Molecular Phylogenetic Relationships among Sichuan Snub-nosed Monkeys (*Rhinopithecus roxellanae*) Inferred from Mitochondrial Cytochrome-b Gene Sequences

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ABSTRACT. The Sichuan snub-nosed monkey (*Rhinopithecus roxellanae*) is a species endemic to China, and its distribution is the widest among all snub-nosed monkeys in China. To clarify whether there is subspecific differentiation within this species, we determined partial sequences of the cytochrome-b gene from four populations of *R. roxellanae*. First, 402bps of the partial sequences from *R. roxellanae* were compared with those from *R. bieti* and *R. avunculus*, and the phylogenetic tree was constructed by the neighbor-joining method. The genetic distance was only 0 – 0.002 among the four populations, and their sequences constituted a monophyletic group. Further, comparison of longer sequences (735bp) among the four populations revealed that there were only four substitutions and the genetic distance was only 0.001 – 0.005 among them. Thus, we suggest that, at least on mtDNA phylogeny, the difference among the four populations does not reach the subspecies level, and that this species should be recognized as a monotypic species.

Key Words: Sichuan snub-nosed monkey; *Rhinopithecus roxellanae*; Mitochondrial DNA; Cytochrome-b; Subspecific divergence.

INTRODUCTION

The Sichuan snub-nosed monkey (*Rhinopithecus roxellanae*) is endemic to China. This species is currently isolated in three separated areas: the northern Hengduan Mountains, the Qinling Mountains, and the Daba Mountains (Fig. 1; ZHANG et al., 1992). In the northern Hengduan Mountains, this species is distributed across 16 counties of Sichuan province and 3 counties of Gansu province. In the Qinling Mountains, this species is found on the northern slope of the mountains in Shaanxi province. In the Daba Mountains, it is found only in Shennongjia.

The Sichuan snub-nosed monkey was first described by MILNE-EDWARDS in 1870. Since then, various taxonomic revisions about this species have been offered, although there are still controversies over classification. Before the 1940s, the Sichuan snub-nosed monkey was recognized as one of four monotypic species according to their morphological differences (ELLIOT, 1913; ALLEN, 1938). During the 1950s and 1960s, several primatologists suggested that all Chinese snub-nosed monkeys should be united into a single species *Rhinopithecus roxellanae* (ELLERMAN & MORRISON-SCOTT, 1951; NAPIER & NAPIER, 1967). While some Chinese primatologists agreed with this view (QUAN & XIE, 1981; ZHANG et al., 1992), others suggested that each of the various local populations of snub-nosed monkeys in China should be classed as a distinct species (PENG et al., 1988; YE et al., 1987). Based on studies using mitochondrial DNA sequencing, ZHANG and RYDER (1997, 1998) also concluded that there were four distinct species in genus *Rhinopithecus*, with the Sichuan snub-nosed monkey as an independent species, *R. roxellanae*.

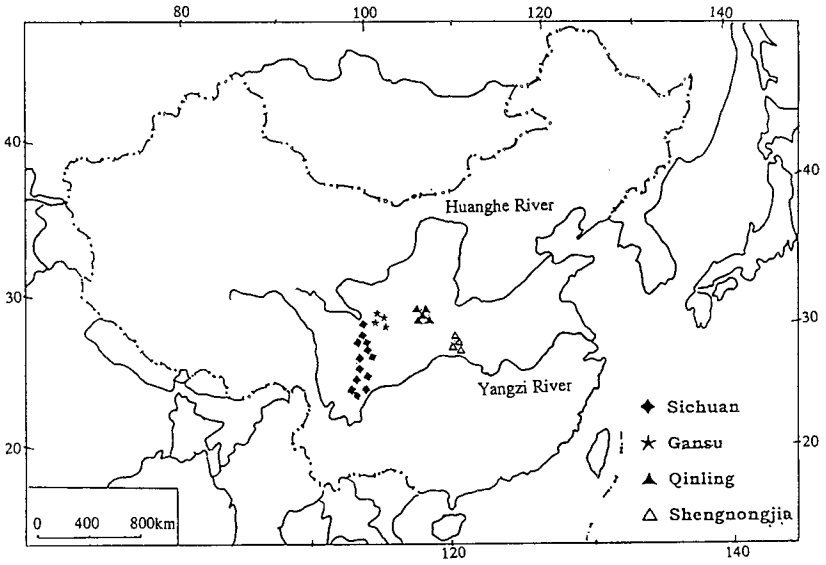

Fig. 1. Distribution of the Sichuan snub-nosed monkey populations in eastern Asia. The marks indicate the distribution of local populations in each region.

Controversy also exists over whether the Sichuan snub-nosed monkey is a monotypic species or a polytypic species. However, very few studies have been conducted on intra-specific variation of Sichuan snub-nosed monkey. WANG, Y. Q. et al. (1995, 1998) found some morphological difference among populations of Sichuan snub-nosed monkey after examining specimens collected in Sichuan, Gansu, and Hubei. They suggested that three local populations should each be classified as a distinct subspecies, namely *R. r. roxellanae* in Sichuan and Gansu; *R. r. hubeiensis* in Shennongjia, western Hubei, and eastern Sichuan; and *R. r. qinlingensis* in the Qinling Mountains, southern Shaanxi (WANG, Y. Q. et al., 1995, 1998). Within this genus, *R. roxellanae* is the most widely distributed species. Most populations are isolated from each other (ZHANG et al., 1992). Thus, it is necessary to develop a conservation management strategy to further explore and clarify the classification of *Rhinopithecus roxellanae*. Molecular genetic studies may shed some light on the problem of classification within this species.

In this paper, we used mitochondrial cytochrome-b (cyt-b) gene sequences from four populations of Sichuan snub-nosed monkeys to study molecular phylogenetic relationships within this species and among the species of genus *Rhinopithecus*.

MATERIALS AND METHODS

SAMPLES

Samples for genetic analysis were collected from four local populations of Sichuan snub-nosed monkeys: two from the Sichuan population (Rrs), four from the Gansu population (Rrg), four from the Shennongjia population (Rrn), and eight from the Shaanxi population (Rrx). The sampled individuals were captured in wild, and reared in the Beijing Center for Breeding Endangered Animals in Daxing County and Wuhan Zoo. Muscles, hairs, and feces samples

were obtained. About 50 g of fresh feces from each individual was collected into a sterile tube and preserved in 80% or absolute ethanol at room temperature (RT). Muscle was stored at −20°C and hairs at RT.

DNA Extraction

Total DNA was extracted from muscle according to Li et al. (1998); total DNA from feces was isolated according to the method of Fang et al. (1994).

Total DNA from hairs was extracted using Chelex 100 (Bio-Rad) (Walsh et al., 1991): five hairs were washed in absolute ethanol and then in double distilled water (ddH_2O). A tip of each hair's root (about 1 cm length) was removed with a sterile scissors and immersed in 200μl ddH_2O in 1.5 ml Eppendorf tubes. The samples were mixed with 20μl Proteinase K (20 mg/ml) and incubated at 55°C for 14 – 16 hr with constant mixing. After mixing to a vortex for 10 – 15 sec, 200μl 10% Chelex 100 was added, and the mixture was boiled for 30 min and mixed to a vortex again for 10 – 15 sec. The mixture was centrifuged at 12,000 rpm for 10 min, and the resultant supernatant (20μl) was used for PCR.

Amplification and DNA Sequencing

A primer pair of L14724 and H15915 (Irwin et al., 1991) was used to amplify a complete cyt-b gene. The PCR amplification was performed in a 100μl reaction volume containing 10xPCR buffer, 8μl 2.5mM dNTP, 0.4μM each primer, 5U of Takara Taq™ DNA polymerase (Takara Biotech) and ddH_2O for 40 cycles, with denaturation for 1 min at 94°C, annealing for 1 min at 42°C, and extension for 1.5 min at 72°C, and finally by a 10-min elongation at 72°C. The product was stored at 4°C. The purified double-stranded DNA was sequenced by an ABI 377 DNA Sequencer according to the manufacturer's protocol.

Data Analysis

DNA sequences were aligned with Clustal W (Thompson et al., 1994), and genetic distances among the four populations were calculated using Kimura's two-parameter method (Kimura, 1980). The phylogenetic analysis was conducted with the neighbor-joining method of the Clustal W package. We used the same methods for comparison of a part of our data with 402 base pair (bp) sequences from other species of snub-nosed monkeys (*R. bieti* and *R. avunculus*) (Zhang & Ryder, 1998) so that we could further explore the question of subspecies within *roxellanae*. The Entellus langur (*Presbytis entellus*) was used as an outgroup in our phylogenetic analysis.

RESULTS

Cyt-B Sequences of Sichuan Snub-nosed Monkeys

The size of the PCR product was 1140 base pairs which covered entire cyt-b gene sequence. We determined a part (735bps) of the PCR product. The sequences from all individuals within same population are identical. All the sequences have been deposited in GenBank under accession numbers AF260643 and AF262358 – AF262360.

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      1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
      4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5
      7 7 7 7 7 7 7 7 8 8 8 8 8 8 8 8 9 9 9 9 9 9 9 9 9 9 0 0 0 0 0 0 0 0 0 0
      3 5 7 7 8 8 8 9 0 3 4 4 5 6 6 6 3 3 4 4 5 5 5 8 8 8 9 0 0 0 0 2 2 2 3 4 5 6 9
      0 4 0 9 2 5 7 0 2 9 3 4 1 2 8 9 4 7 7 9 2 7 8 2 6 7 0 3 6 3 4 7 6 5 2 2 2

Rbe   C C C G G C G T T C G T T C C C C G C T T T T T C T T G C A A C T A G C
Rav   T . T A A . A . . G . C A T A T T A T . . C C C C T C C A T G G . C . . .
Rrs   . T . A . T A C . . C . . T T . . A T C C C C . . T C C A T . . T . G C T
Rrx   . T . A . T A C . . C . . T T . . A T C C C C . . T C C A T . . T . G C T
Rrg   . T . A . T A C . . C . . T T . . A T C C C C . . T C C A T . . T . G C T
Rrn   . T . A . T A C C . C . . T T . . A T C C C C . . T C C A T . . T . G C T
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Fig. 2. Variation in DNA sequences of the golden monkey within 402-base-pair region of the cyt-b gene. Positions of nucleotides in the gene are indicated vertically above the first sequence (Rbe). Dots indicate that nucleotides are identical to the first sequence. Rbe: *R. bieti*; Rav: *R. avunculus*; Rrs: *R. roxellanae* (Sichuan population); Rrx: *R. roxellanae* (Shaanxi population); Rrg: *R. roxellanae* (Gansu population); Rrn: *R. roxellanae* (Shennongjia population).

Figure 2 shows variable sites in the DNA sequences among Sichuan snub-nosed monkey and other snub-nosed monkeys within the 402bp region of the cyt-b gene. In all comparisons of the sequences, we found that there were 37 nucleotide substitutions and that the number of substitutions at the first, second, and third codon positions was 9, 5, and 23, respectively.

The cyt-b sequence differences among all taxa are listed in Table 1. In the region of the 402bp cyt-b gene fragment, the DNA sequence differences between the species were 0.057 – 0.065. The DNA sequence differences among the four populations of *R. roxellanae*, however, were very low (0 – 0.002) and there was only one substitution (Table 2). When longer sequences (735bp) of the cyt-b gene were compared, no intraspecific variation was observed

Table 1. The genetic distance (above diagonal) and transition (TS) / transversion (TV) numbers (below diagonal) for the cyt-b gene sequences (402bp) among Outgroup, *R. bieti*, *R. avunculus*, and four populations of *R. roxellanae*.

Taxa	Pre	Rbe	Rav	Rrs	Rrx	Rrg	Rrn
Pre		0.162	0.164	0.157	0.157	0.157	0.159
Rbe	54/11		0.065	0.057	0.057	0.057	0.060
Rav	52/14	24/2		0.060	0.060	0.060	0.062
Rrs	49/13	21/2	19/5		0	0	0.002
Rrx	49/13	21/2	19/5	0/0		0	0.002
Rrg	49/13	21/2	19/5	0/0	0/0		0.002
Rrn	50/13	22/2	20/5	1/0	1/0	1/0	

See Figure 2 for the abbreviation. Pre: The outgroup, *Presbytis entellus* and its sequence was retrieved from GenBank (AF012470).

Table 2. The genetic distance for the cyt-b gene sequences (735bp) among the four local populations of *R. roxellanae*.

	Rrs	Rrx	Rrg	Rrn
Pre	0.173	0.174	0.173	0.174
Rrs		0.004	0	0.001
Rrx			0.004	0.005
Rrg				0.001
Rrn				

See Figure 2 for the abbreviaiton.

	1	1	1	1
	4	5	5	5
	8	1	2	4
	0	4	0	0
	2	7	0	8
Rrs	T	A	A	C
Rrx	.	G	G	T
Rrg	.	.	.	.
Rrn	C	.	.	.

Fig. 3. Nucleotide substitution in DNA sequences of the golden monkey within 735-base-pair region of the cyt-b gene. Positions of nucleotides in the gene are indicated vertically above the first sequence (Rrs), according to the nucleotide position by ANDERSON et al. (1982). Dots indicate that nucleotides are identical to the first sequence.

between the Sichuan population and the Gansu population of *R. roxellanae*. There were only four substitutions characterized by variation within *R. roxellanae* based on the 735bp of cyt-b gene (Fig. 3).

PHYLOGENETIC TREES

Figure 4 shows a phylogenetic tree constructed by using the neighbor-joining method. Bootstrap values for nodes within species are not indicated because only one substitution was found within the species comparison, but the four populations of *R. roxellanae* were clustered with a 100% bootstrap value and three populations of Sichuan, Gansu, and Shaanxi were little separated from that of Shennongjia.

DISCUSSION

Traditionally, a subspecies must share a special geographic area or habitat, identical characteristics, a special evolutionary history different from that of other subspecies, and it must be reproductively compatible with other subspecies (O'BRIEN & MAYR, 1991). However, the pri-

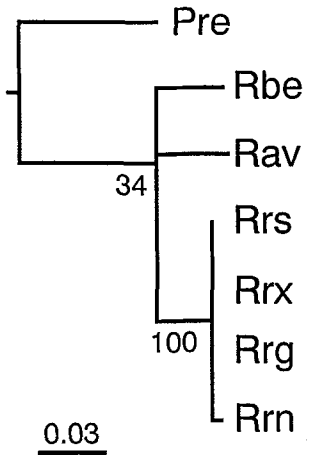

Fig. 4. Phylogenetic tree of the cyt-b gene constructed by the neighbor-joining method. The genetic distance between the taxa, indicated by the scale, was estimated using KIMURA'S two-parameter method. Bootstrap value (%) above branches were derived from 1000 replications.

mary criterion for differentiating taxa is phylogenetic (evolutionary) relatedness in modern taxonomy (AVISE, 1989; O'BRIEN & MAYR, 1991). The classification of subspecies within *R. roxellanae* was based only on some geographic differences and specimen analysis (WANG, Y. Q. et al., 1995, 1998). But GEIST (1991) suggested that morphology may not reflect phylogenetic relationships, and that morphological characteristics may be considerably affected by environmental factors (GEIST, 1987). Differences in size or color of pelage are often used in subspecies classification, but overlapping populations makes the quantification of differentiation difficult. In addition, natural selection can result in rapid divergence of morphology (EHRLICH & RAVEN, 1969; SLATKIN, 1987), or may cause populations that differ in their phylogenetic ancestry to undergo convergence of characteristics, leading to morphological similarity (MAYR, 1963; LANSMAN et al., 1983). Thus, we further analyzed whether or not there is subspecific divergence within *R. roxellanae* using the cyt-b gene sequences in the present paper.

The differences in the cyt-b sequences between *R. roxellanae* and other snub-nosed monkeys were similar to inter-specific differences of 0.051 – 0.062 (253bp) and 0.050 – 0.067 (402bp), respectively, among snub-nosed monkeys (ZHANG & RYDER, 1997, 1998). Our data further support that *R. roxellanae* is a distinct species (JABLONSKI & PENG, 1993). However, the cyt-b sequence of the Sichuan population of *R. roxellanae* was identical to that of the Gansu population of *R. roxellanae* based on 735bp of the cyt-b gene. This seems to support the report of WANG, Y, Q. et al. (1995, 1998) that the populations in Sichuan and Gansu belonged to the same subspecies of *R. roxellanae*, namely, *R. r. roxellanae*. However, the sequence differences were only 0 – 0.002 based on 402bp and 0.001 – 0.005 based on 735bp among the Sichuan-Gansu populations, the Shaanxi population, and the Shennongjia population. These values were much lower than interspecific differences, and also much lower than the 0.024 (6/253bp) and 0.03 (12/402bp) of intraspecific differences within *R. bieti* using cyt-b gene (ZHANG & RYDER, 1997, 1998). Some studies also indicate that the mtDNA sequence differences between populations of the same species are less than 1% in primates (ZHANG & SHI, 1993; WANG, W. et al., 1997; ZHANG & RYDER, 1998). Our result is also in accordance with the previous report by WANG, W. et al. (1997) that there is no obvious subspecific difference between Sichuan population and Shennongjia populations of *R. roxellanae* based on mtDNA ND3-ND4 gene sequences. Moreover, there was also no clear subspecific difference within *R. roxellanae* in comparison with other cercopithecine monkeys using rDNA analysis (WANG, W. et al., 1995). Thus, we suggest that there is no obvious subspecific differentiation within the sample populations of *R. roxellanae* in the cyt-b sequence divergence. In other words, the difference among the four populations does not reach the subspecies level based on DNA data, and this species should be still recognized as a monotypic species.

Variation in mtDNA had been assessed for many species or subspecies of wildlife, but there are some drawbacks. The main drawback of using sequence divergence of mtDNA for quantification of genetic differentiation is that it reflects a very limited part of the gene pool of populations (CRONIN, 1993). MtDNA sequence divergence cannot by itself provide a good assessment of overall genetic differentiation (PAMILO & NEI, 1988). Hybridization can result in mtDNA gene flow between species; intraspecific gene flow can result in the divergence of mtDNA genotype within populations (CRONIN, 1993). So, a combination of mtDNA and nuclear genes is needed for a more complete description of genetic relationships (DESALLE et al., 1987; POWELL, 1991). Thus, further analysis of nuclear genes as well as morphological characteristics are needed to clarify intraspecific divergence of *R. roxellanae*.

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