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METHODS AND RESOURCES ARTICLE

Development of 34 SNP markers developed from the genome resequencing dataset in golden snub-nosed monkey (*Rhinopithecus roxellana*) using high resolution melting (HRM)

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Abstract As single nucleotide polymorphisms (SNPs), known as the third-generation molecular markers, are extensively used in genetic studies. The rapid development of high-throughput sequencing technologies has enabled the development of a large number of SNP markers to be cost-effective and rapid. Based on the genome resequencing dataset of *Rhinopithecus roxellana*, we characterized 34 polymorphic SNP markers using a high resolution melting diversity assay. The observed heterozygosity varied from 0.323 to 0.725, while the expected heterozygosity ranged from 0.352 to 0.638. In addition, the MAF varied from 0.206 to 0.5000, while HWE values varied from 0.060 to 0.725. To the best of our knowledge, this is the largest set of SNP markers developed for *R. roxellana* by far, and could be useful in further geographic populations, analysis of evolutionary relationships and conservation genetics of this species.

Keywords *Rhinopithecus roxellana* · Single nucleotide polymorphisms (SNPs) · High-resolution melting (HRM) analysis

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Introduction

The golden snub-nosed monkey, *Rhinopithecus roxellana*, is an endangered primate endemic to the broadleaf and mixed temperate mountain forests of central China (Li et al. 2003). Over the past several decades, both quality and quantity of suitable habitat available to this species has decreased dramatically, almost entirely due to human commercial factors such as logging, illegal hunting, agricultural expansion or other infrastructures (Guo et al. 2008). Current census data suggest that fewer than 22,000 individuals remain which are divided into three large isolated populations, including ca. 16,000 individuals in Sichuan-Gansu population, ca. 5500 individuals in Shaanxi population, and ca. 1000 individuals in the Shennongjia population (Zhang et al. 2002).

Over the past several decades, the molecular biology techniques have had significant impacts on the fields of ecology, evolution and conservation (Avise 2000). Single nucleotide polymorphisms (SNPs) and short tandem repeats (STRs) are two kinds of genetic markers widely used in phylogenetic analysis, individual identification and paternity analysis (Di Fiore 2003; Morin et al. 2004). However, because the target DNA sequence in SNPs-based genotyping is much shorter (50–70 bp) than that in STRs-based genotyping (80–300 bp) (Morin et al. 2004), SNPs are more appropriate molecular markers than STRs for the degraded DNA samples in the study of endangered wildlife.

In recent years, the rapid development of high-throughput sequencing technologies has enabled identifying candidate SNP markers to be easy and rapid (Baird et al. 2008; Lijavetzky et al. 2007). In the present study, we developed a set of SNP markers based on the genome resequencing data (~6000 million reads from 27 individuals, ~10-fold genome coverage, unpublished) of *R. roxellana* generated on the

Table 1 Characteristics of 34 SNP markers in *Rhinopithecus roxellana*

Locus ID	Primer	Allele	Size (bp)	Tm (℃)	Ho	He	MAF	PHW
2	F: AGAAAGACATAGGAGACCTC R: GAAGCCTGGCTCTGATGTTC	C/T	43	60	0.323	0.352	C 0.438	0.543
3	F: GGTTCTAACTTACTGGGTTT R: TGCTATCAGCAATGTTCTCA	G/A	43	60	0.427	0.476	G 0.412	0.175
8	F: TGTCTGCATCACATGGAGT R: ATTGCTTTACCACATGCTC	T/G	39	60	0.421	0.437	T 0.341	0.190
14	F: GAGAAACCTGTAGTGTTTGG R: GGTCCCTTTGATCTCATTC	A/G	42	60	0.459	0.402	A 0.413	0.417
15	F: AGCCAGATGGAAGGGAAGC R: CGGTGACCAGGATGAAGC	T/C	43	60	0.528	0.481	T 0.291	0.632
17	F: CTTTCAGAAAGGCAGCAG R: ACAGCTCTGTCTCCCTGG	C/A	40	60	0.725	0.638	C 0.317	0.234
20	F: CATTAGTACAGTAGTAACAGCTA R: CATTGCTGTGAGCATTAGT	T/C	48	58	0.433	0.417	T 0.363	0.413
22	F: CTCCCAGGGGCTGAGCCT R: CCTTCCTGACCCTATCCA	A/G	39	58	0.487	0.445	A 0.500	0.376
29	F: TGTCTGCTCCATGCTGTG R: CAATCAGGATCTTGCCAT	A/G	43	58	0.446	0.499	A 0.354	0.326
39	F: GAGGGGCTCCTTGTCCAT R: GGTTTCCAGTGACTGATTAT	C/T	43	60	0.465	0.421	C 0.268	0.592
43	F: TCTTCTGCCCTTTTGTCA R: GCAACTTTGGCATCTTTT	G/A	45	60	0.562	0.552	G 0.311	0.341
46	F: GGAATAGGGGTGCCAGGACAA R: TGGAAATGAACTCTGACAAGG	C/T	84	60	0.423	0.505	C 0.380	0.552
51	F: GTTGTAGGGGCTAGGTATACTG R: AATAGAAGATGGCGAGCCTG	G/A	45	60	0.431	0.402	G 0.291	0.674
53	F: CAGAAATGTTGACAGAAA R: TCATCTGGTATTTCACTT	C/T	43	58	0.372	0.371	C 0.317	0.406
61	F: AGGTTCCATTATCACACACT R: ACCTAGAGGCAGGTTTTG	C/T	44	60	0.427	0.467	C 0.241	0.126
63	F: CAGCCTCACCTAATGTCC R: GAGACTCATCTAGTTTCAA	C/T	44	60	0.498	0.415	C 0.400	0.313
64	F: GTACACCTGGTAGGACTCTGA R: TGTGAAGATAGAGGAAATCTG	T/C	48	60	0.525	0.49	T 0.218	0.470
66	F: TCCTTGATGCTGACTCTT R: TTATTCATACAGCATGTT	C/A	37	60	0.443	0.421	C 0.460	0.365
68	F: TCCTCTGAGACAGGTGGGG R: CTACACCTGCATCTCCATC	C/T	40	60	0.385	0.353	C 0.314	0.210
74	F: TTTGCTTCCAGCCAGCC R: GTGGAGAGCCCTAACCG	T/C	39	60	0.450	0.387	T 0.318	0.430
79	F: GGGATGGTAACAAGCGAAAT R: TTCCATTGTTTAAGGAACCG	A/G	45	60	0.448	0.471	A 0.302	0.223
87	F: CAGCCTAAGCCCTGGAAT R: TGCAAAACAGTTTGATGC	G/A	43	60	0.561	0.473	G 0.161	0.069
88	F: AAGCACAGAAAATAGCCA R: GTGCAAAGCATCTCCCTC	T/C	38	60	0.454	0.421	T 0.383	0.554
94	F: CTAGGGTTCTAGGATGTC R: ACAGGAACTGAGACTCAACT	T/C	45	60	0.362	0.325	T 0.212	0.060
95	F: CCCAACCACCAGAAGAGG R: CTCCTCACAGAATCTGCA	A/G	39	60	0.515	0.447	A 0.427	0.168
98	F: TTGGGGCAAGTTTCTGGG R: CTGCCTACATCTAAATGACA	C/T	44	60	0.424	0.474	C 0.193	0.616
103	F: TAGACTCCGGGTGTAGCC R: CACTCAATTCCTGAGCCA	A/G	39	60	0.518	0.489	A 0.500	0.511

Table 1 (continued)

Locus ID	Primer	Allele	Size (bp)	Tm (℃)	Ho	He	MAF	PHW
111	F: GAACATTGTTCTGGATTTCA R: TATCATGTCCCTGGGCTTGT	T/C	45	60	0.44	0.479	T 0.334	0.423
112	F: GATAACTGGTTCTGTTCTCC R: GTCGCAGCACAGTCAAGATG	G/A	43	60	0.346	0.381	G 0.318	0.347
113	F: GCAAGGGAGCATGGAAAC R: TCCAAGACCGTCCGAAAT	T/G	57	60	0.565	0.491	T 0.378	0.224
114	F: CTGCCCTCCAAGGTAAATCAC R: GTATAGTCAAATCTTTCATCC	C/T	43	60	0.494	0.459	C 0.249	0.216
115	F: TAATGGCTTCATTGTATTTTAC R: AACATAAGTGCATTTTCCAC	T/C	45	58	0.420	0.426	T 0.305	0.725
116	F: TGAGGATGCACCAAGAAGC R: ACATTGGAGTTAAGGGAGAAA	T/C	58	60	0.455	0.431	T 0.416	0.413
124	F: GCAGGTTTGCACTCTTACT R: AAGCAACGAGTCATGTAAG	A/G	47	60	0.454	0.432	A 0.206	0.139

Tm the anneal temperature, *Ho* observed heterozygosity, *He* expected heterozygosity, *MAF* the minor allele frequency, *HWE* Hardy–Weinberg equilibrium

Illumina HiSeq 2500 platform. A total of 85 putative SNP loci were randomly chosen to design the primers using Primer Premier 5.0 with the criterion of an optimum target amplicon length of 50 bp, maximum length of 100 bp and target Tm of 60 °C. High-resolution melting (HRM) analysis which has proven to be a highly sensitive method for the SNP genotyping is used to detect the SNPs. Genomic DNA was extracted from 15 fecal samples using the QIAamp® DNA Stool Kit (Qiagen) according to manufacturer's instructions. Extracted DNA was quantitated using the ratio of absorbance at 260 and 280 nm on a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Amplification of primers was screened using a normal PCR reaction in 25 μL volumes containing 30 ng DNA, 1× PCR buffer, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase (Takara), 0.2 μM each primer. The amplification reaction was performed by denaturing at 95 °C for 5 min; followed by 40 cycles of denaturing at 95 °C for 30 s, annealing temperature gradient between 54 and 64 °C for 30 s, and extending at 72 °C for 30 s and a final extension at 72 °C for 5 min. Following amplification, the PCR products were analyzed by 2% agarose gel electrophoresis to verify the presence of a single band at the expected product size. Primer pairs which failed to be amplified or showed non-specific bands were omitted from further tests.

Polymorphism of the remaining 37 SNP loci was subsequently tested in 15 fecal samples using HRM on a Roche LightCycler 480 to verify the ability of these markers in degraded DNA. The reaction mixture contained 10 ng DNA, 5 μL LC480 HRM Master Mix (Roche, Germany), 0.2 μM of each primer and adjusted with RNase-free water to a final volume of 10 μL. A negative control containing sterile water in place of template DNA was added during each run of the PCR to check for contamination. The amplification was achieved by the following protocol: an initial denaturation step at 95 °C for 10 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C or 58 °C for 20 s, and extension at 72 °C for 15 s. For HRM genotyping, melting program included three steps: denaturation at 95 °C for 1 min, renaturation at 40 °C for 1 min, and a subsequent melting cycle with a continuous fluorescent reading from 65 to 90 °C at a rate of 25 acquisitions per degree celsius. Melting curve analysis was performed using the LightCycler 480 Gene Scanning Software Version 1.5 with default settings. The statistical analysis of genetic variations, including the minor allele frequency (MAF), observed heterozygosity (Ho), expected heterozygosity (He), and Hardy–Weinberg equilibrium (HWE) was performed using PopGene 32 (Yeh et al. 2000).

Of the 37 pairs of primers, 34 (91.9%, Table 1) pairs were genotyped successfully by HRM, and 3 loci could not be distinguished due to more than one SNP were found in the PCR product. The observed heterozygosity varied from 0.323 to 0.725, while the expected heterozygosity ranged from 0.352 to 0.638. In addition, the MAF varied from 0.206 to 0.5000, while HWE values varied from 0.060 to 0.725.

Individual identification is an important step in the noninvasive population genetics and behavioral studies of endangered wildlife. The power of SNPs for individual identification is about two to four times less than that of multi-allelic STRs markers (Glaubitz et al. 2003). Therefore, it is important to test the power of these 34 loci of SNP markers to identify all individuals in *R. roxellana* populations. Computer simulations were performed with the MATLAB software Version 6.1 (Math Works Inc., Natick, MA, USA) to estimate a given number of SNPs to uniquely

identify all individuals within 95 of 100 simulated populations of a specified size. Simulation results show that this marker set was expected to uniquely identify all members of *R. roxellana* populations with less than 50,000 individuals. In consideration of a total population of *R. roxellana* is less than 22,000 individuals, there is no problem for the 34 SNP markers to be used in the study of *R. roxellana*.

In conclusion, we have developed a set of 34 SNP markers in *R. roxellana* from the genome resequencing dataset. These SNP markers could be useful in the research on geographic populations, analysis of evolutionary relationships and conservation genetics of this species.

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References

Avise JC (2000) Phylogeography: the history and formation of species. Harvard university press

Baird NA et al (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. PloS One 3:e3376

Di Fiore (2003) Molecular genetic approaches to the study of primate behavior, social organization, and reproduction. Am Phys Anthr 122:62–99

Glaubitz JC, Rhodes OE, DeWoody JA (2003) Prospects for inferring pairwise relationships with single nucleotide polymorphisms. Mol Ecol 12:1039–1047

Guo S, Ji W, Li B, Li M (2008) Response of a Group of Sichuan Snub-Nosed monkeys to commercial logging in the Qinling mountains, China. Conserv Biol 22:1055–1064

Li B, Jia Z, Pan R, Ren B (2003) Changes in distribution of the snub-nosed monkey in China. In: Primates in fragments. Springer, pp 29–51

Lijavetzky D, Cabezas JA, Ibáñez A, Rodríguez V, Martínez-Zapater JM (2007) High throughput SNP discovery and genotyping in grapevine (Vitis vinifera L.) by combining a re-sequencing approach and SNPlex technology. BMC Genom 8:1

Morin PA, Luikart G, Wayne RK (2004) SNPs in ecology, evolution and conservation. Trends Ecol Evol 19:208–216

Yeh F, Yang R, Boyle T, Ye Z, Xiyan J (2000) PopGene32, Microsoft Windows-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada

Zhang Y, Chen L, Qu W, Coggins C (2002) The primates of China: biogeography and conservation status Asian. Primates 8:20–22