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ORIGINAL PAPER

Isolation and characterization of 11 microsatellite loci for the Sichuan snub-nosed monkey, Rhinopithecus roxellana

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Abstract We report on the isolation and characterization of 11 microsatellite loci in the Sichuan snub-nosed monkey (*Rhinopithecus roxellana*) from AC-enriched genomic libraries. 20 microsatellite loci were screened from the libraries, of which 11 were polymorphic. The number of observed alleles among 32 samples of snub-nosed monkey ranged from three to nine. The observed and expected heterozygosity were 0.071–0.815 and 0.201–0.819, respectively. The high variability revealed in this study should be useful tools for further study on social structure and population genetics of snub-nosed monkey in the future.

Keywords *Rhinopithecus roxellana* · Microsatellite · Polymorphism · Sichuan snub-nosed monkey

Sichuan snub-nosed monkey (*Rhinopithecus roxellana*), one species of the genus *Rhinopithecus*, is widely known for its shining golden-haired coat and funny upturned nose. It is confined to very limited areas in isolated mountainous regions in Sichuan, Gansu, Shaanxi and Hubei provinces (Li et al. 2001; Li et al. 2002), with approximate population size of 20,000 (Ma and Wang 1988; Wang et al. 1998; Quan and Xie 2002). Now, fragmented and deteriorating habitat has severely threatened the survival of this species. Sichuan snub-nosed monkey has been recognized as ''vulnerable'' in the 2006 IUCN List category (http://www.redlist.org/), and has drawn a lot of attention in various conservation efforts. As molecular markers, microsatellite markers are powerful tools for investigating genetic diversity and mating patterns. To date, almost all microsatellite markers for genetic study on Sichuan snub-nosed monkey were from those of humans (Pan et al. 2005). However, we found that a lot of microsatellite markers from human were not powerful enough for some studies on the snub-nosed monkey such as social structure analysis. Clearly, there is a requirement to isolate the specific microsatellite marker for Sichuan snub-nosed monkey study in the future. Here, we report the isolation and characterization of 11 novel microsatellite loci in *R. roxellana*.

A genomic library enriched for AC repeat was constructed by using enrichment protocols modified from those described in Hamilton et al. (1999) and Liu et al. (2005). Thirty-two samples including blood, muscle and skin were collected from the wild and zoos. Genomic DNA was extracted from blood using a standard phenol–chloroform method (Sambrook et al. 1989) and used for microsatellite isolation. DNA was digested with restriction enzyme *Sau*3A1 and the resulting fragments ranging from 300 to 1,000 bp were isolated and ligated to linkers made by annealing equimolar amounts of SAULA (5′-GCG GTA CCC GGG AAG CTT GG-3′) and SAULB (5′-GAT CCC AAG CTT CCC GGG TAC CGC-3′). The ligated fragments were amplified and made double-stranded by polymerase chain reaction (PCR) (5 min 72°C, then 30 cycles of 30 s denaturation at 94°C, 30 s annealing at

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Table 1 Characterization of 11 *Rhinopithecus roxellana* microsatellite loci, including motif, primer sequences, annealing temperatures (*T*a) are given for each locus. Allele size ranges, number of alleles, observed heterozygosity (H_O), expected heterozygosity (H_E) and *P*-value were estimated from genotyping 32 samples (boldface numbers indicate significant deviation from HWE) (see text)

Locus	Primer sequence(5′–3′)	Array	Size range (bp)	No. of alleles	T_a (°C)	H_O	H_E	*P* value	GenBank accession no.
GM209	F:ATC TGA ATG ATG TGT GGA TGT R:TAG AGT AGC ATT GCC T	$(CA)_{13}$	146–158	5	53	0.179	0.427	**0.000**	DQ911151
GM219	F:GTG TAT TGT GGG GCT ATC R:GTG GGC TCT GAC CTA GGA ATC	$(TG)_{12}$	183–187	3	53	0.179	0.201	1.000	DQ911152
GM108	F:CAG CGT AAG CCA GTT GCC R:GGA AAA GTC TGA AAC CCA CGA	$(TG)_{19}$	123–137	7	53	0.815	0.819	0.010	DQ911153
GM228	F:ACC AGC CTC CAA AAT TAT GTG R:GAG GGG TGA CTG AGT CAA A	$(TG)_{22}$	165–175	4	52	0.071	0.264	**0.000**	DQ911154
GM213	F:GCC CTA GCA GAA CAT GAC ACT R:AGC CCA TGC GTA TTG AGT	$(AC)_{12}$	182–198	6	55	0.786	0.654	0.027	DQ911155
GM105	F:CGG ATC ATT GTT GCT C R:AGA TGG GAA GGT GTG TCT ACA	$(AC)_{20}$	161–175	6	55	0.600	0.727	0.190	DQ911156
GM227	F:CAG AAG CCA CCG AAA TG R:AAT TCT CTC CCA AGG AAT ATG	$(TG)_{15}$	148–174	5	52	0.370	0.603	**0.001**	DQ911157
GM214	F:GGG CAA CAG AGC GAG ACT G R:TGC AAA GAT GTG AAC GGA AAT	$(AC)_{16}$	134–154	9	53	0.464	0.727	0.008	DQ911158
GM206	F:GGT GCT ACC AGA TCA TTG TT R:CAG ATG GGA AGG TGT GTC TAC	$(AC)_{21}$	163–183	7	54	0.690	0.795	0.001	DQ911159
GM109	F:GGT GGA GGA GGG CCT AAC R:CTG ATG TCC ATA GGC GAC CAT	$(AC)_{19}$	138–164	7	54	0.613	0.755	0.033	DQ911160
GM220	F:CCC TTC TCT GTG ACC TTG T R:TGA GTC CTC AGA CAC CAA TCA	$(CA)_{18}$	165–179	6	52	0.480	0.755	**0.000**	DQ911161

67°C and 30 s extension at 72°C) using SAULA as the PCR primer. After denaturation, single-strand DNA fragments of the PCR products were hybridized to biotin-labelled probe [biotin-ATAGAATAT $(CA)_{15}$] (Kandpal et al. 1994) and the targeted genomic fragments were recovered using streptavidin-coated MagneSphere (Promega). The eluted fragments were amplified by using SAULA primer and cloned in the pGEM-T Easy Vector (Promega), transfected into *Escherichia coli* DH5α competent cells, following the manufacturer's instruction. Positive colonies were screened for the presence of a repeat-insert using universal M13 primers and the nonbiotin-labelled AC nucleotide repeat primer. Approximately 576 colonies were screened and a total of 65 recombinants that potentially contained microsatellite sequence were obtained. Forty three positive colonies were chosen randomly and subjected to sequencing by using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI 377 automated DNA sequencer. The sequencing results showed that 35 out of 43 colonies contained ten repeats or more and had suitable flanking sequences. Twenty primer pairs were designed from the candidate 35 microsatellite sequences using the software program OLIGO^{TM} 6.0 (Molecular Biology Insights) for further polymorphism analysis.

We tested them to confirm a distinct amplification with a gradient PCR over a range of annealing temperature between 50 and 60°C. PCR amplifications were performed in a 20 μl reaction volume containing approximately 50 ng of template DNA, 10 × PCR buffer, 0.2 mM of each primer, 0.2 mM of each dNTP, and 0.5 U of Ex *Taq* DNA polymerase (TaKaRa). Conditions for PCR included: an initial denaturing step of 5 min at 94°C followed by 35 cycles of 94°C for 30 s, annealing temperature (T_a in Table 1) for 30 s, and 72°C for 30 s, with a final extension 5 min at 72°C. Microsatellite polymorphism was screened using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and alleles were designated according to the PCR product size relative to GeneScan ROX 500 internal size standard. The genotyping analysis was carried out with GENESCAN version 2.0 (Applied Biosystems).

Of 20 loci succeeded in PCR amplification, only 11 loci were polymorphic. Population genetic parameters, deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) test were estimated with ARLEQUIN version 3.01(Laurent et al. 2006). The characteristics of the 11 microsatellite loci were listed in Table 1. All of the 11 loci were highly polymorphic with an average of 5.91 alleles per locus (range from three to nine), expected heterozygosity (H_E) ranged from 0.201 to 0.819, while observed heterozygosity (H_O) ranged from 0.071 to 0.815. The HWE test revealed that 4 loci significantly deviated from Hardy–Weinberg expectations ($P < 0.001$), all of them exhibited heterozygote deficiency, because the samples came from different regions and we have no information about the relationship among them, probably due to the presence of null alleles (Pemberton et al. 1995) as well as the inbreeding–produced sample analysis. Among the 11 loci, only six pairs of loci showed linkage (GM209-GM228, GM209-GM109, GM219-GM228, GM228-GM227, GM228-GM206, GM227-GM109), which indicate these loci should be used selectively. The stability of genotypes was confirmed by repeating up to four times. Contribution of the 11 microsatellite markers developed in this study will provide necessary molecular tools for further studies on Sichuan snub-nosed monkey in the future.

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