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Genetic diversity and taxonomic status of *Gymnocypris chilianensis* based on the mitochondrial DNA cytochrome *b* gene

J.P. Zhang[1], Z. Liu[1], B. Zhang[2], X.Y. Yin[2], L. Wang[3], H.N. Shi[1] and Y.J. Kang[1]

[1]College of Animal Science and Technology, Gansu Agricultural University, Lanzhou, China
[2]The Fisheries Bureau of Jiuquan City in Gansu, Jiuquan, China
[3]Gansu Agro-Ecological Environment Protection Agency, Lanzhou, China

Corresponding author: Z. Liu
E-mail: liuz@gsau.edu.cn

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ABSTRACT. In order to study the genetic diversity and taxonomic status of *Gymnocypris chilianensis* on a molecular level, the mitochondrial DNA cytochrome *b* gene was sequenced for 74 individuals of *G. chilianensis* from two locations (Heihe River and Shule River) and 42 individuals of its affinis species *Gymnocypris przewalskii*. Analyses of genetic diversity and sequence differences were conducted for these samples, combined with the analysis of 30 homologous sequences of another affinis species *Gymnocypris eckloni*, which were downloaded from GenBank. The results showed that both the haplotype diversity (h = 0.9820) and nucleotide diversity (π = 0.0039) of the Shule River *G. chilianensis* were lower than the other populations, thus, the Shule River *G. chilianensis* should be prioritized for protection because of its lower genetic diversity level. The results of sequence analysis showed that the genetic distance between the Heihe River *G. chilianensis* population and the Shule River *G. chilianensis* population was 0.0064, and the

genetic distance between these two populations and the *G. przewalskii* population was 0.0838 and 0.0810, respectively. The genetic distance between the two *G. chilianensis* populations and the *G. eckloni* population was 0.0805 and 0.0778, respectively. Analysis of sequence differences indicates that *G. chilianensis* is sufficiently diverged from *G. przewalskii* and *G. eckloni* to the extent that it has reached species level, thus, *G. chilianensis* can be considered an independent species of *Gymnocypris*.

Key words: *Gymnocypris chilianensis*; Cytochrome *b*; Genetic diversity; Taxonomic status

INTRODUCTION

Polymorphism analysis of mitochondrial DNA (mtDNA) is a powerful tool for research on conservation biology and evolutionary biology (Wilson et al., 1985). The structure and function of the cytochrome *b* gene (Cyt *b*) is the most clearly understood among the 13 protein-coding genes of mtDNA. Because of its simple structure, bare recombination, and faster evolution speed, Cyt *b* has been considered as one of the creditable molecular markers to solve problems on classification and phyletic evolution (Irwin et al., 1991), and it is widely used in the study of genetic diversity and interspecific or intraspecific phyletic evolution of vertebrates (Zhou et al., 2003; O'Bryan et al., 2010).

Gymnocypris chilianensis belongs to the genus *Gymnocypris*, the subfamily Schizothoracinae, and the family Cyprinidae. It is only distributed in inland river basins of the Hexi Corridor in China (Li et al., 1974; Wu and Wu, 1992). The Hexi Corridor is located at the junction of the Loess Plateau, the Tibetan Plateau, and the Inner Mongolian Plateau. Depending on the quantity of rain and snow water from the Qilian Mountains, three inland rivers are formed, the Heihe River, the Shule River, and the Shiyang River, which become the main distribution areas of *Gymnocypris* in China. The source of the three inland rivers in the Hexi Corridor is an alpine glacier from the Qilian Mountains. Because of the attenuation of the function on climate regulation, precipitation, and water retention year by year, the water supply of inland river basin decreases continually, which intensifies the overbalance of ecological environment in the middle and lower reaches of inland rivers. Concurrently, a significant proportion of *G. chilianensis* habitat has been lost and its natural distribution area has been substantially reduced, as a result of factors such as artificial damming and new hydropower engineering. Influenced by both natural and artificial factors, the wild resource of *G. chilianensis* is declining sharply, and its genetic diversity is diminishing progressively.

Genetic diversity is the foundation for evolution and acclimatization of organisms. The more abundant the population genetic diversity is, the stronger the ability to adapt to environmental changes will be. Furthermore, a lack of genetic diversity is a great threat to organisms living in unbalanced ecological environments (Xiao et al., 2013). Therefore, in order to develop reasonable and efficient measures of resource conservation, it is necessary to study the genetic diversity and population structure of *G. chilianensis*. To date, only Zhao et al. (2011) have studied the phylogeography of *G. chilianensis* in the inland river basins of the Hexi Corridor in China, while other research on genetic diversity has not yet been

reported.

Moreover, there are always disagreements on the taxonomic status of *G. chilianensis*, and the taxonomic results based on morphology by several researchers have differed (Wu, 1964; Li et al., 1974; Zhao, 1986). In this research, we analyzed the genetic diversity of *G. chilianensis* and its genetic relationship with *Gymnocypris eckloni* and *Gymnocypris przewalskii*, by focusing on *G. chilianensis* and using the Cyt *b* gene as a marker and *G. eckloni* and *G. przewalskii* as controls, with the aim of providing a theoretical basis for resource conservation and phyletic classification of *G. chilianensis*.

MATERIAL AND METHODS

Material

A total of 74 individuals of *G. chilianensis* were collected, of which 44 individuals were collected from the Heihe River and 30 individuals from the Shule River, and 42 individuals of *G. przewalskii* were collected from the Qinghai Lake [permit No. (Gan) SYXF (2010) 12, issued by the government of Gansu Province). The sampling information is shown in Figure 1. Part of the caudal fin was cut from each specimen after all specimens were identified to species level based on external characteristics, and then preserved in 95% ethanol and stored at -20°C. An additional 30 Cyt *b* sequences of *G. eckloni* were downloaded from the GenBank database, of which the specimens were derived from the mainstream of the Yellow River in Qinghai Province, China.

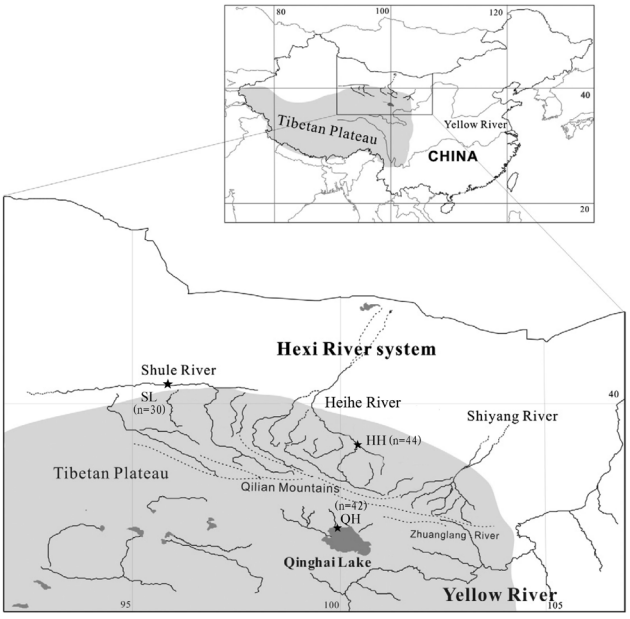

Figure 1. Sampling locations, sample size, and sample codes of *Gymnocypris* populations in China. HH = Heihe River *Gymnocypris chilianensis*; SL = Shule River *G. chilianensis*; and QH = Qinghai Lake *Gymnocypris przewalskii.*

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted by the routine method of phenol-chloroform extraction (Green and Sambrook, 2012). The sequences of the primers are as follows: F5'-CTCCGATCTCCGGATTACAAGAC-3' and R5'-GACTTGAAAACCACCGTTG-3'. The PCR amplification was performed in a total volume of 50 μL containing 50 ng template DNA, 2.5 mM 1X PCR Buffer, 1 U *Taq* DNA polymerase (Takara, Dalian, China), 100 μM dNTPs (Takara), 0.4 μM of each primer, and 19.3 μL ddH_2O. The PCR amplification was carried out under the following conditions: initial denaturation at 95°C for 3 min, 30 cycles consisting of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 10 min.

Cloning and sequencing

The PCR product was purified with the Universal DNA Purification Kit (TianGen, Beijing, China), then cloned into the pMD19-T vector (Takara). The recombinant plasmid was introduced into the *Escherichia coli* DH5α strain (TianGen). After being selected by LacZ blue-white selection and identified by double digestion with *Eco*RI and *Pst*I, the positive clones were sequenced by bi-directional DNA sequencing (Sangon Biotech, Shanghai, China).

Statistical analysis

The original data of the sequences were obtained using the Chromas 1.45 software (http://www.technelysium.com.au), aligned using the ClustalX 1.83 program (http://www.clustal.org/) with default parameters and 1000 bootstraps, and checked by eye. Polymorphic sites, number of haplotypes, haplotype diversity index (h), and nucleotide diversity index (π) were calculated using the DnaSP v5 software (http://www.ub.edu/dnasp/). Base composition and genetic distance among the four populations (the average genetic distances based upon Kimura's two-parameter model) were calculated using the MEGA 5.0 software (http://www.megasoftware.net/). *Gymnodiptychus pachycheilus* (GenBank accession No. JQ082349) and *Ptychobarbus dipogon* (GenBank accession No. JQ082345) were taken collectively as the out-group. The distance matrix of Kimura's two-parameter model was used during the analysis. The phylogenetic tree was created using the neighbor-joining method (NJ), and statistical support was estimated using 1000 bootstrap replicates.

Nucleotide sequence accession Nos.

The Cyt *b* partial sequences newly defined in the current study have been deposited in the GenBank database under the accession No. KM371119-KM371228.

RESULTS

Gene mutation

A total of 146 homologous sequences of 1140 bp were used for the analysis of genetic

diversity and gene mutations, and a total of 336 polymorphic sites were detected, accounting for 29.5% of the total analyzed sites, including 266 transitions and 70 transversions. The transition/transversion ratio was 3.8, which was significantly higher than 2.0 and showed that the mutagenesis was not saturated, and weighted analysis was not needed in the phylogenetic analysis (Knight and Mindell, 1993). The nucleotide content averaged 30.9% T, 26.5% C, 26.1% A, and 16.5% G, showing obvious G-reversed bias. The content of G + C (43%) was less than A + T (57%), showing the common characteristic of the Cyt *b* gene (Su et al., 2014).

Genetic diversity

A total of 132 haplotypes were recovered from 146 aligned sequences and the haplotype diversity and nucleotide diversity indices are shown in Table 1. The haplotype diversity of the Heihe River *G. chilianensis* population was the highest (h = 1.0000), compared with the lowest haplotype diversity of the Shule River *G. chilianensis* (h = 0.9820). While the nucleotide diversity of *G. eckloni* was the highest (π = 0.0067), compared with the lowest nucleotide diversity of Shule River *G. chilianensis* (π = 0.0039).

Table 1. Genetic diversity indices of the four *Gymnocypris* populations studied.

Population	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (h)	Nucleotide diversity (π)
HH	44	116	1.0000	0.0057
SL	27	63	0.9820	0.0039
QH	37	105	0.9930	0.0055
HB	24	40	0.9840	0.0067
Total	132	336	0.9980	0.0414

HH = Heihe River *Gymnocypris chilianensis*; SL = Shule River *G. chilianensis*; QH = Qinghai Lake *Gymnocypris przewalskii*; and HB = Yellow River *Gymnocypris eckloni*.

Genetic differentiation

Genetic distance within and among populations was estimated using the Kimura's two-parameter model, with values ranging from 0.0039 to 0.0068 and from 0.0064 to 0.0838, respectively (Table 2), suggesting that the genetic distances within populations were higher than among populations. The lowest genetic distance was observed between the Heihe River *G. chilianensis* population and the Shule River *G. chilianensis* population (0.0064), followed by the genetic distance between the *G. przewalskii* population and the *G. eckloni* population

Table 2. Genetic distance between the four *Gymnocypris* populations (below diagonal), genetic distance within populations (bold values at diagonal), and standard error (above diagonal).

Population	HH	SL	QH	HB
HH	**0.0061**	0.0017	0.0056	0.0055
SL	0.0064	**0.0039**	0.0054	0.0053
QH	0.0838	0.0810	**0.0055**	0.0019
HB	0.0805	0.0778	0.0079	**0.0068**

HH = Heihe River *Gymnocypris chilianensis*; SL = Shule River *G. chilianensis*; QH = Qinghai Lake *Gymnocypris przewalskii*; and HB = Yellow River *Gymnocypris eckloni*. Bold numbers at diagonal represent the genetic distance within per *Gymnocypris* population.

(0.0079), while the highest genetic distance was observed between the Heihe River *G. chilianensis* population and the *G. przewalskii* population (0.0838).

Phylogenetic analysis

The phylogenetic analysis showed that 132 haplotypes diverged into two main branches. One main branch contains the Heihe River *G. chilianensis* and the Shule River *G. chilianensis*, while the other branch contains *G. przewalskii* and *G. eckloni*. Bootstrap values

Figure 2. Neighbor-joining phylogenetic tree constructed from the 132 Cyt *b* haplotypes from the *Gymnocypris* populations. HH = Heihe River *Gymnocypris chilianensis*; SL = Shule River *G. chilianensis*; QH = Qinghai Lake *Gymnocypris przewalskii* and HB = Yellow River *Gymnocypris eckloni*.

were 100% (Figure 2).

DISCUSSION

The nucleotide diversity index is a significant indicator used to estimate the genetic variation of populations, where the value indicates the abundance or scarcity of the genetic diversity of a population. The nucleotide diversity index of the Shule River *G. chilianensis* population was 0.0039, which was lower than the other populations in our study, but much higher than the result of research on the Shule River *G. chilianensis* (π = 0.0002) by Zhao et al. (2011). As a consequence, the Shule River *G. chilianensis* population should be prioritized for protection, considering its lower genetic diversity (π < 0.0047) according to the range of the nucleotide diversity index, which was proposed by Lan and Shi (1993) to estimate genetic diversity. Moreover, the combination of high haplotype diversity (h = 0.9820) with low nucleotide diversity (π = 0.0039) of the Shule River *G. chilianensis* population reveals the possible mechanisms for the formation of this population, which rapidly derived from a small effective population (Avise, 2000), similar to the research by Yang et al. (2008) on *Anabarilius grahami*.

According to Wu (1964), differences in the morphological traits exist between the scaleless carp of the Hexi region and *G. eckloni* from the Yellow River, based on the lower number of gill rakers, wider hypopharyngeal bone, and wider pupil diameter. Morphological classification has shown that the scaleless carp of the Hexi region, which was named *G. chilianensis*, is an independent species of *Gymnocypris* (Li et al., 1974). The species status of *G. chilianensis* was confirmed by Zhang et al. (2013) using the Cyt *b* gene sequence. However, Zhao (1986) suggested that *G. chilianensis* should be classified as a subspecies of *G. eckloni* from the Yellow River, considering the less obvious morphological variation of *Gymnocypris* as the habitat approaching. For years, several studies of vertebrates, using the Cyt *b* gene as a molecular marker, showed that the intraspecific sequence difference ranged from 0 to 0.0406, with differentiation of species appearing obvious in individuals where the sequence difference was greater than 0.0600 (Xuegan et al., 2002; He et al., 2012). In our research, the genetic distances of the Cyt *b* sequence between the Heihe River *G. chilianensis* population and the *G. przewalskii* and *G. eckloni* populations were 0.0838 and 0.0805, respectively. The genetic distance of the Cyt *b* sequence between the Shule River *G. chilianensis* population and the *G. przewalskii* and *G. eckloni* populations were 0.0810 and 0.0778, respectively. All genetic distances were greater than 0.0600, indicating that the genetic relationship between *G. chilianensis* and *G. przewalskii* and *G. eckloni* is distant, implying that the differentiation has reached species level. The NJ phylogenetic tree showed that the two populations of *G. chilianensis* cluster on one branch, which then clusters with *G. eckloni* and *G. przewalskii*, with bootstrap values of 100%. Therefore, we suggest that *G. chilianensis* is an independent species, the view that is widely accepted (Wu, 1964; Li et al., 1974; Zhang et al., 2013), but different from the view of *G. chilianensis* as a subspecies of *G. eckloni* put forward by Zhao (1986). We also found that the genetic distance between *G. przewalskii* and *G. eckloni* was 0.0079, a differentiation that has not reached the species level. The NJ phylogenetic tree showed that *G. przewalskii* and *G. eckloni* clustered together, forming one branch, but without forming a monophyletic group. As a consequence, *G. przewalskii* is thought to a subspecies of *G. eckloni*, however, further study is required.

Conflicts of interest

The authors declare no conflict of interest.

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