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Development of twenty-one polymorphic tetranucleotide microsatellite loci for *Schizothorax o'connori* and their conservation application

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ABSTRACT

Schizothorax o'connori, of the subfamily Schizothoracinae, is an endemic fish in the Yarlung Tsangpo River in Tibet, China. To evaluate the population genetic diversity as a means of devising conservation strategies, a total of 21 polymorphic tetranucleotide microsatellite loci were isolated from *S. o'connori* using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol. The characterization of these loci was assessed against a panel of 42 individuals. The number of alleles per locus ranged from 4 to 29 with an average of 17.76. Expected locus-specific heterozygosity and the multilocus Shannon–Wiener diversity index ranged from 0.114 to 0.951 and from 0.280 to 3.131, with an average of 0.851 and 2.338, respectively. The cross-species amplification and applicability of these loci were tested against the two other *Schizothorax* species from the same river. These microsatellite loci will be valuable for further studies of population genetic diversity and genetic structure, and assessments of the artificial propagation release effect of *S. o'connori* and other related species.

1. Introduction

Schizothorax (Teleostei: Cyprinidae) is a genus of more than 60 valid species from central and eastern Asia. In China, more than 30 *Schizothorax* species are widely distributed in different rivers and lakes on the Qinghai-Tibetan Plateau and its adjacent area (Cao et al., 1981; He and Chen, 2006), but only 15 *Schizothorax* species are found in Tibet, of which only three species, *Schizothorax o'connori*, *Schizothorax waltoni* and *Schizothorax macropogon*, are distributed in the Yarlung Tsangpo River Basin (Fisheries Bureau of Tibet Autonomous Region, 1995). Karyotype analysis and DNA content measurement indicate that the different species of the subfamily Schizothoracinae may be tetraploid, hexaploid and octoploid (Zan et al., 1985, 1986; Li et al., 1987; Yu et al., 1987, 1990; Wu et al., 1999). This may be associated with the complicated environment during the uplift of the Qinghai-Tibetan Plateau (Zan et al., 1985; Yu et al., 1990). *S. o'connori* is an endemic tetraploid Schizothoracine fish

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that is distributed in the main streams and tributaries along the upper and middle reaches of the Yarlung Tsangpo River Basin in Tibet, China (Fisheries Bureau of Tibet Autonomous Region, 1995; Wu et al., 1999). It is characterized as a cold-adapted, low growth rate, late sexual maturity and low fecundity species. Because of these life-history characteristics, *S. o'connori* is now threatened as a consequence of sharp declines in population size due to overfishing and biological invasions by species such as crucian carp (*Carassius auratus*) and topmouth gudgeon (*Pseudorasbora parva*) in the past few decades (Chen and Chen, 2010). Conservation and sustainable exploitation of natural populations have become a primary concern.

Previous studies (more than 200 reports and papers) of *S. o'connori* have focussed on the biology (Yao et al., 2009; Ma et al., 2010, 2011), phylogenetics and biogeography (He and Chen, 2006, 2009; Yang et al., 2012) and evolution of trophic morphologies (Qi et al., 2012). Recently, artificial propagation of *S. o'connori* has been successful and artificial releasing of juveniles is expected to help to enhance the local stock (Xie and colleagues, work in progress). However, there is limited information about the genetic diversity and population genetic structure of this species. Microsatellites or simple sequence repeats (SSR) are co-dominant genetic markers that have been widely used in many studies of genetics because of their high variability, polymorphism, ease and reliability of scoring (Zane et al., 2002). To assist in the conservation and recovery of *S. o'connori* through future population genetic studies, it is needed to develop sufficient microsatellite markers for this species.

In this study, we report on the development of 21 polymorphic tetranucleotide microsatellite loci in *S. o'connori* and characterization of these loci by genotyping 42 individuals collected from the Yarlung Tsangpo River. Additionally, cross-species amplification and applicability were carried out in two other *Schizothorax* species from the same river, *S. waltoni* and *S. macropogon*. We specifically targeted the development of tetranucleotide microsatellite markers because they tend to be more informative and more stable under PCR conditions than di- or tri-nucleotide repeats.

2. Materials and methods

2.1. Sample collection and DNA extraction

Specimens of *S. o'connori* ($n = 42$), *S. waltoni* ($n = 10$) and *S. macropogon* ($n = 10$) were collected in 2012 from the Xaitongmoin section of the Yarlung Tsangpo River in Tibet, China. Fin clips were collected and preserved in 95% ethanol. Genomic DNA was extracted using the traditional phenol-chloroform extraction protocols. Concentration and quality of DNA were estimated using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc.).

2.2. Isolation of microsatellites

Genomic libraries enriched for microsatellite motifs were constructed using the fast isolation by AFLP of sequences containing repeats (FIASCO) method (Zane et al., 2002) with minor modification. The methods for constructing the $(AGAT)_n$-microsatellite enriched library and the screening positive clones followed Xu et al. (2013). The positive clones were identified by PCR using vector primers. Sequencing of positive clones was conducted by the Sangon Biotech Company (Shanghai, China) using an ABI PRISM 3730 sequencer (Applied Biosystems, USA). Primer pairs for each microsatellite locus were designed using Primer Premier 5.0 (Premier Biosoft).

2.3. PCR amplification and genotyping

Each primer pair was screened for reliable amplification and polymorphisms of all microsatellite loci were assessed in 42 individuals of *S. o'connori*. The PCR amplifications were carried out in 10 μL volumes containing 1 × *Taq* reaction buffer (Fermentas, EU), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.5 μM of each primer, 0.5 U *Taq* polymerase (Fermentas, EU), and 40 ng genomic DNA. PCR conditions were as follows: an initial denaturation step of 5 min at 94 °C, followed by 25–30 cycles of 30 s at 94 °C, 30 s at locus-specific annealing temperature (see Table 1), and 45 s at 72 °C, followed by a final extension at 72 °C for 10 min. PCR products were separated on 8% non-denaturing polyacrylamide gel and visualized by silver staining. A 50 bp DNA ladder (Takara, China) was used as a standard to identify allele size. Cross-species amplification was performed for *S. waltoni* and *S. macropogon* under the same PCR conditions as described above. These two species were chosen because they are the only other members of the genus in the same river system as *S. o'connori*.

2.4. Data analysis

Up to four alleles at a single locus in *S. o'connori* individuals were found in this study, indicating that this species is a tetraploid. The number of alleles (N_A), expected heterozygosity (H_E) and Shannon–Wiener diversity index (H') were estimated using ATETRA version 1.2 software that was developed to analyze microsatellite data for tetraploid species (Van Puyvelde et al., 2010).

3. Results and discussion

In total, 116 positive clones were sequenced and 77 sequences contained microsatellite repeats. Twenty-eight pairs of primers were designed, of which 21 pairs amplified the DNA to yield the expected PCR products. We found that all 21