Conservation Genet Resour
DOI 10.1007/s12686-012-9810-7

TECHNICAL NOTE

Isolation and characterization of eleven novel microsatellite loci of *Brachymystax lenok tsinlingensis*, a threatened fish endemic to Shaanxi, China

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Received: 22 September 2012 / Accepted: 29 October 2012

Abstract *Brachymystax lenok tsinlingensis* is an endangered freshwater fish confined to cold-water mountain streams and rivers in Qinling Mountains of Shaanxi province, China. We isolated and characterized 11 microsatellite loci for *Brachymystax lenok tsinlingensis* and examined them for 42 individuals from Xushui River in Qinling Mountain. The number of alleles per locus ranged from 3 to 8, with an average of 5.3. The expected (He) and observed (Ho) heterozygosities were 0.3483–0.8874 and 0.4183–0.8421, respectively. Two loci deviated significantly from Hardy–Weinberg Equilibrium after Bonferroni correction. We expect these novel microsatellite markers from *B. lenok tsinlingensis* to be useful for population genetic conservation studies of the species.

Keywords *Brachymystax lenok tsinlingensis* · Endangered species · FIASCO · Microsatellites

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Brachymystax lenok tsinlingensis, a small carnivorous fish belongs to the family Salmonidae, is only found in the Heihe, Shitou, Xushui and Taibai Rivers segments of the Qinling mountains' cold-water streams in Shaanxi province, China (Li 1996; Song 1987). Due to environmental degradation of its limited distribution regions and illegal overfishing, the population size of the fish significantly declined in recent years (REN Jian 2004). Therefore, *B. lenok tsinlingensis* has been listed as a second class state protected wild animal in China Red Data Book of Endangered Animals (Yang et al. 1999). The local government has established national natural reserve to protect its living environmental condition, but information about genetic diversity of the fish has no report. Microsatellite DNA markers are powerful tools for the investigations of genetic structure and population genetic assessments. In this study, 11 microsatellite novel markers from *B. lenok tsinlingensis* had been isolated.

Microsatellite loci were isolated using the method of fast isolation by AFLP of sequences containing repeats (FIASCO) (Zane et al. 2002). Genomic DNA was extracted from samples of muscle tissue using a standard traditional phenol-chloroform procedure (Sambrook 1989). A total of 250 ng genomic DNA was completely digested with restriction enzyme *Mse*I (Biolabs), and ligated to *Mse*I AFLP adaptors (5′-GACGATGAGTCCTGAG-3′/ 5′-TACTCAGGACTCAT-3′) using T4 DNA ligase (Fermentas). Diluted digestion-ligation mixture (1:10) was amplified using *Mse*I-N adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′) with following conditions:denaturation 3 min at 94 °C follow by 25 cycles of 30 s at 94 °C, 55 °C at 30 s, 1 min at 72 °C with a final extension 72 °C for 8 min. Approximately 1,000 ng of amplified PCR products were hybridized with 200 pmol of 5′-biotin-labeled oligonucleotide $(AC)_8$ probes in 250 μl

Published online: 06 November 2012

hybridization solution containing 4.2× SSC, 0.07 % SDS, denatured it at 95 °C for 5 min, and then incubated at 60 °C for 2 h. The hybridized DNA was mixed with 600 µl of Streptavidin MagneSphere (Promega) at room temperature for 30 min to separate and capture the potential repeat fragments by the magnetic field, and then washed magnetic beads three times in TEN 100 and three times in stringent washes (SSC 0.2x and 0.1 % SDS). The fragments containing microsatellites were released from the bead-probes with 50 µl TE at 95 °C for 5 min. The microsatellite enriched fragments were amplified for 30 cycles with *Mse*I-N adaptor-specific primers using same program as above. After electrophoresis on a 1.5 % agarose gel, the fragments of 400–1200 bp were purified using Gel Extraction Kit (Omega), and cloned into the pMD18-T plasmid vector (TaKaRa), and then transformed into *Escherichiacoli* competent cells (TOP10, Invitrogen). Positive clones were selected using blue/white screening. Insert-positive clones were tested by PCR using M13 universal primers.

A total of 96 positive clones with fragments of different insert length were screened and sequenced (Sangon Biotech Co., Ltd), in witch 28 with five or more repeats and suitable flanking sequences were suitable for primers design. Primers designed with the software Primer 3 (Rozen and Skaletsky 2000). Characterization of these microsatellites was assessed in a sample of 42 individuals collected from Xushui River (Shaanxi province, China), which located at the southern foot of the Qinling mountain, PCR amplification was performed in a 10 µl volume containing the following components: 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM each primer, 1×Taq buffer and 0.5 unit Taq polymerase (Takara). PCR conditions were as follows: denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation 50 s at 94 °C, 30 s at a locus-specific annealing temperature (Table 1), and 50 s at 72 °C, with a final extension of 8 min at 72 °C. Amplified products were separated on 8 % non-denaturation polyacrylamide gel and visualized by silver staining. The observed (H_O) and expected heterozygosities (He) were estimated using POPGENE 32 software. The Markov chain method (Guo and Thompson 1992) was used to estimate the probability of significant deviation from Hardy-Weinberg equilibrium (HWE). We tested for linkage disequilibrium (LD) using GENEPOP program (online version through http://genepop.curtin.edu.au/) with default parameters. The micro-checker software (Van Oosterhout et al. 2004) was used to infer the most probable technical cause of HWE departures, including null alleles, misscoring and allelic dropout. Finally, all results were adjusted for multiple simultaneous comparisons using sequential Bonferroni correction (Rice 1989).

Table 1 Primer sequences, PCR conditions and characteristics for 11 microsatellite loci in *B. lenok tsinlingensis*

Locus	Repeat motifs	Primer sequences (5′–3′)	*Ta* (°C)	Size range (bp)	No. of alleles	*He*	*Ho*	GenBank acc. no.
TL01	$(CA)_{40}$	F: TGAGCCCATCGGATATACC	51	88–168	8	0.5963	0.4340	JN835585
		R: CAAAAGGAATTGTAGTTCAGTCAG						
TL03	$(TG)_7$ $(CT)_6$ $(TC)_5$	F: GATGAGTCCTGAGTAACCCACA	59	157–193	5	0.3483	0.4906	JN835586
		R: TTCATCTTCTCCAGGGCCTCACT						
TL05	$(GT)_5$ $(GT)_{12}$ $(GT)_9$ $(TG)_9$	F: TACATTTCAGTTTGAATCACGCAAC	56	164–234	6	0.3819	0.4183	JN835587
		R: CGTGATGCAGCCCTGCAT						
TL08	$(GT)_{11}$ $(GT)_5$ $(GT)_{26}$	F: AGGAAATGTTAGGATGATAGAGGCA	57	130–214	8	0.7726	0.6781	JN835588
		R: TTGGTTATTTAATGCGGTTGGG						
TL09	$(CA)_5$ $(CA)_{10}$	F: AATGAGAAGAATGCATGGCGGTT	59	132–162	4	0.8660	0.8421	JN835589*
		R: CTGTTTCCTGCCTAGATGCATTTCA						
TL10	$(GT)_6$ ATGA $(GT)_5$ ATGA $(GT)_7$ GCGG $(GA)_{15}$	F: AGCCTACCTCTTCTGTCTAGTGAGG	54	124–190	4	0.7040	0.7302	JN835590
		R: TGTGCAAATAGTTCAAGAACAAAAG						
TL12	$(AC)_9$ $(GT)_6$ TT $(TG)_5$	F: CTGCAGACTGGATCTTATCAGGAGC	58	168–208	5	0.4185	0.5421	JN835591
		R: GCATACAAGTACGCACGCCGA						
TL14	$(AC)_7$ $(CA)_5$	F: AGGCAGACTAACTGTGTGTATC	46	170–190	3	0.7383	0.6927	JN835592
		R: AGTTAGTGTGAACTCTGTTGTGT						
TL15	$(GT)_{12}$ C $(TG)_6$ TC $(TG)_{10}$ TT $(TG)_{18}$	F: AGCGACAGTGTGTGTGAGTAAGTGG	55	91–183	7	0.6552	0.6844	JN835593
		R: CCTCAAACCTGATGACCTCACACA						
TL21	$(AG)_{25}$	F: GAGATTAGATTGGGAGACTATGGTG	51	91–163	5	0.8874	0.7919	JN835594
		R: ATGGTTGTGTTCTCACTCTCTCTTT						
TL23	$(AC)_{12}$	F: TCCAGTGAGACCTGCCCAGTAACAT	53	138–162	3	0.7950	0.7842	JN835595*
		R: ATTTGTGCATGAGTGCAGCATGTG						

Ta, annealing temperature; *He*, expected heterozygosity; *Ho*, observed heterozygosity.* Significant deviation from HWE after sequential Bonferroni correction ($P < 0.0235$)

Among the 28 microsatellite primers synthesized, only eleven primer pairs were successfully amplified polymorphic DNA fragment with size matching expectation based on the size of initially cloned (Table 1). The number of alleles per locus varied from 3 to 8, with an average of 5.3 (±1.8 SD). The H_E ranged from 0.3483 to 0.8874; and the H_O ranged from 0.4183 to 0.8421. Two loci (TL09 and TL23) tested showed a very significant deviation ($P < 0.05$) from HWE after sequential Bonferroni correction. Further, null alleles were found in four loci (TL03, TL05, TL08 and TL15) detected with MICRO-CHECKER utility ($P < 0.05$), but no evidence for stuttering and allelic dropout were found in all loci ($P > 0.05$). No evidence was found for linkage disequilibrium among loci at a 5 % significance level.

These polymorphic microsatellite markers have been isolated for the first time from *B. lenok tsinlingensis*, and it may be useful for population genetic structure studies and conservation strategies design.

Acknowledgments This research was funded by the open project from Key Laboratory of freshwater biodiversity conservation and utilization, ministry of agriculture.

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