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Short Communication

Comparative phylogeography of the Yellow River schizothoracine fishes (Cyprinidae): Vicariance, expansion, and recent coalescence in response to the Quaternary environmental upheaval in the Tibetan Plateau

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ARTICLE INFO

Article history:

Received 20 February 2009

Accepted 11 March 2009

Available online 11 April 2009

1. Introduction

The biotic consequences of Quaternary environmental change have been an intensely studied topic during the last few decades. The fact that the Pleistocene climate upheaval resulted in profound shifts in the ranges of habitat has led to the development of well-supported hypotheses for the Quaternary biogeography in Europe and America by studies of genetic variation across a myriad of taxa. The uplift of the Tibetan Plateau, which began in the Pliocene and continued through the Quaternary glaciations, considerably influenced the evolution and distribution of many species. Through the application of phylogeography and modern molecular techniques, studies have begun to reveal the genetic patterns of some species endemic to the Tibetan Plateau (Peng et al., 2006; Yang et al., 2006; Jin et al., 2008). The detailed phylogeographic study of more species from this region is critical for the collective understanding of the genetic consequences of Quaternary topographic and climate changes in the Tibetan Plateau.

The schizothoracine fishes are the largest and most diverse taxon of the Tibetan Plateau ichthyofauna (Cao et al., 1981; Wu and Wu, 1992). The three stages of uplift of the Tibetan Plateau have been hypothesized to account for speciation of these fishes (Cao et al., 1981). The biogeography of the schizothoracines emphasizes the role of the drastic environmental perturbations on the Tibetan Plateau, which forced the species that persisted in this area to contend with repeated isolation and

coalescence (Cao et al., 1981). These demographic processes have left genetic ‘footprints’ in modern descendent biotas. Nevertheless, this process is poorly understood below the species level since there have been few intraspecific phylogeographic studies on the species due to the inaccessibility of samples. Here, we present a comparative biogeographical study of the species endemic to the Tibetan Plateau in a large water system, the Yellow River, and focus on all species of schizothoracine fishes across their entire distribution range. These data were used to make inferences regarding the impact of the Quaternary period on their genetic ‘footprint’.

The schizothoracines in the Yellow River are restricted within the limits of the head-water area in the northeast Tibetan Plateau. The history and diversity of the Yellow River schizothoracines have attracted scientific interest at least for two reasons. First, the head-water area of the Yellow River was dramatically affected by the geological and climatic upheaval during the Pleistocene (Chen, 1988; Zhang et al., 2003). Second, it is the most dominant group of Yellow River ichthyofauna and is currently characterized by a high species endemism, with two of its five genera and four of six species endemic (Wu and Wu, 1992), suggesting an unusual evolutionary history.

The Yellow River endemic genera *Platypharodon* and *Chuanchia* are both monotypic, and *Platypharodon extremus* is only found in the reaches upstream from Maqu (MQ) (Fig. 1) (Wu and Wu, 1992). *Schizopygopsis pylzovi* and *Gymnocypris scoliostomus* also are endemic, but the latter is confined to Lake Sunmucuo (Fig. 1) (Wu and Wu, 1984). Only *Gymnocypris eckloni* and *Gymnodiptychus pachycheilus* are shared between the Yellow River and other river drainages (Wu and Wu, 1992). However, prior to this study, no genetic surveys have been conducted to determine the population genetics of *Pl. extremus*, *Chuanchia labiosa*, and *Gd. pachycheilus*.

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The initial genetic study for Yellow River schizothoracine fishes was based on a mitochondrial marker (cytochrome *b* gene) and a small number of samples (58 individuals collected in 1999–2003; this study was based on 199 samples collected in 2008 and using the combining control region and cytochrome *b* sequences) and was restricted to the genus *Gymnocypris* (Zhao et al., 2005); here, we also included samples from all of the other genera. In addition, the species *Gymnocypris przewalskii*, which is endemic to Lake Qinghai adjacent to the Yellow River, is also included in this study, which may be helpful for understanding the phylogeographic process of the schizothoracines of the Yellow River.

2. Materials and methods

2.1. Samples and laboratory analyses

Total six species from five genera (176 individuals) were collected from the Yellow River and Lake Qinghai. In order to identify the most suitable outgroup of each taxon, we collected four representative species (five individuals) from two other genera of schizothoracine fishes (*Herzensteinia microcephalus* collected from Tuotuo River, Qinghai), *Ptychobarbus kaznakovi* (Zaqu River, Qinghai), *Ptychobarbus chungtienensis* (Jinsha River, Yunnan), and *Ptychobarbus dipogon* (Yarlung Zangbo River, Tibet). We also added a species (one individual) from the genus *Gymnodiptychus* (*Gd. integrigymnatus* from Longchuan River, Yunnan) because it was suggested by He et al. (2004) as a closer relative of the Yellow River schizothoracine fishes.

The total genomic DNA was extracted from the muscle tissue according to the phenol/chloroform extraction procedure. The complete mitochondrial cytochrome *b* gene (1140 bp) was amplified using the 'universal' primers L14724 (5'-GAC TTG AA AAA CCA CCG TTG-3') and H15915 (5'-CTC CGA TCT CCG GAT TAC

AAG AC-3') (Xiao et al., 2001). A total of 754 bp from the mitochondrial control region was amplified and sequenced with 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 purified DNA was sequenced with a Perkin-Elmer BigDye DNA Sequencing Kit according to the manufacturer's protocol with the primers used in the PCR.

We sequenced 182 individuals for the control region and cytochrome *b* gene. Sequences were initially aligned using the program ClustalX 1.8 (Thompson et al., 1997), resulting in an alignment of 1894 bp of the mitochondrial genome, and 95 different haplotypes were detected. To obtain more information of the genetic diversity, we added 32 haplotypes from published sequence data, including 12 haplotypes from *G. przewalskii*, 11 haplotypes from *G. eckloni*, six haplotypes from *G. scoliosomus*, and three haplotypes shared by *G. eckloni*, and *G. scoliosomus* (Zhao et al., 2009). Thus, a total of 127 haplotypes representing 267 individual samples from 12 species of seven genera of schizothoracine fishes, were used in this study (the haplotype assignment, sampling localities and haplotype frequency see Fig. 2, and the GenBank accession numbers are given in Appendix S1).

All species from the Yellow River distribute in 12 localities, and these localities are further divided into three groups corresponding to the three reaches on the upper Yellow River (Upper, Middle and Lower reaches, which are divided geographically by two large gorges, Guancang Gorge and Lajia Gorge) (Fig. 1 and Table 1).

2.2. Data analysis

Phylogenetic analyses of gene trees were conducted at interspecies and intraspecies levels, respectively. First, the interspecies relationships of all the studied species were constructed using maximum parsimony (MP) and Bayesian inference (BI) approaches,

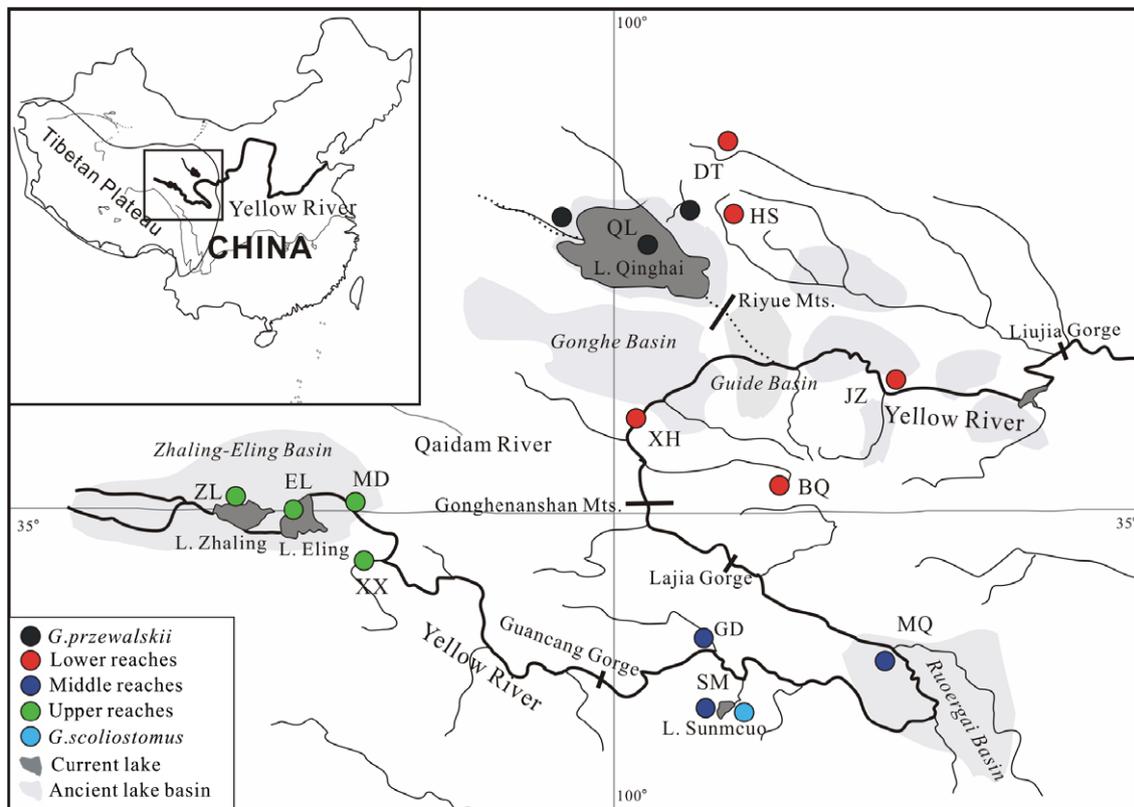
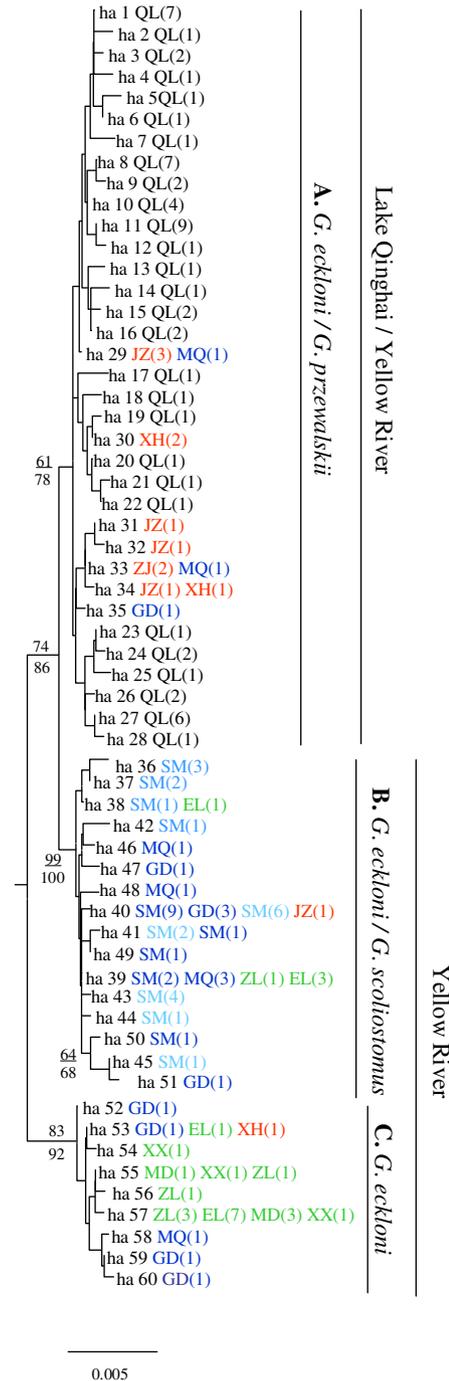
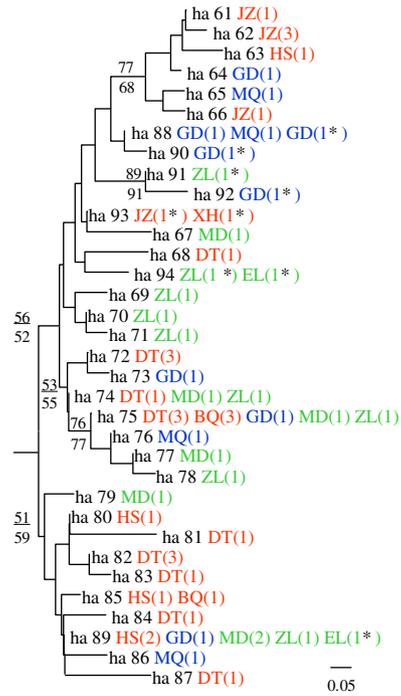


Fig. 1. Map of the head-water area of the Yellow River, showing sampling sites and their corresponding codes, as listed in Table 1. Dashed lines represent the head-water of the ancient Yellow River before the 'Gonghe Movement' event of the Tibetan Plateau in the late Pleistocene (according to Chen, 1988; Li et al., 2001; Zhang et al., 2003).

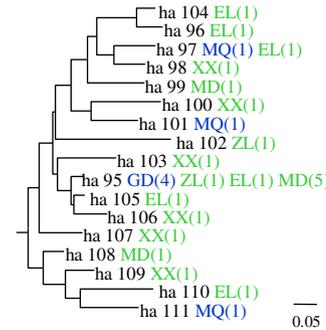
a *Gymnocypris* complex



b *Sy. pylzovi* and *Ch. labiosa* complex



c *Pl. extremus*



d *Gd. pachycheilus*

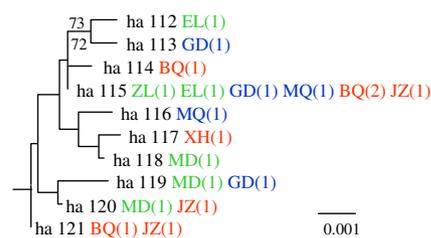


Fig. 2. Neighbor-joining (GTR + I + G model) topologies based on combining the control region and cytochrome *b* sequences (1894 bp) for all the schizothoracine fishes species from the Yellow River. The haplotype designations given at each twig (e.g., ha31 correspond to the Appendix S1) are followed by the localities and numbers of individuals from each locality having that haplotype (e.g., JZ(3)). The geographic origins of the haplotypes are illustrated by color codes and site labels referring to Fig. 1 and Table 1. The individuals marked by asterisks are from *Ch. labiosa*. Bootstrap support values (>50%) from NJ analyses are indicated above branches; those from maximum parsimony (MP) are below branches.

and a haplotype published of *Barbus barbuis* (GenBank Accession number AB238965: Saitoh et al., 2006) was utilized as an outgroup whose genus was proposed as the closest possible relative of the schizothoracine fishes (Zardoya and Doadrio, 1999; Li et al., 2008). On this tree, the clear sister taxon of each species of the Yellow River schizothoracine fishes may be identified. Second, intra-species gene trees were constructed for each species or species

complex of the Yellow River schizothoracines using the MP and neighbor-joining (NJ) approaches, in which a sister taxon identified in the interspecies phylogenetic analyses was used as an outgroup. The MP and NJ approaches were implemented by using PAUP* v4b10 (Swofford, 2002), and the Bayesian inference (BI) of likelihood used MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with different parameter estimations for the control region

Table 1
Populations and numbers of individuals used in this study for each species from the Yellow River and Lake Qinghai. Abbreviations: CH, *Chuanchia labiosa*; PL, *Platypharodon extremus*; GE, *Gymnocypris eckloni*; GS, *Gymnocypris scoliostomus*; GY, *Gymnodiptychus pachycheilus*; SC, *Schizopygopsis pylzovi*; GP, *Gymnocypris przewalskii*.

Code	Location	CH	PL	GS	GE	GY	SC	GP
Upper reaches	Yellow River							
ZL	Zhaling Lake, Maduo, Qinghai	2	2		6	1	7	
EL	Eling Lake, Maduo, Qinghai	2	6		12	2		
MD	Yellow River, Maduo, Qinghai		7		4	3	7	
XX	Lake Xingxing, Maduo, Qinghai		6		3			
Middle reaches	Yellow River							
SM	Lake Sunmcuo, Jiuzh, Qinghai			21	14			
GD	Yellow River, Gande, Qinghai	3	4		10	3	5	
MQ	Yellow River, Maqu, Gansu		3		8	2	4	
Lower reaches	Yellow River							
BQ	Baqu River, Tongde, Qinghai					4	4	
XH	Yellow River, Xinghai, Qinghai	1			4	1		
JZ	Yellow River, Jianzha, Qinghai	1			9	3	5	
HS	Huangshui River, Qinghai						5	
DT	Datong River, Qilan, Qinghai						15	
Lake Qingha	Lake Qingha							
QL	Qinghai Lake, Qinghai							62
Total		9	28	21	70	19	52	62

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. For BI, the posterior distributions were obtained by Markov Chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. Samples of trees and parameters were drawn every 100 steps from a total of 1,000,000 MCMC generations. Furthermore, we used the program Network 4.0 (Bandelt et al., 1999) to construct median joining networks to visualize relationships among haplotypes within lineages.

Arlequin 2.000 (Schneider et al., 2000) was used to calculate all pairwise genetic differentiation (*F*_{st}) values between the reaches partitioned geographically in this study. Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed at different hierarchical levels: among the three reaches and among populations within each reach. Mismatch distributions and Fu's *F*_s test (Fu, 1997) were subsequently carried out using Arlequin 2.000 and the timing of the most important expansion in each species was calculated (Buhay et al., 2007; Huang et al., 2007).

The Bayesian skyline plot approach of Drummond et al. (2005) implemented in the program BEAST 1.4 (Drummond and Rambaut, 2007) was used to calculate the divergence age of the proposed major lineages. In this study, the evolutionary model suggested by Modeltest 3.7 and a length chain of 100,000,000 generations were utilized. Based on the calibration point of the fossil record of the genus *Barbus* from the Pliocene (11–15 million years ago, Ma) of Europe (Zardoya and Doadrio, 1999). The evolutionary rates were estimated here to be 1.67% per million years (confidence interval, CI 1.30–2.08) for the combined sequences and 1.86% per million years (CI, 1.32–2.41) for the cytochrome *b* sequences, and yielded a divergence time of the mtDNA lineages within the *Gymnocypris* species complex around 0.23 Ma (CI, 0.14–0.33) between lineage A and lineage B, and 0.30 Ma (CI, 0.17–0.44) between lineage C and the remaining lineages (Fig. 2a).

3. Results and discussion

3.1. Ancient vicariance and recent coalescence in *G. eckloni*, *Sy. pylzovi*, and *Ch. labiosa*

The partition homogeneity test suggests the absence of significant incongruence between the control region and cytochrome *b* gene for all sequences (*P* = 0.33). The dataset of the five genera

from the Yellow River and Lake Qinghai found evidence of four distinct lineages, and three genera (*Gymnocypris*, *Gymnodiptychus*, and *Platypharodon*) showed clear monophyly of mtDNA lineages, respectively (Appendix S2).

Three species (*G. przewalskii*, *G. eckloni*, and *G. scoliostomus*) from the genus *Gymnocypris* constituted a species complex with three deeply subdivided lineages (lineages A, B, and C) (Figs. 2a and 3A). The pairwise population *F*_{st} comparisons of *G. eckloni* revealed that there were substantial differences (*P* < 0.01) between the three reaches of the Yellow River (*F*_{st} values were 0.5324, 0.43, and 0.4978 for Upper–Middle reaches, Middle–Lower reaches, and Upper–Lower reaches, respectively). AMOVA was consistent with the pairwise *F*_{st} for *G. eckloni*, showing that 47.12% of the total variation was distributed among the three reaches, while only 9.26% variation was within the reaches. Three distinct lineages within *G. eckloni* are related to the division of the major reaches of the river by two large gorges (Fig. 1). In addition, we found three clear star-like haplogroups (Fig. 3B) in each of the species *Sy. pylzovi* and *Ch. labiosa*. These observations indicate that a possible scenario might to be invoked to explain a genealogical divergence within these species, which those ancient lineages might have originated from a former population that fragmented into isolated populations in the head-water area of the Yellow River.

Stratigraphic evidence shows that prior to the development of the Yellow River on the Tibetan Plateau, the limits of the head-water area of the Yellow River existed as a series of ancient lake basins (Chen, 1988; Zhang et al., 2003) (Fig. 1). The ancient Yellow River emerged at the northeastern edge of the Tibetan Plateau around 1.2 Ma (Li et al., 2001). Originally, the two ancient lake basins, Lake Qinghai Basin and Guide Basin, were linked each other (Li et al., 2001), and Lake Qinghai may have been the head-water of the ancient Yellow River (Chen, 1988) (Fig. 1). The 'Gonghe Movement' event of the Tibetan Plateau in the Late Pleistocene (about 0.15 Ma) led to the Yellow River capturing the Gonghe Basin and caused the uplift of the present barrier (the Riyue Mountains), which separated Lake Qinghai from the upper Yellow River (Fig. 1) (Zhang et al., 2003). Since 0.03 Ma, the Yellow River has further cut through the Gonghenanshan Mountains and more recently captured the Ruoergai Basin and Zhaling–Eling Basin, reaching upwards to the present headwaters (Fig. 1) (Zhang et al., 2003). Therefore, the current hypothesis is that the Yellow River primarily developed in the Holocene (Chen, 1988).

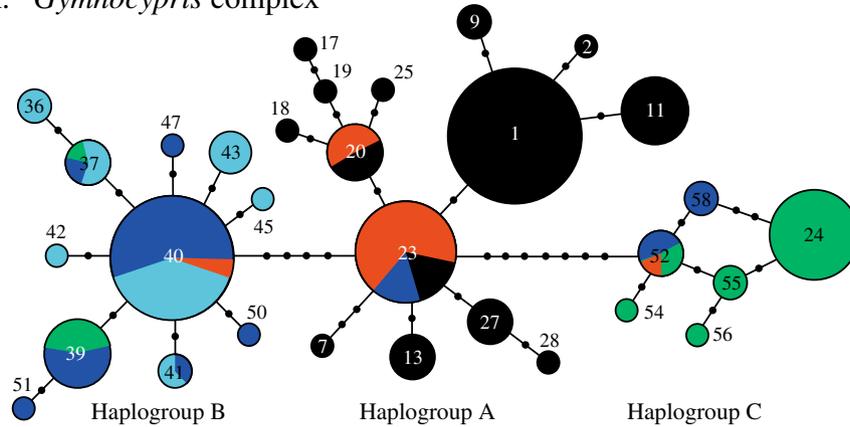
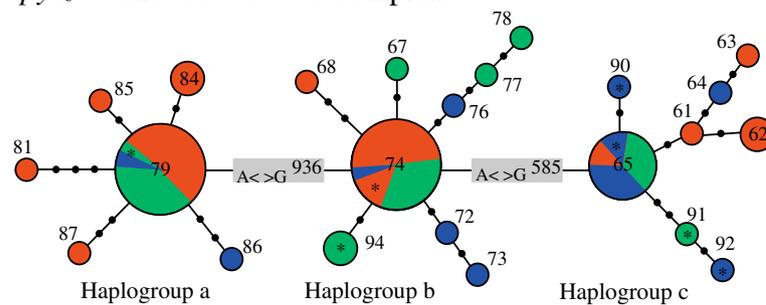
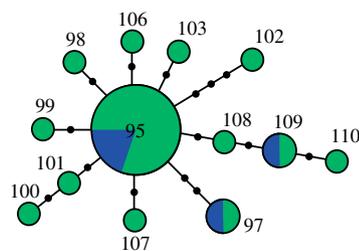
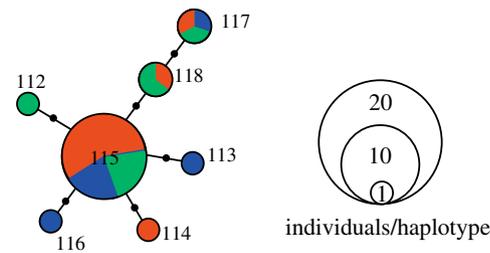
A. *Gymnocypris* complexB. *Sy. pylzovi* and *Ch. labiosa* complexC. *Pl. extremus*D. *Gd. pachycheilus*

Fig. 3. Median-joining network based on the cytochrome *b* sequences. Geographical origins of haplotypes are illustrated by the colors shown in Figs. 1 and 2. Mutations characterizing transitions between the three central haplotypes for *Sy. pylzovi* and *Ch. labiosa* complex are shown, and the species of *Ch. labiosa* are marked by asterisks. The sizes of the haplotypes reflect the number of specimens sharing the same haplotype (see scale in the lower right corner). The black dots are nucleotide substitutions inferred in that branch. Haplotype designation corresponds to the Appendix S1.

In this study, we noted a striking picture in which only one of the three lineages of the Yellow River, *G. ecklon*, shared lineage A with all *G. przewalskii* individuals from Lake Qinghai (Fig. 2a). This branching order suggests that the genetic differentiation within the Yellow River *G. eckloni* could have existed far before the time when Lake Qinghai separated from the Yellow River (about 0.15 Ma). On the other hand, all *G. scolioostomus* individuals endemic to Lake Sunmucuo only occur in lineage B of the *Gymnocypris* complex (Fig. 2a), while the estimated divergence times within this complex (0.23 and 0.30 Ma) are also long before the capture event of the Yellow River (since 0.03 Ma). Thus, we favor a scenario which those very old and large ancient lake basins might have acted as fish evolutionary reservoirs, offering sufficient geographical structure for the existence of barriers to gene flow, followed by a later coalescence as the Yellow River captured those ancient basins.

In contrast to the analysis for *G. ecklon*, the slight genetic differentiation (Figs. 2b and 3B) with nonsignificant geographic structure (F_{st} values were 0.0217, 0.002, and 0.0321, for Upper–Middle reaches, Middle–Lower reaches, and Upper–Lower reaches,

respectively; $P > 0.05$) was observed in *Sy. pylzovi*. The weaker subdivision in *Sy. pylzovi* might be associated with its distinctly ecological characteristics, such as dispersal ability. *Sy. pylzovi* is endemic with the widest distribution in the Yellow River and shows strong dispersal potential (Tsao and Wu, 1962). They dominate the headwaters of all tributaries, including many torrential mountain streams, and they also occur in large rivers and lakes (Wu and Wu, 1992). In contrast, *G. ecklon* is mainly confined to wide valleys and deep lakes, which indicates a relatively sedentary habit (Tsao and Wu, 1962).

Generally, most species with high dispersal potential lack sharp geographical differentiation (Roman and Palumbi, 2004; Ritz et al., 2008), and the headwater taxa are potentially highly susceptible to river capture (Koblmüller et al., 2008). Therefore, for *Sy. pylzovi* we infer that ancient lake basin boundaries did not contribute to deeper genealogical divergence within the population than that within *G. ecklon*, and the lack of geographic structure of *Sy. pylzovi* might indicate substantial later intermix due to their high dispersal ability.

No geographic structure was found in *Ch. labiosa* either, but the sampling was too limited to exclude possible geographical heterogeneity. Most striking is that two species, *Sy. pylzovi* and *Ch. labiosa*, clustered into a monophyletic species complex, and two mitochondrial haplotypes (haplotype 88 and 89) were shared between them (Figs. 2b and 3B). The lack of the respective monophyly supports the hypothesis of a common origin. Clearly, a detailed study, including genetics, ecology, and reproductive isolation will be necessary.

3.2. Allopatric vicariance and later colonization in *Pl. extremus* and *Gd. pachycheilus*

Nonsignificant levels of genetic structuring were found within *Pl. extremus* and *Gd. pachycheilus* (Figs. 2 and 3), and no geographical heterogeneity ($P > 0.05$) was found for either species (F_{st} value in *Pl. extremus* was 0.0269 between Upper and Middle reaches, and in *Gd. pachycheilus* were -0.1229 , -0.0694 , and -0.0741 for Upper–Middle reaches, Middle–Lower reaches, and Upper–Lower reaches, respectively). However, the haplotype network of each species gave a singly clear star-like clustering pattern that reveals a surprisingly high level of gene flow within the species (Fig. 3C and D). The large-scale uniformity in *Pl. extremus* and *Gd. pachycheilus* imply local extinctions in some ancient lake basins or the allopatric speciation in a single water body with relatively recent colonizations.

The *Platypharodon* is a monotypic genus endemic to the Yellow River, but most abundant in Lake Zhaling and Lake Eling on the uppermost reaches of the Yellow River, and it exhibits a geographical trend of decreasing numbers from the upper reaches towards the middle reaches. It is apparently absent from the lower reaches and from Lake Sunmucuo (Wu and Wu, 1992). This is consistent with a stepping stone colonization, which suggests a recent dispersal starting from the upper reaches to the middle reaches. In addition, the population expansion of *Pl. extremus* is estimated to have occurred around 0.086 Ma (Appendix S3). There is evidence that originally the two large lakes, Lake Zhaling and Lake Eling, comprised a single water body, the ‘Zhaling–Eling Basin’. After the late Pleistocene (since 0.03 Ma), the ‘Zhaling–Eling Basin’ separated into two lakes due to the Yellow River capture that drained a large portion of the basin (Zhang et al., 2003). Therefore, we believe that the speciation and the rapid radiation event of *Pl. extremus* occurred before the capture event of the Yellow River and might have been maintained in the large ‘Zhaling–Eling Basin’ in the uppermost Yellow River. At present, Lake Zhaling and Lake Eling still remain connected by a river, which serves as the main breeding sites of *Pl. extremus* and promotes gene flow within species (Wu and Wu, 1992). Such ecological environmental and habitat homogeneity might be more prone to preserve ancestral characters and to aggregate them into a larger panmictic population.

In contrast, *Gd. pachycheilus* has wide distribution in the Yellow River with good dispersal ability (Wu and Wu, 1992); however, this species also occurs in the Yangtze River drainage. Therefore, it is difficult to determine the origin of *Gd. pachycheilus* using in the pattern of *Pl. extremus*. We cannot exclude the possibility that the Yellow River *Gd. pachycheilus* was colonized from the Yangtze River.

3.3. Pleistocene refugia in the Yellow River schizothoracine fishes

In the case using cytochrome *b* haplotypes, significant negative values for Fu’s F_s and unimodal mismatch distributions of the frequencies of pairwise differences were tested in most lineages (Appendix S3). These coupled with the star-like phylogenies (Fig. 3), all indicated that the Yellow River schizothoracine fishes

have passed through recent demographic expansions at several locations after the bottleneck. We estimate that the population expansions occurred at about 0.132–0.062 Ma, and were related to the climatic oscillations of the late Pleistocene due to the large uplift events of the Tibetan Plateau (Li et al., 2001). 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 (Shi, 2002; Zheng et al., 2002). The recent rapid increase of the population sizes of the Yellow River schizothoracine fishes is consistent with the expansion that has occurred after the extensive glacial period in the Tibetan Plateau. This implies that those larger ancient lake basins in the Yellow River head-water area acted as the Pleistocene glacial refugia.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (NSFC) 30225008, 30300036, and 30530120, and by Key Innovation Plan KSCX2-SW-106 and National Basic Research Project (2005cb422005) in China. Z.P. was supported by National Natural Science Foundation of China (30600062).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.03.034.

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