Phylogeography of the endemic *Gymnocypris chilianensis* (Cyprinidae): Sequential westward colonization followed by allopatric evolution in response to cyclical Pleistocene glaciations on the Tibetan Plateau

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ARTICLE INFO

ABSTRACT

The schizothoracine *Gymnocypris chilianensis* is restricted to the Shiyang, Ruoshui and Shule Rivers, listed from east to west, along the northeast edge of the Tibetan Plateau. This distribution provides a valuable system to test hypotheses about postglacial colonization. We used mitochondrial DNA sequence data (a control region and the cytochrome *b* gene; 1894 bp) to assess the phylogeographic structure of this species based on 278 specimens sampled from throughout the species' entire geographical range. We found three lineages corresponding geographically to the three rivers, suggesting three independent glacial differentiation centers within the northeast edge of the Tibetan Plateau. The phylogenetic analysis suggested that the Shiyang River population forms a lineage that separated from the other populations of *G. chilianensis* at the basal phylogenetic split within this species. The molecular data further demonstrated a clear pattern of decreasing genetic diversity from the eastern Shiyang River towards the central Ruoshui River and western Shule River lineages, a pattern consistent with sequential western colonization. We therefore propose a phylogeographic scenario for *G. chilianensis* of a gradual westerly expansion from the Shiyang River population along the northeast edge of the Tibetan Plateau, with subsequent allopatric evolution at approximately 0.37 and 0.05 million years ago (Ma), through at least two glacial maxima. Together with the genetic evidence reported in other species, our findings suggest that this common biogeographic pattern emphasizes the importance of the northeastern edge region of the Tibetan Plateau as a hotspot of genetic diversity for some taxa.

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1. Introduction

Studies on the biotic consequences of Quaternary environmental change have increased rapidly during the last decade. The Pleistocene glacial and interglacial cycles have resulted in profound shifts in the ranges of the biota. Through the application of phylogeography and modern molecular techniques, these patterns have been repeatedly demonstrated across myriad taxa in Europe and America (Dick and Heuertz, 2008; Elderkin et al., 2008; Peters et al., 2008; Streicher et al., 2009; Phillipsen and Metcalf, 2009; Acosta and Premoli, 2010). The Tibetan Plateau is the youngest plateau on Earth, which has made it an important

region for the study of evolution of endemic specialized plateau species. In contrast to the large number of studies investigating the phylogeography of widespread taxa, far less attention has been given to the taxa endemic to the Tibetan Plateau. There is a lack of phylogeographic studies below the species level, partly due to the inaccessibility of samples in this region. Despite this, a few such studies on the plant, fish, and avian species endemic to the Tibetan Plateau have provided empirical evidence of a common biogeographic pattern of glacier-induced fragmentation. These studies indicate that the genetic divergence observed in most taxa is due to the isolation of populations in refugia during the Quaternary glacial advances (Zhao et al., 2005, 2007; Zhang et al., 2005; Liu et al., 2006; Yang et al., 2006; Qi et al., 2007; Jin et al., 2008; Jin and Liu, 2010). The topographical diversity of the Tibetan Plateau might have created networks of refugia during glaciations, thus forming barriers to the subsequent expansion of endemic species (Hewitt, 2004; Qu et al., 2005).
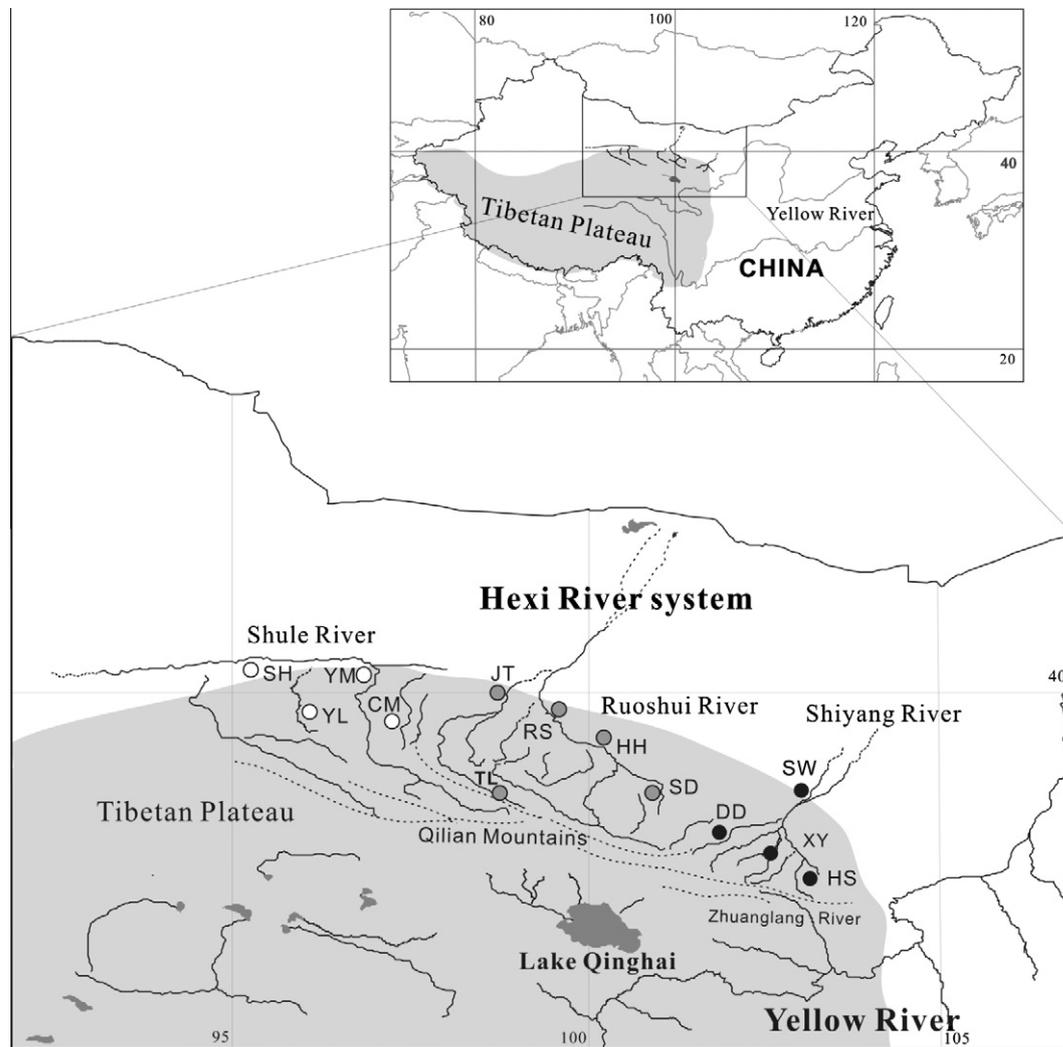

Fig. 1. A map of the Hexi River system showing the sampling sites (see Table 1) and the geographical distribution of the phylogenetic lineages (see Fig. 2). Black represents the Shiyang River lineage, grey represents the Ruoshui River lineage, and white represents the Shule River lineage. The dashed lines represent the ridge of the Qilian Mountains in the northeast Tibetan Plateau.

The complex plateau conditions and the diversified climate could allow for different patterns of postglacial colonization to predominate in the species endemic to the Tibetan Plateau. One such pattern is indicated in phylogeographical studies of two endemic species: the red-necked snow finch *Pyrgilauda ruficollis* and the tree species *Juniperus przewalskii*. These studies reveal that the two species colonized the plateau using a similar route from a single refugium in the northeast and eastern edge of the Tibetan Plateau after the extensive glacial period (Qu et al., 2005; Zhang et al., 2005). Whether the pattern of genetic variation observed in *P. ruficollis* and *J. przewalskii* is shared among the other Tibetan Plateau fauna remains to be determined.

The Hexi River system in the northeastern edge of the Tibetan Plateau comprises the Shiyang, Ruoshui, and Shule Rivers, listed from east to west (Fig. 1). All three rivers derive from the Qilian Mountains in the northeast plateau, flow north into the Hexi region, and finally disappear in the desert. Stratigraphic evidence shows that the northeast Tibetan Plateau was dramatically affected by the geological and climatic upheaval caused by the large-scale uplift of the plateau during the Pleistocene (Chen, 1988; Pan, 1994; Pan et al., 1996; Zhang et al., 2003). Thus, the fauna that inhabit the Hexi River system are a model group well suited for studying the patterns of glacial refugia and postglacial coloniza-

tion. This study uses phylogeographic methods to infer the genetic structure and population history of a schizothoracine fish species endemic to the Hexi River system, *Gymnocypris chilianensis*.

Gymnocypris chilianensis is restricted to the Hexi River system, and it is the only schizothoracine fish in the system (Li and Chang, 1974; Wu and Wu, 1992). This species has been found at altitudes ranging from 1500 to 2500 m (Zhao, 1991). We recently found that *G. chilianensis* also inhabits mountain streams at altitudes of 3800 m, showing irregular altitudinal movement and remarkable adaptability. The species status of *G. chilianensis* has been disputed, and is currently classified as a subspecies of *Gymnocypris eckloni* (Zhao, 1986; Chen and Cao, 2000). Recent evidence suggests that the morphological differences between *G. chilianensis* and *G. eckloni* may be corroborated by genetic data, with a high mean sequence divergence of 2.5% in mtDNA (the combined control region and cytochrome *b* gene) (Zhao et al., 2009). However, prior to this study, genetic surveys had not been conducted across the entire distribution range of *G. chilianensis*. The initial genetic study of *G. chilianensis* was based on a small sample (17 individuals) and was restricted to one river and two localities (Zhao et al., 2009). The present study is based on 278 samples from 13 locations across the three rivers, covering the entire range of the species.

Table 1
The sampling locations, number of specimens (*n*), and river systems for the samples of *G. chilianensis* used in this study. Coordinates: eastern longitude/northern latitude. The location codes correspond to those in Fig. 1.

Lineages	Location codes	Coordinates	Sampling location	*n*
Shiyang River	SW	103°10′/38°62′	Shiyang River, Wuwei	17
	HS	103°04′/37°77′	Hongshui River, Wuwei	16
	XY	101°99′/38°04′	Xiying River, Wuwei	20
	DD	102°41′/38°01′	Dongda River, Sunan	18
Ruoshui River	SD	100°85′/38°88′	Shandan River, Shandan	21[a]
	RS	99°46′/39°82′	Ruoshui River, Gaotai	20[a]
	HH	100°40′/39°19′	Heihe River, Zhangye	16
	JT	98°90′/39°99′	Tuole River, Jinta	18
	TL	98°61′/38°71′	Tuole River, Qilian	32
Shule River	CM	96°80′/39°90′	Changma, Yumen	23
	YM	96°94′/40°50′	Yumenzhen, Yumen	23
	SH	95°53′/40°56′	Shule River, Anxi	29
	YL	95°98′/39°96′	Yulin River, Anxi	25

[a] Eight specimens from the Shandan River (SD) and nine specimens from the Ruoshui River (RS) were provided in a previous work and were utilized in this study (see Appendix 1 for the GenBank accession numbers; Zhao et al., 2009).

This study aims to analyze mitochondrial DNA sequence data (the control region and cytochrome *b* gene) to examine the population genetic structure and evolutionary history of *G. chilianensis*, and to present a hypothesis of population dynamics explaining the impact of Quaternary events on its current distribution.

2. Materials and methods

2.1. Samples and laboratory analyses

Specimens were collected during the dry season of 2008 from the Shiyang, Ruoshui, and Shule Rivers at an altitude of 1000–2500 m (Fig. 1), covering the entire known distribution of this species. In this process, a new locality (the upper Tuole River) was discovered that extended the geographic range of this species to 3800 m in elevation. The samples were collected with gill nets or cast nets, and all of the specimens were preserved in 95% ethanol for the laboratory analyses. In this study, a total of 278 individuals of *G. chilianensis* were used for the phylogenetic and population-genetic analyses (Table 1; Appendices 1–3). The cytochrome *b* and control region sequences for 17 specimens were provided in a previous work (Zhao et al., 2009) and were again utilized in this work (Table 1; Appendices 1 and 3). Gene trees were constructed using all of these specimens, using as outgroups endemic taxa of the genus *Gymnocypris* distributed in the northeast Tibetan Plateau. Outgroup haplotypes include representatives of *Gymnocypris przewalskii przewalskii* (GenBank accession number FJ494958 for cytochrome *b* and FJ534424 for the control region) and *Gymnocypris przewalskii ganzihonensis* (FJ494963 and FJ534434) from Lake Qinghai, *Gymnocypris eckloni eckloni* from the Yellow River (*G. e. eckloni* 1–3: FJ534388 and FJ534445, FJ534385 and FJ534444, and FJ534384 and FJ534441, respectively), and *Gymnocypris eckloni scolistomus* from Lake Sunmcuo on the upper Yellow River (*G. e. scolistomus* 1–2: FJ534412 and FJ534469, and FJ534409 and FJ534467, respectively) (Zhao et al., 2009). These species (subspecies) have been proposed as the closest known relatives of *G. chilianensis* by classical taxonomy (Zhao, 1986; Wu and Wu, 1992). Voucher specimens were deposited in the Northwest Plateau Institute of Biology, the Chinese Academy of Science in Xining (Appendix 3).

Total genomic DNA was extracted from muscle tissue using phenol/chloroform extraction (Sambrook et al., 1989). The complete mitochondrial cytochrome *b* gene (1140 bp) was amplified using the 'universal' primers L14724 (5′-GAC TTG AAA AAC CAC CGT TG-3′) and H15915 (5′-CTC CGA TCT CCG GAT TAC AA GAC-3′) (Xiao et al., 2001). A total of 754 bp from the mitochondrial control region (including the first and second hypervariable sequences) was amplified and sequenced with the new specific primers GEDL200 (5′-CAC CCC TGG CTC CCA AAG CCA G-3′) and GEDH860 (5′-AGG GGT TTG ACA AGA ATA ACA GGA-3′). The PCR reaction contained approximately 100 ng of template DNA, 1 μL of each primer, 5 μL of 10× reaction buffer, 2 μL of dNTPs (2.5 mM each), and 2.0 U of *Taq* DNA polymerase in a total volume of 50 μL. The PCR conditions were identical for both genes, with an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58–64 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The amplified DNA was fractionated by electrophoresis in 0.8% low-melting agarose gels, recovered from the gels, and purified using a BioStar Glassmilk DNA Purification Kit according to the manufacturer's instructions. The purified DNA was sequenced with a Perkin–Elmer BigDye DNA Sequencing Kit according to the manufacturer's protocol with the primers used for PCR.

2.2. Phylogenetic analyses

The sequences were aligned using the program ClustalX 1.8 (Thompson et al., 1997). The phylogenetic congruence of the control region and cytochrome *b* datasets was examined in PAUP* v4b10 (Swofford, 2002). The phylogenies were reconstructed using neighbor-joining (NJ) and maximum parsimony (MP) approaches using PAUP* v4b10 (Swofford, 2002), and the Bayesian inference (BI) was calculated using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with different parameter estimations for the control region and cytochrome *b*. The most appropriate model of DNA substitution (GTR + I + G model) identified by Modeltest 3.7 (Posada and Crandall, 1998) was implemented in the NJ and BI analyses. To assess the statistical support for the hypothesized lineages, a bootstrap analysis was performed with 1000 replicates for the NJ and MP analyses. For the BI analysis, the posterior distributions were obtained by a Markov Chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. The maximum-likelihood model employed six substitution types (Nst = 6), with a proportion of the sites held invariant while the rates for the remaining sites were drawn from a gamma distribution (rates = invgamma). Samples of trees and parameters were drawn every 100 steps from a total of 1,000,000 MCMC generations. Three additional analyses were run beginning with random trees. A consensus of all post-burns (a burn-in of 25%) in each of the generations was computed from all four runs. Finally, we used the program Network 4.0 (Bandelt et al., 1999) to construct
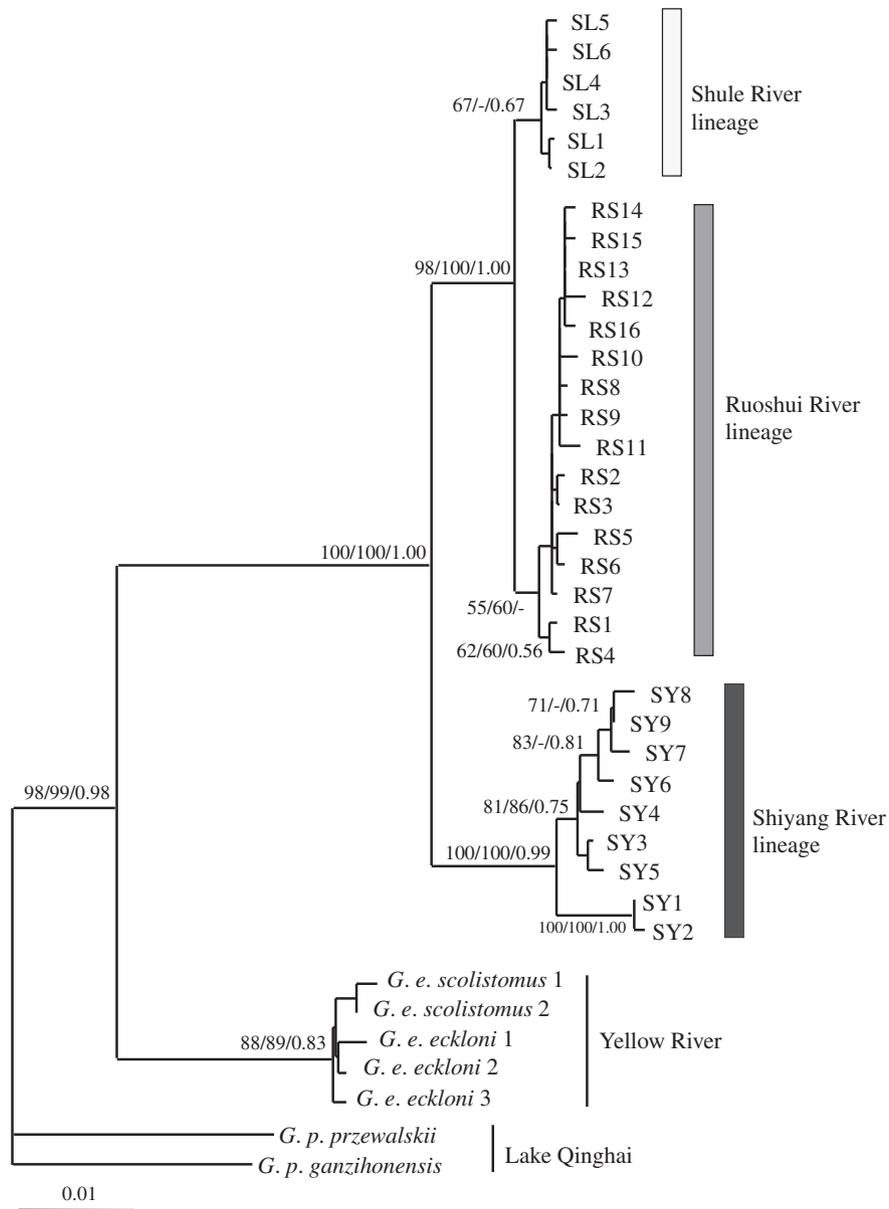
Fig. 2. The neighbor-joining tree (GTR + I + G model) obtained by combining the control region and cytochrome *b* sequences (1894 bp). The haplotype numbers and their geographical origins are shown in Table 1, Fig. 1 and the Appendices. The numbers on the branches correspond to bootstrap support ⩾ 50% obtained in the NJ and MP analyses and posterior probabilities > 0.5 in the Bayesian analyses, respectively.

median-joining networks to visualize the relationships among the haplotypes within the lineages.

2.3. Population-genetic analyses

The nucleotide diversity (π) and haplotype (h) indices (Nei, 1987) with standard errors were calculated in Arlequin 2.000 (Schneider et al., 2000). Arlequin was also used to calculate all of the pairwise genetic differentiation (Fst) values between all populations (Reynolds et al., 1983; Slatkin, 1995). The overall mean divergence among sequences and the net divergence between lineages were according to Nei (1987) and implemented in MEGA VERSION 3 (Kumar et al., 2004), with the standard errors estimated by bootstrapping using 10,000 replicates. An analysis of molecular variance (AMOVA) was used to assess the population configuration and the geographical pattern for the distribution of the main genetic variation.

2.4. Demographic history

The molecular-clock hypothesis was tested using the likelihood-ratio statistic (LR = 2[ln $L1$ − ln $L2$], Huelsenbeck and Crandall, 1997). We used the BEAST 1.4 program (Drummond and Rambaut, 2007) to estimate the divergence times under a model of uncorrelated but lognormally distributed rates of molecular evolution (Drummond et al., 2006). In this study, the evolutionary model (GTR + I + G) suggested by MODELTEST 3.7 (Posada and Crandall, 1998) was used with a chain length of 100,000,000 generations.

The expansion time, or the time passed from the ancestral haplotype, was estimated using the ρ statistic (Forster et al., 1996) based on the median-joining network, and the confidence intervals were calculated (Saillard et al., 2000). We assumed the previously established average substitution rate of 1.69% per million years (Zhao et al., 2009), which has been calibrated for the control region and the cytochrome *b* gene in the schizothoracine fish.
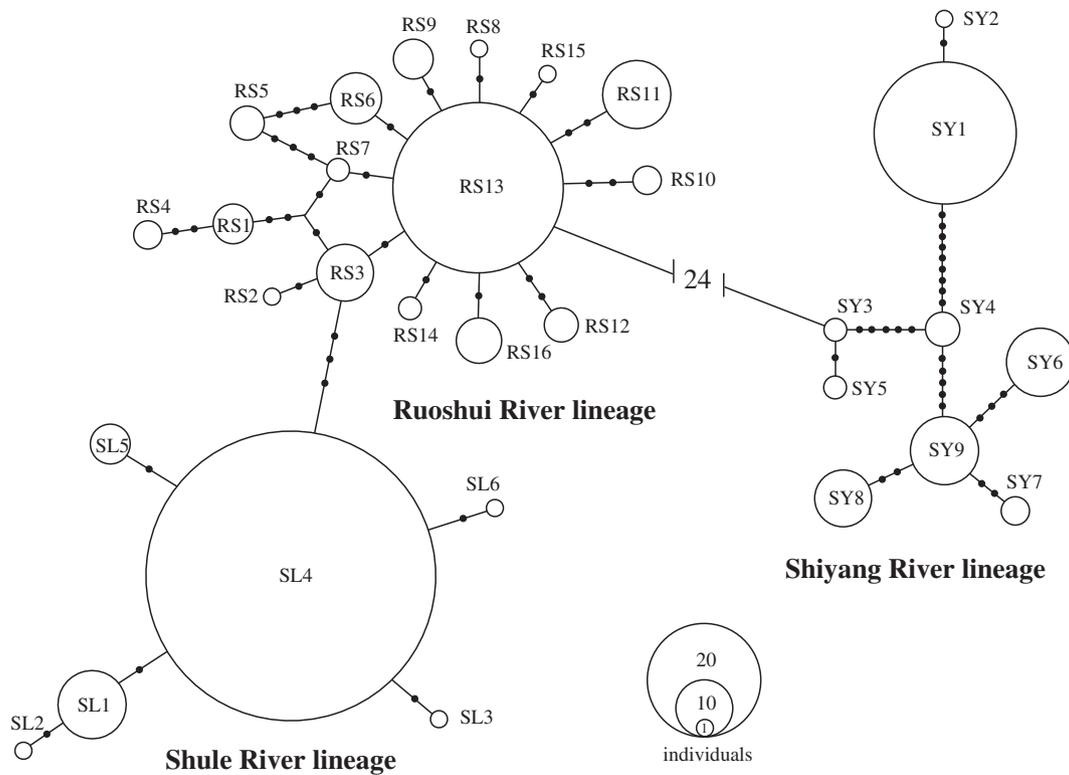
Fig. 3. The median-joining network based on the combined sequence data for the 31 haplotypes of *G. chilianensis*. The haplotype numbers and their geographical origins are shown in Fig. 2 and the Appendices. The circle sizes roughly represent the number of individual(s), and the scale is given below the figure. The black dots are the nucleotide substitutions inferred in that branch. The number of mutational steps joining the clades is indicated along the connecting branches.

Table 2
The *F*st values among the populations of *G. chilianensis*. The significant pairwise differences are shown in italics ($P < 0.05$) and bold ($P < 0.01$). The location codes correspond to those in Fig. 1 and Table 1.

Lineage	Code	Shiyang River lineage				Ruoshui River lineage					Shule River lineage		
		SW	HS	XY	DD	SD	RS	HH	JT	TL	CM	YM	SH
Shiyang River	HS	−0.03											
	XY	−0.04	−0.05										
	DD	0.01	0.01	0.00									
Ruoshui River	SD	**0.84**	**0.85**	**0.85**	**0.83**								
	RS	**0.83**	**0.85**	**0.84**	**0.83**	−0.02							
	HH	**0.84**	**0.85**	**0.85**	**0.84**	−0.02	0.01						
	JT	**0.85**	**0.86**	**0.86**	**0.85**	−0.03	0.00	0.03					
	TL	**0.88**	**0.89**	**0.88**	**0.87**	**0.08**	**0.09**	**0.14**	*0.06*				
Shule River	CM	**0.89**	**0.89**	**0.89**	**0.88**	**0.69**	**0.71**	**0.74**	**0.75**	**0.75**			
	YM	**0.89**	**0.90**	**0.89**	**0.88**	**0.68**	**0.71**	**0.73**	**0.74**	**0.74**	−0.02		
	SH	**0.90**	**0.91**	**0.91**	**0.89**	**0.73**	**0.76**	**0.77**	**0.79**	**0.77**	0.02	0.03	
	YL	**0.89**	**0.91**	**0.90**	**0.88**	**0.71**	**0.73**	**0.75**	**0.77**	**0.76**	−0.02	0.00	−0.02

3. Results

3.1. Sequence data

The aligned control region sequences of *G. chilianensis* were 754 bp long and had 22 variable sites, 14 of which were parsimony-informative with one insertion/deletion site, and they had a Ti/Tv ratio of 4.8. The aligned cytochrome *b* sequence (1140 bp) had 33 variable sites, 23 of which were parsimony-informative. No stop codons, insertions, or deletions were found in the cytochrome *b* sequence.

The partition-homogeneity test of 285 individuals (including seven published haplotypes from two other species) revealed no significant incongruence between the control region and cytochrome *b* ($P = 0.43$). The apparent phylogenetic congruence justified the combination of the two partial sequences in a phylogenetic analysis of 1894 bp of the mitochondrial genome. A total of 31 haplotypes were identified among all of the sequences from *G. chilianensis* (Appendices 1 and 2). For the combined sequence data, the mean divergence among haplotypes was 0.80%. However, the cytochrome *b* divergence among haplotypes (mean divergence 0.87%) exceeded the divergence for the control region (mean divergence 0.69%).

3.2. Phylogenetic analyses

Based on the combined data, the three phylogenetic methods used (NJ, MP and Bayesian) yielded trees with very similar topolo-

gies. Consequently, only the NJ tree (GTR + I + G model) is shown here (Fig. 2). All of the analyses strongly supported monophyly of the mtDNA haplotypes of *G. chilianensis*, and revealed three highly divergent groups of haplotypes within *G. chilianensis* (Fig. 2). These groups were well supported and showed a strong geographical association. The first group (71 individuals) corresponds to the Shiyang River, with a mean sequence divergence of 1.54% ± 0.28 and a net divergence of 1.25% ± 0.25 from the other groups. The second group (107 individuals) corresponds to the Ruoshui River, and the third group (100 individuals) corresponds to the Shule River. The mean sequence divergence between the second and third groups was 0.31% ± 0.11, and the net divergence was 0.18% ± 0.09.

The Ruoshui River and Shule River groups form a haplotype clade (bootstrap support of 98–100%) (Fig. 2). The distinctiveness of the lineages is highly supported for the Shiyang River lineage and modestly supported for the Ruoshui River and Shule River lineages (Fig. 2). In the phylogenetic trees, further subdivisions within the Shiyang River lineage showed no clear geographical structuring (Fig. 2).

The haplotype network very clearly showed three clusters (the Shiyang River, Ruoshui River, and Shule River lineages, Fig. 3), a finding consistent with three phylogenetic groups (Fig. 2). The Shiyang River cluster is separated from the Ruoshui River cluster by 24 mutational steps, while the Ruoshui River and Shule River clusters are separated by three mutational steps. However, this haplotype-network approach permits a fine-grained reconstruction of the evolutionary histories of the *G. chilianensis* species with some differences in topologies among the three lineages, suggesting different demographic histories. The Shiyang River cluster showed the greatest disparity among haplotypes, with a maximum distance between haplotypes of 18 mutational steps. Parts of the network within the Ruoshui River and Shule River clusters showed maximum distances among haplotypes of eight and three mutational steps, respectively. The star-like structure of the haplotype lineages was detected from a central common haplotype of both the Ruoshui River and Shule River lineages, indicative of a recent expansion.

3.3. Nucleotide diversity and genetic structure

The nucleotide (π) and haplotype (h) diversities were high for all three lineages (h = 0.8875 ± 0.013; π = 0.0070 ± 0.004), but there were significant differences among the main genealogical lineages. The nucleotide diversity was highest in the Shiyang River lineage (π = 0.0035 ± 0.018), lowest in the Shule River lineage (π = 0.0002 ± 0.0001), and intermediate in the Ruoshui River lineage (π = 0.0012 ± 0.0007). The maximum divergence between the haplotypes was estimated at 0.69% for the Shiyang River lineage, 0.37% for the Ruoshui River lineage, and 0.16% for the Shule River lineage.

Pairwise comparisons of genetic differentiation (Fst) based on the combined data detected strong differences between all three lineages (Table 2). However, these comparisons failed to find any significant differentiation between the locations sampled within the three lineages, except between one specific location, the upper Tuole River (TL), and other locations within the Ruoshui River lineage (Fst = 0.06–0.14; Table 2). The AMOVA was consistent with the pairwise Fst, showing that most of the total mtDNA variation (85.51%) observed was distributed among the three main lineages, with 0.18% of the variation observed among the populations within lineages.

3.4. Demographic history

The molecular-clock hypothesis was not rejected by the LRT test (LR = 33.05, df = 29, P = 0.27). The net distances indicated a diver-gence time of about 0.37 (±0.073) Ma between the Shiyang River lineage and the remaining lineages and a divergence time of 0.053 (±0.027) Ma between the Ruoshui River and Shule River lineages. The application of the BEAST 1.4 program yielded similar estimates of about 0.387 (confidence interval, CI: 0.564–0.192) and 0.065 Ma (CI: 0.041–0.078), respectively.

The star-like structure of haplotype networks within both the Ruoshui River and Shule River lineages suggests expansion events in the population's recent demographic history (Fig. 3). The expansion times were estimated at about 0.059 (±0.014) Ma for the Ruoshui Rive lineage and 0.033 (±0.019) Ma for the Shule River lineage.

4. Discussion

4.1. Demographic processes of G. chilianensis

This study provides the first complete sampling of the geographic range of *G. chilianensis*. Our analyses identified three lineages correlated with geography that had patterns of genetic diversity likely produced by past demographic and dispersal events. Such a pattern indicates considerable demographic and biogeographical dynamics during the species' evolution after these lineages originated by allopatric fragmentation (Avise, 2000; Krystufek et al., 2007). This study provides strong evidence for the existence of three geographic isolates within the northeast edge of the Tibetan Plateau. It is possible that a hydrological barrier between the rivers promoted allopatry of these lineages.

However, the genetic structure of *G. chilianensis* revealed three distinct patterns, suggesting that the evolutionary history of *G. chilianensis* is more complex than previously assumed. The most remarkable of these is the clear geographic pattern of decreasing genetic diversity from the eastern Shiyang River lineage (π = 0.0035 ± 0.018) towards the central Ruoshui River (π = 0.0012 ± 0.0007) and western Shule River lineages (π = 0.0002 ± 0.0001). This pattern suggests that the Shiyang River is likely to be the region where *G. chilianensis* first arose, followed by west ward expansion preceding allopatric fragmentation separating the three rivers.

We therefore consider rare Pleistocene dispersal across hydrological boundaries with subsequent range expansion a more likely explanation of the distribution of these populations than the theory that these lineages are relics of a pre-Pleistocene species that was widespread along the northeastern edge of the Tibetan Plateau. A gradual westward colonization process is expected to yield a decrease in genetic variation along the colonization path (Ibrahim et al., 1996; Ramachandran et al., 2005; Krystufek et al., 2007). Interestingly, the morphological study of *G. chilianensis* also indicated a geographical cline from east to west in the character of the horny ridge at the inner margin of the lower jaw, with a weak horny ridge in the Shiyang River population, trending towards modest in the Ruoshui River population and strong in the Shule River population (Zhao, 1986). This observed pattern was attributed to ecological adaptation to different environments after exploiting new habitats (Zhao, 1986).

G. chilianensis is the most northerly species in the geographical range of the genus *Gymnocypris*, and also no fish of the genus *Gymnocypris* have been found in drainages west of the Shule River, which is consistent with the relatively small divergence among haplotypes within the Shule River population. *G. e. eckloni* and *G. e. scolistomus*, the closest relatives of the *G. chilianensis*, are from the Yellow River system with a geographical range that extends further east and south from the Shiyang River, consistent with our inference that *G. chilianensis* originated in the eastern part of its current range. Stratigraphic evidence shows that tectonic

activity caused by the intermittent uplifts of the Qilian Mountains around 2.94–0.14 Ma led to the origin of the Hexi River system (Fang et al., 2005). In addition, there is evidence that the Shiyang River originally drained to the southeast and was linked with the ancient Yellow River by the Zhuanglang River (Fig. 1) (Feng, 1981). The separation and subsequent flow reversal of the Shiyang River could have occurred as result of intermittent uplift of the Qilian Mountains (Feng, 1981).

Lastly, we propose a temporal framework for the westward expansion of *G. chilianensis* from the Shiyang River region. An early lineage colonized the Ruoshui River (approximately 0.37 Ma) with subsequent allopatric evolution of the lineage, and this was followed by a later colonization further west to the Shule River (approximately 0.05 Ma), which gradually diverged (again in allopatry) into the present Shule River lineage. The distinct geographical trend of decreasing within lineage sequence variation is concordant with a range expansion in response to the multiple glaciations during the late Pleistocene. The northeast Tibetan Plateau was dramatically affected by the geological and climatic upheaval due to the large-scale uplift of the plateau, and it has been suggested that the connections between these rivers occurred in the late Pleistocene, presumably caused by a humid period during the Tibetan Plateau interglacial age (Feng, 1988).

Since the Pleistocene, the Tibetan Plateau has undergone four or five glaciations (Zheng et al., 2002). The largest glacier development in the Tibetan Plateau occurred during the middle Pleistocene (0.5 Ma), while glacial retreat has occurred since 0.17 Ma (Zhuo et al., 1998; Zhang et al., 2000; Zheng et al., 2002). The Tibetan Plateau entered the last glacial age at approximately 0.075 Ma, continuing until 0.01 Ma, after which the plateau has experienced short glacial cycles with three warmer periods (Jing et al., 2004; Yi et al., 2005). Because climate change throughout the Quaternary was cyclical, glacial refugia would have occurred episodically throughout the epoch during periods of glacial maxima (Spellman et al., 2007). The differences in divergence times among the three allopatric phylogenetic lineages could be because these populations originally colonized new habitats during different glacial maxima, while the typical signature of the expansion events suggests that the populations have undergone a rapid increase in their population sizes, which might be related to a suitable environment within their confines during the short glacial cycles.

In contrast to the strong differentiation among the three distant rivers, the phylogeographic data show no differentiation among the branches within the rivers. Despite the fact that some branches seem to be completely isolated, there is evidence for continuous gene flow. Geographic isolation within the rivers occurred during the last several hundred years, mainly due to a dry climate and the over-exploitation of the river resources (Feng, 1981, 1988); such isolation is too recent to be evident in patterns of mitochondrial haplotypic variation. One exception to the significant phylogeographic structure was observed between the Tuole River (TL) population and other populations within the Ruoshui River lineage. The upper reaches of the Tuole River have a steep gradient, with a loss of altitude of approximately 1500 m, and the river flows through several large gorges that may have constituted a barrier to the dispersal of fish.

It is surprising that the Shiyang River population of *G. chilianensis* contained deeply divergent but intermixed haplotypes, suggesting both the persistence of local populations (refugial divergence, no extinctions) and subsequent gene flow. These populations most likely survived in isolated habitats and differentiated through genetic drift and selection. These divergent populations could have become interspersed during glacial periods in a secondary admixture of allopatrically evolved lineages. In contrast, within the Ruoshui River and Shule River lineages, the phylogenetic structure was generally shallow. Only six different haplotypes with one

substitution each were recognized in the 100 individuals of the Shule River population, a finding concordant with a recent colonization event and possibly also a very small colonizing population.

4.2. Glacial refugia in the northeast edge of the Tibetan Plateau

There is a growing body of evidence from phylogeographic studies that the Pleistocene climatic cycles and glacial refugia played integral roles in the evolution of the Tibetan Plateau taxa (Liu et al., 2002; Qu et al., 2005; Zhang et al., 2005; Yang et al., 2006; Qi et al., 2007; Jin et al., 2008; Jin and Liu, 2010). In the schizothoracine fish, the patterns of genetic divergence also suggest that these taxa were originally isolated to different refugia due to the same or multiple isolating events during periods of glacial maxima (Zhao et al., 2007; Qi et al., 2007). However, this study supports a different hypothesis, that of a gradual east to west expansion during the late Pleistocene through at least two glacial maxima. This expansion is novel with respect to the Tibetan Plateau freshwater fish, but it has recently been suggested for two other endemic species. A phylogeographic study of a tree species (*J. przewalskii*) revealed that this species colonized the plateau from the northeast edge during the recent postglacial period (Zhang et al., 2005). Additionally, a study of the red-necked snow finch *P. ruficollis* provided evidence that this species expanded west from the eastern margin after the last extensive glacial period (Qu et al., 2005). The recent histories of these three completely different taxa (a tree species, a finch species, and a freshwater fish species) appear to be similar. Their demographic processes suggest a route of westerly range expansion from a common refugium in the northeastern edge of the Tibetan Plateau during the Quaternary glacial advances. We hypothesize that the northeastern edge of the Tibetan Plateau might have acted as the refugium for the major radiations for some taxa that are now widespread in the plateau. However, *P. ruficolis* and *J. przewalskii* have weak phylogeographical structure and no significant genetic divergence. This contrasts with the deeply diverged lineages of *G. chilianensis*, which are only found in a small geographical range. We propose that hydrological boundaries formed after each colonization event constituted significant barriers for secondary contact, thus permitting accumulation of large genetic differences.

The results of this study suggest a gradual east to west expansion along the northeast edge of the Tibetan Plateau during the late Pleistocene, which is a novel view of the freshwater fish phylogeography of the Tibetan Plateau. Furthermore, this study has provided the first evidence for the existence of three independent differentiation centers in the region. Together with evidence of the Pleistocene glacial refugia found in the tree and finch species, it seems that this common biogeographic pattern emphasizes the importance of the northeast edge region of the Tibetan Plateau as a hotspot of genetic diversity for some taxa. Clearly, more detailed phylogeographical studies are needed on a wide range of different species endemic to the Tibetan Plateau to test the generality of the observed patterns.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.02.001.

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