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TECHNICAL NOTE

Twelve novel polymorphic microsatellite loci developed from the Asiatic black bear (*Ursus thibetanus*)

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Abstract Since the number and range of Asiatic black bear (*Ursus thibetanus*) are declining due to habitat loss and illegal trade, it is essential to take effective actions to reinforce the conservation of the remaining bear populations. In order to aid such conservation efforts, we developed 12 novel polymorphic microsatellite loci of Asiatic black bear from genomic DNA-enriched libraries in this paper. The number of alleles per locus in 24 individuals ranged from 3 to 10, the average observed heterozygosity per locus from 0.214 to 0.950, and the average expected heterozygosity per locus from 0.243 to 0.891. Eight loci followed Hardy–Weinberg expectations after Bonferroni correction for multiple comparisons. No significant linkage association was found among all these loci. The 12 polymorphic microsatellite loci will be helpful to the conservation of the Asiatic black bear.

Keywords Asiatic black bear (*Ursus thibetanus*) · Microsatellites · Heterozygosity · Conservation plans

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Introduction

The Asiatic black bear (*Ursus thibetanus*) is widely distributed in Afghanistan, China, India, Indochina, Japan, Korea, Laos, Nepal, Pakistan, Thailand, Russia and Vietnam (Wilson and Reeder 2005). However, the present number and range of Asiatic black bear (*U. thibetanus*) are declining due to habitat loss and illegal trade, and the remaining populations are patchily distributed in fragmented habitat. Phylogeographic analysis suggested that the significant genetic differentiation has occurred among fragmented populations (Ishibashi and Saitoh 2004), which indicated that it is essential to take effective actions to reinforce the conservation of the remaining bear populations. Since the data of genetic diversity within the species will be helpful to the aforementioned conservation efforts (Frankham et al. 2002), and microsatellite markers can be useful in getting the data of genetic diversity within the species because of their abundance, high polymorphism content, co-dominance and bi-parentally inherited characteristics (Li et al. 2002), more microsatellite loci are still needed to estimate the levels of genetic diversity within the species by using noninvasive genetic methods, even though 18 microsatellite markers have been developed for the Asiatic black bear (Kitahara et al. 2000; Shih et al. 2009). In this paper, we report the development and characterization of 12 novel polymorphic microsatellite loci for the Asiatic black bear.

Microsatellites were isolated as the enrichment protocols by Wu et al. (2008) with minor modifications. Briefly, fresh blood samples of Asiatic black bear were collected from *U. thibetanus mupinensis* and whole genome DNA was extracted using standard phenol–chloroform procedures (Sambrook et al. 1989). Approximately 4 μg of genomic DNA was digested with *Mbo*I restriction enzyme

(TaKaRa), and the restriction fragments were ligated to an adaptor generated by annealing together oligo A and oligo B (Refseth et al. 1997). Then the 300–1,000 bp DNA fragments were isolated from the ligated products and enriched by polymerase chain reaction (PCR) using Oliga A as the PCR primer. Enrichment was carried out using $(CA)_{20}$ biotin-labelled probe and streptavidincoated magnetic beads (Promega). The enriched fragments were amplified to double-stranded form, and ligated into pMD18-T vector (TaKaRa). Approximately 496 recombinant colonies were screened by PCR amplification directly from bacterial colonies using CA RPT and universal M13 primers (Lunt et al. 1999). 148 positive clones were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI 3730 Genetic Analyzer.

Sixty-eight sequences were selected for primer design. Primer pairs were designed using the software Primer 3. Unlabelled primers were tested for amplification using DNA obtained from 12 individuals. PCR amplifications were performed in 10 μl reaction volume containing approximately 20 ng of template DNA, 0.2 mM of each dNTP, 0.15 μM of each primer, 1.5 mM Mg^{2+}, 1× PCR buffer, 0.1 μg/μl BSA and 0.15 U of *Taq* DNA polymerase (TaKaRa). The amplification conditions were as follows: 95°C for 5 min, 35 cycles at 94°C for 30 s, *Ta* for 30 s, 72°C for 45 s and a final extension at 72°C 10 min. All reactions were amplified using an MBS Satellite thermal cycler (Thermo Electron Corporation, USA). Finally, a total of 27 primers were selected, and labelled with one of the fluorescent dye (FAM, TAMRA or HEX) for polymorphism detection. PCR reactions were performed as aforementioned but using the optimal annealing temperatures (Table 1). Genotyping of the 27 microsatellite loci for each individual was done by electrophoresis on an ABI Prism 3700 Genetic Analyzer (Applied Biosystems), and fragment length was determined in comparison to an internal size standard (GeneScan ROX 400, Applied Biosystems) using GeneScan version 3.1.2 and Genotyper version 2.5 (Applied Biosystems).

The variability of these 27 loci was assessed in 24 individuals from *U. thibetanus mupinensis* at Southwest

Table 1 Characteristics of 12 polymorphic microsatellite loci in the Asiatic black bear (*Ursus thibetanus*)

Locus	Primer sequence (5′-3′) (F, forward; R, reverse)	Repeat motif	Labelling dye	T_a (°C)	A	Size range (bp)	H_O	H_E	Genbank accession no.
ABB1	F: CTTCTTGTTCCCCATACGCA	(CA)14	TAMRA	50	9	133–153	0.719	0.732	FJ795722
	R: ACAGGAGGAAGGAATCCTCAA								
ABB2	F: TTCCCTGGGACTCCATTC	(CA)12	TAMRA	50	3	147–155	0.230	0.243	FJ795723
	R: GCACAGCGTCATTCAAGAT								
ABB3[a]	F: AAAAGAAAAGGTCGGAAGAA	(GT)21	HEX	48	5	171–181	0.950	0.677	FJ795724
	R: GATACCAACTCATTGAAAGG								
ABB4	F: GATCTGGAGCCAAACACG	(CA)20	TAMRA	50	10	134–152	0.833	0.867	FJ795725
	R: CTGCTCCTGAAGCCATAA								
ABB5	F: AGATAGGCATGTTGGTCTG	(GT)16	TAMRA	50	9	114–136	0.619	0.712	FJ795726
	R: ATGTGGTGAAAAGGGAAA								
ABB6[a]	F: CCTAGCGTGAAGGAACAT	(GT)13	TAMRA	54	9	113–135	0.545	0.819	FJ795727
	R: CCGAACACTTACACCCATAC								
ABB7[a]	F: GAGGAACCTGGCAAAGTGAC	(CA)14	HEX	48	10	149–171	0.529	0.891	FJ795728
	R: GGAGACAGGGCTTGTGATGT								
ABB8[a]	F: TGATTTCGTTCACTTAGCAT	(GT)16	TAMRA	54	4	154–162	0.214	0.672	FJ795729
	R: ATGGATAAAGAAGATGTGGC								
ABB9	F: TTTGTCACCTCCACTTCT	(GT)10	HEX	50	4	183–189	0.468	0.555	FJ795730
	R: TACCCTTAACCACAATCC								
ABB10	F: TCTGTTCCCAGATAAGGTC	(CA)16	HEX	50	8	146–166	0.736	0.800	FJ795731
	R: GGGTGGGGTATGAAGATG								
ABB11	F: GGATAAATTACAAGACAGGAAA	(CA)16	FAM	54	10	192–212	0.889	0.890	FJ795732
	R: GCTCCTCCGCTATGAGTC								
ABB12	F: ATAGTTTTAGTTCAGGTG	(CA)16	HEX	50	9	141–173	0.827	0.863	FJ795733
	R: TGATGGTTTTAGAGTAAT								

T_a, annealing temperature; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

[a] significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$)

China, and 12 polymorphic loci were found. Conditions and characteristics of the 12 loci are given in Table 1. Population genetic parameters were estimated in Genepop version 3.4 (Raymond and Rousset 1995). The number of alleles per locus ranged from 3 to 10 (Table 1). The average observed heterozygosity per locus from 0.214 to 0.950, and the average expected heterozygosity per locus from 0.243 to 0.891 (Table 1). Eight loci followed Hardy–Weinberg expectations after Bonferroni correction for multiple comparisons. No significant linkage association was found among all these loci. The 12 polymorphic microsatellite loci will be helpful to conservation efforts of the Asiatic black bear.

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