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Noninvasive genetic assessment of the population trend and sex ratio of the Shennongjia population of Sichuan snub-nosed monkeys (*Rhinopithecus roxellana*)

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Effective population management relies on assessments of population size and sex ratio. However, these estimates are difficult to obtain for elusive and rare species. Recently, noninvasive genetic census methods have been developed as an alternative to traditional capture-mark-recapture methods. In this study, we estimated the size of the Sichuan snub-nosed monkey (*Rhinopithecus roxellana*) population in the Shennongjia Nature Reserve (SNR) using a noninvasive sampling method based on 16 microsatellite loci. We also used a PCR-based genetic method to sex the sampled individuals and infer the population sex ratio. The population size of *R. roxellana* in the SNR was estimated to be 1044 individuals (95% CI_{TIRM}: 613–1409). The estimated population sex ratio is more female-biased than expected, which we attribute to the sampling biased towards one male units and limited sampling of bachelor male units. Moreover, there is no suggestion that the heavy traffic road through the reserve might block movement of monkeys. The results of this study indicate genetic assessments based on a noninvasive sampling method can provide useful information regarding populations of elusive primates.

snub-nosed monkey, Shennongjia, noninvasive sampling, population size, sex ratio

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The Sichuan snub-nosed monkey (*Rhinopithecus roxellana*), also known as the golden monkey, is an endangered primate endemic to China. Despite a wide distribution in China during the Pleistocene [1], wild *R. roxellana* populations now only occur in 3 isolated mountainous regions. The total population size is estimated to number about 15000 [2,3]. Because of large-scale commercial timber exploitation between the 1950s and early 1980s, the habitats of *R. roxellana* in the Shennongjia Nature Reserve (SNR) became fragmented, and illegal hunting led to the capture of at least 130 monkeys in the 1970s and 1980s [4]. In the SNR, the population size was estimated to be more than 2000 individuals prior to 1970 [5]. However, the population had dropped to an estimated 500 individuals in 1989, a reduction of 75% [4].

Effective conservation management requires data on population size and sex ratio. Traditional capture-mark-recapture (CMR) methods are an efficient tool but may be difficult to apply to rare, elusive and capture-sensitive species [6]. In addition, traditional CMR methods that require trapping or handling of individuals may harm the animals. For difficult-to-observe species, noninvasive genetic surveys have in recent years provided valuable information for the management and monitoring of populations [7–13]. These studies showed that use of molecular census techniques may reduce time and effort and provide more detailed information about elusive species [14]. However, because of the relatively high cost, noninvasive genetic census techniques normally are applied only to small areas

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or small and/or isolated populations [11].

R. roxellana is highly arboreal and only occasionally descend to the ground. Because of their shy nature, quick movements and occurrence in remote and inaccessible areas, achieving estimates of population size is difficult. Unlike some other ape species, which build large nests that are easily detected, *R. roxellana* are not nest builders. The most commonly used census method is individual counts based on line transects. However, traditional line transect methods do not work well for arboreal primates that tend to flee silently through the forest canopy before being counted [15]. In addition, vegetation can often hamper efforts to spot and count individuals. Cui et al. [16] once estimated group sizes of Yunnan snub-nosed monkeys (*Rhinopithecus bieti*) on the basis of feces amount at sleeping sites. However, this method gave a highly uncertain estimate owing to the influence of weeds, shrubs and tangles of fallen branches [16].

In this study, we conducted a pilot project aimed at determining the suitability of noninvasive genetic surveys for *R. roxellana* in the SNR. Our goals were to elucidate the population trend during the past two decades and estimate the sex ratio of the wild *R. roxellana* population in the SNR. We also suggest directions for further research and place our results in the context of conservation of this endangered species.

1 Materials and methods

1.1 Study site and sample collection

Research was conducted in the Shennongjia Nature Reserve (31°21′20″–31°36′20″N, 110°03′05″–110°33′50″E) in Hubei Province, China (Figure 1). The SNR is a mountainous area of ca. 705 km^2. One road passes through *R. roxellana* habitat, which consequently represents a potential hindrance to monkey movement. The SNR was covered originally with primary forest. A large part of the reserve was clear-cut between the 1950s and early 1980s [17]. Presently, 4 types of habitats occur in the reserve: grassland, shrub forest and bamboo, young forest, and primary forest [18].

We collected fecal samples from throughout the study site from November 2007 to October 2008. *R. roxellana* are diurnal animals. Li [19] showed *R. roxellana* in the SNR exhibit 2 travel peaks (morning and afternoon) and a midday rest period with little or no travel. The rest periods for all seasons were identical, of about 1.5 h duration from 12:45 to 14:15. Generally, the monkeys would defecate during rest periods and we could collect the fecal samples under the trees after the monkeys left the rest site. Thus, most of the fecal samples in our study were defecated by the monkeys on the same day, with the exception of the site YJW, which was estimated to be 3 days old. The geographic coordinates of each group sampled were recorded using a GPS. In total, we collected 337 fecal samples at 8 sites (Figure 1), which were desiccated using the two-step ethanol-silica procedure [20] and stored at room temperature.

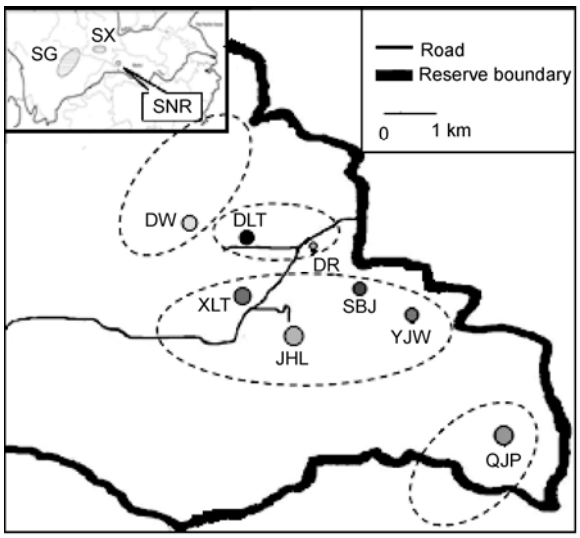

Figure 1 Location of the Shennongjia Nature Reserve study area. Collection sites are indicated by circles with different colors representing different sample periods. The size of the circles is directly proportional to the number of fecal samples collected. Dashed ellipses indicate the seasonal distribution ranges of golden monkeys in the SNR.

1.2 DNA extraction and amplification

Fecal samples were extracted using the QIAmp Stool Kit (QIAGEN). Two negative extraction controls were processed along with 10 fecal extractions. The amount of amplifiable DNA in the extracts was estimated by means of a quantitative polymerase chain reaction (PCR) assay as previously described [21]. Extracts that contained less than 10 pg DNA/μL were not analyzed further.

Three independent amplifications from each DNA extract were performed for 16 loci originally characterized in humans and used for studies of other primates (D1S533, D1S1656, D1S1665, D2S442, D5S1457, D6S474, D6S493, D6S1056, D7S817, D7S1826, D7S2204, D10S611, D10S676, D10S1432, D13S321 and D14S306) together with a minimum of 6 negative controls (in which 5 μL H_2O rather than DNA was added to the well). A two-step multiplex PCR method was used as described by Arandjelovic et al. [22]. Multiplex PCRs were conducted in a 20-μL reaction volume containing 1× SuperTaq buffer (HT Biotechnology), 1.75 mmol/L $MgCl_2$, 0.15 mmol/L of each forward (unlabelled) and reverse (unlabelled) primer, 110 μmol/L of each dNTP, 16 μg bovine serum albumin, 0.5 U SuperTaq (HT Biotechnology) premixed 2:1 with TaqStart Antibody (BD Biosciences), and 5 μL template DNA. The PCR protocol was as follows: initial denaturation for 9 min at 94°C, 30 cycles of 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and a final extension of 5 min at 72°C. Singleplex PCRs were carried out as above but with the following modifications: 5 μL of 1:100 diluted multiplex PCR product was used as template, half the amount of $MgCl_2$ (0.875 mmol/L)

was added, and only 0.35 U of SuperTaq (HT Biotechnology) premixed 2:1 with TaqStart Antibody was used. Moreover, each singleplex PCR only contained a single primer pair: 0.25 mmol/L of a FAM, HEX, or NED fluorescently labeled forward primer and 0.25 mmol/L of a reverse primer. To guard against contamination, all steps of the PCR set-up for DNA samples (except the addition of template) were performed under a hood that was irradiated with ultraviolet light before and after use.

Up to 3 different PCR products were combined and electrophoresed on an ABI PRISM 3100 Genetic Analyser and allele size was determined relative to an internal size standard (ROX-labeled HD400) using GeneMapper version 3.7 software (Applied Biosystems). Genotypes were confirmed with 99% certainty by observation of each allele twice in two or more independent reactions for heterozygote genotypes, while homozygous genotypes were ascertained by up to 5 independent observations depending on the quality of DNA in the extract.

1.3 Sex identification

Villesen and Fredsted [23] designed a PCR assay to reliably sex ape and monkey DNA samples. The PCR yields fragment sizes of 180 bp (X) and 209 bp (Y), respectively. Males were identified by two bands (the X and Y fragments) and females were identified by a single band (the X fragment). DNA of one known male and one known female monkey from the SNR were used as positive controls. Cycling conditions were 94°C for 5 min, and 45 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s, and a final extension of 72°C for 10 min. Each sample was amplified 3 times and PCR fragments were separated on 2.5% agarose gels (100 V, 1 h). A sample was identified as male if all amplifications showed the Y band, and as female if no Y band was produced.

1.4 Error control

Because of the low quality and/or quantity of DNA derived from noninvasive genetic samples, genotyping errors are difficult to eliminate and may lead to incorrect genotypes and consequently to overestimated population size [24]. To avoid erroneous results arising out of genotyping error caused by nonreplicable results or the nonamplification of an allele in heterozygotes ('allelic dropout'), all results were subject to the appropriate amount of replication given the quantity of DNA present as detailed by Arandjelovic et al. [22]. However, such a 'multitube' approach cannot detect errors that may be created when scoring or transcribing data into a database [25]. We therefore also used two screening methods, the examining bimodality and difference in capture history tests, implemented in the DROPOUT package to detect genotyping errors [26]. We also used MICRO-CHECKER to look for genotyping errors owing to null alleles, short allele dominance (large allele dropout) and scoring errors because of stuttering [27].

1.5 Genotype data analysis

We used CERVUS 3.0 software [28] to find matching genotypes and to assess the probability of full siblings or unrelated individuals having an identical multilocus genotype (PID_{sib} and PID). To determine with 99.9% confidence that two matching samples originated from the same individual, we determined the minimum number of loci necessary to obtain a PID_{sib} value of $\leqslant 0.001$ [29]. Matching samples were given a consensus ID and genotype for use in subsequent analysis. Genotypes from different samples mismatching at three or fewer loci were re-examined for possible scoring or transcribing data errors. CERVUS 3.0 was also used to calculate the number of alleles per locus (A), observed (H_O) and expected (H_E) heterozygosities for each locus, and to test for deviations of used loci from Hardy-Weinberg equilibrium. Genotypic disequilibrium between loci within the population was tested using FSTAT version 2.9.3.2 [30]. Significance values were adjusted by Bonferroni correction for multiple testing, as implemented in the software.

1.6 Population size estimation

Grouping all samples into a single-sampling session scheme, we then calculated genetic capture-recapture estimates in 3 models. Miller et al. [31] developed a maximum likelihood estimator implemented in the CAPWIRE software. Unlike the traditional mark-recapture method, this method deals efficiently with data inferred from multi-observations of individuals within a sampling session. The even capture model (ECM) assumes there is no capture heterogeneity in the data set, whereas the two innate rates model (TIRM) assigns individuals as having unequal capture probability. We used both ECM and TIRM models to estimate population size, as we could not be certain whether capture heterogeneity existed in our data. Both models assume a closed population (no births, deaths, or migration) and a recapture probability equaling the capture probability. Miller et al. [31] found one heterogeneity estimator in the program CAPTURE (M_h-Chao) [32] performed much better when dealing with populations larger than 100 individuals. Given our expected large census population size, we also used M_h-Chao to estimate population size. The M_h-Chao Model is implemented in the program CAPTURE [33]. Algorithms in CAPTURE also assume the population is closed to births, deaths, emigration and immigration. All of the three models yield population size estimates and a 95% CI of this estimate.

1.7 Birth data collection

Birth data were collected from a provisioned free-ranging

group at the DLT locality between 2006 and 2010. The individual monkeys in the DLT group were provided with food from 2005 so that we could achieve better observation. Apples, chopped radishes, lichen and peanuts were scattered on the ground randomly three times per day (9:00, 12:00, and 16:30). To minimize the effect of artificial feeding on their natural diet, we controlled the quantity of provisioning for each monkey. Individuals in this group were accustomed to the approach of researchers. Our observations were performed at distances between 0.5 and 50 m. Observations from such close distance enabled us to identify each individual and to distinguish the sex of the new-born infants.

2 Results

We attempted to genotype 337 DNA extracts at 16 loci. Nine extracts did not yield any genotypes, 15 extracts yielded genotypes at fewer than 5 loci, and 26 extracts yielded genotypes at 5 to 10 loci. Among the remaining 287 extracts, 196 (68.3%) extracts provided a complete genotype at the 16 loci. The DNA concentration of most unsuccessful extracts was low (<35 pg/uL). The locus D13S321 deviated significantly from the Hardy-Weinberg equilibrium. After applying Bonferroni correction, none of the paired loci significantly deviated from genotypic disequilibrium. Overall, the microsatellite loci used were polymorphic with an average of 4.5 alleles. The average observed and expected heterozygosity of 16 loci was 0.591 and 0.589, respectively (Table 1). The probability of two full siblings sharing the same multilocus genotype at the 16 loci was 2.11×10^{-5} (PIDsib) and the corresponding probability for two unrelated individuals was 2.71×10^{-11}.

Table 1 Characteristics of microsatellite loci used in this study[a)]

Locus	*A*	*N*	H_O	H_E
D14S306	4	153	0.575	0.590
D7S2204	6	151	0.728	0.699
D5S1457	6	148	0.601	0.638
D6S1056	5	149	0.497	0.507
D1S1656	5	150	0.660	0.629
D6S493	4	151	0.702	0.695
D10S611	5	148	0.669	0.718
D6S474	3	150	0.633	0.578
D1S1665	4	146	0.610	0.653
D2S442	7	140	0.750	0.742
D10S1432	7	150	0.673	0.661
D1S533	3	152	0.592	0.569
D7S817	3	152	0.507	0.441
D7S1826	3	151	0.377	0.333
D10S676	3	152	0.572	0.549
D13S321	4	143	0.315	0.421
Mean	4.5	149	0.591	0.589

a) *A*, Number of alleles per locus; *N*, number of samples; H_O, observed heterozygosity; H_E, expected heterozygosity.

MICRO-CHECKER analysis provided no evidence of null alleles, large allele dropout or scoring error because of stuttering in our final data set, with the exception of D13S321, which showed evidence of null alleles and possible scoring error causd by stuttering. DROPOUT analysis indicated genotyping errors were reduced to a non-significant level.

Among the 287 extracts that yielded genotypes, 153 unique individuals were identified, which represented the minimum number of individuals using the area. The number of captures/recaptures per individual ranged from 1 to 3. The ECM produced the lowest point estimate of 934 (95% CI_{ECM}: 603–1484) monkeys, whereas the TIRM gave the highest point estimate of 1044 monkeys and the smallest confidence interval (95% CI_{TIRM}: 613–1409). The M_h-Chao model gave an intermediate point estimate of 970 monkeys and the largest confidence interval (95% CI M_h-Chao: 583–1704). Considering the entire 705 km^2 study area, the density of *R. roxellana* in the SNR was estimated to be 1.32 monkeys/km^2 (95% CI: 0.86–2.1) with the ECM, 1.48 monkeys/km^2 (95% CI: 0.87–2) with the TIRM, and 1.38 monkeys/km^2 (95% CI: 0.83–2.42) with the M_h-Chao model.

Most monkeys were genetically sampled ('captured') only once, but 11 monkeys were sampled twice and one monkey was sampled 3 times. One individual was sampled at 3 different localities (XLT, JHL, and SBJ) and an additional 6 monkeys were samples at both XLT and JHL. Three individuals were sampled at both XLT and SBJ, and 2 at both SBJ and YJW. Given that groups of *R. roxellana* were relatively stable and not characterized by periods of fission and fusion [34], our results suggested that the individuals sampled at those 4 sites (JHL, YJW, XLT, and SBJ) belong to the same group. These collection localities span the road in the reserve, and so further indicate that *R. roxellana* were able to cross the road. One additional individual was identified at both the DR and DW sampling localities, but additional sampling would be necessary to estimate the composition of additional groups.

Five extracts failed to amplify using the sexing assay. Among the remaining 148 samples we identified 91 females and 57 males. The sex ratio (M/F) for all individuals was about 1:1.6. For the DLT group, 36 infants (17 males and 19 females) were born from 2006 to 2010. The sex ratio (M/F) in this group was approximately 1:1.12 (Table 2).

Table 2 Sex of new-born infants and sex ratio in the DLT group from 2006 to 2010

Year	Sex of new-born infants		Sex ratio (M:F)
	Male	Female	
2006	2	4	1:2
2007	3	2	1:0.67
2008	6	6	1:1
2009	3	2	1:0.67
2010	3	5	1:1.67
Total	17	19	1:1.12

3 Discussion and conclusions

Small and island populations have a higher extinction risk than mainland populations [35]. However, direct estimates of extinction risk are frequently unavailable. O'Grady et al. [36] proposed that population size was the best predictor of extinction risk and was therefore the most cost-effective data to collect on threatened species. Population monitoring is essential to evaluate the success of conservation efforts, yet standard survey methods may be ineffective because animals flee silently before they are seen [15]. Population estimation using a noninvasive genetic census technique does not require visual or physical contact with the individuals, and allow for larger sample sizes to be collected than would be possible with traditional trapping methods [24]. However, several sources of potential error are associated with this method, including failure to identify individuals and incorrect assignment of individual genotypes [24]. Population estimates have tended to be biased downward because of the 'shadow effect' [37], a phenomenon of multiple individuals showing identical tags caused by using too few loci or loci with low heterozygosity. In this study, the set of 16 microsatellite loci was enough to differentiate between the individuals in the area. Therefore, the shadow effect was not a serious problem in our study. Overestimation because of genotyping errors is much more difficult to control. In this study, we used a two-step multiplex PCR method, which can significantly increase the success rate and efficiency of genotyping and substantially reduce the average allelic dropout rates [22]. Analysis with DROPOUT also indicated genotyping errors in our study decreased to a negligible level and did not affect the estimate of population size.

This study demonstrated the utility of genetic capture-recapture to estimate the size of *R. roxellana* population. All of the 3 models gave consistent point estimates. However, the ECM model is known to be biased downwards when capture heterogeneity exists [6]. Therefore the M_h-Chao and TIRM models might provide the most robust estimates. Our results indicated the population size of *R. roxellana* in the SNR substantially increased from 500 to 1044 individuals during the past two decades. Based on a direct count, Yang et al. [38] estimated the SNR population size to be 1282 individuals, a value larger than our estimates but still within our 95% CI. Because of the preliminary nature and limited sampling effort in the present study, we could only obtain a rough estimate of population size and a wide confidence interval. Previous studies have shown that the sampling intensity influences the confidence of the estimate [31]. The difficulty of finding *R. roxellana* in the field is the major reason we were unable to collect additional fecal samples in this study. *R. roxellana* usually travel to remote and inaccessible areas rendering it difficult for researchers to locate them. For instance, the QJP group was untraced for more than 6 months in 2008. In addition, because of the small population size, it took considerable searching time to locate monkeys in such a large area. However, the focus in this study was not to obtain a precise estimate of population size but to elucidate the population trend. Thus, sampling effort can be reduced to the point that will provide enough statistical power to detect the trend [6]. To achieve an accurate estimate of population size in future, the number of samples collected should be approximately three times the 'assumed' number of individuals [6].

Sex ratio is also a key parameter to monitor population health, because it can dictate mate competition/choice and affect population growth rates [39]. In the field, it is very difficult to distinguish the sex of infant and subadult *R. roxellana* (subadult males: 3–5 years old, subadult females: 3–4 years old, [34]). Therefore, previous field studies only described the sex ratio of adults, while in the present study we do not know the ages of the monkeys and so report the sex ratio for all sampled individuals. For the SNR population, the adult sex ratio (M/F) differed in previous studies from 1∶1.42 in ref. [4] and 1∶2.8 in ref. [34]. The sex ratio (M/F) of all individuals of 1∶1.6 in the present study was similar to that previously reported for adults, which showed a bias toward females. Qi et al. [40] found slightly more male infants than female were born based on four years of investigation in the Qinling mountains. However, observation of the DLT group for five years suggests that in SNR the sex ratio of new-born infants does not significantly differ from 1∶1 (M/F = 1∶1.12) (Table 2). There are at least two possible explanations for our observed female-biased sex ratio in the SNR. One possibility is that the social structure of *R. roxellana* resulted in the collection of a biased set of samples. *R. roxellana* groups are composed of two basic units: one male units (OMUs) and all male units (AMUs). An OMU is composed of one adult male, several adult females, several subadults and infants. An AMU contains several adult and subadult males. The sex ratio (M/F) in OMUs was estimated previously as 1∶7 and so is highly biased toward females [34]. Field investigations showed the individuals in AMUs are consistently located at the periphery of the group whenever resting or moving [34]. This spatial distribution of individuals can result in unequal capture possibilities for the two sexes. The female-biased sex ratio suggests we collected more samples from OMUs than from AMUs. An alternative, nonmutually exclusive explanation for our results is that the population genuinely exhibits a female-biased sex ratio. Deviations from an equal sex ratio are common in mammalian populations and, according to a complex framework of models and hypotheses, either adaptive mechanisms (social or parental control of sex ratio from conception onward [41,42]) or nonadaptive ones, e.g., unpredictable environmental events leading to different mortality rates in the sexes [43,44], can shift primate sex ratios from parity [45]. Future work employing a more extensive genetic census of the population will serve to refine the estimate of the population sex ratio.

Natural and anthropogenic landscape features, such as

rivers, mountain ranges and roads, can provide complete or semipermeable barriers that alter animal dispersal paths and movement patterns [46]. Roads have been shown previously to affect animal movement patterns and typically impede dispersal and reduce gene flow [47]. In the present study, we showed that the heavy traffic road did not present a barrier to movement of *R. roxellana* groups, which is probably because of the relatively high mobility of *R. roxellana*.

The Sichuan snub-nosed monkey is a flagship species for conservation in China, and is an object of public interest and national pride. Protection of Sichuan snub-nosed monkeys as 'umbrella' populations is both a biologically and culturally effective way to preserve whole ecosystems [48]. Although the population experienced a slight increase during the past two decades, the outlook for the species is still worrisome. In the winter of 2008, the worst snowfall in the last 5 decades hit large portions of southern and central China [49]. The huge storm and extremely low temperature killed 73 individuals in one group, a reduction of 27.2% [50], an impact consistent with the high vulnerability of island populations to extreme environmental changes. Reed et al. [51] defined minimum viable population sizes (MVPs) that could ensure a 99% probability of population persistence for 40 generations. These authors estimated the mean MVPs to generally be 7316 adults, an estimate that was far higher than the current size of the SNR *R. roxellana* population. Given the small population size and vulnerability to environmental changes, we suggest that this population merits special concern. To ensure long-term population survival, it is imperative to facilitate gene flow among groups and stop deforestation and fragmentation of habitats.

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