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Comparative Mitogenomic and Phylogentic Analyses of a Schizothoracine Fish, *Gymnodiptychus dybowskii* from Two Water Systems in Xinjiang

Wei Meng[1,3], Tianyan Yang[2,*], Yunguo Liu[1], Mahmut Halik[1] and Tianxiang Gao[2]

[1]*College of life Science and Technology, Xinjiang University, Urumqi 830046, China*
[2]*Fisheries College, Zhejiang Ocean University, Zhoushan 316004, China*
[3]*Xinjiang Fisheries Research Institute, Urumqi, 830000, China*

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TY conceived and designed the work. YL performed the experiments. WM analyzed the data and wrote the manuscript. MH and TG revised the manuscript.

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***Gymnodiptychus dybowskii*, Complete mitochondrial genome, Phylogenetic analysis.**

ABSTRACT

Gymnodiptychus dybowskii belonging to subfamily Schizothoracinae is a rare and endangered aboriginal fish in Xinjiang. In this study, the complete mitochondrial genomes of *G. dybowskii* from Tarim River system (16, 677bp) and Ili River system (16, 667bp) were sequenced. Besides, their genetic characteristics were also identified and compared simultaneously. Genetic distance and sequence differentiation suggested that great genetic variation existed within species and the sample from Kaidu River in South Xinjiang might be a cryptic species or subspecies of *G. dybowskii*. The phylogenetic analyses from 12 concatenated H-strand-encoding protein genes were conducted by Neighbor-Joining method to reveal the evolutionary relationships within subfamily Schizothoracinae. Three different grades of schizothoracine fishes were well recognized from each other in branching diagram. The primitive group and the specialized group + the highly specialized group constituted a sister relationship with strong supports.

INTRODUCTION

Schizothoracine fishes are characterized by a line of specialized anal scale on both sides of the anus and anal fin. They are members of the family Cyprinidae and consist of 15 genera and more than 100 species all over the world (Mirza, 1991). In China, their distribution pattern presents Qinghai-Tibet Plateau-centered characteristics (Chen and Cao, 2000). As a most diverse group of ichthyofauna in Xinjiang, it contains about 11 species belonging to 5 genera accounting for over 15% of the Chinese schizothoracine species (Wang, 1998; Guo *et al*., 2012).

Xinjiang is located in the western border of China and is the largest province with a land mass of 1.66 million square kilometers, accounting for a total area of 1/6 China. Tianshan Mountains span across the central part of Xinjiang in the middle from east to west, dividing it into two parts: south Xinjiang and north Xinjiang (Sabit, 2012). *Gymnodiptychus dybowskii* is a rare and endangered aboriginal fish in Xinjiang, which has been listed as Class I wild aquatic protected animal of Xinjiang in 2004. It mainly distributes in Ili River system and river systems in Junggar basin on the northern slope of Tianshan Mountains with the altitude 1500-2900 m. In Tarim River system of south Xinjiang, it can be only found in upper and middle reaches of Kaidu River (Guo *et al*., 2012). As one of specialized schizothoracine fishes, it is nearly esquamate, only covered with shoulder scale, anal scale and ateral-line scale. These changes of morphological characteristic are in close connection with adaptation of the plateau environment, and it also reveals the evolutionary direction of whole subfamily Schizothoracinae (Cao *et al*., 1981).

Population genetic studies based on the mitochondrial Cyt *b* gene showed a low genetic variation of *G. dybowskii* in Ili River system, but strong genetic divergence between individuals from Kaidu River and Ili River. To further confirm the differences, we amplified and compared the complete mtDNA sequences of *G. dybowskii* collected from south and north Xinjiang in this study. Both selection pressure test and phylogenetic analysis were performed to detect the natural selection and species differentiation during the uplift of the Qinghai Tibet Plateau. The results are expected to provide useful references for the adaptive evolution and biogeography studies of schizothoracinae fishes.

MATERIALS AND METHODS

Sample collection and DNA extraction

In the present study, the specimen of *G. dybowskii*

* Corresponding author: hellojelly1130@163.com
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were collected in Kaidu River and Kunes River in 2014, respectively (Fig. 1). Fresh tail fins were preserved in 95% ethanol immediately, and the total genomic DNA was extracted from caudal fin using standard phenol-chloroform methods (Maniatis *et al*., 1982).

PCR amplification and sequencing

Fragments of COI, Cyt *b*, 16S rRNA and ND4 gene were amplified by universal primers FishF1/FishR1 (Ward *et al*., 2005), L14724/H15915 (Xiao *et al*., 2001), 16Sar/16Sbr (Palumbi, 1996) and ND4F/ND4R (Xiao *et al*., 2005), respectively. Based on the obtained partial sequences and consulting the mitochondrial genomes of closely related species, LA-PCR (long and accurate polymerase chain reaction) primer pairs were designed by software Primer 5.0 to amplify *G. dybowskii* mtDNA (Table I). PCRs were performed in an Eppendorf thermal cycler. LA-PCR amplification was performed within a reaction mixture containing 0.5 μL LA Taq DNA polymerase (5U/μL), 5 μL 10×LA PCR buffer II (Mg^{2+} Plus), 8 μL dNTP mixture (2.5 mM each), 1 μL each primers (20 μM) and 2.5 ng template DNA (50 ng/μL). Sterile distilled H_2O was added to reach a total volume of 50 μL. PCR was carried out with an initial denaturation at 95°C for 1 min, followed by 30 cycles of 94°C for 30 s, 57-64°C for 15 s, and a final extension at 72°C for 1min/kb. Negative controls were conducted to confirm the absence of contaminants. Products were detected by 1.0% agarose gel electrophoresis, purified with the Gel Midi purification Kit (Tiangen Biotech) and then sequenced by Sanger method.

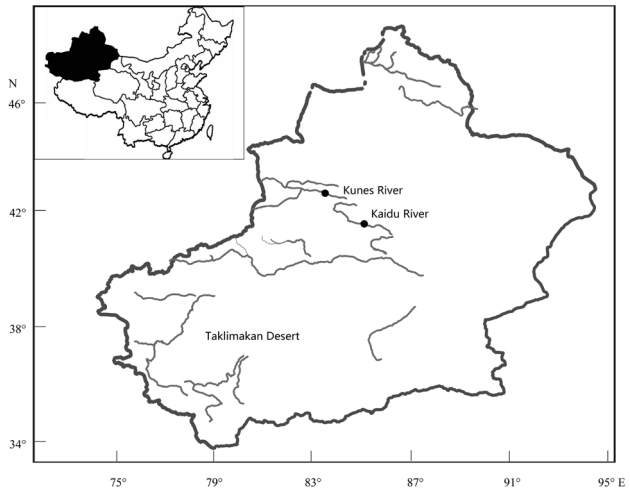

Fig. 1. The sampling locations of *G..dybowskii.*

Data analysis

The corresponding gene locations were determined by BLAST comparison with either nucleotide or amino acid sequences of other Schizothoracinae fishes. EasyCodeML software was employed to analyze the selection pressure of 13 protein-coding genes (PCGs) (Gao and Chen, 2016). The relative influence of natural selection acting on PCGs can be detected by comparing the rates of non-synonymous

Table I.- Primers used in amplifying complete mitogenomes of *G. dybowskii.*

Sample name	Source	Primer		Nucleotide sequence (5'-3')	Annealing temperature (°C)
SLCC	Kaidu River (South Xinjiang)	Lna2	Forward	CGAACTCAACCCAAGAGAGCAATG	60
			Reverse	ACAAGTCAGTTTCCAAATCCCCCG	
		Lnac4	Forward	TCCTCCTCGCCGTGTTTACAGTCG	64
			Reverse	AGGTGTTCTCGGGTGTGGAATGGT	
		Lnc2	Forward	TTTGTCTGGCTAATACCGCATACG	59
			Reverse	TCGTAAAATAGCGTAGGCGAACAG	
		p	Forward	CAGTAGATAACGCCACGCTAACACG	62
			Reverse	TAATAGCACGCCAGTGTGGGGGTA	
NLCC	Kunes River (North Xinjiang)	Lba1	Forward	CAGCCTGCCCAGTGACGATAAGTT	63
			Reverse	TGTGAGGTCTACTGATGCTCCCGC	
		Lbab	Forward	ACTACCCCCATCATTCCTGTTAC	57
			Reverse	GTTTCGGTCTGTGAGAAGCATTG	
		Lbb1	Forward	GCCTCATCAATCCTGGGGGCTATC	61
			Reverse	GCTAATGGGTTCAGGAGCGATGTG	
		Lbbc	Forward	GTCCACAGGATTTTCAACAGCCC	59
			Reverse	TCGTATGCGGTATTAGCCAGACA	
		Lbca2	Forward	CACCTCAGACATTTCAACCGCCTT	61
			Reverse	TTTTCTTTCCTCCGTGGTCGCCCC	
		Lbc1	Forward	TTGCCTACTCATCCGTAAGCCATA	57
			Reverse	TGTTGCGTTATCTACTGAAAAGCC	

substitutions (*Ka*) versus synonymous substitutions (*Ks*), which indicates the net balance between deleterious and beneficial mutations (Yang and Bielawski, 2000; Hurst, 2002; Nielsen, 2005). We examined three pairs of null and alternative hypothesis models to assess the *Ka*/*Ks* ratio (or ω, *dN*/*dS*) for all codon sites. The likelihood ratio tests (LRTs) were performed to compare the fit of three pairwise models: M0 (one-ratio) vs. M3 (discrete), M1a (nearly neutral) vs. M2a (positive selection), and M7 (β distribution) vs. M8 (β distribution and *Ka*/*Ks* ratio).

In order to illustrate phylogenetic relationship of *G. dybowskii* in Schizothoracinae fishes, forty-eight related species belonging to ten genera were used to draw dendrogram, with six taxa under genus *Barbus* regarded as the out-groups. All sequences were available from NCBI Genbank and aligned by MUSCLE 3.6 (Edgar, 2004). Neighbor-Joining (NJ) method and Kimura 2-parameter model performed with Mega6.0 were used to construct phylogenetic trees based on concatenated amino acids sequences of the 12 PCGs excluding ND6 gene.

Table II.- The mitochondrial genome characteristics of *G. dybowskii*.

Gene	Strand	Position	Size (bp)	Intergenic region	No. of proteins	Start codon	End codon	K2P distance
tRNA^{Phe}	H	1-69	69					0.015
12S rRNA	H	70-1024	955					0.018
tRNA^{Val}	H	1025-1096	72					0.029
16S rRNA	H	1097-2778	1682					0.022
tRNA$^{Leu(UUR)}$	H	2779-2854	76	1				0.000
ND1	H	2856-3830	975	4	324	ATG	TAG	0.130
tRNA^{Ile}	H	3835-3906	72	-2				0.044
tRNA^{Gln}	L	3905-3975	71	3/2				0.014
tRNA^{Met}	H	3979-4047/3978-4046	69					0.077
ND2	H	4048-5092/4047-5091	1045		348	ATG	T--	0.135
tRNA^{Trp}	H	5093-5163/5092-5162	71	1				0.000
tRNA^{Ala}	L	5165-5233/5164-5232	69	1				0.045
tRNA^{Asn}	L	5235-5307/5234-5306	73	-4				0.014
$_{O}$L	L	5304-5351/5303-5350	48	-11				0.021
tRNA^{Cys}	L	5341-5407/5340-5406	67	-1				0.000
tRNA^{Tyr}	L	5407-5477/5406-5476	71	1				0.060
COI	H	5479-7029/5478-7028	1551		516	GTG	TAA	0.062
tRNA$^{Ser(UCN)}$	L	7030-7100/7029-7099	71	3				0.029
tRNA^{Asp}	H	7104-7175/7103-7174	72	12/13				0.029
COII	H	7188-7878	691		230	ATG	T--	0.091
tRNA^{Lys}	H	7879-7954	76	1				0.055
ATP8	H	7956-8120	165	-7	54	ATG	TAG	0.085
ATP6	H	8114-8797	684	-1	227	ATG	TAA	0.100
COIII	H	8797-9581	785		261	ATG	TA-	0.079
tRN^{AG}ly	H	9582-9653	72					0.000
ND3	H	9654-10002	349		116	ATG	T--	0.102
tRNA^{Arg}	H	10003-10072	70					0.029
ND4L	H	10073-10369	297	-7	98	ATG	TAA	0.056
ND4	H	10363-11743	1381		460	ATG	T--	0.114
tRNA^{His}	H	11744-11812	69					0.078
tRNA$^{Ser(AGY)}$	H	11813-11881	69	1				0.061
tRNA$^{Leu(CUN)}$	H	11883-11955	73	3				0.014
ND5	H	11959-13782	1824	-4	607	ATG	TAA	0.107
ND6	L	13779-14300	522		173	ATG	TAA	0.119
tRNA^{Glu}	L	14301-14369	69	4				0.030
Cyt *b*	H	14374-15514	1141		380	ATG	T--	0.105
tRNA^{Thr}	H	15515-15585/15515-15586	71/72	87/74				0.691
tRNA^{Pro}	L	15673-15742/15661-15730	70					0.044
D-loop	H	15743-16677/15731-16667	935/937					1.804

Forward slashes denote values of SLCC/NLCC; otherwise, both are identical. Negative numbers indicate overlapping nucleotide.

RESULTS

Mitochondrial genome origination and sequence differentiation

The length of 16, 677 bp (Genbank accession no. KT588613) and 16,667 bp (Genbank accession No. KX688545) complete mitogenome sequences of *G. dybowskii* from Kaidu River (SLCC) and Kunes River (NLCC) were obtained and analyzed, respectively (Table II). They consisted of 13 PCGs (Cyt *b*, ATP6, ATP8, COI-COIII, ND1-ND6 and ND4L), two ribosomal RNA genes (12S rRNA and 16S rRNA), 22 tRNA genes and two non-coding regions (CR and O_L) (Table II). The location and arrangement order of the 37 genes were considered to be relatively conserved just like most metazoan and determined by comparison of DNA or amino acid sequence with other Schizothoracinae fish mitochondrial genomes (Gong *et al*., 2012; Jiang *et al*., 2014; Yang *et al*., 2016; Tong *et al*., 2017).

The pairwise genetic distance between different genes was calculated from 0.000 ($tRNA^{Leu(UUR)}$, $tRNA^{Trp}$, $tRNA^{Cys}$ and $tRNA^{Gly}$) to 1.804 (D-loop). The similarity of whole nucleotide sequences between two mitochondrial genomes was 92.6%, even much lower than that between different species by BLAST in NCBI. The level of homology of 13 PCGs and two ribosomal RNA genes between two individuals ranged from 87.9% (ND2) to 98.2% (12S rRNA), confirming that 12S rRNA gene was the most conserved gene in *G. dybowskii* mtDNA. Control region was the most rapidly evolving region of mtDNA, with 5- to 10- fold higher substitution rate than the rest part. It was one of the most commonly used markers for addressing evolutionary relationships of closely related species or subspecies (Moore, 1995). The gene variation analysis suggested that besides D-loop gene, ND2, ND1 and ND6 genes were also ideal molecular markers in genetic studies, which could be used to analyze the genetic diversity among different populations.

Selection tests

In order to test for the possibility of selection mode on mtDNA protein-coding sequences, we adopted site model to detect the *Ka*/*Ks* ratio in 13 PCGs for two *G. dybowskii*, as well as other four schizothoracine fishes (*Aspiorhynchus laticeps*, *Diptychus maculates*, *Schizothorax biddulphi* and *S. pseudoaksaiensis*) distributed and collected in Xinjiang. ND6 nucleotide sequence was reverse complement in order to infer the correct amino acid sequence.

The LRTs under the first model (M0 vs. M3) showed that 12 PCGs were significant except for ND4L gene (*P*=0.639440891), but no positive site was detected for all 13 PCGs. The LRTs under the second model (M1a vs. M2a) showed that only COII gene was significant (*P*=0.029621169), but no positive site was detected. The LRTs under the third model (M7 vs. M8) showed that four genes (COIII, ND3, ND4, ND5) were significant, and 1, 2, 3 and 7 positive sites were detected, respectively, but the posterior probability did not reach 95% level. The ω values of four genes were 1, 1.69083, 1.23762 and 1.96092, respectively (Table III).

Phylogenetic analysis

Multiple alignments of 12 concatenated heavy-strand encoded gene sequences were concatenated to conduct phylogenetic analysis by NJ method. The ND6 gene was not included because of being encoded on the light-strand and had a strikingly different nucleotide composition relative to other mitochondrial PCGs (Arnason *et al*., 2010).

Table III.- Results of site model on COIII, ND3, ND4 and ND5 genes.

Gene	Model	np	Ln L	Estimates of parameters	Model compared	LRT P-value	Positive sites
COIII	M7	15	-2178.710959	p=0.01824, q=0.11945			
	M8	17	-2175.434750	p0=0.97398, p=0.04179, q=0.54830, (p1=0.02602), ω=1.00000	M7 vs. M8	0.037771176	220 I 0.554
ND3	M7	15	-1087.956641	p=0.04120, q=0.26936			
	M8	17	-1084.151680	p0=0.97153, p=0.05064, q=0.43842, (p1= 0.02847), ω=1.69083	M7 vs. M8	0.022260065	83 A 0.917, 84 D 0.578
ND4	M7	15	-4562.036585	p=0.36527, q=5.61137			
	M8	17	-4557.178766	p0=0.98783, p=0.66527, q=13.26254, (p1= 0.01217), ω=1.23762	M7 vs. M8	0.007767406	43 S 0.744, 86 R 0.865, 189 D 0.623
ND5	M7	15	-5663.404398	p=0.13861, q=1.14597			
	M8	17	-5659.281408	p0=0.98935, p=0.03112, q=0.19861, (p1= 0.01065), ω= 1.96092	M7 vs. M8	0.016196016	27 E 0.837, 29 A 0.767, 30 K 0.937, 273 P 0.798, 486 S 0.813, 573 A 0.759, 580 G 0.567

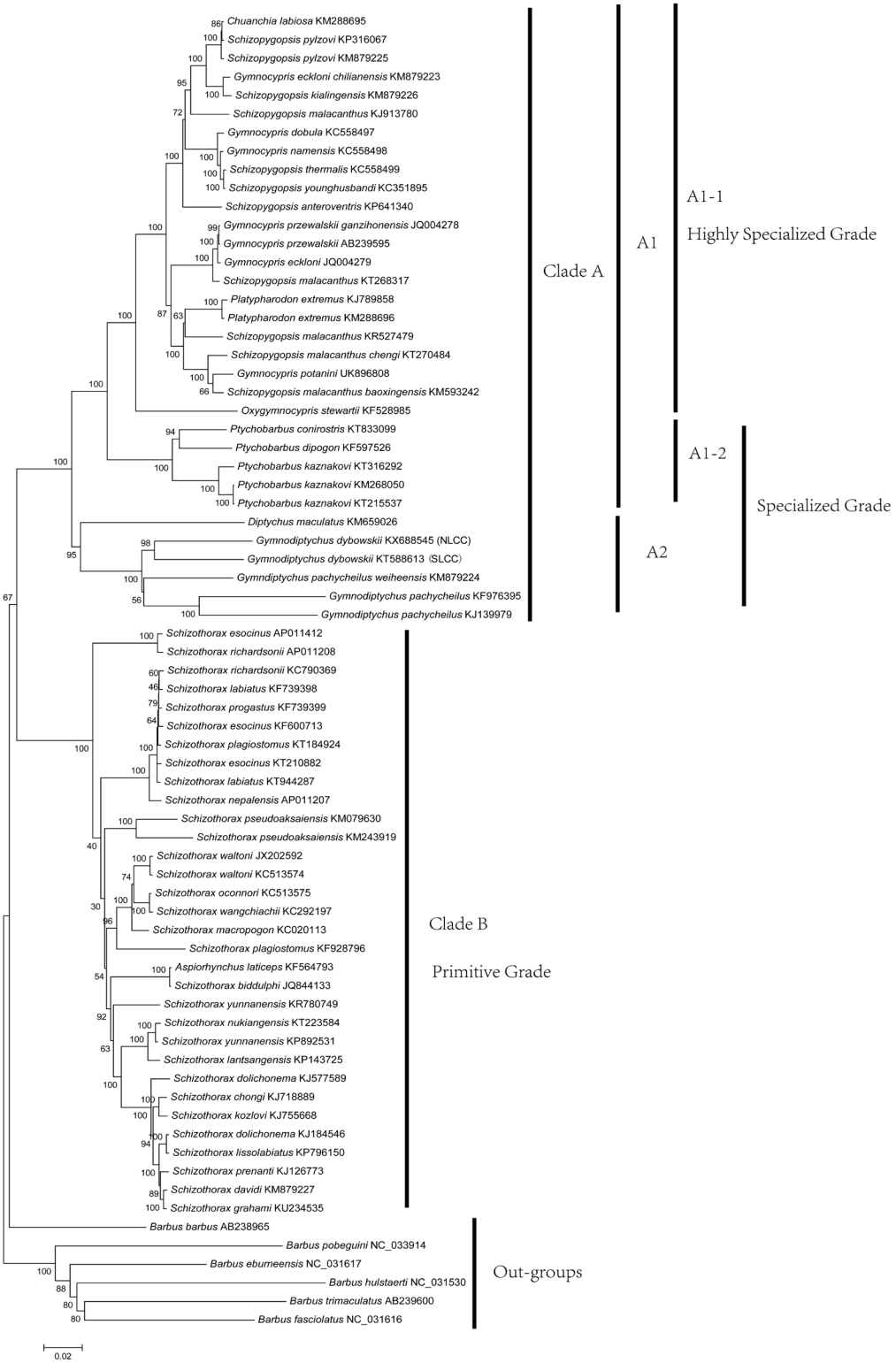

Fig. 2. NJ phylogenetic tree of 48 schizothoracine species constructed by 12 concatenated PCGs except for ND6 gene. The integer labeled on each node correspond to bootstrap support value, and numbers written behind Latin name presented the GenBank accession numbers for the mitochondrial genome of the related species.

A total of 65 individuals from 48 published sub species mitochondrial genomes that represented 10 genera (*Oxygymnocypris*, *Platypharodon*, *Chuanchia*, *Gymnocypris*, *Schizopygopsis*, *Diptychus*, *Gymnodiptychus*, *Ptychobarbus*, *Aspiorhynchus* and *Schizothorax*) and 6 out-group taxa (*Barbus barbus*, *Barbus pobeguini*, *Barbus eburneensis*, *Barbus hulstaerti*, *Barbus trimaculatus* and *Barbus fasciolatus*) were assembled to investigate the phylogenetic relationships of Schizothoracinae subfamily (Fig. 1). The NJ tree was divided into two clades (Fig. 2). Clade A consisted of highly specialized schizothoracine fishes and specialized schizothoracine fishes, while clade B contained primitive schizothoracine fishes.

DISCUSSION

Hebert *et al*. (2003) examined COI sequence divergences for 13320 animal species belonging to 11 phyla, and revealed that 98% interspecific genetic distance was greater than 2%, with the mean divergence value of 11.3%. The result validated the ability of COI sequences to diagnose species in certain taxonomic groups as DNA barcoding. Furthermore, closely related species of vertebrates ordinarily showed from 2% to 5% sequence divergence at mitochondrial cytochrome *b* gene (Avise, 1994; Avise and Walker, 1999). Here, we found that the K2P distance based on COI and Cyt *b* gene between two *G. dybowskii* was 6.2% and 10.5%, respectively, which was much higher than the threshold value and implied potential existence of a cryptic species or subspecies. The existing molecular phylogeography researches revealed by partial mtDNA and nDNA genes had also strongly confirmed it. We examined the population genetic differentiation of *G. dybowskii* from both South and North Slope of Tianshan Mountain, and found that the genetic distance between them was even more than 10% based on Cyt *b* gene, exceeding the divergence level of species suggested by Avise (Meng *et al*., 2015). Li *et al*. (2016) used two mtDNA genes (Cyt *b* and 16S rRNA) combined with a nuclear gene (RAG-2) to evaluate the phylogeography and historical demography of *G. dybowskii* across three northern Qinghai-Tibetan Plateau river systems: the Kaidu River, Ili River and Junggar Basin. Results showed that they have resolved three reciprocally monophyletic clades which should be treated as species or minimally, as evolutionarily significant units (ESUs).

Through the selection pressure analysis of 13 PCGs, positive sites were detected in four genes, of which three were NADH dehydrogenase genes and one was Cytochrome c oxidase gene. Mitochondria is the center of cellular energy metabolism. NADH dehydrogenase is one of the "entry enzymes" of cellular respiration or oxidative phosphorylation in the mitochondria. It is used in the electron transport chain for generation of ATP. Cytochrome c oxidase is the terminal enzyme of the respiratory chain in the mitochondria. It catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen. These two genes participate in oxidative respiratory chain and are likely to play an important role in hypoxia adaptation, which is critical for the plateau adaption. However, all the positive sites were not significant in this study. On the one hand, it may be related to the fewer species (only 5 species in Xinjiang were selected). On the other hand, plateau adaptation is a complex evolutionary process, many nuclear genes may play a more direct role, and natural selection will favor these genes much more.

Although phylogenetic interrelationships of Schizothoracinae subfamily have been extensively investigated in the perspective of molecular biology in previous studies (He *et al*., 2004, 2016; He and Chen, 2006, 2007; Qi *et al*., 2006, 2015; Haysa *et al*., 2014; Zhang *et al*., 2016), however, analyses mainly focusing on schizothoracine fishes distributed in Xinjiang based on the whole mtDNA sequences were rare. A broad range of taxa were chosen for phylogenetic analysis here. The topology structure was identified with previous molecular research conclusions and supported the morphological classification of Cao *et al*. (1981). Primitive grade schizothoracine fishes were in the base of tree, followed by the specialized grade schizothoracine fishes, and highly specialized schizothoracine fishes placed on the top of the phylogenetic tree. This arrangement structure was coincidence with their evolutionary status. Clade A can be further divided into two well-supported (BP=100) subclades A1 and A2. Three species belonging to *Ptychobarbus* genus (branch A1-2) which should have been categorized into specialized grade schizothoracine fishes, were clustered together with highly specialized grade (branch A1-1). This phenomenon was also found in phylogeny analyses by partial mitochondrial DNA genes and complete mitogenome (Haysa *et al*., 2014; Zhang *et al*., 2016). As a monophyletic group, we conjectured that *Ptychobarbus* genus might be a transitional group between highly specialized and specialized grade schizothoracine fishes. According to the classification based on morphological specificities, for example, maxilla bone, the 2nd prosethmoid bone and so on, Wu (1984) considered that *Ptychobarbus* and *Gymnodiptychus* were grouped as sister taxon and had close relationships. But our research results didn't agree with the morphological classification because they situated in independent subclades (A1 and A2) and didn't gather together.

Species of *Gymnocypris* and *Schizopygopsis*

were cross-clustering and did not form a monophyletic group separately. Mitochondrial DNA data did not fully support the morphological findings (He and Chen, 2007). *Oxygymnocypris stewartii* located in the root of branch A1-1 and was a sister to other highly specialized grade schizothoracine fishes with high bootstrap value. In subclade A2, *Gymnodiptychus pachycheilus* and *G. pachycheilus weiheensis* first gathered and then clustered with *G. dybowskii*, with *Diptychus maculates* placing outside of them. That meant perhaps *Diptychus* was the earlier diverged genus of this group.

The taxonomic status of species within genus *Schizothoracinae* have been confused and disputed since Heckel (1838) erected this genus. Chen and Cao (2000) deemed the features of mandible bone as ecological bases for subgenus classification, and divided *Schizothorax* genus into two subgenus: *Schizothorax* and *Racoma*. But cluster analysis found there weren't any one-to-one genetic relationships between each other, not upholding the subgenus division of *Schizothorax* fishes. He and Chen (2006) investigated biogeography and molecular phylogeny of the genus *Schizothorax* in China inferred from cytochrome *b* sequences, and their results also not congruent with the traditional subdivision of the genus *Schizothorax*. They gained a clear geographical structure across drainages and suggested species from the same drainage often shared the same evolutionary lineage. As sympatric species belonging to two different genus, the genetic distances between *A. laticeps* and *S. biddulphi* based on Cyt *b* and COI gene were all less than the intergeneric genetic threshold values recommend by Kartavtsev and Lee (2006) and Ward *et al*. (2005), respectively, which showed relatively close phylogenetic relationships with each other. As anadromous migration species, reproductive time of *A. laticeps* (late April to mid May) and *S. biddulphi* (April to early July) overlaps to some extent in natural water (Guo *et al*., 2012). Besides, the artificial hybridization experiment only for scientific purposes has succeed before. Maybe the discrepancy could be explained in terms of introgressive hybridization or incomplete lineage sorting. Therefore, some academics speculated that *A. laticeps* should be a specialized species within the *Schizothorax* genus (Yang *et al*., 2011; Haysa *et al*., 2014). But the accuracy remains to be further tested and investigated from the whole genome level.

CONCLUSION

In this report, we sequenced the complete mitogenomes of *G. dybowskii* collected from two distinctively different water systems in Xinjiang. The organization and gene arrangement of the mitogenome was similar to those reported from other schizothoracine fishes. However, comparative genomic analysis revealed that the mtDNA genomic features were apparently different in two *G. dybowskii* individuals, with 7.4% sequence divergence between them, indicating a potential cryptic species or subspecies of *G. dybowskii* in Kaidu River. It was speculated that geographical isolation of Tianshan Moutains caused and increased the genetic differentiation of *G. dybowskii* populations. In addition, the phylogenetic relationships of *G. dybowskii* together with 48 related species were analyzed by Neighbor-Joining method. The topological structure showed that schizothoracine fishes form a strongly supported monophyletic group that was a sister taxon to *Barbus barbus*. The primitive grade schizothoracine fishes formed monophyletic group, and constituted sister group relationships with specialized grade schizothoracine fishes + highly specialized schizothoracine fishes. The findings could hopefully provide the basis of the genetic conservation management of *G. dybowskii* in Xinjiang.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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