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Molecular phylogeny and taxonomic status of the red goral by cyt *b* gene analyses

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Abstract. The phylogeny and taxonomic status of the red goral (*Naemorhedus baileyi*) is still unclear. We sequenced the complete cyt *b* (1140bp) gene extracted from hair samples from three animals from the Shanghai Zoo, and compared them with thirty sequences of *Naemorhedus* downloaded from GenBank. Our results show three distinct lineages within *Naemorhedus*. Two closely related *N. baileyi* haplotypes were found. They belong to the most basal group within *Naemorhedus* together with *N. griseus* haplotype 2 from Thailand, which is a sequence likely to be misidentified.

Key words: *Naemorhedus baileyi*, *Naemorhedus*, genetic distances

Introduction

The red goral (*Naemorhedus baileyi*, Caprinae, Bovidae) is under first class protection in China, classified as Vulnerable on the IUCN Red List (Duckworth & MacKinnon 2011), and is listed in Appendix I of CITES (2012). The red goral inhabits the southeastern Tibet and the northwestern Yunnan Province, and is also found in northern Myanmar and northeastern Arunachal Pradesh in India (Smith 2009). However, the red goral is threatened by hunting and habitat loss (Wang 1998). Wilson & Mittermeier (2011) suggest that fewer than 10000 red goral are believed to survive today, and fewer than 1500 red goral are thought to live in China. Guo (2004) estimated that almost 500 red goral had been hunted every year in Medog, Tibet.

Because of its small population size and distribution range, research into the red goral is sparse. We sequenced the red goral mtDNA cyt *b* genes, and compared them with cyt *b* sequences from other species including long-tailed goral (*N. caudatus*), Himalaya goral (*N. goral*) and Chinese goral (*N. griseus*), which are generally considered as separate species in *Naemorhedus* (Wilson & Reeder 2005, Smith 2009), to investigate the relationships among them.

Material and Methods

We collected hair samples from three red gorals using a non-invasive sampling method from the Shanghai Zoo. The three red gorals were descendants of the red goral captured in the wild from Milin in 1981, and they were bred in captivity (Wang 1998). All the samples were preserved at –20 °C. The other thirty sequences analyzed in this study were downloaded from GenBank (Table 1).

DNA extraction and PCR amplification

DNA was extracted using a modified phenol-chloroform method (Chen 2006). The primers used for specific amplification were as follows (Irwin et al. 1991, Kocher et al. 1989):

L14724

5′-CGAAGCTTGATATGAAAAACCATCGTTG-3′

H15915

5′-GGAATTCATCTCTCCGGTTTACAAGAC-3′

Thermal cycling was performed on a PTC-200 thermocycler (MJ Research, Inc. USA). The PCR amplifications were carried out in 25 µl volumes using 7 µl of DNA extract, 2.5 µl of 10 × PCR buffer, 2.5 µl of $MgCl_2$ (25 mM), 2 µl of dNTPs (25 mM), 0.7 µl of each primer at 10 µM, and 0.2 µl of 5U rTaq (Takara, Dalian, China). The PCR program had 35 cycles with 95 °C for 30 s, 54 °C for 30 s and 73 °C for 60 s. All reactions were started with a denaturation step at 95 °C for 2 min and the last cycle was followed by a 5 min extension at 72 °C.

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Table 1. Sequence information.

Common name	Species	Haplotype code	GenBank Accession numbers	Reference
long-tail goral	*Naemorhedus caudatus*	*N.c* 1	AY356357	Min et al. Unpublished
		N.c 2	FJ469673	Jang & Hwang 2010
		N.c 3	U17861	Groves & Shields 1996
Korean goral	*Nemorhaedus caudatus raddeanus*	*N.c* 1	EU259195	An et al. Unpublished
		N.c 1	EU259196	An et al. Unpublished
		N.c 1	EU259197	An et al. Unpublished
		N.c 1	EU259198	An et al. Unpublished
		N.c 1	EU259099	An et al. Unpublished
		N.c 1	EU259100	An et al. Unpublished
		N.c 1	EU259101	An et al. Unpublished
		N.c 1	EU259102	An et al. Unpublished
		N.c 1	EU259103	An et al. Unpublished
		N.c 1	EU259104	An et al. Unpublished
		N.c 1	EU259105	An et al. Unpublished
		N.c 1	EU259106	An et al. Unpublished
		N.c 1	EU259107	An et al. Unpublished
		N.cr 1	EU259117	An et al. Unpublished
		N.cr 2	EU259115	An et al. Unpublished
		N.cr 3	EU259113	An et al. Unpublished
		N.cr 4	EU259108	An et al. Unpublished
		N.cr 4	EU259109	An et al. Unpublished
		N.cr 4	EU259110	An et al. Unpublished
		N.cr 4	EU259111	An et al. Unpublished
		N.cr 4	EU259112	An et al. Unpublished
		N.cr 5	EU259116	An et al. Unpublished
		N.cr 6	EU259114	An et al. Unpublished
Himalaya goral	*Naemorhedus goral*	*N.go*	EU259118	An et al. Unpublished
Chinese goral	*Naemorhedus griseus*	*N.gr* 1	FJ207532	Hassanin et al. 2009
		N.gr 2	JN632664	Hassanin et al. 2012
red goral	*Naemorhedus baileyi*	*N.b* 1	JN632663	Hassanin et al. 2012
		N.b 1	JX506309	This study
		N.b 1	JX506311	This study
		N.b 2	JX506310	This study

Sequencing

The PCR products were visualized using 2 % agarose gel electrophoresis, then purified and sequenced at Shanghai Map Biotech Co., Ltd.

Data analysis

The sequences of cyt *b* genes were aligned in ClustalX 2.0 (Larkin et al. 2007) and then manually collated. The total genetic divergence and its standard error (uncorrected *p*-distances) was calculated in MEGA 4.0 (Kumar et al. 2008). Haplotype diversity and nucleotide diversity were explored using DnaSP 5 (Librado & Rozas 2009). Maximum likelihood (ML) phylogenetic tree was constructed using Phyml 3.0 (Guindon & Gascuel 2003). We used Modeltest 3.7 (Posada & Crandall 1998) to test the optimal substitution model. The optimal model selected by the Akaike Information criterion (AIC) is GTR + I (ln*L* = –3621.1016, K = 9, AIC = 7260.2031). One hundred bootstrap replications were used for the ML analysis. We used *Ovis aries* (accession No. FR873153), *Oreamnos americanus* (accession No. AF190632) and *Budorcas taxicolor* (accession No. FJ207524) as outgroup species.

Results

The sequence fragment was 1140bp long, no insertions, deletions or stop codons were observed in the coding sequence. The fragment contained the entire mitochondrial cyt *b* gene because all the sequences began with ATG and terminated with the stop codon AGA (Hassanin et al. 1998).

Comparison of all sequences in *Naemorhedus* is listed in Table 2. Genetic divergence between the red goral and long-tailed goral, Himalaya goral and Chinese goral was 0.090, 0.092 and 0.072, respectively. The haplotype diversity and nucleotide diversity of the red goral was comparable to that found in the long-tailed goral, which is the only species represented by more than two individuals (Table 3).

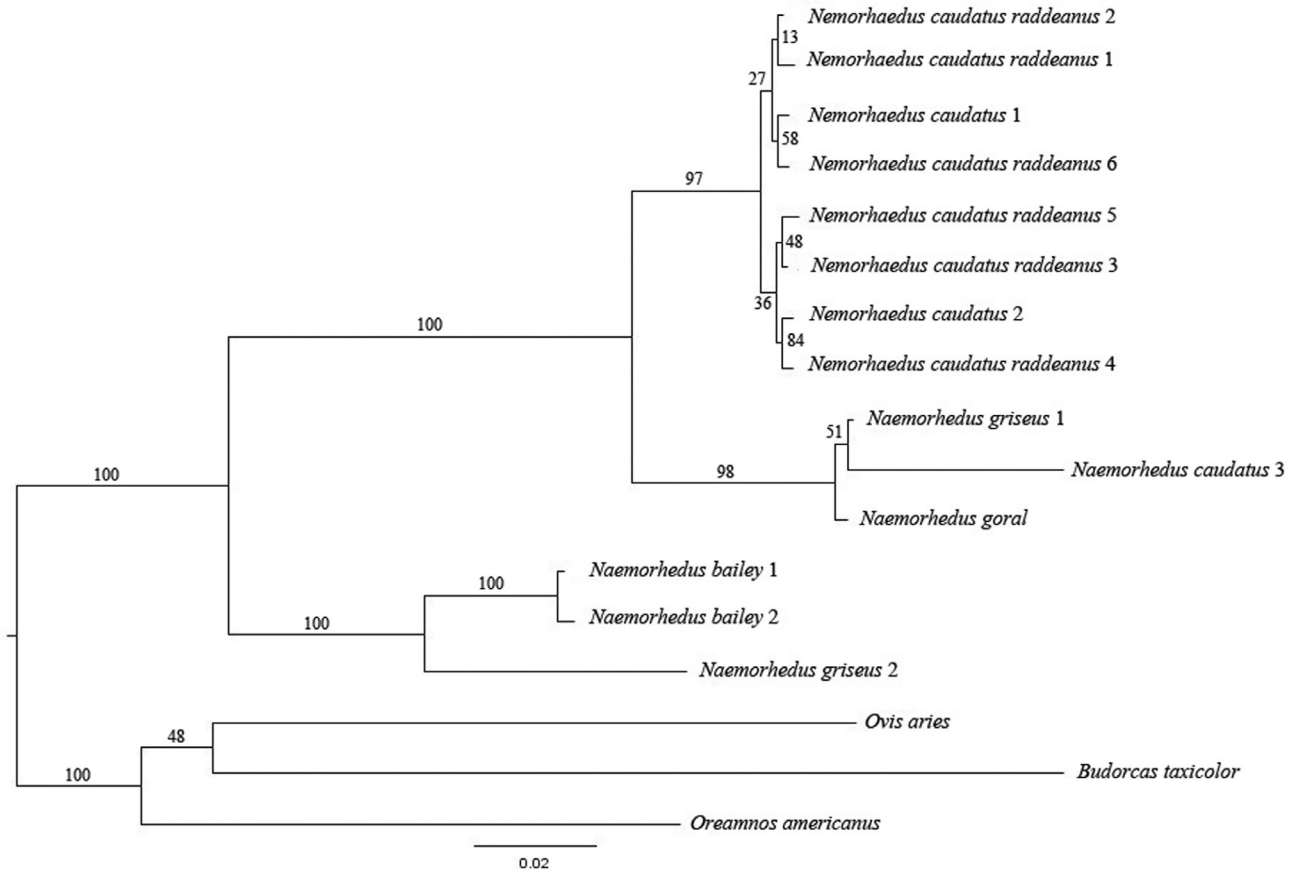

Fig. 1. Maximum Likelihood tree based on cytochrome *b* sequence data from *Naemorhedus* species and three outroups.

Table 2. Genetic divergence (*p*-distance) (below diagonal) and standard error (above diagonal) for cytochrome *b* nucleotide sequences of animals examined in this study.

	Naemorhedus caudatus	*Naemorhedus goral*	*Naemorhedus griseus*	*Naemorhedus baileyi*
Naemorhedus caudatus	-	0.006	0.006	0.008
Naemorhedus goral	0.044	-	0.004	0.008
Naemorhedus griseus	0.008	0.051	-	0.006
Naemorhedus baileyi	0.090	0.092	0.072	-

Table 3. Haplotype diversity and nucleotide diversity of all sequences.

Species	Number of sequences	Number of haplotypes	Haplotype diversity Hd	Nucleotide diversity Pi	Std. deviation of Hd	Std. deviation of Pi
Naemorhedus caudatus	26	9	0.689	0.00620	0.090	0.00440
Naemorhedus goral	1	-	-	-	-	-
Naemorhedus griseus	2	2	1.000	0.10263	0.050	0.05132
Naemorhedus baileyi	4	2	0.500	0.00088	0.265	0.00047

The Maximum likelihood phylogenetic tree is shown in Fig. 1. The ML phylogeny showed polyphyletic relationships of *Naemorhedus* species. The most basal supported group contained the red goral and Chinese goral from Thailand (Hassanin et al. 2012). Next diverged group containing most long-tailed goral sequences and a group that included Himalaya goral, Chinese goral from an unlisted locality (Hassanin et al. 2009) and long-tailed goral from San Diego Zoo, USA (Groves & Shields 1996).

Discussion

This study provided insights into the taxonomic status of *Naemorhedus*. The results indicate that the red goral is an independent species of *Naemorhedus*. The four sequences are closely related and they belong to

two haplotypes. The red goral sequence downloaded from GenBank was collected from the Rotterdam Zoo (Hassanin et al. 2012). Because of high similarity, the red goral in Shanghai Zoo and Rotterdam Zoo may have the same origin. The known distribution range of the red goral includes the southeastern Tibet, northwestern Yunnan Province, northern Myanmar and northeastern Arunachal Pradesh in India. Although Groves & Grubb (1985) distinguished two subspecies of the red goral (Tibetan red goral *N. b. baileyi* and Burmese red goral *N. b. cranbrooki*), our sampling does not allow assessing their status. More data from multiple regions in the distribution area of the red goral are needed to clarify the subspecies status of the red goral, but our results refute Rabinowitz's (1999) view recognizing the red goral as long-tailed goral's subspecies.

It is generally considered that there are four separate species in *Naemorhedus*: red goral (*N. baileyi*), long-tailed goral (*N. caudatus*), Himalaya goral (*N. goral*) and Chinese goral (*N. griseus*) (Wilson & Reeder 2005, Smith 2009). However, their phylogenetic relationships were not resolved based on cyt *b*. The phylogenetic analyses showed three major evolutionary clades, whose monophyly was supported by high bootstrap values. The long-tailed goral and the Chinese goral were polyphyletic in the tree. This might indicate either misidentification of some samples or lack of lineage sorting of mitochondrial genomic lineages in *Naemorhedus*.

The lineage that is well defined in our data included long-tailed goral individuals sampled from the wild. They are found in eastern Russia, northeastern China and Korean Peninsula (Smith 2009). The Korean goral (*N. c. raddeanus*) is considered a separate subspecies, but cyt *b* data do not indicate diversification within the lineage that would merit the subspecies classification. The sister lineage to the long-tailed goral from the wild is enigmatic. It groups three species with little divergence. Here, the long-tailed goral individual with haplotype *N.c* 3 from San Diego Zoo seems to carry different mtDNA, which might be an introgression. Sequences of the Himalaya goral and the Chinese goral are very similar indicating conspecificity, introgression or sample misidentification.

The most basal lineage of *Naemorhedus* includes the red goral and Chinese goral from Thailand. Hassanin et al. (2012) admitted that the haplotype *N.gr* 2 from the sample collected in Thailand by local hunters probably suffers from species misidentification. Their reason was the high divergence between the *N.gr* 2 and the haplotype *N.gr* 1 reported by Hassanin et al. (2009). The Chinese goral from Thailand was described as a subspecies of *N. griseus* by Hassanin et al. (2012), and the authors suggested that Chinese goral in Thailand could be elevated to a full species. Detailed systematic evaluation of the Chinese goral from Thailand is necessary as in this study, we show that they are closely related to the red goral.

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