ONLINE RESOURCES

Isolation and characterization of twenty-one polymorphic microsatellite loci from *Schizothorax o'connori* and cross-species amplification

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Introduction

Schizothorax o'connori (Cyprinidae: Schizothoracinae) is an endemic fish that is distributed in the main streams and tributaries along the upper and middle reaches of Yarlung Tsangpo River basin in Tibet, China. It is a cold-adapted, long-lived, low growth rate, late sexual maturity and low fecundity species (Chen and Cao 2000). These life-history characteristics make it particularly susceptible to adverse effects of human activity. *S. o'connori* became threatened as a consequence of sharp decrease in population size due to overfishing and biological invasions in the past few decades, and conservation and sustainable exploitation of natural populations of the species have become a major concern (Chen and Chen 2010).

Previous studies of *S. o'connori* have focussed on the biology (Yao *et al.* 2009; Ma *et al.* 2010, 2011), phylogeography (He and Chen 2009; Yang *et al.* 2012) and evolution of trophic morphologies (Qi *et al.* 2012). There is limited information about population genetic variation and the conservation needs of the species. Microsatellites or simple sequence repeats (SSR) have proved to be useful markers for the study of genetic diversity, population genetic structure and genetic resource assessment in many fishes because of their codominance, high mutation rates and ease of scoring (Garcia de Leon *et al.* 1997; Huang *et al.* 2012). However, there is presently no information about microsatellite variation in *S. o'connori*.

In this study, 21 polymorphic microsatellite loci were first isolated and characterized in *S. o'connori*. Additionally, cross-species amplification and applicability of these loci were tested in five additional Schizothoracinae species, *S. waltoni*, *S. macropogon*, *Ptychobarbus dipogon*, *Schizopygopsis younghusbandi* and *Oxygymnocypris stewartii*.

Materials and methods

Sampling and isolation of microsatellites

Specimens of *S. o'connori* ($n = 46$), *S. waltoni* ($n = 10$), *S. macropogon* ($n = 10$), *P. dipogon* ($n = 10$), *Schizopygopsis younghusbandi* ($n = 10$) and *O. stewartii* ($n = 10$) were collected in 2012 from Xaitongmoin section of the Yarlung Tsangpo River. Fin clips were collected and preserved in 95% ethanol. Genomic DNA was extracted using the phenol–chloroform extraction method (Sambrook and Russell 2002). Genomic libraries enriched for microsatellite motifs were constructed as described in detail by Zane *et al.* (2002) with minor modifications. Genomic DNA was digested with *Mse*I (Fermentas, Vilnius, Lithuania) at 37°C for 3 h and ligated to *Mse*I adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using T4 DNA ligase (TaKaRa, Dalian, China). The digested–ligated product was diluted (1:10) and then amplified using *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) as primer. The amplified products were hybridized to 5′-biotinylated $(AC)_{10}$ probes at 48°C for 90 min. Probe-bound DNA fragments were then enriched by streptavidin-coated magnetic beads (Promega,

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Keywords. microsatellite; genetic diversity; Schizothoracinae; *Schizothorax o'connori*.

Table 1. Characteristics of the 21 polymorphic microsatellite loci isolated from *Schizothorax o'connori*.

Locus	Accession no.	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	Size range (bp)	N_A	H_O	H_E	PIC
Scho01	KC247930	$(TG)_{12}$	F: TAATGATAATGCCGTGTCGTA R: GAAACAGAAAACAGCCCAGAT	57	237–286	7	0.587	0.479	0.447
Scho02	KC247931	$(AC)_{21}$	F: CCGTGTGGGTAGAGGTGACTG R: CTGGCATCTGTGAGGGGCA	55	140–180	7	0.935	0.701	0.656
Scho03*	KC247932	$(AC)_9CG(CA)_{18}$	F: TCCTCATAAGTCACCTTCTCC R: TTGTGTGTTTTCTTGCTACG	52	145–170	6	0.422	0.758	0.712
Scho04*	KC247933	$(TG)_{12}$	F: TAACCGAGTCTTGCTTTCTAT R: AGGCTGCTGTGGTATTGG	55	185–220	5	0.957	0.626	0.548
Scho05*	KC247934	$(TG)_5TA\ (TG)_{10}$	F: ATGTTGGCACCCATAGCA R: CCTGAGTCTCCCGTCTTGT	56	205–222	5	0.543	0.462	0.400
Scho06	KC247935	$(TG)_{17}$	F: CGGTGACTGTATCTGTATCCA R: CAAAGAGCCGTTGTGTGC	55	163–194	6	0.870	0.654	0.617
Scho07*	KC247936	$(TG)_{33}\ (AG)_5$	F: CAGAGCAATAGATGTGTAACG R: GGGAGACCATAAGCAAAAG	50	191–271	19	0.689	0.906	0.887
Scho08*	KC247937	$(CA)_{18}$	F: ATTGGACAGGCAGTGGAGATG R: AGCGAGAATGAGAGAGGGGA	60	200–264	15	1.000	0.846	0.820
Scho09*	KC247938	$(AC)_{14}$	F: TAAGTATTCCCTCCCTGTCCT R: CAAACCCCAGAAGTGAAAGTG	58	205–243	12	0.587	0.883	0.860
Scho10*	KC247939	$(AC)_{31}$	F: GGTGGAGCAGAGGAAACTAC R: ATCTCTCACGCACGAACAG	55	150–207	18	0.957	0.925	0.909
Scho11	KC247940	$(AC)_{33}$	F: GATGTTGTGATTGGACAGAGG R: GTAGGATGGAGAAGGCGTG	57	181–233	13	0.935	0.892	0.871
Scho12	KC247941	$(TG)_{21}$	F: ATTTCCTCCCTCTTCGTC R: CTTCAGGCATACTCATTAGC	52	190–234	7	0.978	0.798	0.762
Scho13	KC247942	$(TG)_{23}$	F: TTCTTGTTTACAGGTTGCTCA R: CGGAATAATAATCAGTGGCTC	52	206–240	7	0.711	0.543	0.497
Scho14	KC247943	$(AC)_{17}$	F: GTGAAAGATGCGACTGAGC R: ATGTGGCGTTATGATGTGAT	55	177–219	14	0.913	0.870	0.846
Scho15	KC247944	$(CT)_{12}...(CA)_{13}$	F: CGAAGGCGTGAACGAGAGT R: GTGCGTCAATGAGTCCAGATG	59	210–280	14	0.674	0.799	0.766
Scho16	KC247945	$(TG)_{11}$	F: GATGAGATGGGGTCCAGATGA R: GTCCTCCGAGAAAACAGCAGA	62	140–170	6	0.630	0.793	0.750
Scho17	KC247946	$(AC)_{19}$	F: CAGTAGGGTTTGAGATGACA R: GAACAGTTAATGCGAGGAG	58	191–250	10	0.750	0.846	0.818
Scho18*	KC247947	$(TG)_{25}$	F: AGGAATAGACCCTTGTTGT R: AATACCTCTGTCATACCCAT	59	202–271	21	0.951	0.936	0.920
Scho19	KC247948	$(TA)_5(TG)_{14}$	F: TGGAGGTGTAAGTGTTGTCAT R: GAGGTGGTGTTGGAAGGT	59	138–172	6	0.913	0.641	0.564
Scho20*	KC247949	$(TG)_{10}$	F: GCTGACCTTCACGCCACA R: GGGTTGACACCATCCACACT	59	119–142	5	1.000	0.557	0.450
Scho21*	KC247950	$(GT)_8$	F: ATTACCACCAATCATACGCC R: CTCCTGTTTGCCACCATCT	60	193–242	10	1.000	0.786	0.745
Mean	–	–	–	–	–	10.14	0.810	0.748	0.707

T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphism information content.
* indicates significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($\alpha = 0.05$).

Table 2. Cross-species amplification of the 21 microsatellite loci in five Schizothoracinae species.

Locus	*Schizothorax waltoni*			*Schizothorax macropogon*			*Ptychobarbus dipogon*			*Schizopygopsis younghusbandi*			*Oxygymnocypris stewartii*		
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
Scho01	4	0.600	0.500	6	0.700	0.721	6	0.500	0.879	14	1.000	0.968	4	0.100	0.626
Scho02	10	1.000	0.895	2	1.000	0.526	4	0.700	0.626	3	0.900	0.616	5	1.000	0.779
Scho03	6	0.600	0.811	4	0.700	0.611	–	–	–	–	–	–	–	–	–
Scho04	8	1.000	0.837	4	1.000	0.684	2	1.000	0.526	4	1.000	0.621	1	0.000	0.000
Scho05	2	1.000	0.526	3	1.000	0.574	3	0.500	0.679	6	0.600	0.632	1	0.000	0.000
Scho06	5	0.800	0.600	4	0.900	0.674	–	–	–	–	–	–	–	–	–
Scho07	6	0.400	0.784	10	0.700	0.821	3	0.400	0.353	2	1.000	0.526	–	–	–
Scho08	8	0.900	0.884	2	0.500	0.395	–	–	–	–	–	–	–	–	–
Scho09	7	1.000	0.842	6	1.000	0.826	7	0.900	0.784	5	1.000	0.774	5	0.700	0.768
Scho10	9	0.778	0.915	8	0.800	0.863	2	1.000	0.526	–	–	–	7	0.900	0.821
Scho11	7	0.800	0.805	10	0.900	0.911	–	–	–	–	–	–	–	–	–
Scho12	4	0.400	0.363	3	0.200	0.195	–	–	–	12	1.000	0.942	1	0.000	0.000
Scho13	5	1.000	0.711	4	1.000	0.616	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
Scho14	2	0.900	0.521	3	0.300	0.279	2	1.000	0.526	3	1.000	0.574	1	0.000	0.000
Scho15	5	0.300	0.505	8	0.800	0.795	–	–	–	–	–	–	–	–	–
Scho16	11	1.000	0.932	2	1.000	0.526	–	–	–	–	–	–	–	–	–
Scho17	2	0.444	0.471	–	–	–	–	–	–	–	–	–	–	–	–
Scho18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Scho19	3	0.500	0.468	7	0.300	0.842	–	–	–	–	–	–	–	–	–
Scho20	2	0.200	0.189	2	0.900	0.521	2	0.200	0.189	3	0.143	0.385	–	–	–
Scho21	5	0.500	0.511	6	0.500	0.763	1	0.000	0.000	3	0.500	0.426	–	–	–
Mean	5.55	0.706	0.653	4.95	0.747	0.639	3.00	0.564	0.463	5.09	0.740	0.588	2.89	0.300	0.333

N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; –, indicates no cross-amplification.

Madison, USA). Captured DNA fragments were amplified again with primer *Mse*I-N and then ligated into the pMD18-T vector (TaKaRa) and transformed into *Escherichia coli* DH5α competent cells. The positive clones were identified by PCR using vector primers. Sequencing of positive clones was conducted using an ABI PRISM 3730 sequencer (Applied Biosystems, Foster City, USA). Primer pairs for each microsatellite locus were designed with PRIMER 3 program (Rozen and Skaletsky 2000).

PCR amplification and genotyping

Each primer pair was screened for reliable amplification, and polymorphisms of all isolated loci were assessed in 46 individuals of *S. o'connori*. The PCR amplifications were carried out in 10 μL volumes containing 1× *Taq* reaction buffer, 1.5 mM $MgCl_2$, 250 μM dNTPs, 0.5 μM of each primer, 0.5 U *Taq* polymerase (Fermentas), and 40 ng genomic DNA. PCR thermal conditions were an initial denaturation 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, and a final extension at 72°C for 10 min. PCR products were separated on 8% nondenaturing polyacrylamide gel electrophoresis and visualized by silver staining. Fifty base pair DNA ladder (Tiangen, Beijing, China) was used as a standard for identifying allele size. Cross-species amplification was investigated in five additional Schizothoracinae species under the same PCR conditions.

Data analysis

Micro-Checker ver. 2.2.3 (van Oosterhout *et al.* 2004) was used to assess genotyping data for scoring errors. The number of alleles, and the observed and expected heterozygosity at each locus was estimated using Popgene ver. 1.32 (Yeh and Boyle 1997). Cervus ver. 3.0.3 (Kalinowski *et al.* 2007) was used to determine the polymorphic information content (PIC) for each locus. Tests for Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were conducted using Arlequin ver. 3.11 (Excoffier *et al.* 2005). All *P* values were adjusted for multiple tests using sequential Bonferroni corrections (Rice 1989).

Results and discussion

A total of 24 primer pairs were designed, of which 21 (87.50%) were successfully amplified to yield the expected PCR products. We found that these 21 microsatellite loci were polymorphic in 46 individuals of *S. o'connori*. Their characteristics are described in table 1. The number of alleles per locus ranged from 5 to 21, with an average of 10.14. The observed and expected heterozygosity ranged from 0.422 to 1.000 and from 0.462 to 0.936, with an average of 0.810 and 0.748, respectively. The PIC per locus ranged from 0.400 to 0.920. Ten loci (Scho03, Scho04, Scho05, Scho07, Scho08, Scho09, Scho10, Scho18, Scho20 and Scho21) deviated from HWE after Bonferroni correction ($P < 0.05$). Micro-Checker testing indicated that three of the loci (Scho03, Scho07, and Scho09) exhibited an excess of homozygotes and potential evidence of null alleles. Null alleles are found in most taxa, and the presence of null alleles can sometimes be detected as an excess of homozygotes leading to deviations from HWE (Dakin and Avise 2004). Additionally, other factors such as nonrandom mating, reduction in effective breeding population or locus-specific selective pressure may be the causes for the deviations from HWE (Garcia de Leon *et al.* 1997). Genotypic linkage disequilibrium was found between one pair of loci (Scho04/Scho20) after Bonferroni correction ($P < 0.05$).

Cross-species amplification was investigated in five additional Schizothoracinae species from the Yarlung Tsangpo River (table 2). Seven of 21 loci amplified successfully in all five species under the PCR conditions developed for *S. o'connori*. Only locus Scho18 did not amplify in all five species. Twenty and 19 loci amplified and showed polymorphism in *S. waltoni* and *S. macropogon* in genus *Schizothorax*, respectively. On the other hand, 11 or 9 loci amplified in three other species of which there were two, one and five monomorphic loci in *P. dipogon*, *Schizopygopsis younghusbandi* and *O. stewartii*, respectively. This high level flanking sequence similarity among related species in genus *Schizothorax* is consistent with microsatellite DNA results obtained from a diverse group of organisms (Jones *et al.* 2004; Huang *et al.* 2012).

In conclusion, the microsatellite markers described here will be useful for assessing the genetic diversity and population genetic structure of *S. o'connori* and other related Schizothoracinae species, which may help in the effective conservation of these species.

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