Conserv Genet (2009) 10:523–525
DOI 10.1007/s10592-008-9556-7

TECHNICAL NOTE

Isolation and characterization of 12 novel microsatellite loci for the red panda (*Ailurus fulgens*)

Hua Wu · Xiang-Jiang Zhan · Yu Guo · Zejun Zhang · Lifeng Zhu · Li Yan · Ming Li · Fu-Wen Wei

Received: 20 February 2008 / Accepted: 7 March 2008 / Published online: 21 March 2008

Abstract The information on dispersal patterns and mating systems of red pandas is quite important for the understanding of the genetic diversity and divergence of this species. And microsatellite marker is an ideal tool to analyze dispersal patterns and mating systems. Thus, we describe in this paper the isolation and characterization of 12 microsatellite loci in the red panda from genomic DNA-enriched libraries. These loci were highly polymorphic with numbers of alleles per locus in 24 individuals ranging from 2 to 14, observed heterozygosity from 0.143 to 0.864 and expected heterozygosity from 0.297 to 0.872. All loci except for RP6 locus followed Hardy–Weinberg expectations. No significant linkage association was found among all these loci. The 12 novel polymorphic microsatellite loci will be of use in studying dispersal patterns and mating systems of red pandas.

Keywords Red panda (*Ailurus fulgens*) · Microsatellites · Heterozygosity · Dispersal patterns · Mating systems

The red panda (*Ailurus fulgens*), an endangered and special herbivorous carnivore, is distributed in the bamboo forests of Burma, Nepal, Sikkim and China (Wei et al. 1999). Population genetics studies showed that the red panda displayed a high level of genetic diversity and there was no obvious genetic divergence between the Sichuan population and the Yunnan population (Su et al. 2001; Li et al. 2005). Although many factors, such as habitat fragmentation, habitat features and climates, can affect the genetic diversity level and the genetic divergence degree by restricting the gene flow among populations (Manel et al. 2003), dispersal patterns and mating systems which determine group composition, distribution of genetic variation, and effective population size within populations, are the most important factors (Chesser 1991; Ross 2001). Accordingly, the information on dispersal patterns and mating systems of red pandas is quite important for the understanding of the genetic diversity and divergence of this species.

Microsatellite marker is an ideal tool to analyze dispersal patterns and mating systems (Ritland 2000; Ross 2001). Although some microsatellite loci have been developed for red panda (Liu et al. 2005; Liang et al. 2007; Zhang et al. 2008), more microsatellite loci are still needed to study dispersal patterns and mating systems of this species (Blouin 2003). In this paper, we report the isolation and characterization of 12 novel microsatellite loci in the red panda.

Microsatellites were isolated as the enrichment protocols by Hamilton et al. (1999) with slight modifications as follows. Whole genome DNA was extracted from a muscle sample of red panda using standard phenol–chloroform procedures (Sambrook et al. 1989). Approximately 9 μg of genomic DNA was digested with *Mbo*I restriction enzyme (TaKaRa) and 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 streptavidin-coated magnetic beads (Promega). First, 4 μg of the adaptor-ligated size fractionated genomic DNA was hybridized to

H. Wu · X.-J. Zhan · Y. Guo · Z. Zhang · L. Zhu · L. Yan · M. Li · F.-W. Wei (✉)
Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, People's Republic of China
e-mail: weifw@ioz.ac.cn

2 μg of probe in 6× SSC at 50°C overnight, and the complex was added to 600 μl of streptavidin coated beads and the two were mixed for 1 h at 43°C. The enriched fragments that released from the probe were amplified to double-stranded form, and ligated into pMD18-T vector (TaKaRa) and transformed into competent Top10 cells. Approximately 496 recombinant colonies were screened by PCR amplification directly from bacterial colonies using CA RPT and universal M13 primers (Lunt et al. 1999). About 170 positive clones were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI 3730 Genetic Analyzer.

Eighty sequences having adequate flanking regions for primer designing and showing no homology to microsatellite markers previously published for this species were selected for primer design. Primer pairs were designed using the software Primer 3 (http://www.genome.wi.mit.edu/genome_software/other/primer3.html). Unlabelled primers were used to determine optimal amplification conditions. 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 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 Corp., USA). Finally, a total of 31 primers were selected, and one of each of the 31 primer pairs was labelled with one of the fluorescent dye (FAM, TAMRA or HEX) for polymorphism detection. PCR reactions were performed as above but using the optimal annealing temperatures (Table 1). Genotyping of the 31 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).

Twelve polymorphic loci were tested in 24 individuals from the Sichuan population and the Yunnan population at Southwest China. Population genetic parameters were

Table 1 Characteristics of 12 polymorphic microsatellite loci in the red panda (*Ailurus fulgens*)

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.
RP1	F: CTGCTGTAGGCATTGGTTAT	$(GT)_{37}$	TAMRA	52	14	84–138	0.565	0.859	EU487201
	R: CCTCTTGGAAATGAATAGTAAA								
RP2	F: AAGGCAATGAAATACGAAAT	$(GT)_{12}$	TAMRA	52	3	150–156	0.143	0.297	EU487202
	R: TTAGAAGAGGAAGAATGGAT								
RP4	F: CTTTCTATAAATCAGGTCTG	$(GT)_{19}$	FAM	48	8	198–212	0.818	0.842	EU487203
	R: CTACTTCATACACTGCTTCC								
RP5	F: AGGGTGACATGAGTAAATT	$(CA)_{23}$	TAMRA	50	7	110–130	0.722	0.817	EU487204
	R: ATCACTGAAGGGAAAGAA								
RP6	F: ACTGAGGCGAAGAAAGGAGC	$(CA)_{13}$	HEX	52	5	155–167	0.429	0.681	EU487205
	R: CATGGGCATTGAAGATGGTG								
RP7	F: TACTCAGTGGAAGGGTTG	$(CA)_{28}$	TAMRA	50	12	122–156	0.850	0.872	EU487206
	R: TTCTGTTCTTCAGGCTCT								
RP8	F: CTTATTCTCCGCTTGTCTATCA	$(CA)_{28}$	HEX	52	6	163–173	0.739	0.757	EU487207
	R: CACTTCCCTGCCCAGCCTCA								
RP9	F: ACAAACTGGAATGTAAAGG	$(CA)_{20}$	TAMRA	52	8	125–143	0.818	0.821	EU487208
	R: CACATGCTTATGTTATAGGG								
RP10	F: TGCCCTGGTCCCCATTTA	$(CA)_{21}$	FAM	50	2	234–240	0.542	0.434	EU487209
	R: TAGGCACCCCAGGCTTTA								
RP11	F: TGAATGTTGCCTTGCTCT	$(CA)_{12}$	TAMRA	50	8	105–127	0.762	0.862	EU487210
	R: CACCACCTCTTACTGTTCTC								
RP12	F: TCAAAGGCAATTTCCTAG	$(CA)_{16}$	TAMRA	50	9	131–147	0.667	0.833	EU487211
	R: TATCACATTTACGGTGGC								
RP13	F: TCCCTTACGCTTCCTCCTTT	$(GT)_{18}$	HEX	50	14	181–211	0.864	0.872	EU487212
	R: GCAGGCGGAGAATTGGTTGG								

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

estimated in Genepop version 3.4 (Raymond and Rousset 1995). The 12 loci were highly polymorphic with numbers of alleles per locus ranging from 2 to 14, observed heterozygosity from 0.143 to 0.864 and expected heterozygosity from 0.297 to 0.872 (Table 1). All loci except for RP6 locus followed Hardy–Weinberg expectations. No significant linkage association was found among all these loci. The 12 novel polymorphic microsatellite loci will be of use in studying dispersal patterns and mating systems of red pandas.

Acknowledgements This study was supported by projects of the National Basic Research Program of China (973 Program, 2007CB411600), National Natural Science Foundation of China (No.30620130432 and 30770399) and CAS 100 Talent Programme.

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