

Huangshan population of Chinese *Zacco platypus* (Teleostei, Cyprinidae) harbors diverse matriline and high genetic diversity

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ABSTRACT

Six main mitochondrial DNA (mtDNA) lineages have been described in minnow (*Zacco platypus*) samples obtained from northern, western and southern China. Perdices et al. (2004) predicted that further sampling of other tributaries might discover more lineages of this species. In this study, we collected 26 *Zacco platypus* individuals in the Huangshan area of eastern China and determined the cytochrome *b* (*cytb*) sequence variations. Combined with reported data in GenBank, we identified ten matriline (Zacco A-J) in a total of 169 samples, with relatively high molecular divergence found among them. The Huangshan population had the greatest genetic variation among all sampled regions and hosted six of the ten matriline. Our results highlight the significance of the Huangshan area for the conservation of *Zacco platypus*.

Keywords: *Zacco platypus*; Matriline; Huangshan; Phylogenetics; Diversity

INTRODUCTION

Zacco platypus is a common minnow that occurs in sympatry with most Chinese cyprinids (Deng et al., 2013). Topographical barriers may restrict its life history and drive cryptic diversity. The species' distribution encompasses all major river systems in mainland China, as well as the Korean Peninsula and Japan

(Chen, 1998). Perdices et al. (2004) analyzed the genetic diversity of *Z. platypus* sampled in the upper and middle Changjiang (Yangtze River) and found four major matriline that may harbor multiple species. Long-term interruption of dispersal is thought to have driven this diversity. Perdices and Coelho (2006) further studied samples from the Pearl River and northern drainages, and obtained six matriline in China. Using nuclear DNA data, Berrebi et al. (2005) identified four genetic groups within *Z. platypus* from Sichuan, Hunan and Guangxi provinces in China.

Although Perdices et al. (2004) predicted that exhaustive sampling of other tributaries might discover other lineages of *Zacco*, few specimens have been sampled in eastern China. The Huangshan area in eastern China is a mosaic of mountains with elevations lower than 2 000 m, and exhibits a complex geological history that includes tectonic movements, orogenesis, and periodic climatic change (e.g., Ju et al., 2007; Rüber et al., 2004; Zhang et al., 1990). Based on patterns of intraspecific genetic variation and buffer-zone models, Huangshan hosts refugia of eastern Asian conifers, frogs, non-migratory birds and Asian salamanders (Gao et al., 2007; Li et al., 2009; Murphy et al., 2000; Wu et al., 2013; Zhang et al., 2008).

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In view of modern genetics, genetic diversity in a given species is closely related to its adaptability, variability, and evolutionary potentiality, with genetic variation considered a prerequisite for organisms to cope with environmental uncertainty (Conrad, 1983). Herein, we report on the genetic diversity of *Z. platypus* from eastern China based on extensive sampling of the Huangshan area together with prior *cytb* sequence data from mainland China, Taiwan (Perdices et al., 2004; Perdices & Coelho, 2006; Wang et al., 2007), and Japan (He et al., 2004; Kawamura et al., 2014; Kitamura et al., 2012; Sasaki et al., 2007; Wang et al., 2007). We further evaluated the matrilineal diversity of *Z. platypus* and revealed the possible ecological significance of the Huangshan area.

MATERIALS AND METHODS

Sampling

We evaluated 169 sequences in total, including 26 from five Huangshan counties (Table 1), 137 from Perdices et al. (2004) and Perdices & Coelho (2006), one from Wang et al. (2007), and five from Japan (AF309085, He et al. (2004); AB198972, Sasaki et al. (2007); AY958194, Wang et al. (2007); AB620130, Kitamura et al. (2012); and AB366543, Kawamura et al. (2014)). Our new samples were preserved and deposited in the Museum of Huangshan University (Voucher numbers: HUM201201–26). Sampling sites in this study are shown in Figure 1.

PCR amplification and sequencing

Fresh dorsal muscle tissues were removed from the 26 Huangshan individuals and immediately preserved in 95% ethanol for sequencing complete mitochondrial *cytb*. Total DNA was extracted from tissues using standard phenol/chloroform techniques (Sambrook et al., 1989). *Cyt b* was amplified using polymerase chain reaction (PCR) with the following sets of primers: LCB1 (5'-AATGACTTGAAGAACCACCGT-3') and HA (5'-CAACGATCTCCGGTTTACAAGAC-3') (Brito et al., 1997; Schmidt & Gold, 1993). Reagents included 100 ng of template DNA, 1 μ L of each primer, 5 μ L of 10 \times reaction buffer, 2 μ L dNTPs (each 2.5 mmol/L), and 2.0 U of Taq DNA polymerase. The reactions were cycled as follows: an initial preheating at 94 $^{\circ}$ C for 3 min, 30 cycles of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 55 $^{\circ}$ C for 40 s, extension at 72 $^{\circ}$ C for 1 min, and a final extension at 72 $^{\circ}$ C for 5 min. We next obtained nucleotide sequences through fractionation, purification, and sequencing according to Tiangen's protocols. Newly obtained haplotype sequences were deposited in GenBank under Accession Nos. KM491716-35 (Table 1).

Matrilineal genealogy and population structure

We used 916 bp out of 1 140 bp of the *cytb* sequences in the following analyses. The newly obtained sequences and those downloaded from GenBank were aligned using Clustal X (Thompson et al., 1997). For phylogenetic reconstruction, two closely related species, *Zacco temminckii* and *Candidia barbatus* (Mayden et al., 2007; Wang et al., 2011), were chosen as outgroups.

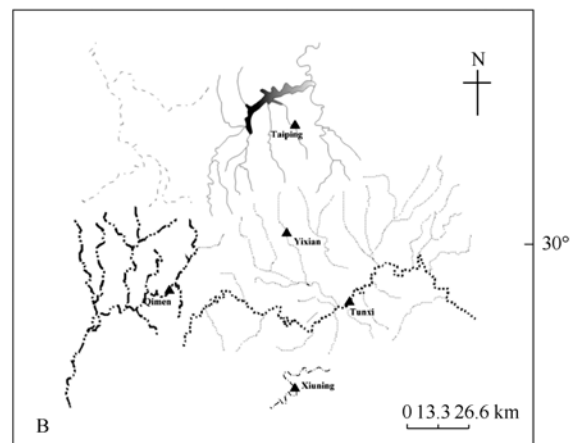
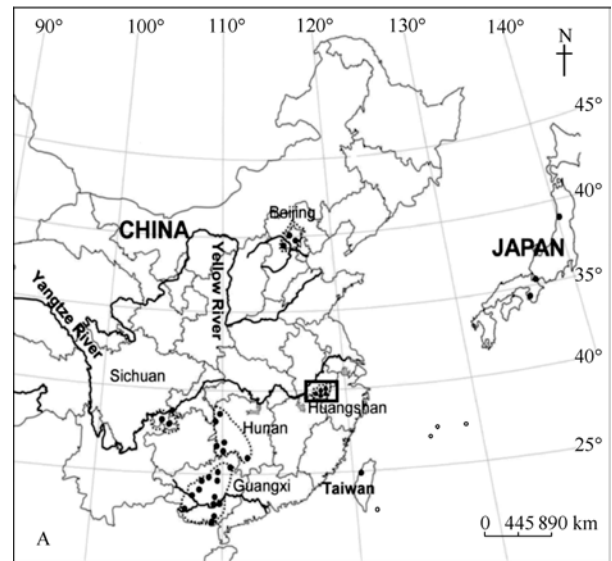


Figure 1 Sampling localities (A) and main drainages of the Huangshan area (B)

Black dots refer to samples from GenBank, and triangles are from this study. This map shows the seven geographic units grouped according to geographic distances and similarities. Xiuning, Qimen, and Taiping drainages belong to the Yangtze River, while Tunxi and Yixian drainages belong to the Qiantang River.

Bayesian inference (BI) and maximum likelihood (ML) were used to reconstruct a bifurcating tree using MrBayes v3.0 (Huelsenbeck & Ronquist, 2001) and RAxML at the CIPRES Science gateway (<http://www.phylo.org/portal2/login!input.action>), respectively. JModelTest v. 0.1.1 (Posada, 2008) was used to find the best model of nucleotide evolution for ML based on the Akaike Information Criterion (AIC) and for BI based on the Bayesian Information Criterion (BIC). Analyses selected the TN93+G model. Bayesian posterior probabilities (BPP) and the frequencies of nodal resolution were obtained by Markov Chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. The BI analysis used 10 000 000 generations,

with sampling every 1 000 generations and discarding the first 3×10^6 generations as burn-in. We ran four analyses starting

with random trees and a consensus of the resulting 36 000 trees was computed from all four runs.

Table 1 Information for samples newly obtained in this study, including localities, rivers, sample sizes, haplotypes, coordinates, voucher specimens and GenBank accession numbers

Localities	Sample Size (n)	Rivers	Coordinates	Haplotypes	Voucher specimens	GenBank Accession No.
Qimen	4	Chang Jiang	N29°84'51", E117°71'77"	Qimen1	HUM201212	KM491716
				Qimen2	HUM201213	KM491717
				Qimen3	HUM201214	KM491718
				Qimen4	HUM201215	KM491719
Xiuning	8	Jiangwan He	N29°43'20", E118°16'75"	Xiuning1	HUM201201	KM491733
				Xiuning2	HUM201202-03	KM491731
				Xiuning3	HUM201204-06	KM491732
				Xiuning4	HUM201207	KM491735
				Xiuning5	HUM201208	KM491734
Tunxi	3	Xinan Jiang	N29°70'43", E118°31'16"	Tunxi1	HUM201209	KM491728
				Tunxi2	HUM201210	KM491729
				Tunxi3	HUM201211	KM491724
Yixian	6	Xinan Jiang	N29°92'06", E118°10'13"	Yixian1	HUM201216	KM491730
				Yixian2	HUM201217	KM491727
				Yixian3	HUM201218	KM491726
				Yixian4	HUM201219-20	KM491725
				Yixian5	HUM201221	KM491723
Taiping	5	Taiping Hu	N30°36'16", E118°04'65"	Taiping1	HUM201222	KM491722
				Taiping2	HUM201223-25	KM491720
				Taiping3	HUM201226	KM491721

Sampling information of extant sequences is not listed in this table.

Estimation of divergence time

Divergence times among the main lineages of *Z. platypus* were estimated using a Bayesian MCMC approach implemented in BEAST V.1.7.5 based on a strict molecular clock (Drummond & Rambaut, 2007). The parameters were: substitution model, TN93+G; tree prior, Coalescent: constant size; normal distribution; 10 million generations; parameters logged every 1 000; burn-in value=1 000. The molecular clock of cyprinids was assumed to be $1.52\% \text{ site}^{-1} \text{ Ma}$ (million years)⁻¹ (Doadrio et al., 2002) for *cyt b*.

RESULTS

A total of 75 haplotypes were defined from all 169 in-group individuals. The topologies of the BI and ML trees were nearly identical (Figure 2). The haplotypes were grouped into main clade 1 and 2. Clade 1 hosted individuals from Huangshan, Sichuan, Hunan and Guangxi and clade 2 contained specimens from Huangshan, Beijing and Japan. We identified ten matrilineal lineages of *Z. platypus* according to the topology of the phylogenetic tree and the genetic variation between the ten matrilineal lineages.

Six of the ten lineages involved Huangshan individuals, and

four consisted entirely of Huangshan individuals. Moreover, samples from the same Huangshan location were grouped into different clades. For example, Qimen had samples from matrilineal lineages A, F and J, and Xiuning had samples from matrilineal lineages A and I. The genetic divergences of these samples were significant, and the maximum pairwise differences from the same counties reached 6.0% (Xiuning) and 21.9% (Qimen).

We grouped sampling localities into seven geographic units according to geographic distances and then calculated the nucleotide diversity within them. Huangshan showed remarkably high nucleotide diversity relative to other groups (2.5-111.7 times that of others) although the geographical area of Huangshan was less than that of the other units (Table 3).

Divergence times estimated for the in-group nodes are shown in Figure 3. The initial divergence occurred at about 10.67 Ma.

DISCUSSION

Perdices et al. (2004) and Perdices & Coelho (2006) divided *Z. platypus* sampled in southern, western and northern China into matrilineal lineages A-F. They suggested that the long-term interruption

of gene flow might have caused the diversification and an underestimation of the number of species. Our analyses identified ten matrilineal lineages of *Z. platypus* in Chinese and some Japanese populations. This confirms the prediction of Perdices

et al. (2004, page: 9) that “exhaustive sampling of other tributaries might evidence other *Zacco* lineages”. This is also in accordance with that found for *Opsariichthys bidens*, a sympatric species of *Z. platypus* (Perdices et al., 2005).

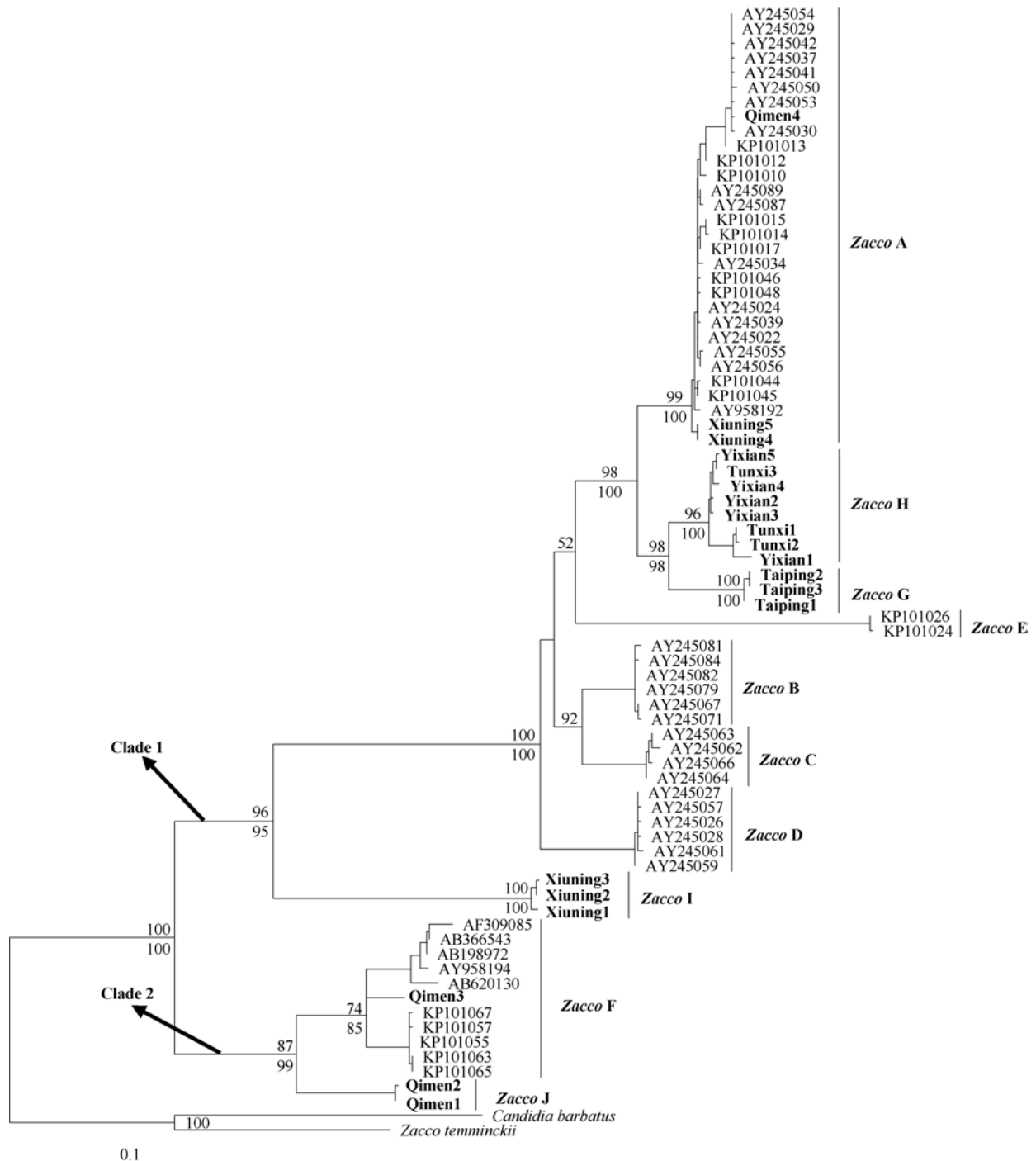


Figure 2 Phylogenetic tree derived from the maximum likelihood of the *cyt b* sequences

Values above branches represent the support level of ML (BSP) and values below branches represent the popularity rating of BI (BPP). Vertical bars indicate the mtDNA lineage assignment (A-J). *Zacco* A-F follow the nomenclature of Perdices & Coelho (2006) and the others follow the alphabet. Bold types are Huangshan populations.

Table 2 Matrix of pairwise genetic variation between matriline (A-J) of *Z. platypus*

	A	B	C	D	E	F	G	H	I	J
A										
B	0.067									
C	0.078	0.044								
D	0.076	0.059	0.065							
E	0.118	0.112	0.109	0.108						
F	0.146	0.148	0.163	0.158	0.174					
G	0.052	0.073	0.074	0.074	0.122	0.152				
H	0.059	0.076	0.091	0.08	0.128	0.158	0.044			
I	0.157	0.157	0.158	0.146	0.184	0.155	0.152	0.155		
J	0.157	0.151	0.156	0.151	0.181	0.067	0.153	0.164	0.135	

Table 3 Matriline, haplotype (h), and nucleotide diversity (π) with standard errors (SE) for each geographic unit

Geographic unit	mtDNA lineage	No. of samples (n)	$h \pm SE$	$\pi \pm SE$
Huangshan	A, F, G, H, I, J	26	0.9631±0.0219	0.085751±0.042501
Beijing	F	23	0.6087±0.0761	0.000768±0.000670
Hunan	A, C, D	49	0.9092±0.0262	0.034648±0.017036
Sichuan	B	19	0.6374±0.1045	0.001098±0.000863
Guangxi	A, E	46	0.6963±0.0740	0.029668±0.014733
Taiwan	A	1	—	—
Japan	F	5	—	0.011681±0.007511

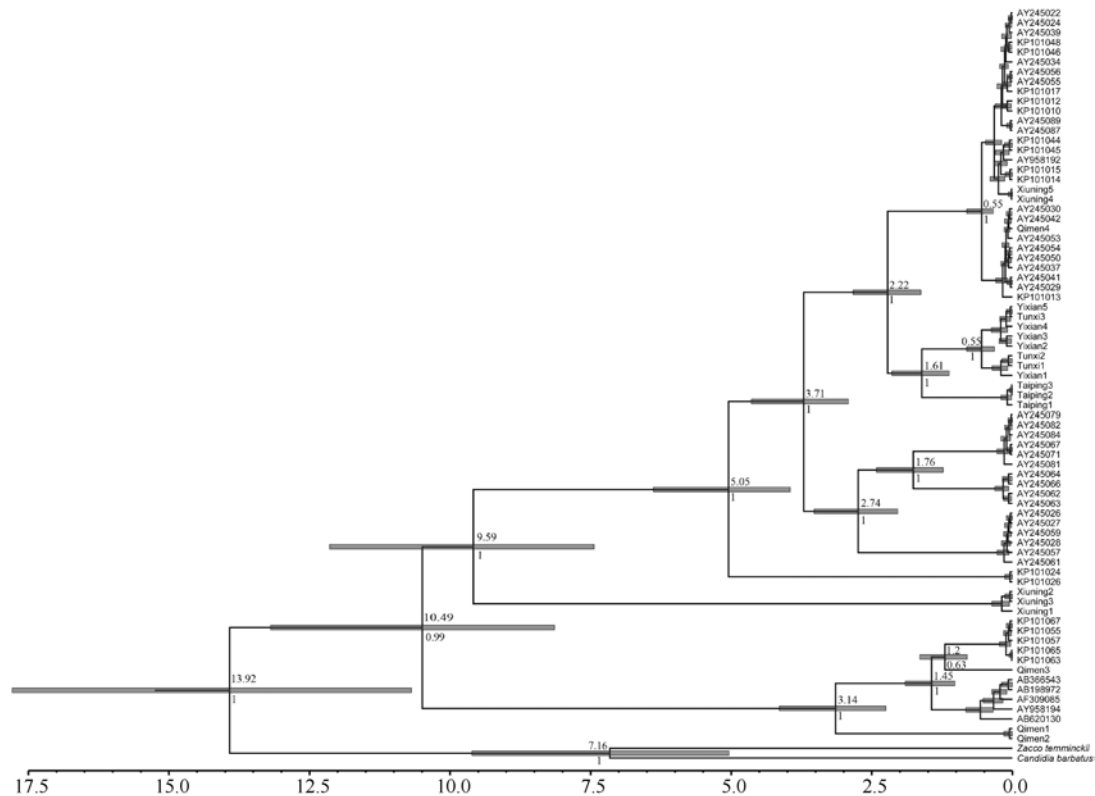


Figure 3 Time tree of *Z. platypus*

Tree topology derived from BEAST analyses of all 77 haplotypes. Numbers above branches represent node age and values below are support rates. Gray bar represents 95% posterior credible intervals.

Some drainages still await sampling, such as the Yellow River, one of the most important drainages in China. Future research should detect additional matrilineal lines of *Zacco*, while morphological analyses may help differentiate morphological differences of taxonomic significance.

Grant & Bowen (1998) interpreted four basic population history scenarios based on haplotype and nucleotide diversities, which can also be used to clarify the history of *Z. platypus* populations. Our results revealed a pattern of high haplotype and nucleotide diversity in the Huangshan population (Table 3), which likely indicates large stable populations with long evolutionary histories or secondary contact between differentiated lineages (Grant & Bowen, 1998). The highest levels of genetic variation may occur in the region of origin. For example, Savolainen et al. (2002) claimed an East Asian origin for the domestic dog in part due to the area having the highest level of genetic diversity.

Genetic variability in mtDNA has been reported in fish species. Several scenarios have been proposed to explain the maintenance of high haplotype diversity within populations, including large population size, environmental heterogeneity, and life history traits that favor rapid population increase (Han et al., 2008; Ju et al., 2013; Yang et al., 2012). Huangshan has a heterogeneous topography, with mountains of elevation lower than 2 000 m maintaining stable climatic conditions during the Pleistocene. This condition likely provided glacial refugia for many species (Gao et al., 2007; Li et al., 2009; Qian & Ricklefs, 2000; Wu et al., 2013; Zhang et al., 2008). At least three other species or species groups have high levels of nucleotide diversity in the Huangshan area, including the Chinese giant salamander, sharp-snouted pit viper and Asian salamander (Huang et al., 2007; Murphy et al., 2000; Wu et al., 2013). These co-occurrences indicate that Huangshan hosts old lineages.

We only used mtDNA for genetic analyses. Therefore, it will be necessary to gather and analyze nuclear DNA data in the future to assess population structure and gene flow and thus better inform the demographic history of this fish species.

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