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TECHNICAL NOTE

Thirty-three microsatellite loci for noninvasive genetic studies of the giant panda (*Ailuropoda melanoleuca*)

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Abstract Limited microsatellite markers useable in noninvasive genetic methods have hampered the studies of dispersal patterns and mating systems of giant pandas. Therefore, we describe in this paper the characterization of 15 novel microsatellite loci from genomic DNA-enriched libraries and 18 redesigned microsatellite loci from published papers on the giant panda. The number of alleles per locus in 60 individuals ranged from 2 to 13, the average observed heterozygosity per locus from 0.168 to 0.800, and the average expected heterozygosity per locus from 0.152 to 0.882. All loci followed Hardy-Weinberg expectations. Four pairs of significant linkage association were found among all these loci. Moreover, the 33 microsatellite loci showed high amplification successes rate in noninvasive samples, which indicated that these loci will be of use in studying dispersal patterns and mating systems of giant pandas using noninvasive genetic methods.

Keywords Giant panda (*Ailuropoda melanoleuca*) · Microsatellites · Heterozygosity · Dispersal patterns · Mating systems · Noninvasive samples

The giant panda (*Ailuropoda melanoleuca*), a critically endangered species, is originally distributed over southern and eastern China, extending to northern Burma and northern Vietnam (Hu 2001). But habitat loss from deforestation rapidly reduced the giant panda's distribution. Currently, giant pandas are restricted to Qinling, Minshan, Qionglai, Daxiangling, Xiaoxiangling and Liangshan Mountains in the eastern edge of the Tibetan plateau (Hu 2001). Recent studies indicated that significant genetic differences had occurred between Qinling population and other populations (Lu et al. 2001; Wan et al. 2003, 2005; Zhang et al. 2007). Although many factors, such as habitat fragmentation, habitat features and climates, can affect the genetic divergence degree by hampering the gene flow between 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 the giant pandas is quite important for the understanding of the genetic divergence of this species. However, because the giant panda lives in bamboo forests, it is difficult to obtain the information on dispersal patterns and mating systems of this species by direct observation of the behavior of individuals. In recent years, with the development of many new statistical methods for analyzing kin relationships from molecular data, it is possible to infer dispersal patterns and mating systems of species by analyzing the spatial distribution of molecular genotypes and constructing pedigree of individuals of natural populations (Blouin 2003; Zhan et al. 2007).

Microsatellite marker is an ideal tool to analyze kin relationships among individuals in natural populations (Ritland 2000; Ross 2001). Although 63 microsatellite loci have been developed for giant panda (Zhang et al. 1995, 2008; Lu et al. 2001; Shen et al. 2005, 2007), some of them could not be used in analysis of noninvasive samples (Zhan et al. 2006; He et al. 2008). Since limited microsatellite markers useable in noninvasive genetic methods

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have hampered the studies of dispersal patterns and mating systems of the giant panda, more microsatellite loci are still needed to address above questions (Blouin 2003). Therefore, we report in this paper 15 novel microsatellite loci and 18 redesigned microsatellite loci of the giant 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 blood sample of giant panda using standard phenol-chloroform procedures (Sambrook et al. 1989). Approximately 5 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 300–1000 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). First, 4 μg of the adaptor-ligated size fractionated genomic DNA was hybridized to 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). 142 positive clones were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI 3730 Genetic Analyzer.

Table 1 Characteristics of 15 polymorphic microsatellite loci in the giant panda (*Ailuropoda melanoleuca*)

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.
GP1	F: TGCTACAAACACTTTCCACCAT	(CA)20	TAMRA	54	8	124–138	0.658	0.767	EU604083
	R: TTGTTCTCCCAGGCCACA								
GP2	F: TGAGAATGGAACCCTGAG	(GT)22	HEX	54	7	123–139	0.633	0.794	EU604084
	R: GTCCTCTTCCAGAGCCAC								
GP3	F: GATCAAGCCCCAAGACGAGC	(CA)16	FAM	54	7	153–171	0.657	0.772	EU604085
	R: TGGGGAGTCTGCTTGAAGAG								
GP4	F: CCTGGCATAATGTGAGCAAC	(CA)12	TAMRA	54	6	120–138	0.658	0.704	EU604086
	R: AGGATGTGGAGACCACGACT								
GP5	F: TCAGACCCTAGATTTCATTC	(GT)16	HEX	54	7	130–142	0.715	0.727	EU604087
	R: GAAGAGCCATACCACAGAG								
GP6	F: GCTTATGGGTCCATTTCG	(GT)11	FAM	54	4	172–182	0.168	0.152	EU604088
	R: GCACGTTGTCAGCCTCTAT								
GP7	F: CCCCTGCGTGAGTATGTG	(CA)14	TAMRA	54	7	110–136	0.609	0.668	EU604089
	R: GAGAAGATGCTGGGTGGA								
GP8	F: AGAAAGTCAGTAAGGCATT	(CA)16	FAM	54	6	140–154	0.610	0.710	EU604090
	R: ATCAAGAGGCAGAGGACT								
GP9	F: AATGGCACATCCTACTATCTT	(CA)10	FAM	54	5	194–204	0.735	0.760	EU604091
	R: TCCGGGACGTGTCTACCT								
GP10	F: ACACCAGGAAATGTAGCT	(CA)18	HEX	54	6	136–150	0.610	0.662	EU604092
	R: GTTGTCATGCCTCAAGTT								
GP11	F: TTCGGATCATCCAGCACT	(GT)17	TAMRA	52	10	122–142	0.724	0.709	EU604093
	R: ACAGCTCCCATTCGGTCC								
GP12	F: ATGGAACCCTGAGGAACA	(GT)22	FAM	50	10	158–176	0.718	0.788	EU604094
	R: CACATCTCCAGCAGACCC								
GP13	F: ATACAAACATGGGTACGA	(CA)20	TAMRA	48	8	110–126	0.720	0.818	EU604095
	R: CTCATTCTAATTTCCTGAT								
GP14	F: TTTTGCTAACACGGTCCTCA	(GT)12	FAM	54	9	161–183	0.676	0.741	EU604096
	R: ACCCTACCAAGCTCCCATAC								
GP15	F: TTCTCAAGGCTGACTAATA	(GT)18	HEX	54	5	124–132	0.733	0.725	EU604097
	R: TAGCCAAGATATGGAAAG								

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

Table 2 Characteristics of eighteen redesigned microsatellite loci in the giant panda (*Ailuropoda melanoleuca*)

Locus	Primer sequence (5′–3′) (F, forward; R, reverse)	Repeat motif	Labelling dye	T_a (°C)	A	Size range (bp)	H_O	H_E	References
Ame-μ11	F: CTCTTTGCATCTCAAATTCA	(CA)12	TAMRA	48	10	108–126	0.775	0.831	Lu et al. (2001)
	R: TGAGATAGGAAAGAGTGGGT								
Ame-μ24	F: TATTTGTCATGGGGTTTACT	(CA)15	FAM	48	7	108–122	0.665	0.734	Lu et al. (2001)
	R: CCTACCTCACATTCTACTCC								
AY79	F: GAGCACTCTTGGGTTTTGTTA	(GT)20	TAMRA	48	9	140–160	0.415	0.459	Shen et al. (2005)
	R: TGGTGGGCAGGAATGAT								
AY83	F: TCTGTCCACGTACACGTAAT	(CA)12	FAM	50	4	178–188	0.495	0.502	Shen et al. (2005)
	R: ATTCTCATGCTGTTGTATCTC								
AY85	F: TGTGATCTGACTTCTTGGTT	(TG)21	HEX	58	9	150–168	0.771	0.826	Shen et al. (2005)
	R: GATTTAACAGGTACAGGGTG								
AY87	F: AATCTTTCCTTTTCTGGCTGTT	(CA)21	HEX	48	3	180–186	0.545	0.528	Shen et al. (2005)
	R: TGGAGGAAGGGTAGAGAAATAA								
AY90	F: GGCCCAAGTGTCCACTGACT	(CA)24	FAM	58	7	144–158	0.750	0.823	Shen et al. (2005)
	R: CTGGCTGCATCCATGTTGTT								
AY95	F: GCATGAAAGCCAGAAAACAG	(CAA)12	FAM	48	2	144–147	0.615	0.647	Shen et al. (2005)
	R: GTAAAGAAGCCAGCCCAACT								
AY99	F: CCGTCCTCTTTGCACCTA	(GTT)12	TAMRA	48	4	158–164	0.441	0.394	Shen et al. (2005)
	R: ATCCCTGATTCAACCTCT								
AY217	F: CTCATGTGCTCATTCACT	(TATC)10	FAM	48	6	104–120	0.697	0.697	Shen et al. (2005)
	R: TAGGGAGAAACAGATACAT								
Aime-1	F: CTGGAGGAACAGGGTATT	$(AC)_{23}$	HEX	48	10	133–151	0.798	0.825	Zhang et al. (2008)
	R: AGGTGCCAGAGGTCAGTA								
Aime-2	F: GTTTGCTACCATCCTGCTT	$(AC)_{22}$	FAM	48	7	127–141	0.774	0.809	Zhang et al (2008)
	R: TGTCCCTGCTGAACTCAC								
Aime-4	F: ACCATTTCACAGCGAAGG	$(GT)_{23}AT$	HEX	48	8	156–194	0.739	0.768	Zhang et al. (2008)
	R: TGTCACTCCCAAATTCATTC	$(GT)_{12}$							
Aime-10	F: ATACCTGGAAACTTCATA	$(AG)_{10}$	FAM	44	10	182–220	0.794	0.856	Zhang et al. (2008)
	R: GGTATTTTGTCCTTAGCG								
Aime-12	F: ACCCTTAGCTCAGTATCAA	$(GA)_{22}$	HEX	48	13	144–172	0.759	0.791	Zhang et al. (2008)
	R: TAAACATCCACCTTCACC								
Aime-13	F: TTTAGCCTCCTGGGTATT	$(AC)_{10}$	TAMRA	48	12	129–163	0.783	0.882	Zhang et al. (2008)
	R: TCTAGCGGTGTTTGTCTT	$(AG)_{23}$							
Aime-15	F: TTGCATGCTCTATGGATT	$(AC)_{21}AG$	FAM	48	10	147–171	0.800	0.849	Zhang et al. (2008)
	R: CATTTCTTTGTGCTTACTC	$GA(AG)_{21}$							
Aime-16	F: CTTGGCACTCTACCAGGAC	$(AG)_{30}$	TAMRA	48	6	176–192	0.671	0.687	Zhang et al. (2008)
	R: CTAGAAGCTCCGCACTTT								

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

Seventy sequences having adequate flanking regions for primer designing and showing no homology to microsatellite loci 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.). In addition, because the amplification failure rate and genotyping error rate of loci with alleles longer than 200 bp in analysis of noninvasive samples is usually higher than that of loci with alleles shorter than 200 bp (Frantzen et al. 1998; Taberlet et al. 1999; Pompanon et al. 2005; Zhan et al. 2006), we redesigned the PCR amplification primers of 18 microsatellite loci (Table 2) from published papers to make the amplification fragment of these loci shorter than 200 bp (Lu et al. 2001; Shen et al. 2005; Zhang et al. 2008).

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, 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 Corp., USA). Finally, a total of 46 primers were selected, and one of each of the 46 primer pairs was 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 (Tables 1 and 2). Genotyping of the 46 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 33 polymorphic loci were tested in 60 individuals (Tables 1 and 2). Population genetic parameters were estimated in Genepop version 3.4 (Raymond and Rousset 1995). The number of alleles per locus ranged from 2 to 13, the average observed heterozygosity per locus from 0.168 to 0.800, and the average expected heterozygosity per locus from 0.152 to 0.882 (Tables 1 and 2). All loci followed Hardy–Weinberg expectations. Four pairs of significant linkage association (GP2 and GP12, GP9 and GP11, GP9 and Aime-12, GP2 and Aime-16) were found among all these loci ($P < 0.05$).

In addition, the amplification success rates of 33 loci were tested in 48 feces samples from the Qinling Mountains, and the results showed that these loci had high amplification success rates. The amplification success rates of 6 loci (GP4, GP6, GP7, GP15, Ame-μ24 and *Aime*-1) were more than 90%, 9 loci (GP1, GP5, GP8, GP10, GP11, AY79, AY95, *Aime*-2 and *Aime*-12) more than 80%, 15 loci more than 70% and 3 loci (AY85, AY87 and *Aime*-13) more than 60%. These results indicated that the 33 polymorphic loci will be of use in studying dispersal patterns and mating systems of giant pandas using noninvasive genetic samples.

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