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Ten polymorphic microsatellite markers characterized for *Schizothorax pseudaksaiensis* and applied for population genetic analysis

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Introduction

Schizothorax pseudaksaiensis is one of the 10 indigenous fish species in the Ili river (Xinjiang, China), mainly distributed in the Ili river – Balkhash lake drainage (Fisheries Bureau of Xinjiang Uygur Autonomous Region 1979; Ren 1998). In recent decades, the stock of *S. pseudaksaiensis* declined rapidly due to overexploitation and deterioration of aquatic environment. Currently, it has been listed as a second-class protected animal in Xinjiang Uygur Autonomous Region (Cai *et al.* 2014) and VU (vulnerable) species by IUCN (Ministry of Environmental Protection of the people's Republic of China 2015, *Red list of China's biodiversity*). Previous studies of *S. pseudaksaiensis* have focussed primarily on the morphology (Cai *et al*. 2011) and histoembryology (Niu *et al.* 2011). However, little is known about the genetic diversity and population structure of this species.

Simple sequence repeats (SSR) or microsatellites are widely used as a kind of molecular marker owing to prominent advantages such as variability, convenient and reliability of scoring (Zane *et al.* 2002). High conservatism in the flanking regions of microsatellite loci allows us to develop primers, and these primers can be utilized to amplify the same locus in other closely-related species. Generally, the development of new microsatellite primers is expensive and time-consuming, whereas this alternative option is cheaper and faster. The method of cross-species amplification has been successfully applied in some fish species (Scribner *et al*. 1996; Zardoya *et al*. 1996; Mohindra *et al*. 2001; Lal *et al*. 2004; Lin *et al*. 2008).

In the present study, 10 polymorphic microsatellite loci were characterized first for *S. pseudaksaiensis* through cross-species amplification methodology and then applied to the analysis of its genetic structure from three populations along the Ili river drainage. The overall statistics showed that the number of alleles in each locus, observed heterozygosity and expected heterozygosity ranged from 2 to 26 (averaged 10.97), 0.063 to 0.818 and 0.061 to 0.943, respectively. Yining population indicated more genetic diversity than the other two populations. The genetic distance and similarity demonstrated that genetic differentiation had occurred among three populations. Since the level of genetic diversity revealed by microsatellite markers is much higher and more convincing than those obtained by phenotypic markers (Triantafyllidis *et al.* 2002; Corujo *et al.* 2004; Zarraonaindia *et al.* 2009), this study could provide more precise perspective for population genetic analysis of *S. pseudaksaiensis* and contribute to the conservation management of the species.

Materials and methods

Specimens of *S. pseudaksaiensis* were collected from the Ili river from May 2013 to October 2014. Ili river is a cross-border river between China and Kazakhstan, with the largest runoff in Xinjiang Autonomous Region, northwest China (Ren *et al.* 1998), and consists of three main tributaries (Tekes, Kunes and Kashi rivers). Unlike other two branches (from east to west) of the Ili river, the Tekes river flows from south to north. According to the flow direction of Ili river (from east to west), three populations were ascertained and originated from the upper, middle and lower reaches of Ili river. Along the Ili river, *S. pseudaksaiensis* has been found from three sites, including Zhaosu (N 42°57′, E 80°57′), Yama Ferry (N 43°37′, E 81°47′) and Yining (N 43°50′, E 80°39′) (figure 1). No specimen was detected from other sites in figure 1. The Yining site is situated at the mainstream of Ili river, Yama Ferry located at the intersection of three main tributaries (Tekes, Kunes and Kashi rivers), whereas Zhaosu

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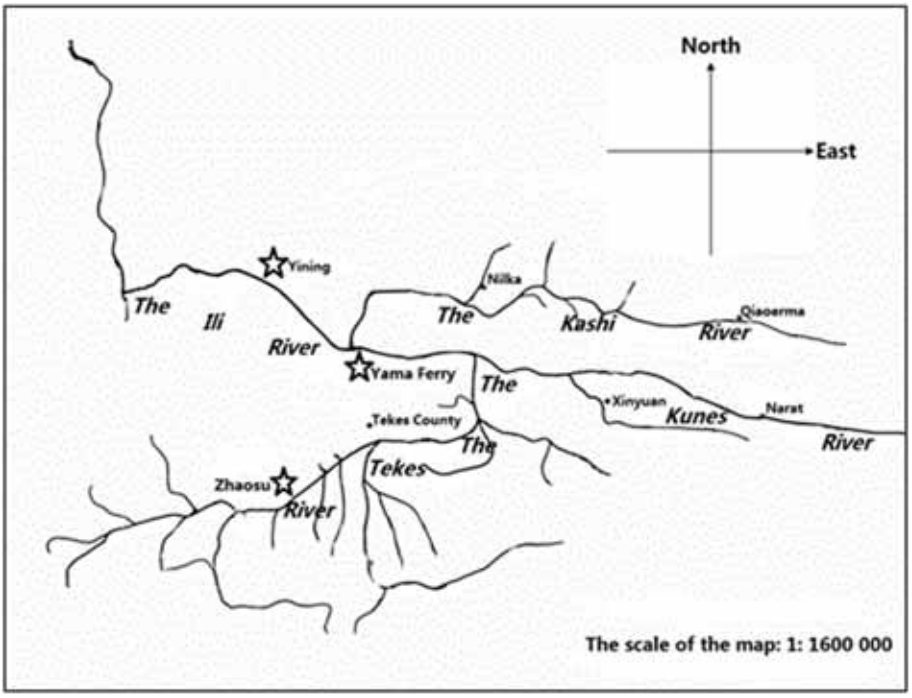

Figure 1. Hydrologic map of Ili river drainage. Open pentagram represents the location of sampling sites.

at the branch (Tekes river). A total of 75 individuals were collected from the three sampling sites, with 32, 32 and 11 specimens from Yining, Yama Ferry and Zhaosu, respectively. All dorsal fin clips were preserved in 95% ethanol and then refrigerated at −20°C until analysis. Besides, the morphological indices such as fish length and weight were determined in field work.

To obtain microsatellite markers of *S. pseudaksaiensis*, we referred to its closely-related species *S. biddulphi* and *S. o'connori* in the same genus. For these two fish species, their microsatellite markers have been developed by high-throughput sequencing (Luo *et al.* 2012) and traditional method with constructing microsatellite-enriched libraries (Guo *et al.* 2013). Thirteen markers from *S. biddulphi* and 19 markers from *S. o'connori* were used to test the efficiency of cross-amplification. The agarose gel electrophoresis of 1% concentration was used as a preliminary selection and polyacrylamide gel electrophoresis for further screening.

Genomic DNA from fin tissue was extracted using a modified ammonium acetate precipitation protocol (Li *et al.* 2011). Polymerase chain reaction (PCR) amplifications were conducted in a 10 μL volume containing 1× Taq buffer (Takara, Dalian, China), 0.5 μmol/L each of primer, 200 μmol/L dNTPs, 0.5 U *Taq* DNA polymerase (Takara) and 50 ng genomic DNA under the following programme: a predenaturation at 94°C for 5 min, followed by 32 cycles of denaturation at 94°C for 30 s, locus-specific annealing temperature for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min. Amplified products were electrophoresed in 4% nondenaturing polyacrylamide gel and subsequently visualized by silver staining. A pUC18/Msp I marker (Tiangen, Beijing, China) was used as the standard to identify the sizes of alleles. PopGene 32 (Yeh *et al.* 2000) and Convert 1.3 were used to calculate the number of alleles, observed heterozygosity, expected heterozygosity as well as genetic distance and similarity. Polymorphism information content (PIC) for each microsatellite marker was calculated by means of biological software PIC_Calc 0.6 based on traditional computational method (Botstein *et al.* 1980).

Results and discussion

For the 13 markers from *S. biddulphi* and 19 markers from *S. o'connori*, eight (61.54%) and 12 (63.16%) loci could be amplified successfully in *S. pseudaksaiensis*. As a result, 10 polymorphic markers were characterized for *S. pseudaksaiensis*, with two (15.38%) from *S. biddulphi* and eight (42.11%) from *S. o'connori* (table 1). The closer genetic relationship of tested species contribute better to cross-species amplification (Lin *et al.* 2008). Our result possibly suggest that *S. pseudaksaiensis* has more closer genetic relationship with *S. o'connori* than *S. biddulphi*. However, further research is necessary to make it clear.

Genetic diversity is one of the inherent biological characteristics and considered as a result which adapts to the environment and evolutes for a long time. Higher genetic diversity represents more potential for breeding and genetic improvement. PIC and genetic heterozygosity are indispensable indices to evaluate the level of genetic diversity. Present study shows that the overall 10 microsatellites loci were polymorphic with 2–26 alleles (averaged 10.97). Particularly, *Schs3* exhibited low N_E value (only one) at each population, probably suggesting lower availability and polymorphism for *Schs3* in contrast with other nine microsatellite markers.

Table 1. Characteristics of 10 microsatellites isolated for *S. pseudaksaiensis* from closely-related species.

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Size (bp)	N_A	H_O	H_E	Accession number
Schs1	F: CCACTTACTAGAAAAGGGAT R: TGGGAACCAGAGACTTATT	$(AGAC)_9 \ldots (AGAT)_{19}$	58	170–288	20	0.78	0.93	KC902768
Schs2	F: CGTCTATTGTCTGCTCATCA R: ATCTGCTTACGCCCCAT	$(ATAG)_{14}$	56	142–188	12	0.59	0.87	KC902770
Schs3	F: CAGAGAACTGACCACCGC R: AAGGGACTCCCGTATTGTA	$(ATCC)_{10}$	56	154–187	10	0.62	0.87	KC902771
Schs4	F: TCTTGAGCACTTGGAAATG R: GTCTACAGCCAGCAGAAAAT	$(AGAC)_9$	56	181–287	27	0.89	0.95	KC902773
Schs5	F: AGTTTTCTCTCTTTGCTCTTCT R: ATCAGGGTAGTGCGTCATT	$(ATCT)_{17}$	60	128–215	25	0.85	0.93	KC902781
Schs6	F: AACTCTCTGGATTTGGATTG R: CAGCACAACATCAACACTCT	$(ATCT)_{15}$	54	234–310	22	0.88	0.94	KC902782
Schs7	F: TAGAGGAGGATGGGTGAGAA R: CCAACACTGCGAACGATAG	$(TCTA)_9$	54	198–291	21	0.72	0.91	KC902783
Schs8	F: TGTCTCTAATTGATTTCGGGT R: CATAGGTGCCAGTGACTTGA	$(AGAC)_5$	60	220–300	24	0.77	0.94	KC902784
Schs9	F: CGCCAGCGTCTGCCACAA R: GCCGCCATCTTCACCCAC	$(AGC)_5$	58	220–316	6	0.64	0.75	JX473043
Schs10	F: TGCCTCAAGGAACTGGTG R: GAGCATTAGAGTATCGTGGT	$(CGACG)_5$	50	154–174	2	0.39	0.39	JX473044

T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. *Schs1* to *Schs8* were selected from *S. o'connori*, while *Schs9* and *Schs10* were from *S. biddulphi*.

According to the standard proposed by Botstein *et al.* (1980), eight of the markers are highly polymorphic (PIC values > 0.5) except *Schs3* and *Schs10*. A higher PIC value (averaged 0.723) was observed in Yining population and implied a more abundant genetic diversity in Yining.

Genetic heterozygosity which includes observed heterozygosity and expected heterozygosity, reflects the genetic variation of loci across groups. The observed heterozygosity ranged from 0.094 to 0.719 (averaged 0.494) in Yining population, 0.063 to 0.688 (averaged 0.50) in Yama Ferry population and 0.091 to 0.818 (averaged 0.573) in Zhaosu population (table 2). The expected heterozygosity ranged from 0.089 to 0.943 (averaged 0.742), 0.061 to 0.912 (averaged 0.715) and 0.087 to 0.905 (averaged 0.721) for populations Yining, Yama Ferry and Zhaosu, respectively. Yining population had higher genetic variation, and this was consistent with the consequence of genetic diversity revealed by PIC value (table 2).

In the 1970s, *S. pseudaksaiensis* was an economically important fish with an annual yield of 50 t (Wang 1998). Due to the influence of human activities, stocks of this species have declined sharply and could not form sustainable yield. Hence, it is urgent to protect the germplasm resources, especially for populations with lower genetic diversity (e.g. Yama Ferry and Zhaosu populations).

Unlike genetic diversity, genetic differentiation analysis could determine whether the phylogenetic relationship among populations is far or near. Thus, the pairwise genetic distance and genetic similarity were evaluated (table 3) according to Nei's (1978) formula. Our study suggests that pairwise genetic distance varied from 0.2754 to 0.3924 across populations, while genetic similarity ranged between 0.6754 and 0.7593. Specifically, Yama Ferry and Zhaosu populations showed the lowest pairwise genetic distance (0.2754) and the highest pairwise genetic similarity (0.7593), respectively. Slight genetic differentiation was found across

Table 2. Basic results for 10 microsatellite markers applied in three populations of *S. pseudaksaiensis.*

	Yining population (32 individuals)					Yama Ferry population (32 individuals)					Zhaosu population (11 individuals)				
Locus	N_A	N_E	H_O	H_E	PIC	N_A	N_E	H_O	H_E	PIC	N_A	N_E	H_O	H_E	PIC
Schs1	22	15	0.625	0.934	0.93	17	11	0.625	0.909	0.902	10	9	0.636	0.893	0.882
Schs2	11	3	0.5	0.736	0.699	10	6	0.688	0.837	0.82	8	4	0.455	0.793	0.767
Schs3	2	1	0.094	0.089	0.085	2	1	0.063	0.061	0.059	2	1	0.091	0.087	0.083
Schs4	25	16	0.75	0.941	0.938	17	9	0.75	0.899	0.891	13	9	0.636	0.888	0.879
Schs5	19	9	0.469	0.898	0.89	14	8	0.563	0.889	0.878	9	6	0.546	0.831	0.813
Schs6	20	14	0.719	0.931	0.926	16	11	0.531	0.912	0.906	13	10	0.636	0.905	0.897
Schs7	26	17	0.594	0.943	0.94	11	7	0.438	0.869	0.855	8	6	0.546	0.843	0.824
Schs8	12	4	0.125	0.778	0.75	7	4	0.406	0.76	0.724	6	3	0.546	0.653	0.622
Schs9	9	2	0.438	0.598	0.574	8	4	0.688	0.792	0.763	7	5	0.818	0.801	0.776
Schs10	4	2	0.625	0.574	0.502	3	1	0.25	0.225	0.21	3	2	0.818	0.517	0.422
Mean	15	8.3	0.494	0.742	0.723	10.5	6.2	0.5	0.715	0.701	7.9	5.5	0.573	0.721	0.697

N_E, effective number of allels; PIC, polymorphism information content.

Table 3. The indices of genetic distance (below diagonal) and genetic similarity (above diagonal) of *S. pseudaksaiensis* among three populations.

Population	Yining	Yama Ferry	Zhaosu
Yining	–	0.6965	0.6754
Yama Ferry	0.3617	–	0.7593
Zhaosu	0.3924	0.2754	–

three populations, Yama Ferry and Zhaosu populations indicated closer genetic relationship. Such results were ascribed primarily to geographical environment. Yama Ferry and Zhaosu populations were from the tributaries of Ili river and may show similar biotope. In contrast, Yining was located in the mainstream of Ili river and the biotope differed from those in Yama Ferry and Zhaosu. Cai *et al.* (2014) have studied the genetic differentiation of *S. pseudaksaiensis* along the Ili river using mitochondria DNA markers, and also revealed closer relationship between Yama Ferry and Zhaosu populations. Although, a slight genetic differentiation occurred, it is just limited to individual differences and did not reach the subspecies level (Cai *et al.* 2014). Such result was in agreement with the present study.

Ten polymorphic microsatellite markers were characterized for *S. pseudaksaiensis* for the first time. Our findings proved that cross-species amplification provides a useful tool to develop microsatellite markers for those related species in *Schizothorax* genus. Further study is necessary for the population genetic structure of *S. pseudaksaiensis* and will help conservation management of the species.

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