_{ST}$  (below diagonal, \* $P < 0.001$ ) between populations and haplotype diversity within population ( $h \pm$  SD) of *Schizopygopsis pylzovi*<sup>†</sup>

Population	QG	QT	HB	HD	HH	HN	YR	YZ	TU	Haplotype diversity
QG	0.0018 ± 0.0012	0.40	0.54	0.47	0.86	0.71	0.52	0.55	0.62	0.83 ± 0.09
QT	0.1966*	0.0024 ± 0.0015	0.51	0.46	0.77	0.65	0.43	0.46	0.51	0.88 ± 0.06
HB	0.3241*	0.2216*	0.0041 ± 0.0023	0.47	0.76	0.58	0.60	0.68	0.70	0.98 ± 0.02
HD	0.3036*	0.2157*	0.0669*	0.0032 ± 0.0019	0.65	0.58	0.56	0.61	0.60	0.92 ± 0.04
HH	0.6150*	0.5431*	0.4327*	0.4283*	0.0041 ± 0.0024	0.73	0.87	0.91	0.75	0.90 ± 0.05
HN	0.4615*	0.3640*	0.1216*	0.2334*	0.3696*	0.0045 ± 0.0026	0.73	0.82	0.81	0.91 ± 0.05
YR	0.5477*	0.3681*	0.3452*	0.4175*	0.5578*	0.4008*	0.0031 ± 0.0023	0.60	0.63	0.83 ± 0.22
YZ	0.1942*	0.0666	0.2565*	0.2379*	0.5129*	0.3747*	0.3349*	0.0038 ± 0.0022	0.63	0.86 ± 0.09
TU	0.6286*	0.5098*	0.4994*	0.4978*	0.5278*	0.5300*	0.6131*	0.4512*	0.0021 ± 0.0013	0.85 ± 0.07

<sup>†</sup> Average sequence distance between populations based on Kimura 2-parameter model.

isolation by distance, contiguous range expansion and past gradual range expansion followed by fragmentation (Table 6). The inferences from the three of the two-step (clades 2–12, 2–13 and 2–15) and one of the three-step (clade 3–2) clade levels revealed a pattern of restricted gene flow with isolation by distance. The inference key (Templeton, 2004) suggested that contiguous range expansion among the four tributaries of the Huangshui River (rivers Beichuan, Dongdatan, Heishuitan and Nanmenxia) was the likely process to explain the pattern of geographical variation in clades 2–17 and 3–1. Contiguous range expansion was also proposed to explain the distribution of clade 3–3. The oldest event corresponding to the total cladogram was past gradual range expansion followed by fragmentation with the diverged clades, 3–1, 3–2, 3–3 and 3–4, distributed in the inflow (e.g. Germu River) and outflow (e.g. Huangshui River and Yellow River) drainages and isolated lakes (e.g. Tuosu Lake and Tuosuo Lake). No conclusive results were inferred for clades 1–29 and 3–4 because significant  $D_c$ ,  $D_n$  or I-T distances could not be detected or tip/interior status could not be determined.

#### Population expansion

To investigate the hypothesis of a recent population expansion in *S. pylzovi*, the distribution of pairwise differences among the 133 sequences (mismatch distribution) was computed using the DnaSP program (Fig. 4). The pairwise differences ranged from 0 to 32 substitutions. Interestingly, the mismatch distribution analysis showed unimodal distribution reflecting a model of a sudden population expansion. This finding was supported also by Tajima's  $D$  and Fu's  $F_s$ -test of neutrality when all the 133 sequences were considered as one population. Tajima's  $D$  and Fu's  $F_s$  were  $-1.79$  ( $P < 0.01$ ) and  $-24.91$  ( $P < 0.01$ ) for the complete data set respectively, which indicated a recent population expansion as suggested by the mismatch distribution analysis. However, when the four drainage systems (Qiadam Basin, Huangshui River, Yellow River and Tuosuo Lake) were analysed separately, the  $F_s$ -values of three (Qiadam Basin, Huangshui River and Tuosuo Lake) were still negative while the Yellow River was positive, which suggested that the Yellow River group seems to be relatively stable, and shows a tendency for a population growth (Fu, 1997). In addition, the estimated



Groups	Source of variation	Percentage of variation	$\Phi$ statistic	<i>P</i>
None specified	Among drainage systems	17.0	$\Phi_{CT} = 0.1709$	0.025
	Among populations within drainage systems	24.6	$\Phi_{SC} = 0.2986$	<0.001
	Within populations	58.4	$\Phi_{ST} = 0.4185$	<0.001
Species	Among species	5.2	$\Phi_{CT} = 0.0517$	0.172
	Among populations within species	35.3	$\Phi_{SC} = 0.3727$	<0.001
	Within populations	59.5	$\Phi_{ST} = 0.4051$	<0.001

**Table 4** Hierarchical analysis of molecular variance of mtDNA haplotypes of *Schizopygopsis pylzovi*



**Fig. 3** Maximum parsimony network and corresponding nested design for cytochrome *b* haplotypes observed in nine populations of *Schizopygopsis pylzovi*. Each line in the network represents a single mutation. Small empty circles represent the inferred, non-detected interior haplotypes. The number inside each circle identifies the detected haplotypes, as in Table 2. One-step clades are contained within thin-lined boxes, 2-step clades are contained within medium-lined boxes and 3-step clades are contained within thick-lined boxes. The overall cladogram is a single 4-step clade.

**Table 5** Nested contingency analysis of geographical associations for cytochrome *b* data from *Schizopygopsis pylzovi*. Clades with no genetic or geographical variation are excluded (no test is possible within such nested categories)

Clade	Permutational $\chi^2$ statistic	P-value
1-2	4.0	0.310
1-3	1.4	1.000
1-7	3.8	0.558
1-15	2.0	1.000
1-17	7.0	0.279
1-23	3.0	0.329
1-29	20.9	0.033
1-31	4.0	0.477
1-33	5.0	0.612
1-36	2.0	1.000
1-42	7.0	1.000
2-1	2.4	0.756
2-3	0.8	1.000
2-5	6.0	0.330
2-6	0.9	1.000
2-7	3.4	0.266
2-8	0.8	1.000
2-12	23.5	0.038
2-13	21.7	0.014
2-15	8.3	0.048
2-17	20.0	<0.001
3-1	21.8	0.002
3-2	11.0	0.002
3-3	55.5	<0.001
3-4	16.8	<0.001
3-5	9.1	0.381
Total cladogram	229.1	<0.001

value of  $\tau$  inferred from mismatch distribution analysis was 6.77. Based on the molecular clock calibration rates of 0.91% per million years of the schizothoracine fishes derived from well-dated geological events (He et al., 2004) and a generation time of 3 years, this analysis suggests that the population of *S. pylzovi* expanded around 110 000 years ago.

## Discussion

The specific status of populations of *S. pylzovi* from different geographic units or drainage systems has been controversial for some time (Wu, 1964, 1984; Wu & Wu, 1991; Yue et al., 2000). The phylogenetic trees constructed from the complete mtDNA *cyt-b* gene clearly show that individuals from the Qiadam Basin do not form a reciprocal monophyletic clade distant from the clades formed by individuals in the outflow drainage systems. This information coupled with results of AMOVA show that only a small amount of

variation could be explained by specific boundaries when genetic data were partitioned by species, and reveal that *S. kessleri* is not a separate species but a synonym of *S. pylzovi*.

In general, high levels of genetic variability in a given population can be maintained if the population is large enough and stable for a long period of time. It is often assumed that when a population goes through a severe bottleneck, random genetic drift will induce a massive loss of genetic variability. In the present study, the nucleotide diversity in nine populations of *S. pylzovi* was lower than the average values of other Cyprinidae fishes (Perdices, Cunha & Coelho, 2004; Perdices, Sayanda & Coelho, 2005), while the populations from the Qiadam Basin exhibited the lowest nucleotide diversity (0.0018–0.0021). Historically, the Qiadam Basin experienced an extremely harsh and dry event during the late Pleistocene (Jing et al., 2001). During that time, the Germu River was intermittent and the population of *S. pylzovi* suffered a high degree of fragmentation and high mortality. The Tuosuo Lake was subjected to several shrinkages despite a connection with many streams. Therefore, it is more likely that the low nucleotide diversity assessed in the populations from the Qiadam Basin is related to the paleoenvironmental fluctuation in the Qiadam Basin, and that the populations have experienced severe bottleneck events in history.

Although the phylogenetic tree construction in the present study indicates that individuals from different populations do not form reciprocal monophyly, the populations from the adjacent drainages cluster geographically. For example, all individuals of populations from the inflow drainage (Germu River and Tuosuo Lake) in the Qiadam Basin, Tuosuo Lake and Zhaling Lake were placed in clades I and II, whereas most individuals of populations from Huangshui River are clustered in clades III and IV. In the same fashion, AMOVA reveals that a higher genetic variance is caused by differences among populations both within and among drainage systems (Table 3). The nested clade analysis indicates that historical processes are very important in the observed geographical structuring of *S. pylzovi*, i.e. contiguous range expansion, fragmentation and restricted gene flow. These results, together with the conclusion that the speciation and radiation of the species *S. pylzovi* occurred around 370 000 years ago (phylogenetic data of the genus *Schizopygopsis*, Qi, Guo, Yang, Wang, Li & Zhao

**Table 6** Results of nested clade analysis showing clade ( $D_c$ ), nested ( $D_n$ ) and interior-tip (I-T) clade distances. Only clades with significant permutational chi-squared probabilities for geographical structure have been included in the table. Inferred pattern indicates the steps taken in the inference key to reach the conclusion (Templeton, 2004)

Nesting	Clade no.	Location	$D_c$	$D_n$	Inferred pattern
1-29	5	Tip	0	168.9	1-2-11-17 NO: inconclusive outcome
	10	Tip	0	113.3	
	16	Tip	0	445.2	
	4	Interior	146.1	146.5	
2-12		I-T	146.1	-63.7	1-2-3-4 NO: restricted gene flow with isolation by distance
	1-25	Tip	0	115.6	
	1-26	Interior	0	115.6	
	1-27	Tip	0	169.7	
	1-28	Tip	0 <sup>S</sup>	115.6 <sup>S</sup>	
2-13	1-29	Interior	155.9	162.9 <sup>L</sup>	1-2-3-4 NO: restricted gene flow with isolation by distance
		I-T	137.6	27.0	
	1-18	Tip	0	204.0	
	1-19	Tip	0	251.4	
	1-20	Tip	0	142.9	
2-15	1-21	Tip	0	142.9	1-2-3-4 No: restricted gene flow with isolation by distance
	1-22	Tip	0	251.4	
	1-23	Interior	120.0	234.7	
	1-24	Interior	0	142.9	
		I-T	90.1 <sup>L</sup>	7.1	
2-17	1-2	Tip	21.4 <sup>S</sup>	52.4	1-2-3-4 No: restricted gene flow with isolation by distance
	1-3	Interior	40.0	48.9	
		I-T	18.7	-3.4	
3-1	1-6	Tip	0 <sup>S</sup>	44.0	1-2-11-12 No: contiguous range expansion
	1-7	Interior	29.3 <sup>S</sup>	43.2	
		I-T	29.3 <sup>L</sup>	-0.9	
3-2	2-1	Interior	25.3 <sup>S</sup>	35.9 <sup>S</sup>	1-2-11-12 No: contiguous range expansion
	2-2	Tip	0	69.5 <sup>L</sup>	
	2-3	Tip	26.4	31.1	
	2-4	Tip	0	27.3	
3-3		I-T	14.0	-11.1 <sup>S</sup>	1-2-3-4 No: restricted gene flow with isolation by distance
	2-5	Interior	40.5	73.6 <sup>L</sup>	
	2-6	Tip	9.6 <sup>S</sup>	43.5 <sup>S</sup>	
3-4		I-T	30.9 <sup>L</sup>	30.1 <sup>L</sup>	1-2-11-12 No: contiguous range expansion
	2-7	Interior	95.3 <sup>S</sup>	154.4 <sup>S</sup>	
	2-8	Tip	113.8	199.4	
	2-9	Tip	0	142.7	
	2-10	Interior	0 <sup>S</sup>	142.7 <sup>S</sup>	
3-5	2-11	Tip	0 <sup>S</sup>	322.5 <sup>L</sup>	1-2-11-17 No: inconclusive outcome
		I-T	39.2	-107.1 <sup>S</sup>	
	2-12	Interior	148.7 <sup>S</sup>	192.4	
Total cladogram	2-13	Interior	181.1	229.8	1-2-3-5-6-13-21 No: past gradual range expansion followed by fragmentations or long-distance movement
	3-1	Interior	41.2 <sup>S</sup>	195.2 <sup>S</sup>	
	3-2	Tip	55.3 <sup>S</sup>	239.2	
	3-3	Tip	202.9	225.5	
	3-4	Interior	209.2	260.0 <sup>L</sup>	
	3-5	Tip	45.6 <sup>S</sup>	223.6	
		I-T	55.3 <sup>L</sup>	12.9	

<sup>S</sup>, <sup>L</sup>Indicates that the distance measures are significantly smaller and larger ( $P < 0.001$ ) respectively, than expected under random distribution of haplotypes.

unpublished), suggest that the contemporary population structure and population differentiation of *S. pylzovi* may be consistent with the historical tectonic events that occurred in the course of uplifts of

the Qinghai-Tibetan Plateau. Previous geological studies (Li & Ying, 1998; Li *et al.*, 2000; Zhu *et al.*, 2003) suggest that the rising of the Qinghai-Tibetan Plateau is a process of multistage, heterogeneity, and

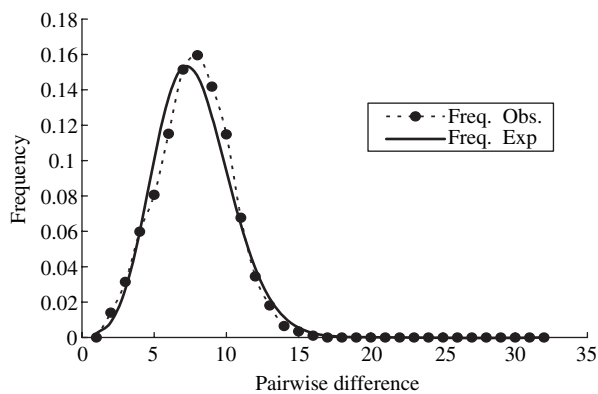


Fig. 4 The mismatch distribution of the 133 mtDNA *cyt-b* sequences of *Schizopygopsis pylzovi* in the Qinghai–Tibetan Plateau.

that in particular, the tectonic events that took place in the Kunlun-Huanghe Movement (middle Pleistocene) and the Gonghe Movement (150 000 years ago) had a significant impact on the evolution and formation of the drainage systems in the northern and north-eastern plateaus. The fluctuations of the ecogeographical environment and major hydrographic formation might have promoted contiguous range expansion of freshwater fish populations, whereas geological barriers among drainages have resulted in the fragmentation of population (e.g. the total cladogram). The geographical connections of the haplotypes distributed in the Qiadam Basin (i.e. Germu River and Tuosu), the Zhaling Lake and the Yellow River suggest the presence in the past of connections among these rivers and lakes. In fact, the tectonic events that occurred during the Kunlun-Huanghe Movement resulted in a large eastward outflow drainage system in the Qiadam Basin, which connected the Germu River and Tuosu Lake in the Qiadam Basin with Tuosuo Lake, Zhaling Lake and Yellow River (Li & Ying, 1998; Li *et al.*, 2000). During that time, the connection might have promoted the range expansion and dispersal of *S. pylzovi* I and II mtDNA lineages. However, the geographical barriers formed in the Gonghe Movement (Li *et al.*, 2000; Zhu *et al.*, 2003), which resulted in inflow drainage system (Germu River) and many isolated lakes such as Tuosuo Lake and Tuosu Lake, made the populations of *S. pylzovi* in the north-west plateau (i.e. QG, QT, YZ and TU) isolated from each other and the gene flow among populations was limited. In spite of the geological complexity of the north-west plateau, it is accepted that a high-tectonic activity during the Gonghe

Movement (around 150 000 years ago) in the north-west plateau seems to have transformed river trajectories (Li & Ying, 1998; Zhu *et al.*, 2003). These most likely recent changes on river configurations and hydrography might have promoted contiguous range expansion of current *S. pylzovi* populations on previously established lineages in the north-east plateau (e.g. Huangshui River) as suggested by clades 2–17 and 3–1 in the NCA (nested clade analysis) analysis. Nevertheless, population isolations may have occurred too recently for the populations from different drainages to have attained reciprocal monophyly.

Estimation of population expansion requires several assumptions (Su *et al.*, 2001; Pearse & Crandall, 2004); hence, we evaluated several methods, including mismatch distributions, Tajima's D-test and Fu's  $F_s$ -values. The Tajima's D-test put more weight on ancient mutation, thus revealing ancient population events, while the Fu's  $F_s$ -test is sensitive to recent population events, and the  $F_s$ -value tends to be negative when there is an excess of mutations (Su *et al.*, 2001). In the present study, the complete data set of *S. pylzovi* had a significantly large negative  $F_s$ -value ( $-24.91$ ,  $P < 0.01$ ), which is significantly larger than the Tajima's D ( $-1.79$ ,  $P < 0.01$ ), indicating that the population of *S. pylzovi* has undergone a sudden/recent population expansion as inferred from the mismatch distribution characterised by unimodal distributions with a mean of seven substitutions. The mismatch distribution suggests that the population has expanded significantly from a much smaller population around 110 000 years ago. This result might be consistent with the Gonghe Movement estimated to have occurred around 150 000 years ago, which had a significant impact on the evolution and development of the contemporary drainage systems (Li & Ying, 1998; Li *et al.*, 2000; Zhu *et al.*, 2003), and promoted range expansion and population dispersal of *S. pylzovi*.

The fact that the Yellow River runs through Zhaling Lake compelled us to group the individuals from Zhaling Lake and Yellow River into one population (Yellow River drainage) in the present study. However, when the four drainage systems (Qiadam Basin, Huangshui River, Yellow River and Tuosuo Lake) were analysed separately in Fu's  $F_s$ -test of neutrality, the  $F_s$ -values of three drainage systems (Qiadam Basin, Huangshui River and Tuosuo Lake) were still negative while the Yellow River was positive, suggesting that the population of the Yellow River

drainage is larger and more stable than that of the other populations (Fu, 1997). Therefore, historically, the Yellow River might be the home-drainage of *S. pylzovi*, and population growth might have led to north-westward and north-eastward expansion to the Qiadam Basin and the Huangshui River.

Generally, conservation of the genetic diversity and integrity of a species relies on identifying the critical genetic units and then managing these units in a coordinated manner (McCauley, 1991; Alpers *et al.*, 2004). These critical units are often referred to as evolutionarily significant units (ESUs) and an ESU is defined as a population that exchanges few migrants with others and that has been geographically isolated for long enough to be genetically distinct and independent (Avice, 1989; Moritz, 1994). In the present study, although AMOVA indicates significant genetic variance is caused by differences among populations both within and among drainage systems, both the phylogenetic and NCPA analyses consistently suggest that there is not a genetically distinct and independent population that could be considered as an ESU. Considering that most populations are geographically isolated and the species has declined rapidly over the last decade and is now endangered, it is proposed that, for the time being, all populations should be managed and conserved separately and that efforts should be directed towards preserving the genetic integrity of each population. The Huangshui River and Qiadam Basin have just begun to suffer the impact of human activities associated with river flow management; this means that suitable conditions for this species to continue to inhabit these regions may soon change. The implications of the restricted gene flow and population fragmentation report here for *S. pylzovi* may affect the long-term survival of this species. We suggest that if local extinctions occur, the chances of recolonisation would be difficult. Supporting this prediction is the very recent and fast decline of *S. pylzovi* from the Qiadam Basin (such as rivers Nuomuhong and Chaidamu). In conclusion, the information obtained from this study may be very important in the development of a suitable management plan for the conservation of *S. pylzovi* in the Qinghai-Tibetan Plateau.

### Acknowledgments

The authors take the opportunity to give special thanks to Dr B. W. Murray (University of Northern British

Columbia, Canada), Prof. Richard Johnson (Chief Editor) and two anonymous reviewers for valuable comments on the manuscript. We also thank Baosheng Xie (Qinghai University, China) for help in field collections. This research was supported by a National Basic Research Project (no. 2005CB422005) in China.

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(Manuscript accepted 3 January 2007)