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Short Communication

Development of Microsatellite Markers for Three *Schizothorax* Species: *S. macropogon*, *S. oconnori* and *S. waltoni*

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HY, LW, GJ, YW, WL and JC conceived and designed the research. QT, XW, YR, MS and XZ wrote the manuscript with contributions. HX, KC and LL analysed the data. HY, HS and ZG carried out the experiment.

Key words
Microsatellite, *Schizothorax macropogon*, *Schizothorax oconnori*, *Schizothorax waltoni*, Population genetics, Genetic tools

ABSTRACT

Schizothorax spp. has experienced a rapid decline in wild population size. Evaluating its genetic diversity and population genetic structure is of critical importance. We developed ten microsatelittes for *S. macropogon*, *S. oconnori*, and *S. waltoni*, which will be useful for genetic analysis. The expected heterozygosity (H_E), observed heterozygosity (H_O), Shannon-Weiner diversity indices (H'), and polymorphic information content (*PIC*) for these 10 microsatellites varied within the ranges of 0.834 to 0.942, 0.643 to 0.899, 1.35 to 1.66, and 0.655 to 0.799, respectively. The 10 microsatellites in this study have shown high allelic polymorphism, suggesting that these markers will compensate for the lack of genetic tools available for the three *Schizothorax* species.

Schizothorax macropogon, belonging to the family Cyprinidae and subfamily Schizothoracinae, is a unique tetraploid fish primarily found in the mid-sections of the Yarlung Tsangpo River in Tibet, China. This species exhibits adaptations to its harsh environment, such as cold-tolerance, slow growth rate, and delayed sexual maturation. Over the past several decades, there has been a precipitous decrease in the wild population of *S. macropogon*. This decline is largely attributable to factors such as overexploitation, dam construction, and the incursion of non-native fish species. Consequently, *S. macropogon* has been classified as a near-threatened species in the IUCN Red List of Threatened Species. *S. oconnori* (Lloyd, 1908), a member of the subfamily Schizothoracinae, family Cyprinidae, is a significant economic fish species native to the Qinghai-Tibet Plateau in China. The primary habitat of this species is the Yarlung Zangbo River drainage in Tibet, China (Ji, 2008), while certain data indicate that *Schizothorax oconnori* populations are locally abundant (Zhang *et al*., 1995) and not currently negatively impacted by local fishing activities. There is growing concern that escalating fishing pressure and water pollution associated with economic development may precipitate a decline in *S. oconnori* populations in future years (Zhang, 2011). The IUCN Red List has classified *S. oconnori* as a species of least concern. Nevertheless, there is a pressing need to gather additional data regarding the population trends of this species (Ng, 2010). Given the current lack of comprehensive information, further research into *S. oconnori* is urgently required. *S. waltoni*, 1905, is predominantly found in the main body and tributaries of the Yarlung Zangbo River, from Xigaze to the Milin section. This species exhibits an omnivorous diet, primarily consuming benthic invertebrates and algae (Lin *et al*., 2010). Significantly, due to a notable decrease in population numbers, *S. waltoni* has been conferred the status of secondary key protected wildlife at the national level in China.

The conservation status of the three endemic fish species from the Tibetan Plateau, is increasingly of concern due to environmental changes and anthropogenic pressures. However, their habitats are under threat from climate change, overfishing, and habitat fragmentation.

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Effective conservation strategies are urgently needed to prevent further declines. One promising approach to enhance conservation efforts is the application of genetic tools, which can provide valuable insights into the population structure, genetic diversity, and evolutionary history of these species (Hewitt, 2004; Yang *et al*., 2012).

Recent studies have highlighted the critical role of genetic diversity in the resilience of fish populations to environmental changes. For instance, the use of microsatellite markers and mitochondrial DNA analysis has been pivotal in understanding the genetic variability and phylogeographic patterns of various fish species (Kumar *et al*., 2004). Specifically, study on *Schizothorax oconnori* in the Yarlung Tsangpo River revealed significant genetic differentiation, indicating the presence of distinct population groups that require tailored conservation strategies (Yao *et al*., 2009).

Genetic tools also enable the identification and correction of genotyping errors, which is crucial for accurate population genetic studies (van Oosterhout *et al*., 2004). Moreover, the development of software such as MEGA3 and Micro-checker has facilitated the analysis of genetic data, allowing for more precise estimates of genetic diversity and population structure (Kumar *et al*., 2004; van Oosterhout *et al*., 2004). These advancements underscore the importance of integrating genetic tools into conservation planning for *Schizothorax* species.

Environmental factors such as glacial activity and river dynamics have historically influenced the distribution and genetic makeup of these species. Studies on the glacial history of the Tibetan Plateau and the megaflood events in the Tsangpo River gorge provide context for understanding the evolutionary pressures faced by *Schizothorax* species (Li *et al*., 1986). Additionally, climatic oscillations during the Quaternary period have left a significant imprint on the genetic landscape of these fish, further complicating conservation efforts (Hewitt, 2004).The integration of genetic data with ecological and environmental studies offers a comprehensive approach to the conservation of three *Schizothorax* species (Hubert *et al*., 2007).

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are segments of DNA consisting of motifs that range from one to 10 nucleotides in length, repeated between 5 and 50 times (Vieira *et al*., 2016). These genetic markers are invaluable in investigating population structure and genetic diversity, thereby informing supportive stocking programs. Microsatellites can be utilized to assess animal breeding practices and establish pedigree animal populations, thereby facilitating genetic improvement (Weising *et al*., 1997). They have been extensively employed for genetic analysis in aquatic species, including *Acipenser sinensis* (Hu *et al*., 2020), *Exopalaemon carinicauda* (Zhang *et al*., 2020), and *Oreochromis niloticus* (Zhao *et al*., 2016). However, the application of microsatellites in the study of *Schizothorax* species has been limited. To aid in their conservation, we have developed a set of microsatellite loci for population genetic research. This will enable the estimation of gene flow, genetic diversity, and the potential existence of metapopulations within these species.

Materials and methods

Total 30 individuals of *S. macropogon*, *S. oconnori*, and *S. waltoni* (10 individuals of each species) were obtained from aquaculture farms in Tibet, China and their fins were preserved in alcohol. Genomic DNA was isolated through proteinase-K digestion and phenol/chloroform extraction, and its integrity was confirmed via 1% agarose gel electrophoresis.

The microsatellite identification tool was utilized to discern sequences within the *Schizothorax lantsangensis* genome harboring perfect tetranucleotide repeat motifs, following the protocol delineated by Beier *et al*. (2017). The parameters of the tool were set to detect sequences with a minimum of 6 repeat units. Upon pinpointing these sequences, microsatellite primers were engineered using the Primer Premier 5.0 software. The primer pairs were designed to encompass each microsatellite, with an optimal annealing temperature established at 56°C. The anticipated length of the PCR products was configured to fall within the range of 100 to 400 base pairs.

The genomic DNA derived from the three *Schizothorax* species was harnessed to probe for polymorphic microsatellites and to optimize the amplification conditions. The PCR amplification was undertaken in 25μl reaction mixtures, each encompassing 0.25μM of each primer, 1.5 mM $MgCl_2$, 0.25U of Taq polymerase (TaKaRa Taq), 0.25μM of the PCR buffer (TaKaRa Taq), 0.25μM of dNTPs, roughly 50-100 ng of template DNA, and ultra-pure water to adjust the volume. The amplification regimen was as follows: An initial phase at 94ºC for 3 min, followed by 35 cycles of 30 sec of denaturation at 94ºC, annealing for 30 sec at 56ºC, and extension for 30 sec at 72ºC. This was succeeded by a final elongation phase at 72ºC for 10 min.

The resultant PCR products were scrutinized using 10% polyacrylamide gel electrophoresis (PAGE). The pBR322 DNA/Mspl marker (Takara) was utilized as a molecular size benchmark for the electrophoresis.

The ATetra1.2 software was leveraged to calculate the observed heterozygosity (H_O), mean expected heterozygosity (H_E), and Shannon-Weiner diversity indices (H') in accordance with the procedure delineated by Van *et al*. (2010). The polymorphic information content (*PIC*) was ascertained using the equation (Equation 1) where Pi and Pj denote the frequencies of the I and J alleles at each

microsatellite locus, respectively.

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 P_i^2 P_j^2 \quad ...(1)$$

Allele sizes were determined using the Gene Marker software, whereas genotypic data were analyzed using Microsoft Office Excel 2007. A dendrogram illustrating the allele phenotypes of ten randomly chosen *S. macropogon* individuals was constructed employing MEGA software, adhering to the method of Kumar *et al*. (2004).

The FaMoz software was utilized to compute the exclusion and combined exclusion probabilities based on allele frequencies, following the strategy outlined by Gerber *et al*. (2003). It is imperative to avert the manifestation of allele dropouts and false alleles during the genetic data analysis stage of the experiment.

Results and discussion

In this study, a suite of 200 microsatellite primers was designed with the intention of formulating PCR reactions. These primers were trialed on 10 specimens of *Schizothorax macropogon*, *Schizothorax oconnori*, and *Schizothorax waltoni*, among which 10 microsatellites demonstrated distinct polymorphism (Table I). The number of discerned alleles for these 10 microsatellites oscillated between 12 and 16.

Table I. Characterization of PCR reactions in *S. macropogon*, *S. oconnori*, and *S. waltoni* annealing temperature (°C).

Locus	Primer sequences (5′-3′)	Repeat motif (s)
LFY1	F AATGTGGGCTGATGGG	TGTC
	R TGCGTGCGTCTGTCTG	
LFY2	F GAATGAATGAACAAGCAA	AAAT
	R AAATGGCACTGAATAACA	
LFY3	F TGCATTCGATAAGACAGA	TCTA
	R CATTGTTAAGTGGTTGCTAA	
LFY4	F GGTTGGTTTGGATAGTTG	CTAT
	R TCCCAGAATCCCTTGA	
LFY5	F CTCGATGACATCAGAAACTA	AATA
	R CACCGAACCCAAACTT	
LFY6	F AGCCGCAGAAATGTAG	TCAT
	R CACTGGTGGTGGTTGA	
LFY7	F AAACTCAGGACGAGGTT	GAGT
	R ATCTACAGCACAGACGG	
LFY8	F GGTTCCTGAAGGGTCT	AAAT
	R CTGCTTATTGTAAAGTGTTA	
LFY9	F CTGCGTAGGTGAGTGC	GTGA
	R CCATCAACTAAACGACTG	
LFY10	F TGCTGGAGGGTGAGAC	GACA
	R GACAGAAAGATGGACGGA	

The expected heterozygosity (H_E), observed heterozygosity (H_O), Shannon-Weiner diversity indices (H'), and polymorphic information content (PIC) for these 10 microsatellites varied within the confines of 0.834 to 0.942, 0.643 to 0.899, 1.35 to 1.66, and 0.655 to 0.799, respectively (Table II).

Grounded on the results procured for the 10 microsatellites from the *S. macropogon*, *S. oconnori*, and *S. waltoni* specimens, the PCR reactions yielded stable PCR products. This evidence intimates that these 10 microsatellites can function as valuable markers for genetic analysis in the three *Schizothorax* speices.

Table II. Genetic diversity of ten microsatellites in PCR reactions of *S. macropogon*, *S. oconnori*, and *S. waltoni*.

Locus	Na	H_E	H_O	H'	PIC
LFY1	13	0.917	0.851	1.46	0.673
LFY2	12	0.867	0.643	1.66	0.675
LFY3	14	0.895	0.699	1.46	0.713
LFY4	12	0.834	0.746	1.47	0.693
LFY5	13	0.911	0.778	1.35	0.723
LFY6	13	0.856	0.744	1.56	0.714
LFY7	16	0.942	0.899	1.66	0.799
LFY8	15	0.888	0.823	1.46	0.655
LFY9	14	0.876	0.861	1.54	0.684
LFY10	12	0.894	0.769	1.51	0.655

H_E, expected heterozygosity; H_O, observed heterozygosity; H′, Shannon-Weiner diversity indices; PIC, polymorphic information content

Cross-species amplification was investigated in the three *Schizothorax* speices. Ten of 200 loci amplified successfully in all three *Schizothorax* species under the PCR conditions. H_E, H_O, H′, and PIC for these 10 microsatellites varied within the ranges of 0.834 to 0.942, 0.643 to 0.899, 1.35 to 1.66, and 0.655 to 0.799, respectively (Table II). The ten microsatellites reported in this study showed polymorphism in *Schizothorax* spp.

To better contextualize our findings, we compared the developed markers with microsatellite markers from similar studies on other fish species. For instance, in a study on *Schizothorax prenanti,* researchers identified microsatellites with lower polymorphism levels, with heterozygosity values averaging around 0.50 (Wu *et al*., 2012). Additionally, studies on *Cyprinus carpio* (common carp) reported microsatellites with moderate polymorphism (heterozygosity ranging from 0.40 to 0.75), demonstrating that our markers for *Schizothorax* species are among the more polymorphic identified for fish species in general (Thai *et al*., 2007).

The high allelic richness observed in our study may be attributed to the ecological and evolutionary factors specific to the three *Schizothorax* species. These species are distributed across a diverse range of habitats in the Qinghai-Tibet Plateau, a region known for its complex geological history and high biodiversity. The high polymorphism levels might reflect the adaptive genetic variation necessary for survival in such a varied environment.

Microsatellite markers have been recognized as indispensable instruments in the genetic preservation and administration of fish species, with a plethora of studies corroborating their effectiveness in broodstock management and evaluation of population structure (Wozney *et al*., 2011; Boscari *et al*., 2014). In this investigation, we successfully developed ten new microsatellites, which demonstrated utility for genetic analysis in three *Schizothorax* speices. These values indicate a high degree of genetic differentiation, suggesting that these loci are suitable for characterizing population structure.

Nonetheless, given the polyploid characteristic of the three species, it is impracticable to determine the exact copy number per locus. As a result, we were unable to execute conventional tests for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium. Future studies utilizing complementary genetic tools and techniques, such as next-generation sequencing, may help address these limitations and provide a more comprehensive understanding of the genetic architecture in these species.

The development of these highly polymorphic microsatellite markers is important for understanding the genetic diversity is crucial for conservation management in formulating strategies to protect genetic resources and manage fishery stocks. Secondly, these markers can be utilized to study the evolutionary history and phylogeography of the *Schizothorax* genus, offering insights into the processes that have shaped their current distribution and genetic diversity.

DECLARATIONS

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Ethical statement

All experiments were carried out in accordance with relevant guidelines and regulations. The study was carried out in compliance with the ARRIVE guidelines.

Statement of conflict of interest

The authors have declared no conflict of interest.

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