Int J Primatol (2008) 29:265–272
DOI 10.1007/s10764-007-9175-z

Identification of 13 Human Microsatellite Markers via Cross-species Amplification of Fecal Samples from *Rhinopithecus bieti*

Z. J. Liu · B. P. Ren · Y. L. Hao · H. R. Zhang · F. W. Wei · M. Li

Received: 20 January 2007 / Accepted: 9 July 2007 /
Published online: 5 February 2008

Abstract Yunnan snub-nosed monkeys (*Rhinopithecus bieti*) are 1 of 3 snub-nosed monkey species endemic to China. Only *ca.* 1500 individuals remain in high-altitude forests 3000–4500 m above sea level on the Tibetan Plateau, making them the nonhuman primate living at the highest known elevation. It is one of the most endangered 25 primate species in the world. Proper knowledge of the population genetics and social system of *Rhinopithecus bieti* will contribute to more appropriate conservation management decisions. Cross-species amplification of human microsatellite loci has facilitated analysis of the population genetics and reproductive strategies of various primate species. We screened 72 human-derived markers to assess their utility in Yunnan snub-nosed monkeys. Thirteen of them produced reliable results and exhibited moderate levels of polymorphism.

Keywords cross-species amplification · fecal sample · human microsatellite · Yunnan snub-nosed monkey

Introduction

Rhinopithecus comprises 4 species, 3 of which are endemic to China: *R. roxellana* (golden or Sichuan snub-nosed monkey); *R. bieti* (black-and-white or Yunnan snub-

Z. J. Liu · B. P. Ren · Y. L. Hao · H. R. Zhang · F. W. Wei · M. Li (✉)
Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Chaoyang, Beijing 100101, China
e-mail: lim@ioz.ac.cn

Y. L. Hao · H. R. Zhang
Graduate School of the Chinese Academy of Sciences, Beijing 100039, China

M. Li
Jiangsu Key Laboratory for Biodiversity and Biotechnology, College of Life Sciences, Nanjing Normal University, Nanjing 210097, China

nosed monkey); and *R. brelichi* (gray or Guizhou snub-nosed monkey). The fourth species is distributed in northern Vietnam: *Rhinopithecus avunculus* (Tonkin's snub-nosed monkey) (Jablonski 1998a, b). *Rhinopithecus bieti*, widely known as black-and-white snub-nosed monkeys because of a black-and-white coat and striking snub nose, has suffered dramatic reduction in distribution and population in the past (Li *et al.* 2002). Currently, only *ca.* 1500 *Rhinopithecus bieti* live in high-altitude forests 3000–4500 m above sea level, within a narrow area on the Tibetan Plateau between the Yangtze and Mekong Rivers (98°37′–99°41′E, 26°14′–29°20′N), making them the nonhuman species inhabiting the highest known elevation (Li *et al.* 2002; Long *et al.* 1994, 1996). Because of their distinct appearance and rarity, Yunnan snub-nosed monkeys are one of the national icons of China, albeit less famous than the giant panda (*Ailuropoda melanoleuca*). The 19th Congress of the International Primatological Society in Beijing 2002 identified it as one of the 25 most globally endangered primate species.

Previous wild surveys suggested that *Rhinopithecus bieti* has suffered from loss of habitat and has been fragmented into 15 distinct populations, with individual numbers ranging from 50 to 300 in each population. The populations were isolated by clear topographic features such as great rivers, busy highways, human-active regions, and widely bared side slopes, making it impossible for individual exchange between them (Quan and Xie 2002). Also, there are another 3 captive populations in Beijing Zoo, Kunming Zoo, and Kunming Institute of Zoology, Chinese Academy of Sciences. However, a lack of reliable genetic markers and useful samples limited research on genetic variation in *Rhinopithecus bieti* and consequently information on the species.

Cross-species amplification via human microsatellite loci has facilitated analyses of the population genetics and reproductive strategies of other primate species: *Macaca mulatta*, *M. fascicularis*, *M. sylvanus*, *Pan troglodytes verus*, *Gorilla gorilla gorilla*, *Hylobates lar*, and *Cercopithecus aethiops sabaeus* (Bonhomme *et al.* 2005; Bradley *et al.* 2000; Chambers *et al.* 2004; Clisson *et al.* 2000; Hadfield *et al.* 2001; Kanthaswamy *et al.* 2006; Morin *et al.* 1997; Newman *et al.* 2002; Smith *et al.* 2000). We tested 72 human microsatellite loci (Table I) that have proved to be useful in those studies to select reliable polymorphic molecular markers for a population genetic study of *Rhinopithecus bieti*.

Materials and Methods

Samples, DNA Extraction, and Polymerase Chain Reaction (PCR)

We collected blood, muscle, and fecal samples of 3 individuals from the Beijing Wildlife Zoo, and fecal samples of another 40 individuals in 3 wild populations during the course of behavioral observation. We stored fecal samples in 95% ethanol, and banked blood and muscle samples in a –80°C freezer. To prevent contamination during DNA extraction, we cleaned benches and plasticware with 10% bleach and sterile water, and then exposed them to UV light for 30 min. We also exposed the surfaces of muscle samples to UV light for 30 min. We extracted total DNA from blood and muscle samples via a standard phenol-chloroform method (Sambrook *et al.* 1989). We extracted the fecal DNA via the QIAGEN DNA Stool

Table I Locus, amplification condition, and results of microsatellite markers tested in Yunnan snub-nosed monkeys

Locus	Repeat motif	Result	Annealing temp. (°C)
▸D1S207	Tetra	Polymorphic	52
D1S238	Di	Poor*	50
▸D1S533	Tetra	Polymorphic	52
D1S548	Tetra	Poor*	51
D1S549	Tetra	Poor*	52
D1S550	Tetra	Poor*	50
▸D2S1326	Tetra	Polymorphic	55
D2S305	Di	Monomorphic	50
D3S1279	Di	Monomorphic	50
D3S1292	Di	Monomorphic	50
D3S1566	Di	Monomorphic	50
D3S1768	Tetra	Poor*	50
D4S403	Di	Monomorphic	50
D4S1626	Tetra	Poor*	50
D4S2365	Tetra	Poor*	50
D4S2374	Tetra	Poor*	50
D4S243	Tetra	Poor*	52
D5S820	Tetra	Poor*	50
▸D5S1457	Tetra	Polymorphic	50
D5S1470	Tetra	Poor*	55
▸D6S264	Di	Monomorphic	51
▸D6S271	Di	Polymorphic	54
D6S311	Tetra	Polymorphic	50
D6S434	Di	Monomorphic	50
▸D6S474	Tetra	Polymorphic	53
▸D6S493	Tetra	Polymorphic	53
D6S501	Tetra	Polymorphic	50
D7S513	Di	Monomorphic	50
D7S794	Tetra	Poor*	50
D7S1789	Tri	Poor*	50
D7S1826	Tetra	Poor*	55
D7S1830	Tetra	Poor*	55
▸D7S2204	Tetra	Polymorphic	54
D8S272	Di	Monomorphic	50
▸D8S505	Di	Polymorphic	55
D8S1106	Tetra	Poor*	50
D8S1119	Tri	Poor	50
D9S290	Di	Monomorphic	50
D10S1432	Tetra	Poor*	55
D10S611	Tetra	Poor*	50
D10S1688	Di	Monomorphic	50
D11S902	Di	Poor*	50
D11S1366	Tetra	Poor*	54
D11S1902	Di	Monomorphic	50
D11S1975	Tetra	Poor*	50
▸D11S2002	Tetra	Polymorphic	55
D12S67	Tetra	Poor*	51
D12S85	Di	Poor*	50
D12S1617	Di	Monomorphic	50
D13S317	Tetra	Poor*	50
D13S318	Tetra	Poor*	50
D13S765	Tetra	Poor*	50
D13S894	Tetra	Poor*	50
D14S581	Tetra	Poor*	50

Table I (continued)

Locus	Repeat motif	Result	Annealing temp. (°C)
D15S644	Tetra	Poor*	50
D15S1007	Di	Monomorphic	50
D15S644	Tetra	Poor*	50
D17S804	Di	Poor*	55
D17S831	Di	Monomorphic	50
D17S924	Di	Monomorphic	50
▸D17S1290	Tetra	Polymorphic	55
D18S536	Tetra	Poor*	50
D18S537	Tetra	Poor*	50
D18S851	Tetra	Poor*	50
D18S861	Tri	Poor*	50
D19S210	Di	Monomorphic	55
D19S255	Tetra	Poor*	50
D19S571	Di	Monomorphic	50
D20S117	Di	Monomorphic	50
▸D20S206	Tetra	Polymorphic	52
D22S274	Di	Monomorphic	50
D22S280	Di	Monomorphic	50

The polymorphic loci are indicated by black triangles (▸). Poor* means poor amplification in the PCR system.

Mini Kit (Qiagen GMBH, Germany). We tested all DNA samples via agarose gel (1.5%) electrophoresis and quantified them via spectrophotometery. We first amplified 72 microsatellite loci from DNA of blood and muscle samples (Table I). Then, we tested the loci that successfully amplified in 43 fecal samples via PCR using fluorescently labeled primers (FAM, HEX, or TET). We performed PCR amplifications in 20-μL reaction mixtures, consisting of *ca*. 50 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 2.0 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM of each dNTP, and 0.5 U of Hotstart *Taq* DNA polymerase (Qiagen GMBH, Germany). We subjected the reaction mixture to amplification via a GeneAmp PCR System 9700 (Applied Biosystems, USA), with an initial denaturing step of 5 min at 94°C and 35 cycles of 94°C for 30 s, 50–55°C for 30 s, and 72°C for 30 s, followed by 15 min at 72°C. We processed 2 negative extraction controls along with each set of 8–10 fecal extractions, and each set of PCRs contained ≥2 negative PCR controls as well. We repeated amplification 3–4 times for fecal samples. For genotyping, we electrophoresed PCR products along with GeneScan ROX 350 internal size standard on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). We assigned allele sizes against the internal size standard and genotyped individuals via GeneScan version 2.0 (Applied Biosystems).

Data Analysis

We typed samples as heterozygous at 1 locus if both alleles appeared at least twice among replicates and as homozygous if all the replicates showed identical homozygous profiles. If neither case applied, we treated the alleles as missing data. We calculated observed and expected heterozygosities via ARLEQUIN version 3.01 (Laurent *et al.* 2006). We tested all loci for linkage disequilibrium to ensure marker

independence via GENEPOP version 3.4 (Raymond and Rousset 1995; available: http://wbiomed.cutin.edu.au/genepop/).

Results

Of the 72 loci screened, we successfully amplified 34 of them in the samples of *Rhinopithecus bieti*; however, only 13 loci were reliable and polymorphic (Tables I and II). We estimated population genetic parameters for the 13 loci via ARLEQUIN version 3.01 (Laurent *et al.* 2006). The number of observed alleles per locus ranged from 3 to 9, and 5 loci had ≥5 alleles. Observed heterozygosity (H_O) ranged from

Table II Characterization of human microsatellite loci that successfully amplified a polymorphic product from fecal DNA of Yunnan snub-nosed monkeys

Locus	Primer sequence (5′-3′)′	*N*	Annealing temp. (°C)	No. of alelles	Allele Sizes (bp)	H_O	H_E	Success rate (%)
D1S207	F: CACTTCTCCTTGAATCGCTT R: GCAAGTCCTGTTCCAAGTCT	20	52	3	148–156	0.35	0.48	56.5
D1S533	F: CATCCCCCCCAAAAAATATA R: TTGCTAATCAAATAACAATGGG	35	52	5	199–215	0.73	0.77	85.1
D2S1326	F: AGACAGTCAAGAATAACTGCCC R: CTGTGGCTCAAAAGCTGAAT	39	55	4	190–202	0.57	0.61	81.4
D5S1457	F: TAGGTTCTGGGCATGTCTGT R: TGCTTGGCACACTTCAGG	37	50	4	125–137	0.73	0.73	74.2
D6S264	F: AGCTGACTTTATGCTGTTCCT R: TTTTCCATGCCCTTCTATCA	28	51	6	242–256	0.07	0.87	67.5
D6S271	F: CTACTGGCTAAATTGAAGAAGGG R: TTACTTCATTATCTTAGCATACAGAG	32	54	3	235–239	0.00	0.73	73.4
D6S474	F: TGTACAAAAGCCTATTTAGTCAGG R: TCATGTGAGCCAATTCCTCT	23	53	3	126–138	0.33	0.54	51.2
D6S493	F: ATCCCAACTCTTAAATGGGC R: TTCCATGGCAGAAATTGTTT	40	53	4	256–268	0.40	0.41	84.3
D7S2204	F: TCATGACAAAACAGAAATTAAGTG R: AGTAAATGGAATTGCTTGTTACC	32	54	4	284–296	0.69	0.77	75.6
D8S505	F: CAAAAGTGAACCCAAACCTA R: AGTGCTAAGTCCCAGACCAA	41	55	9	144–162	0.56	0.85	86.0
D11S2002	F: CATGGCCCTTCTTTTCATAG R: AATGAGGTCTTACTTTGTTGCC	38	55	5	260–276	0.44	0.63	77.1
D17S1290	F: GCCAACAGAGCAAGACTGTC R: GGAAACAGTTAAATGGCCAA	36	55	5	222–238	0.84	0.78	81.3
D20S206	F: TCCATTATTCCCCTCAAACA R: GGTTTGCCATTCAGTTGAGA	18	52	4	122–134	0.75	0.69	41.9

N = number of individuals genotyped; H_E = expected heterozygosity; H_o = observed heterozygosity.

0.00 to 0.84 and expected heterozygosity (H_E) from 0.41 to 0.87. The average success rate of amplification from fecal DNA was 66.1% (2573 attempts), with the least successful locus (D20S206) at 41.9% (261 attempts) and the most successful locus (D8S505) at 86.0% (184 attempts; Table II). No linkage association was evident from pairwise comparisons of loci ($p>0.05$, Fisher's exact test).

Discussion

Previous studies of genetic variation indicated that the tri- or tetranucleotide repeat markers could reduce occurrence of amplification artifacts such as stutter bands and demonstrated the greater ease of consistent allele identification via automated technology (Bradley *et al.* 2000; Edwards *et al.* 1991). Therefore most of the loci we tested were tri- or tetranucleotide repeat loci. Among the 13 polymorphic loci, 10 are tetranucleotide repeat loci (D1S207, D1S533, D2S1326, D5S1457, D6S474, D6S493, D7S2204, D11S2002, D17S1290, D20S206) and another 3 loci (D6S264, D6S271, and D8S505) are dinucleotide repeats (Table I). Though a powerful marker of population genetics, the use of microsatellite loci is subject to several technical challenges when applied to fecal samples. Fecal samples almost always provide a low amount of degraded DNA with inhibitive factors for amplification, which results in a typically low success rate (Deuter *et al.* 1995; Kohn and Wayne 1997; Monteiro *et al.* 1997; Lathuilliere *et al*. 2001). The success rates obtained with the 13 loci from fecal samples varied from 41.9% to 86.0%, and 10 loci produced success rates >60% (Table II). The loci have moderately-to-highly successful amplification rates, and one could use them in further genetic research on the wild population sampled noninvasively, though we do not recommend D1S207, D6S474, and D20S206 for fecal samples because of their low success rate (Table II). For future research, it might be worthwhile to try a magnesium gradient and more PCR cycles to optimize further the amplification conditions and improve the success rates for the loci on fecal samples.

Chambers *et al.* (2004) compared success rates of human microsatellite cross-species amplification in different primate species. The success rates in Catarrhini (52.9% in *Macaca mulatta*, 34.3% in *Presbytis entellus*, 26.3% in *Hylobates muelleri*, 25.5% in *Cercopithecus aethiops*, 25% in *Papio hamadryas*, and 18.1% *Rhinopithecus bieti*) are higher than those in Platyrrhini (7.9% in *Saimiri boliviensis*; Launhardt *et al.* 1998; Oka and Takenaka 2001; Rogers *et al.* 2000; Smith *et al.* 2000; Witte and Rogers 1999). Ellsworth and Hoelzer (1998) and Zhong *et al.* (1996) also reported poor success rates of cross-species amplification in New World monkeys. It may be preferable to construct specific genomic libraries and to clone new microsatellite loci for New World monkeys. Prior and our results have proved cross-species amplification of human microsatellite loci is a valid method to establish molecular makers for Old World monkeys, and genetic data derived from the reliable loci that we screened will help researchers to investigate the intrapopulation genetic structure and genetic relationships among wild populations of *Rhinopithecus bieti*. In addition, adequate levels of variation at the 13 loci will allow researchers to assess the paternity and other important components such as reproductive strategies in

captive populations, which will establish a sound base of conservation genetics for proper management strategy for Yunnan snub-nosed monkeys.

Acknowledgments The National Basic Research Program of China (973 Program: 2007CB411600), the National Science Foundation of China (30630016 and 30570292), the Innovation Project of the Chinese Academy of Sciences (KSCX2-YW-R-091), and Jiangsu Key Laboratory for Biodiversity and Biotechnology provided funding for this project. We thank Prof. Michael W. Bruford (Cardiff University, U.K.) for technical guidance and data analysis, and Drs. X. J. Zhan, L. F. Zhu, and M. Lu for their laboratory assistance. We express special thanks to Dr. Z. J. Zhang, Beijing Zoo and Beijing Endangered Animal Breeding Center (Daxing, Beijing) for providing Yunnan snub-nosed monkey samples.

References

Bonhomme, M., Blancher, A., & Crouau-Roy, B. (2005). Multiplexed microsatellites for rapid identification and characterization of individuals and populations of Cercopithecidae. *American Journal of Primatology, 67*, 385–391.

Bradley, B. J., Boesch, C., & Vigilant, L. (2000). Identification and redesign of human microsatellite markers for genotyping wild chimpanzee (*Pan troglodytes verus*) and gorilla (*Gorilla gorilla gorilla*) DNA from faeces. *Conservation Genetics, 1*, 289–292.

Chambers, K. E., Reichard, U. H., Moller, A., Nowak, K., & Vigilant, L. (2004). Cross-species amplification of human microsatellite markers using noninvasive samples from white-handed gibbons (*Hylobates lar*). *American Journal of Primatology, 64*, 19–27.

Clisson, I., Lathuilliere, M., & Crouau-Roy, B. (2000). Conservation and evolution of microsatellite loci in primate taxa. *American Journal of Primatology, 50*, 205–214.

Deuter, R., Pietsch, S., Hertel, S., & Muller, O. (1995). A method for preparation of fecal DNA suitable for PCR. *Nucleic Acids Research, 23*, 3800–3801.

Edwards, A., Civitello, A., Hammond, H. A., & Caskey, C. T. (1991). DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*, *49*, 746–757.

Ellsworth, J. A., & Hoelzer, G. A. (1998). Characterization of microsatellite loci in a New World primate, the mantled howler monkey (*Alouatta palliata*). *Molecular Ecology*, *7*, 657–659.

Hadfield, R. M., Pullen, J. G., Davies, K. F., Wolfensohn, S. E., Kemnitz, J. W., Weeks, D. E., *et al*. (2001). Toward developing a genome-wide microsatellite marker set for linkage analysis in the rhesus macaque (*Macaca mulatta*): Identification of 76 polymorphic markers. *American Journal of Primatology, 54*, 223–231.

Jablonski, N. G. (1998a). The evolution of the doucs and snub-nosed monkeys and the question of the phyletic unity of the odd-nosed colobines. In N. G. Jablonski (Ed.), *The natural of the doucs and snub-nosed monkeys* (pp. 13–52). Singapore: World Scientific Publishing.

Jablonski, N. G. (1998b). The response of catarrhine primates to Pleistocene environmental fluctuations in East Asia. *Primates, 39*, 29–37.

Kanthaswamy, S., Dollen, V. A., Kurushima, J. D., Alminas, O., Rogers, J., Ferguson, B., *et al*. (2006). Microsatellite markers for standardized genetic management of captive colonies of rhesus macaques (*Macaca mulatta*). *American Journal of Primatology, 68*, 73–95.

Kohn, M. H., & Wayne, R. K. (1997). Facts from feces revisited. *Trends in Eco1ogy & Evolution, 12*, 223–227.

Lathuilliere, M., Menard, N., Gautier-Hion, A., & Crouau-Boy, B. (2001). Testing the reliability of noninvasive genetic sampling by comparing analyses of blood and fecal Samples in Barbary Macaques (*Macaca sylvanus*). *American Journal of Primatology, 55*, 151–158.

Launhardt, K., Epplen, C., Epplen, J. T., & Winkler, P. (1998). Amplification of microsatellites adapted from human systems in faecal DNA of wild Hanuman langurs (*Presbytis entellus*). *Electrophoresis, 19*, 1356–1361.

Laurent, E., Guillaume, L., & Stefan, S. (2006). Arlequin ver 3.01: An integrated software package for population genetics data analysis. Available at: http://cmpg.unibe.ch/software/arlequin3.

Li, B. G., Pan, R. L., & Oxnard, C. E. (2002). Extinction of snub-nosed monkeys in China during the past 400 years. *International Journal of Primatology, 23*, 1227–1244.

Long, Y. C., Kirkpatrick, C., Zhong, T., & Xiao, L. (1994). Report on the distribution, population, and ecology of the Yunnan snub-nosed monkey. *Primates 35*, 241–250.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K. G., Bonnet, J., Vidal, R., *et al*. (1997). Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. *Journal of Clinical Microbiology, 35*, 995–998.

Morin, P. A., Kanthaswamy, S., & Smith, D. G. (1997). Simple sequence repeat (SSR) polymorphisms for colony management and population genetics in rhesus macaques (*Macaca mulatta*). *American Journal of Primatology, 42*, 199–213.

Newman, T. K., Fairbanks, L. A., Pollack, D., & Rogers, J. (2002). Effectiveness of human microsatellite loci for assessing paternity in a captive colony of vervets (*Chlorocebus aethiops sabacus*). *American Journal of Primatology, 56*, 237–243.

Oka, T., & Takenaka, O. (2001). Wild gibbons' parentage tested by non-invasive DNA sampling and PCR-amplified polymorphic microsatellites. *Primates, 42*, 67–73.

Quan, G. Q., & Xie, J. Y. (2002). *Research on the golden monkey.* Shanghai: Science and Education Publishing House.

Raymond, M., & Rousset, F. (1995). GENEPOP, version 1.2: Population genetics software for exact tests and ecumenicism. *Journal of Heredity, 86*, 248–249.

Rogers, J., Mahaney, M. C., Witte, S. M., Nair, S., Newman, D., Wedel, S., *et al*. (2000). A genetic linkage map of the baboon (*Papio hamadryas*) genome based on human microsatellite polymorphisms. *Genomics*, *67*, 237–247.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual, 2nd edn*. New York: Cold Spring Harbor Laboratory Press.

Smith, D. G., Kanthaswamy, S., Viray, J., & Cody, L. (2000). Additional highly polymorphic microsatellite (STR) loci for estimating kinship in rhesus macaques (*Macaca mulatta*). *American Journal of Primatology, 50*, 1–7.

Witte, S. M., & Rogers, J. (1999). Microsatellite polymorphisms in Bolivian squirrel monkeys (*Saimiri boliviensis*). *American Journal of Primatology, 47*, 75–84.

Zhong, F., Brady, A. G., Shi, J., Abee, C. R., McCombs, C. C., & Michalski, J. P. (1996). Strategy using pooled DNA to identify 56 short repeat polymorphism for the Bolivian squired monkey. *BioTechniques*, *21*, 580–586.