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ONLINE RESOURCES

Isolation and characterization of fourteen novel microsatellite loci from *Brachymystax lenok tsinlingensi*

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Introduction

Brachymystax lenok tsinlingensis, a member of the family Salmonidae, is found only in the Heihe, Shitou, Xushui and Taibai rivers which are segments of the Qinling mountain's cold-water streams in Shaanxi province, China (Li 1966; Song and Fang 1984). Populations of this species are declining rapidly each year due to overexploitation, environmental pollution, low rate of natural regeneration and other causes (Froufe *et al.* 2004). Some populations have virtually disappeared owing to anthropogenic activities and overexploitation (Ren and Liang 2004) . Consequently, the Chinese government has given *B. lenok tsinlingensis* the status of a second-class protected wild animal in the China Red Data Book of Endangered Animals (Yang *et al.* 1999). Though population genetic assessments are important tools for management and conservation, little information is available on the genetic structure and genetic variation of *B. lenok tsinlingensis*. Microsatellites are currently the markers of choice for population genetic studies (Goldstein and Pollock 1997) and these markers have proved very useful for clarifying the evolutionary relationships of closely related populations (Takezaki and Nei 1996). In this study, we described the development of 14 novel polymorphic microsatellite markers of *B. lenok tsinlingensis* to provide more population genetic information on this species.

Materials and methods

Genomic DNA was extracted from samples of muscle tissue according to the simplified method of Laird *et al.* (1991). The repeat-enriched library was constructed following the protocol of Liu *et al.* (2013), with minor modifications. Briefly, ∼1 μg genomic DNA was digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, USA) and visualized on a 1.2% agarose gel. We used a gel extraction kit (TIANGEN Company, Beijing, China) to extract fragments of 400–1200 bps from the agarose gel. We then added the adapter (5′-GACGATGAGTCCTGAG-3′/5′-TACTCAGGACTCAT-3′) to both ends of extracted fragments with T4 DNA ligase (Promega, Madison, USA) at 16°C for 4 h (Liu *et al.* 2013). The diluted digestion–ligation mixture (1:10) was amplified using *Mse*I-N adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′) with following amplification conditions: initial denaturation at 94°C for 3 min; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; and a final extension at 72°C for 7 min. Subsequently, denatured PCR products were hybridized with 5′-biotin-$(AC)_8$-labelled oligonucleotide probes at 95°C for 5 min, and then incubated at 60°C for 2 h. The hybridized DNA with the potential repeat fragments was separated and captured by Streptavidin MagneSphere (Promega) according to the manufacturer's instruction. Five μL of the microsatellite-enriched fragments were amplified by polymerase chain reaction (PCR) with *Mse*I-N adaptor-specific primers using the same amplification conditions as mentioned above. PCR productions were then ligated into PGEM-T Easy vector (Promega) and transferred into DH5α competent cells (laboratorymade). The transformed mixture was plated on agar plates containing ampicillin, X-gal, and IPTG to cultivate at 37°C for 12 h. Subsequently, recombinants were screened by PCR using M13-F/M13-R universal primers (Qin *et al.* 2012).

Bands varying in size from 300 to 800 bp were randomly selected for sequencing. In total, 110 clones were

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sequenced. Microsatellite sequences were searched using the software SSRHUNTER 1.3 (Li and Wan 2005). A total of 34 sequenced clones containing microsatellite motifs were detected, and all motifs contained no less than five dinucleotide repeats. Primer 5.0 software (http://www.premierbiosoft.com/primerdesign/index.html) was used to design primers. The polymorphism of microsatellite loci was evaluated using a screening set of 50 individuals collected from Xushui river (Shaanxi province, China), which is located at the southern foot of the Qinling mountain. The PCR mixture consisted of 2.0 μL of 10× buffer, 0.4 μL of 5 U *Taq* polymerase (Takara, Tokyo, Japan), 0.4 μL of 0.25 mM each dNTPs, 2.0 μL of 2.5 mM $MgCl_2$, 0.4 μL gene-specific primer, and 20 ng template DNA in a total volume of 20 μL. PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 35 cycles of denaturation 45 s at 94°C, 45 s at a locus-specific annealing temperature (table 1), and 50 s at 72°C, with a final extension of 7 min at 72°C. PCR products were electrophoresed and separated on 8% polyacrylamide gel and visualized using silver staining.

The software POPGENE32 ver. 1.32 (Yeh *et al.* 2000) was used to calculate the number of observed alleles per locus (N_A). Observed heterozygosity (H_O), expected heterozygosity (H_E) (Nei 1978) and deviations from Hardy–Weinberg equilibrium (HWE) were determined with Tools for Population Genetic Analyses (TFPGA) ver. 1.3 (Miller 1997). We tested for link-age disequilibrium (LD) using GENEPOP program (online version through http://genepop.curtin.edu.au/) with default parameters. We applied Bonferroni corrections for tests of observed and expected heterozygosity and LD (Rice 1989).

Results and discussion

A total of 82 alleles were identified in 50 individuals and the number of alleles per locus ranged from 3 to 9, with an average of 5.9 (±SD) (table 1). Expected and observed

Table 1. Primer sequences, PCR conditions and characteristics for 14 microsatellite loci in *B. lenok tsinlingensis*.

Locus	Repeat motifs	Primer sequences (5′–3′)	T_m (°C)	Size range (bp)	N_A	H_E	H_O	HWE P	GenBank accession no.
TL24	$(GT)_{15}$	F: CTGAGGTTTCTGGGTGGA R: CTCGGACAAGGTTTACTTACTG	55	127–204	6	0.8857	0.7191	0.3011	KC244168
TL25	$(CA)_7$ $(AC)_{10}$	F: CTGTATTTGCTTCAAAGTCAC R: GAATCATGAGGTTGAACACGT	56	145–192	5	0.8750	0.7864	0.5089	KC244169
TL26	$(CA)_5$	F: AGCCCACGTTTAGAATGCC R: CACAGGGTGACTGGACTGC	58	176–248	7	0.6061	0.7402	0.3279	KC244170
TL27	$(TG)_{24}$	F: CGTGTTGGCCTTTCAG R: CGCATACTAACTCGCTAT	50	96–150	8	0.8430	0.8042	0.8647	KC244171
TL28	$(AC)_7$ $(AC)_5$ $(GT)_{10}$	F: ATGAGCCGTAACAAGGT R: TATACAAACGCAGGGAC	48	143–182	4	0.8992	0.8831	0.4827	KC244172
TL29	$(GT)_{13}A(TG)_8$ $TA(TG)_9$ $(TG)_5$	F: CCCATGAGACCTACTTG R: CACATCCGCATAGTCCA	50	139–280	3	0.8657	0.8825	0.0052	KC244173
TL30	$(GT)_{13}$	F: ATTGATTATGTGGCTTCGC R: ATGTCTCGCAACTTCAGTAA	54	92–165	4	0.6278	0.8231	0.2855	KC244174
TL31	$(GT)_{22}$	F: TAACCTGCAACAAAACATA R: GCGTATTCGTCTTCAGTAA	49	163–240	5	0.6652	0.7428	0.2126	KC244175
TL32	$(AGC)_3(TG)_6$ $(TG)_{44}(TG)_{10}$ $(GT)_7$ $(TG)_{13}$	F: CAGAAGTAGCAGCAGCGTAT R: TAGCGAGTCAGTATGGAACA	54	145–346	7	0.8625	0.7838	0.0185	KC244176
TL33	$(GT)_7A(CG)_7T$ $GC(GT)_{16}$	F: TGTCCCTGCCTTTATGTG R: CCATCTGGGTCTACCTTCA	53	88–195	8	0.7566	0.8443	0.3871	KC244177
TL34	$(TG)_{12}$	F: ACCCTCCTGTTGTATCACC R: GAAATGTTGGCAGCCCTA	55	105–180	4	0.8954	0.7856	0.2471	KC244178
TL35	$(CT)_4TTC$ $(TG)_{10}G(GT)_{17}$	F: AACACGGACCCTTGAACT R: AGGATGGGCAACGGGAAG	60	126–241	7	0.8035	0.7399	0.3488	KC244179
TL36	$(AC)_7(CA)_7$ $(CA)_5(CA)_8$ $(CA)_6(AC)_{11}$	F: GTTCCGTGCCTGAATGTTA R: GCAGTTGCCCAGATTGTTA	56	136–295	5	0.5287	0.6988	0.0501	KC244180
TL37	$(GT)_{21}A(TG)_{18}$	F: TCCCTTCAGCGAATAGAAAGAT R: CAACCAGCCATGTAAACAAACA	59	80–179	9	0.7484	0.7590	0.3792	KC244181

T_m, annealing temperature; N_A, number of alleles per locus; H_E, expected heterozygosity; H_O, observed heterozygosity; HWE P, the test for deviation from HWE.

heterozygosity ranged from 0.5287 to 0.8992 and from 0.6988 to 0.8831, respectively.

Three loci (*TL29*, *TL32* and *TL36*) showed significant deviations from HWE ($P < 0.05$) after sequential Bonferroni correction, which may be due to the small sample size or the presence of null alleles. No evidence was found for LD among loci at a 5% significance level. In addition, two loci (*TL29* and *TL36*) presented null alleles, but no evidence for stuttering and allelic drop-out was found in all loci ($P > 0.05$). In conclusion, we developed 14 polymorphic microsatellite markers for *B. lenok tsinlingensis* and these polymorphic microsatellites will be useful for genetic diversity analysis and maker-assisted breeding for *B. lenok tsinlingensis.*

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