RESEARCH PAPERS

Origin of *Gymnocypris przewalskii* and phylogenetic history of *Gymnocypris eckloni* (Teleostei: Cyprinidae)[*]

Zhao Kai[1,2,3**], Duan Ziyuan[4**], Yang Gongshe[2],

Peng Zuogang[1], He Shunping[1***] and Chen Yiyu[1]

(1. Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China; 2. Laboratory of Animal Fat Deposition and Muscle Development, Northwest Sci-Tech University of Agriculture and Forestry, Yangling 712100, China; 3. Agriculture and Animal Husbandry College, Qinghai University, Xining 810003, China; 4. Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China)

Abstract   The origins and phylogenetic patterns were assessed for *G. przewalskii* and *G. eckloni* by analyzing the complete mtDNA cytochrome *b* gene sequence (1140bp). Phylogenetic analyses further supported that there were three mtDNA lineages (A-C) identified in *G. przewalskii* and *G. eckloni*, demonstrating that outer rakers of the first gill have little significance in the phylogeny of the *Gymnocypris* fishes. The network established showed that *G. eckloni* of the Yellow River specific haplotype A1 was a founder and it radiated all haplotypes of *G. przewalskii* which suggested *G. przewalskii* might only originate from one of two maternals of *G. eckloni* from the Yellow River. Fs test and mismatch analysis showed at least two expansion events in the population of *G. przewalskii* about 0.2734 Ma and 0.0658 Ma, while *G. eckloni* from Qaidam Basin could have experienced severe bottleneck effect about 0.0693 Ma. The population expansion was detected in subclades A1 and A21 with the most recent common ancestor (TMRCA) about 0.2308 ± 0.01 Ma and 0.1319 ± 0.015 Ma, respectively, which were within the geological age range of "Gonghe Movement" event that caused the separation of Lake Qinghai from the upper Yellow River. These results suggested the effect of the fish diversification by rapid uplift of the Qinghai-Tibetan Plateau in the Late Pleistocene.

Keywords: *G. przewalskii*, *G. eckloni*, origin, Cyt *b*, phylogeography.

In Quaternary great geographic changes had taken place in the Chinese continent, for example, a number of lakes and Loess Plateau were shaped in Pleistocene. However, it is considered that the uplift of the Qinghai-Tibetan Plateau mainly caused the current biology pattern in plateau. Based on morphological characters, Cao et al.[1] investigated the fish-fauna in the Qinghai-Tibetan Plateau and suggested that the origin and evolution of schizothoracine fishes is related with the upheaval of the Qinghai-Tibetan Plateau. As the geographic occurrence of the fishes is restricted by the distinct water system, the schizothoracine fishes provided an ideal model for studying underlying biogeographical hypotheses of the Qinghai-Tibetan Plateau.

Lake Qinghai is in the northeastern Qinghai-Tibetan Plateau and is the largest salt water lake in the plateau, where one unique species of Schizothoracinae, *Gymnocypris przewalskii* and its one subspecies, *Gymnocypris przewalskii ganzihonensis* are distributed[2,3]. Another species within *Gymnocypris*, *Gymnocypris eckloni* is unique to the Yellow River and Qaidam Basin[2,3].

We have ever analyzed the molecular phylogenetic relationships of *Gymnesocypris* in Lake Qinghai and adjacent drainages[4]. The result showed that neither *G. przewalskii* nor *G. eckloni* was a monophyletic assemblage, and suggested that the outer rakers of the first gill might have little significance in the phylogeny of the *Gymnocypris* fishes. However, the previous study failed to include some rare individuals with high variance of the gill rakers, and the origins and population history of *G. przewalskii* and *G. eckloni* were not well clarified[4].

Zhu et al.[2] believed that *G. eckloni* of the

Yellow River had a very close relationship with *G. przewalskii* mainly based on the following hypotheses: firstly, the fishe-fauna of Lake Qinghai was quite similar to that of the Yellow River; secondly, *G. eckloni* distributed in the upper Yellow River and Geermu River of Qaidam Basin was the only species of the genus *Gymnocypris* in this area except *G. przewalskii*; and thirdly, Lake Qinghai was once connected with the Yellow River in history. These results provided important clues for us to study the origins and phylogenetic history of *G. przewalskii* and *G. eckloni*.

The geographic data showed that the Yellow River had a young history. About 1.2 Ma ago the Yellow River emerged at the edge of the Qinghai-Tibetan Plateau. About 0.15 Ma the "Gonghe Movement" event of the Qinghai-Tibetan Plateau caused the separation of Lake Qinghai from the upper Yellow River and then the Yellow River began to reach upwards to the present headwaters[5,6].

In the present study, we added some rare samples with high raker variance and some samples from an area of the Yellow River near Lake Qinghai. With these data, we aimed at investigating the details of origins and population history of *G. przewalskii* and *G. eckloni*, and confirming the significance of the raker variance in the phylogeny of the *Gymnocypris* fishes by analyzing the complete mitochondrial cytochrome *b* (Cyt *b*) genes.

1 Materials and methods

1.1 Sample collection

A total of 163 individuals including all natural populations and subspecies of *G. przewalskii* and *G. eckloni* were collected from their distributed regions (Fig. 1 and Appendix A): 53 individuals of *G. przewalskii przewalskii* from Lake Qinghai; 14 individuals of *G. przewalskii ganzihonensis* from Gangzi River; 93 individuals of *G. eckloni* including 70 from the upper Yellow River and 23 from Geermu River of Qaidam Basin. These individuals contained the published data (GenBank accession Nos: DQ058216-DQ058291)[4]. In the added samples, 3 from *G. przewalskii przewalskii* (outer rakers of the first gill were 41, 50 and 51), 3 from *G. eckloni* of the Yellow River (outer rakers of the first gill were 8, 9 and 28) as well as 9 from the Guide, Tongde and Jiuzhi of the upper Yellow River (Fig. 1). All the muscle

tissues were preserved in 95% ethanol and deposited in the permanent Freshwater Fish Museum of Institute of Hydrobiology, Chinese Academy of Sciences in Wuhan.

1.2 PCR amplification and sequencing

Total DNA was extracted from alcohol fixed muscle tissues following the Ausubel's[7] method. The primers L14724 (5'-GACTTGAAAAAC-CACCGTTC-3') and H15915 (5'-CTCCGATCTC-CGGATTACAAGAC-3') were used for amplification and sequencing. Amplification reactions were performed in a 60 μL volume containing approximately 100 ng of template DNA, 0.75 μL dNTP (each 2.5 mmol/L), 1.5 μL each primer, 5.0 μL 10 × reaction buffer and 3 units *Taq* DNA polymerase (Biostar). The thermocycling conditions included an initial denaturation at 95℃ for 3 min; then 30 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 1 min, and extending at 72℃ for 1 min 30 s; followed by 72℃ extending for 7 min. The amplified fragments were fractionated by electrophoresis through 0.8% low-melting agarose gels, re-covered from the gels and purified using Biostar Glass-milk DNA purification kit following the manufacturer's instructions. The purified fragments were directly sequenced in both directions.

1.3 Data analysis

Multiple alignments of sequences were performed using Clustal W[8] and revised by eye. Nucleotide composition was calculated using MEGA 2.1[9]. The phylogenetic analysis was conducted using neighbor-joining (NJ) method in PAPU * 4.0b10[10]. The best fit substitution model was detected (TrN + G, α = 0.1913) with the program Modeltest 3.06[11]. 1000 replicates of bootstrapping analysis were conducted to estimate relative support of nodes for NJ method.

Median-joining network[12] was drawn using the program Network 4.0 to investigate the possible relationships among haplotypes. The nucleotide diversity (π) and haplotypic diversity (h) of populations were estimated by ARLEQUIN Ver. 2.000[13]. Through an analysis of molecular variance (AMOVA)[14], the population was grouped according to geographic occurrence to detect phylogeographic structure of *G. eckloni* from the Yellow River. The analysis was

done with 10000 permutations. Fu's Fs test[15] and mismatch analysis[16] were applied to test population expansion. The approximate time to the most recent common ancestor (TMRCA) for major haplogroups of star-like phylogeny was estimated by calculating coalescent time of a group of Cyt b sequences using the averaged mutational distance[17], while the ages of the major populations were assessed by dividing the nucleotide diversity in each clade[18].
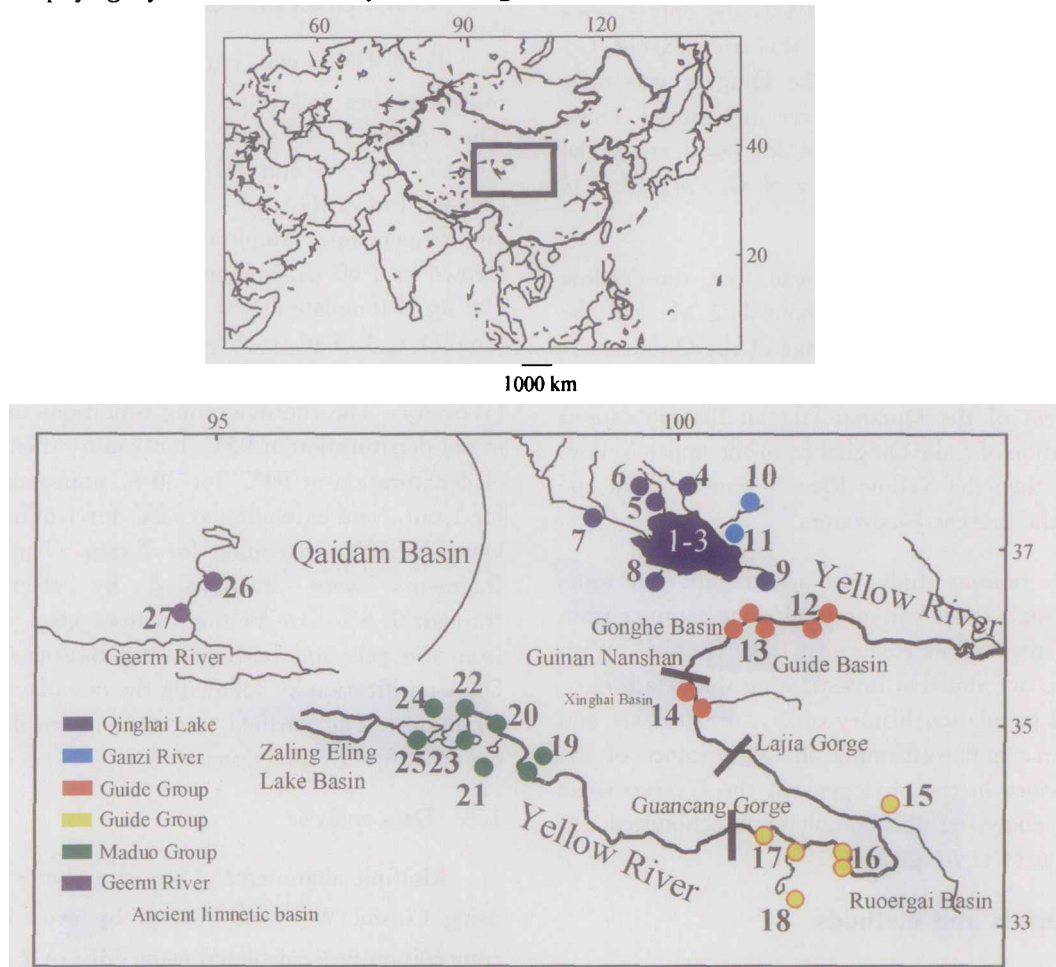

Fig. 1. Geographic localities (1—27) of the *G. przewalskii* and *G. eckloni* samples and distribution map of ancient limnetic basins in the upper Yellow River.

2 Results

2.1 Sequence variation and AMOVA analysis

A total of 105 variable sites were identified in *G. przewalskii* and *G. eckloni* from three drainages, and 83 were parsimony informative sites. 42 haplotypes were identified from 163 individuals (Appendix A). For the added individuals, 5 haplotypes were identified of which 3 were from *G. przewalskii* and 2 from *G. eckloni*. All haplotypes were unique to each population of the *Gymnocypris* fishes from Lake Qinghai, the Yellow River and Qaidam Basin.

For AMOVA analysis[14], the population of *G. eckloni* from the Yellow River was grouped into three groups (Guide Group, Jiuzhi Group, and Maduo Group) according to Guancang Gorge and Lajia Gorge, the two largest gorges on the upper Yellow River (Fig. 1). Result of AMOVA showed the maximum group variation of 18.97% ($p < 0.01$), which revealed a significant geographical structure of mtDNA variation within population of *G. eckloni* from the Yellow River.

2.2 Network of the major clades

Three highly divergent clades (A-C) were clearly discerned in the NJ tree of 42 haplotypes. They included 30, 6 and 6 haplotypes comprising 111, 29 and 23 individuals, respectively. Network also clearly
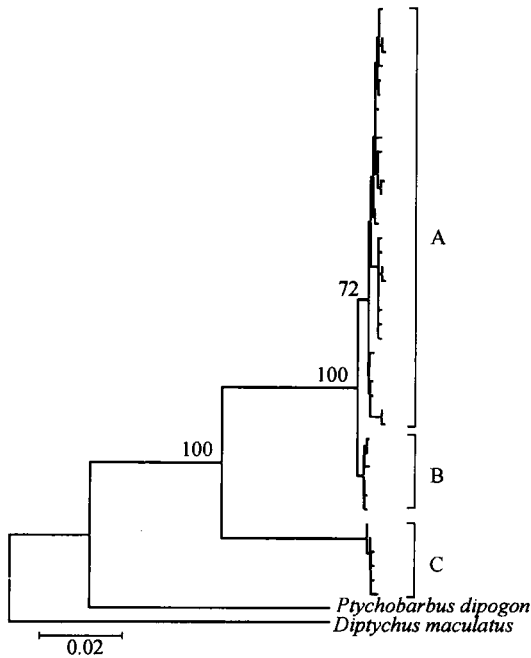
Fig. 2. Neighbor-joining (NJ) tree constructed by 42 haplotypes from 163 individuals of *G. przewalskii* and *G. eckloni* (TrN + G, $\alpha = 0.1286$). Values at the nodes represent 1000 bootstrap replicates. *Ptychobarbus dipogon* and *Diptychus maculatus* are included as outgroups.

clade A) and C1 (17 individuals, 73.9% of the total of clade C) located in the center of each clade or subclade (Fig. 4). Three unique haplotypes with high outer rakers variance did not form a specific clade in network, which further supported that the rakers might have little significance in the phylogeny of the *Gymnocypris* fishes.

2.3 Geographic distribution of the clades

Clade A contained all samples of *G. przewalskii* and partial samples of *G. eckloni* from the Yellow River (59.15% of individuals and 66.67% of haplotypes). Calde B contained the remaining samples of *G. eckloni* from the Yellow River. Clade C was exclusive to *G. eckloni* from Qaidam Basin. Subclade A1 was shared by individuals of *G. przewalskii* and individuals of *G. eckloni* from the Yellow River, but mainly presented in *G. przewalskii* (82.4% of individuals and 90% of haplotypes), while subclade A21 and A29 were only from *G. eckloni* of the Yellow River.

2.4 Population expansions

The mismatch analysis of a complete data set of all *G. przewalskii* showed a clear peak at around three differences (Fig. 5), and *F*s test had a significantly large negative value (Table 1). Although mismatch analysis of *G. eckloni* from Qaidam Basin did not show a clear peak (Fig. 5), *F*s test obtained a significantly negative value (Table 1). These results suggested possible population expansions in the history of *G. przewalskii* and *G. eckloni* from Qaidam Basin.

discerned three clades A-C and clade A was separated from clade B and C by 6 and 77 mutation steps, respectively (Fig. 3). Three subclades (subclade A1, A21 and A29) were found in clade A including 20, 8 and 2 haplotypes comprising 84, 23 and 4 individuals, respectively (Figs. 3 and 4, subclade A29 was not shown). Clade C, subclade A1 and A21 presented a star-like phylogeny, in which three high-frequency haplotypes A1 (14 individuals, 12.6% of the total of clade A), A21 (10 individuals, 9% of the total of
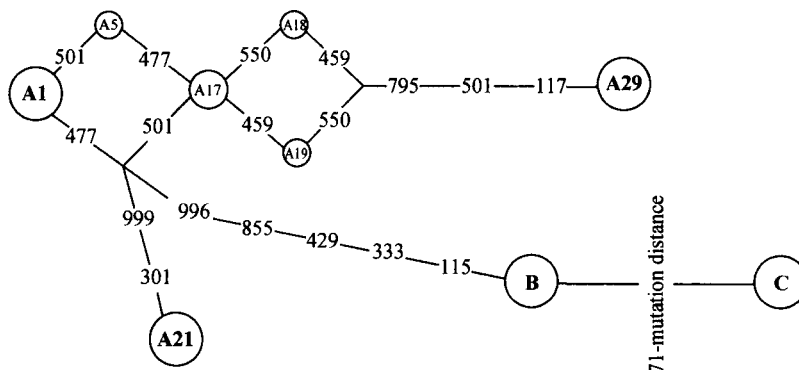

Fig. 3. Relative relationship of the three clades (A-C) from *G. przewalskii* and *G. eckloni*. The numbers in circles represent haplotypes and on lines denote the locus of nucleotide mutations (71 mutations between Clade B and C were not shown).
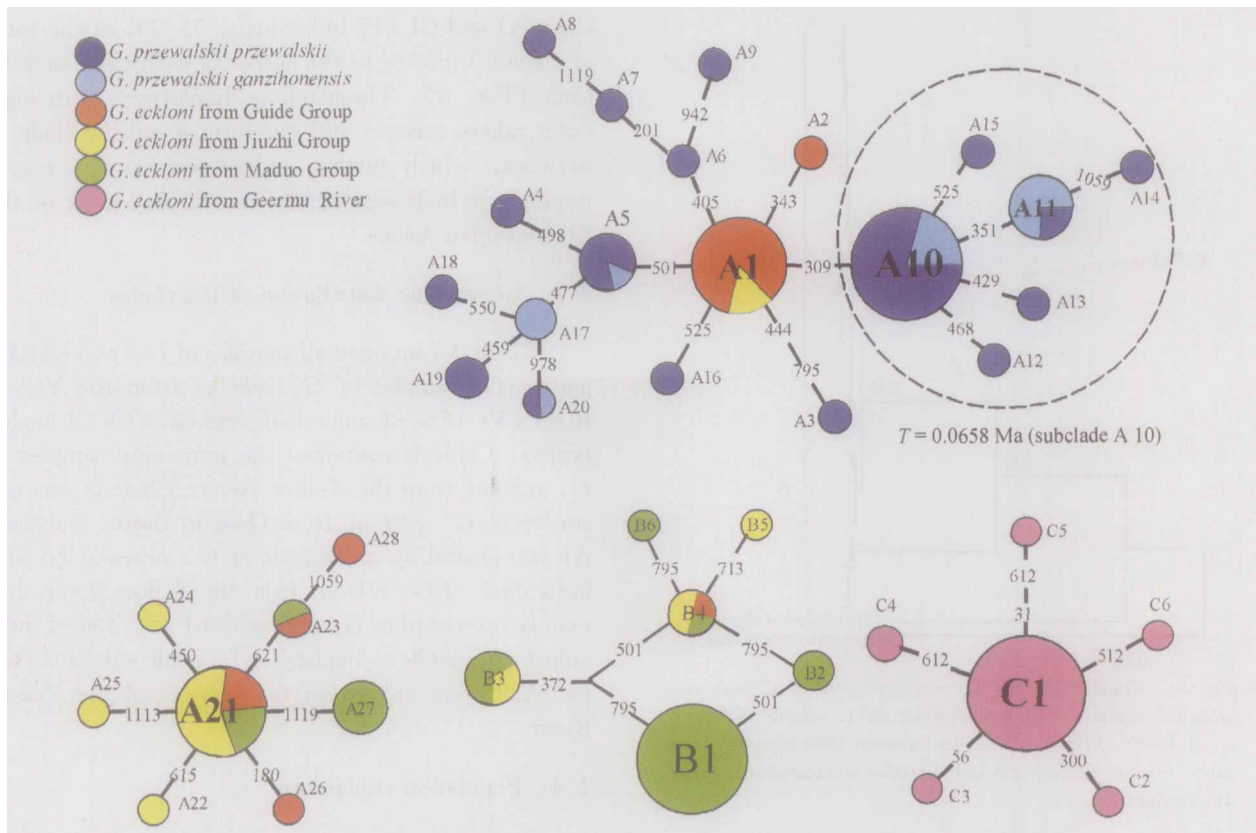
Fig. 4.   Networks of haplotypes from the main clades of *G. przewalskii* and *G. eckloni*. The sizes of circles are proportional to haplotype frequency. The numbers in circles represent haplotypes and on lines denote the locus of nucleotide mutations.
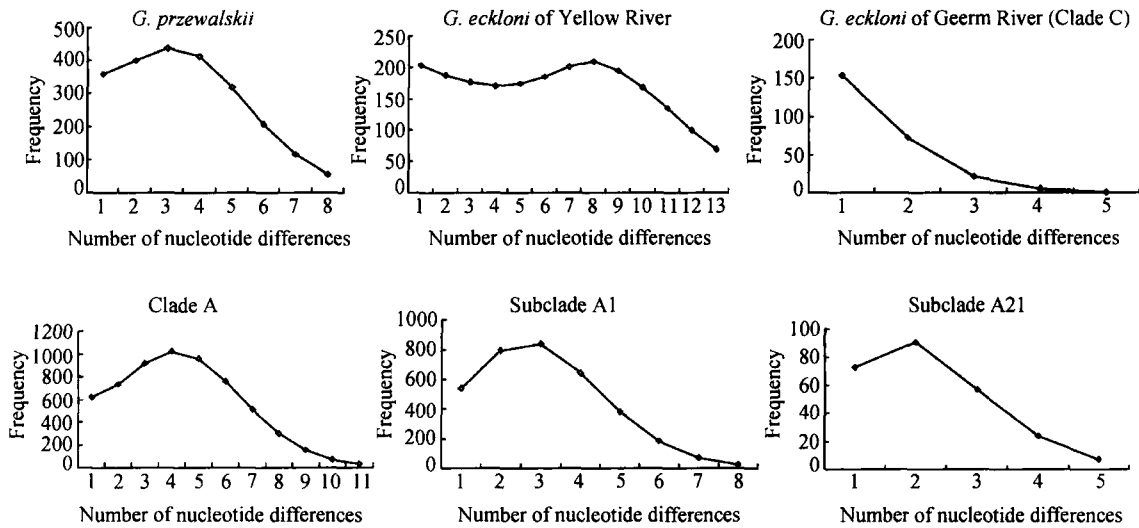

Fig. 5.   Mismatch analysis of the major clades and some populations.

Table 1.   The parameters estimate of neutrality tests from the population in *G. przewalskii* and *G. eckloni*

Population	N	Haplotypes	$\pi \pm SE$	F_s	P	Age (MaBP)
G. przewalskii	69	18	0.002078 ± 0.00127	-7.8096	$P<0.01$	0.2734
G. przewalskii przewalskii	55	17	0.002132 ± 0.00126	-7.4039	$P<0.01$	0.2805
G. przewalskii ganzihonensis	14	5	0.001677 ± 0.00114	-0.0365	$P>0.05$	0.2655
G. eckloni of Yellow River	71	18	0.004823 ± 0.00260	-1.3891	$P>0.05$	0.6346
G. eckloni of Qaidam Basin	23	6	0.000527 ± 0.00049	-3.7001	$P<0.01$	0.0693

To further explore a detailed information on the population history of *G. przewalskii* and *G. eckloni*, we also estimated the *F*s statistic values and mismatch analysis for clades or subclades. The results obtained from the mismatch analysis (Fig. 5) were congruent with those from the *F*s test with *F*s values of -16.6725 ($P<0.01$), -10.6234 ($P<0.01$) and -4.2951 ($P<0.01$) for clade A, subclade A1 and A21, respectively.

2.5   Age estimation

Using the divergent rate of 0.76% per site per million years[19], the estimated ages of the population expansion in *G. przewalskii* and *G. eckloni* from Qaidam Basin were about 0.27434 Ma and 0.0693 Ma, respectively (Table 1), while the age of the TMRCA of major subclades was about 0.2308 ± 0.01 Ma and 0.1319 ± 0.015 Ma for subclade A1 and A21, respectively.

3   Discussion

3.1   Origin of *Gymnocypris przewalskii*

Our previous analysis based on the constructed phylogenetic trees revealed that *G. przewalskii* had not evolved into different lineages[4]. The detailed information on the pattern of genetic variation of *G. przewalskii* is shown in the network (Fig. 4). In samples from *G. przewalskii ganzihonensis*, five haplotypes were identified in which four (haplotype A5, A10, A11 and A20) were shared with *G. przewalskii przewalskii* and dispersedly distributed in clade A. Although another haplotype A17 was unique to *G. przewalskii ganzihonensis*, three haplotypes radiated from it were either unique to *G. przewalskii przewalskii* (haplotype A18 and A19) or shared with *G. przewalskii przewalskii* (haplotype A20) (Fig. 4). Haplotypes of the recent origin occurred preferentially at the tips of a network[20], thus our results did not support that A17 as a recent origin haplotype. On the other hand, AMOVA analysis showed that 94.3% ($P<0.05$) variation was within population of *G. przewalskii* and only 5.7% ($P<0.05$) was between *G. przewalskii przewalskii* and *G. przewalskii ganzihonensis*. These results supported that there was no significant population structure within *G. przewalskii*.

The *G. eckloni* from Geerm River of Qaidam Basin composed a monophyletic group (clade C) in the Neighbor-joining tree (Fig. 2). Network also identified a high mutation distance (77 steps) between clade A and C (Fig. 3), which demonstrated the interruption of gene flow in a long time between *G. przewalskii* and *G. eckloni* from Qaidam Basin. The haplotype A1 (possible founder haplotype) located in the center of subclade A1 was shared by 14 individuals of *G. eckloni* from the Yellow River and radiated all haplotypes of *G. przewalskii*, forming a well star-like phylogeny with 1-mutation along with all haplotypes except 2-mutation between haplotype A1 and A3. This result well demonstrated that the *G. przewalskii* originated from *G. eckloni* of the Yellow River and showed a single origin in this study.

3.2   Lineage diversification of *G. eckloni* of the Yellow River

The network showed that the majority of haplotypes in clade A1 occurred in samples of *G. przewalskii*, and subclade A21 and clade B were exclusive to *G. eckloni* of the Yellow River. The mutation distance between subclade A1 and clade B reached 6 steps, while between subclade A1 and A21 it was only 3 steps at most. This result supported that *G. eckloni* from the Yellow River might have two maternal origins at least[4], while *G. przewalskii* might only originate from one of them.

AMOVA analysis showed that there was a significant geographical structure within the population of *G. eckloni* from the Yellow River. Two large gorges on the upper Yellow River restricted the pervasion of *G. eckloni*, as is shown by the fact that the *Gymnocypris* fishes generally exist in wide valleys. Phylogenetic analysis revealed that the divergence time of *G. eckloni* between clade A and clade B was far earlier than the separation of Lake Qinghai from the upper Yellow River. However, the geological data showed that the Yellow River did not start the headward erosion until Lake Qinghai had been separated from the upper Yellow River[21]. Those suggested that the origin of clade B might not be a result of the restricted gene flow between gorges on the upper Yellow River.

Analyzing the frequencies of haplotypes in different areas of the Yellow River would help us to discern the origin of clade B. The majority of the individuals (88.67%) of *G. eckloni* from the Yellow River in subclade A1 were mainly distributed in Guide Group

(Guide Basin and Gonghe Basin) (Fig. 4 and Appendix A). On one hand, it suggested that Guide Basin and Gonghe Basin were the largest basins on the upper Yellow River before the "Gonghe Movement" event in the late Pleistocene[22], where might be the center of the origin and diffusion for the *Gymnocypris* fishes in this area. On the other hand, it suggested that the ichthyofauna of ancient Lake Qinghai, Guide Basin and Gonghe Basin was restricted with the source area of the Yellow River for a long time. Of the 79.31% of samples in clade B were distributed in Maduo Group of the uppermost Yellow River, which also suggested they had only limited genic communion with the fishes of Guide Basin and Gonghe Basin. This analysis is consistent with a recent opinion that the Yellow River cut through the Nanshan in south Guinan extending upwards to Ruoergai Basin about 0.03 Ma (Fig. 1)[23]. These results implied that the multiple maternal origins of *G. eckloni* of the Yellow River might be relevant to the headward erosion of the Yellow River.

Before the Yellow River extended upwards to the current headwaters area, many ancient limnetic basins, for example, Xinghai Basin, Ruoergai Basin and Zaling-Eling Basin had presented there[21—24]. Cao et al.[1] considered that Schizothoracinae in the Qinghai-Tibetan Plateau originated from Barbinae which had already existed in the plateau earlier. This revealed that schizothoracine fishes had appeared in some ancient limnetic basins before the Yellow River reached its current headwaters area. Especially, in the Quaternary Zaling-Eling Basin of the uppermost Yellow River there had been a large inland lake about two times as large as the current lake and might appear to have acted as fishes genetic reservoirs then. After "Gonghe Movement" event, the Yellow River further extended upward, eroded those ancient limnetic basins serially and finally formed the current water system (Fig. 1)[23,24]. The Yellow River did not develop to reach its current source area until the late Pleistocene or Holocence[21]. Therefore, the diversification of mtDNA lineages of *G. eckloni* of the Yellow River might be originated from the endemic schizothoracine fishes which had existed in the genetic reservoir of the ancient limnetic basins.

3.3 Population history of *G. przewalskii* and *G. eckloni*

The "Gonghe Movement", an important geological event, caused the separation of Lake Qinghai from the upper Yellow River, making the Qinghai-Tibetan Plateau uplifted to the present height and finally shaped current geographic pattern[8]. The estimated time of the population expansion for *G. przewalskii* occurred about 0.2734 Ma (Table 1) and the ages calculated for subclade A1 and A21 from the potential founder haplotype were 0.131 Ma and 0.2308 Ma, respectively, which were within the range of geological time of the "Gonghe Movement" event (0.15 Ma). Network showed a star-like phylogeny to reveal a subsequent expansion in the population of *G. przewalskii*. Neutrality tests also detected population expansion within subclade A10 (27 individuals of *G. przewalskii* shared haplotype A 10, $Fs = 3.1167$, $P < 0.05$) showing a partial expansion in population of *G. przewalskii* about 0.0658 Ma (Fig. 4). After the "Gonghe Movement" event, the quick development of Lake Qinghai might provide more opportunities to facilitate lineage diversification of *G. przewalskii*.

The schizothoracine fishes in Qaidam Basin have only two species. *G. eckloni* distributed in midwest of Qaidam Basin[3]. In the Late Pleistocene, the Qinghai-Tibetan Plateau climate became extremely harsh to cause Qaidam Basin experiencing several times serious aridity which eradicated many species[25—27]. The time of population expansion about 0.00693 Ma suggested that *G. eckloni* of Qaidam Basin might have experienced a very severe bottleneck (Table 2). This result is congruent with the fact of very poor fish diversity of Qaidam Basin. The population expansion times of *G. eckloni* of Qaidam Basin (0.0693 Ma) was very close to that of *G. przewalski* of Lake Qinghai (0.0658 Ma), which were within the range of the most recent uplift times of the Qinghai-Tibetan Plateau in Holocene[23]. They might have important palaeoenvironment significance to reflect the effect of the latest rapid uplift of the Qinghai-Tibetan Plateau in the late Pleistocene on fish diversification.

Appendix A. Sample information of *G. przewalskii* and *G. eckloni* used in this study

Group	Location numbers	Water system	Location	Species/Subspecies	No.	Haplotypes
Lake Qinghai	1	Lake Qinghai	Hubei, Qinghai Lake	*G. przewalskii*	6	A7(1), A9(1), A10(2), A15(1), A19(1)
	2	Lake Qinghai	Hudong, Qinghai Lake	*G. przewalskii*	6	A5(1), A9(1), A10(3), A14(1),
	3	Lake Qinghai	Hunan, Qinghai Lake	*G. przewalskii*	6	A8(1), A10(3), A16(1), A18(1)
	4	Lake Qinghai	Quan ji River, Qinghai Lake	*G. przewalskii*	6	A10(2), A13(1), A18(1), A19(2)
	5	Lake Qinghai	Upper Saliu River, Qinghai Lake	*G. przewalskii*	5	A5(1), A9(1), A10(1), A12(1), A20(1)
	6	Lake Qinghai	Saliu River Bridge, Qinghai Lake	*G. przewalskii*	8	A4(1), A5(1), A6(1), A10(3), A19(2)
	7	Lake Qinghai	Buha River, Qinghai Lake	*G. przewalskii*	6	A5(1), A6(1), A10(3), A11(1)
	8	Lake Qinghai	Heima River, Qinghai Lake	*G. przewalskii*	6	A5(1), A6(1), A10(2), A11(1), A19(1)
	9	Lake Qinghai	Xiaobeihu, Qinghai Lake	*G. przewalskii*	6	A3(1), A10(4), A18(1)
Ganzi River	10	Lake Qinghai	Hadong Bridge, Ganzi River, Qinghai Lake	*G. przewalskii przewalskii*	7	A5(1), A10(2), A11(3), A20(1)
	11	Lake Qinghai	Lower Ganzi River, Qinghai Lake	*G. przewalskii przewalskii*	7	A10(2), A11(3), A17(2)
Guide group	12	Yellow River	Ashigong, Guide, Qinghai	*G. eckloni*	6	A1(5), A28(1)
	13	Yellow River	Luohantang, Guide	*G. eckloni*	6	A1(4), A2(1), A21(1)
	14	Yellow River	Bagou, Tongde, Qinghai	*G. eckloni*	6	A1(3), A23(1), A26(1), B4(1)
Jiuzhi group	15	Yellow River	Maqu, Gansuo	*G. eckloni*	3	A1(2), B5(1)
	16	Yellow River	Jiuzhi, Qinghai	*G. eckloni*	5	A21(2), A25(1), B3(2)
	17	Yellow River	Mentang, Jiuzhi, Qinghai	*G. eckloni*	5	A21(3), B4(2)
	18	Yellow River	Sunmcu Lake, Qinghai	*G. eckloni*	3	A21(1), A22(1), A24(1)
Maduo group	19	Yellow River	Huanghe, Maduo, Qinghai	*G. eckloni*	5	A27(1), B2(1), B3(3)
	20	Yellow River	Huanghe Bridge, Maduo, Qinghai	*G. eckloni*	5	B1(4), A30(1)
	21	Yellow River	Xingxing Lake, Maduo, Qinghai	*G. eckloni*	7	A21(2), B1(1), B2(1), A29(3)
	22	Yellow River	Hubei, Eling Lake, Qinghai	*G. eckloni*	5	A23(1), A27(2), B1(1), B6(1)
	23	Yellow River	Hunan, Eling Lake, Qinghai	*G. eckloni*	5	A27(1), B1(3), B4(1)
	24	Yellow River	Hubei, Zaling Lake, Qinghai	*G. eckloni*	5	A21(1), A27(2), B1(1), B6(1)
	25	Yellow River	Hunan, Zaling Lake, Qinghai	*G. eckloni*	5	B1(4), B3(1)
Geerm River	26	Qaidam Basin	Lower Geerm River	*G. eckloni*	12	C1(10), C2(1), C6(1)
	27	Qaidam Basin	Middle Geerm River	*G. eckloni*	11	C1(7), C3(1), C4(2), C5(1)

The number of individuals shared the same haplotype at each collection site are shown in parentheses.

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