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Simple Assessment of Taxonomic Status and Genetic Diversity of Korean Long-Tailed Goral (*Naemorhedus caudatus*) Based on Partial Mitochondrial Cytochrome *b* Gene Using Non-Invasive Fecal Samples

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ABSTRACT

South Korea presently harbors less than 800 long-tailed gorals (*Naemorhedus caudatus*), an endangered species. I report for the first time on the taxonomic status and genetic diversity of the Korean species using non-invasive fecal sampling based on mitochondrial cytochrome *b* gene sequence analyses. To determine the taxonomic status of this species, I reconstructed a consensus neighbor-joining tree and generated a minimum spanning network combining haplotype sequences obtained from feces with a new goral-specific primer set developed using known sequences of the Korean goral and related species (e.g., Russian goral, Chinese goral, Himalayan goral, Japanese serow, etc.). I also examined the genetic diversity of this species. The Korean goral showed only three different haplotypes. The phylogenetic tree and parsimony haplotype network revealed a single cluster of Korean and Russian gorals, separate from related species. Generally, the Korean goral has a relatively low genetic diversity compared with that of other ungulate species (e.g., moose and red deer). I preliminarily showcased the application of non-invasive fecal sampling to the study of genetic characteristics, including the taxonomic status and genetic diversity of gorals, based on mitochondrial DNA. More phylogenetic studies are necessary to ensure the conservation of goral populations throughout South Korea.

Keywords: Conservation, Cytochrome *b* gene, Endangered species, Feces, *Naemorhedus caudatus*

Introduction

The long-tailed goral (*Naemorhedus caudatus*) is a member of the tribe Rupicaprini in the subfamily Caprinae (An, 2006). The distribution of this species ranges from the northern to central Baekdoodaegan mountain range in South Korea (Choi & Park, 2004; 2005; Yang, 2002). The goral has been listed as an endangered species (Ministry of Environment, 2004) and a natural monument (No. 217; Cultural Heritage Administration of Korea, 1999) by the South Korean government. Internationally, this species is also considered endangered (Baillie & Groombridge, 1996). Commercial trade of this species is prohibited in countries signatory to the Convention on International Trade in Endangered Species (Hutton & Dickson, 2000). The population size of the goral has gradually decreased to fewer than 800 individuals because of illegal hunt-

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Table 1. Summary of invasive and non-invasive sampling of Korean long-tailed goral (*Naemorhedus caudatus*)

No.	Sample	Haplotype	Sample	Individual	Sampling site	Reference
N=24	Non-Soraksan population					
1	Cgrb1612	H2	Feces	A	Yanggu	This study
2	Cgrb1613	〃	〃	A	〃	〃
3	Cgrb1614	〃	〃	A	〃	〃
4	Cgrb1617	〃	〃	A	〃	〃
5	Cgrb1618	〃	〃	A	〃	〃
6	Cgrb1619	〃	〃	A	〃	〃
7	Cgrb1620	〃	〃	A	〃	〃
8	Cgrb1621	〃	〃	A	〃	〃
19	Cgrb1622	〃	〃	C	〃	〃
10	Cgrb1623*	〃	〃	C	〃	〃
11	Cgrb1624*	〃	〃	A	〃	〃
12	Cgrb1625*	〃	〃	B	〃	〃
13	Cgrb1626	〃	〃	C	〃	〃
14	Cgrb1629*	〃	〃	D	〃	〃
15	Cgrb1630	〃	〃	A	〃	〃
16	Cgrb1633	〃	〃	D	〃	〃
17	Cgrb1634	〃	〃	D	〃	〃
18	Cgrb1635	〃	〃	A	〃	〃
19	KG09*	〃	Tissue	-	〃	An (2006)
20	KG12*	〃	〃	-	〃	〃
21	KG02*	〃	〃	-	Samcheok	〃
22	KG03*	〃	〃	-	〃	〃
23	KG23*	〃	〃	-	〃	〃
24	KG30*	〃	〃	-	Donghae	〃
N=31	Soraksan population					
1	Cgrb1640	H1	Feces	-	Injae	This study
2	Cgrb1641	〃	〃	-	〃	〃
3	Cgrb1642	〃	〃	-	〃	〃
4	Cgrb1643	H2	〃	-	〃	〃
5	Cgrb1645*	〃	〃	E	〃	〃
6	Cgrb1646	〃	〃	-	〃	〃
7	Cgrb1647	H1	〃	-	〃	〃
8	Cgrb1653	H2	〃	-	〃	〃
9	Cgrb1654*	〃	〃	F	〃	〃
10	Cgrb1655*	H1	〃	G	〃	〃
11	Cgrb1659	H2	〃	-	〃	〃
12	Cgrb1660*	〃	〃	H	〃	〃
13	Cgrb1665	〃	〃	-	〃	〃
14	Cgrb1666	〃	〃	H	〃	〃
15	Cgrb1668	〃	〃	H	〃	〃

Table 1. Continued

No.	Sample	Haplotype	Sample	Individual	Sampling site	Reference
16	Cgrb1669	〃	〃	-	〃	〃
17	Cgrb1671	〃	〃	-	〃	〃
18	Cgrb1673	〃	〃	-	〃	〃
19	KG05*	〃	Tissue	-	〃	An (2006)
20	KG17*	〃	〃	-	〃	〃
21	KG18*	H1	〃	-	〃	〃
22	KG19*	H2	〃	-	〃	〃
23	KG20*	〃	〃	-	〃	〃
24	KG26*	〃	〃	-	〃	〃
25	KG27*	〃	〃	-	〃	〃
26	KG28*	〃	〃	-	〃	〃
27	KG02*	〃	〃	-	Gosung	〃
28	KG03*	〃	〃	-	〃	〃
29	KG16*	〃	〃	-	〃	〃
30	KG29*	〃	Blood	-	〃	〃
31	KG10*	〃	Tissue	-	Yangyang	〃
N=9	Unknown population					
1	KG01*	H2	Tissue	-	Everland Zoo	An (2006)
2	KG04*	〃	〃	-	〃	〃
3	KG11*	〃	〃	-	〃	〃
4	KG13*	〃	〃	-	〃	〃
5	KG14*	〃	〃	-	〃	〃
6	KG21*	〃	〃	-	〃	〃
7	KG22*	H3	〃	-	〃	〃
8	KG24*	〃	〃	-	〃	〃
9	KG25*	〃	〃	-	〃	〃
N=7	Others					
1	Russian goral	RG	Tissue	-	Russia	An (2006)
2	Chinese goral	CG	GenBank	-	China	U17861
3	Himalayan goral	HG	DNA	-	Singapore Zoo	An (2006)
4	Indian serow	IS	GenBank	-	India	DQ459334
5	Japanese serow	JS	〃	-	Japan	D32191
6	Goat (outgroup)	G	〃	-	〃	D84201
7	Sheep (outgroup)	S	〃	-	〃	D84205

*Fecal samples used in this study. A, B, C, D, E, F, G, and H were previously identified as individuals via microsatellite genotyping analysis (B.J. Kim, unpublished data).

ing, overexploitation, habitat destruction, and habitat fragmentation in South Korea (Ministry of Environment, 2002; Yang, 2002).

In particular, habitat fragmentation is a major issue in the conservation of the goral. For wild populations, habitat fragmentation has caused decreased connectivity, i.e., the degree to which the landscape facilitates or impedes movement between resource patches (Coulon *et al*., 2004; Taylor *et al*., 1993). Heterogeneity caused by fragmentation can create natural barriers against population movement, because unfavorable habitats do not provide cover against predators or because distances between

suitable patches are greater than can be rapidly crossed (Arnold *et al*., 1993). Therefore, the movement ability of wild animals may be altered by habitat fragmentation. Such changes can have negative consequences for wildlife populations, partially due to the reduction of gene flow between populations, which leads to greater inbreeding and loss of genetic diversity within fragments (Coulon *et al*., 2004; Frankham *et al*., 2002). Fragmentation can even lead to the extinction of species, and many studies on habitat fragmentation have focused on fragmentation created by barriers, such as roads, islands, and settlements (Burkey, 1989; Coulon *et al*., 2004; Soulé *et al*., 1992). In the case of wild goral in South Korea, few studies have hitherto been conducted on the ecology and genetics of this species (Choi & Park, 2004; 2005). Suitable goral habitats are expected to significantly decline and/or disappear from South Korea in this century (Lee *et al*., 2019).

Collecting elusive animal samples (e.g., tissue, blood, etc.) in the wild is difficult, but non-invasive sampling methods could overcome such constraints (Kohn & Wayne, 1997). Fecal sampling is the most frequently used method of obtaining wildlife DNA (Wehausen *et al*., 2004). As a useful genetic marker in non-invasive wildlife research, the mitochondrial cytochrome *b* gene has been used for phylogenetic, population, and forensic investigations (Parson *et al*., 2000). The nucleotide sequence of this gene contains species-specific information. Furthermore, the cytochrome *b* gene is located on the mitochondrial genome, making it suitable for forensic PCR-based mitochondrial DNA typing. Taxonomic researches—using invasive samples—based on mitochondrial genes, such as cytochrome *b* (An, 2006; Min *et al*., 2004) gene or D-loop (An, 2006) region have been conducted on the goral in South Korea. A population study on the goral was recently performed using invasive samples based on 12 microsatellite loci (Choi *et al*., 2015). For the first time to my knowledge, the present study assessed the phylogenic status and genetic diversity of the long-tailed goral inhabiting South Korea via a simple method using non-invasive fecal samples.

Materials and Methods

Samples and DNA extraction

A total of 64 goral fecal samples were collected from the wild (n=40) in Soraksan National Park, Injae, Gangwon Province, and a goral farm (n=24) in Yanggu, Gangwon Province, South Korea, during summer (July 4 and 6) 2005 and winter (December 16 and 17) 2006 (Table 1; Fig. 1). The samples were frozen at −70°C until experimentation. Genomic DNA was extracted from fecal samples using the method developed by Gerloff *et al*. (1995). Additional sequences (n=28) were obtained from a previous study (An, 2006). In total, I obtained 36 sequences (10, 17, and 9 for non-Soraksan, Soraksan, and unknown population, respectively; Table 1). All sequences were obtained from different goral individuals identified in another study (B.J. Kim, unpublished data).

Species-specific PCR amplification and sequencing

Partial sequences of the mitochondrial cytochrome *b* gene (337 bp) from the fecal samples were amplified via PCR using a new Korean goral-specific primer set developed in this study (NCF: 5'-atgatcaacatccgaaaaac-3'; NCR: 5'-ataggagggattaccccaata-3'). The PCR analysis was carried out using a 25 μL reaction volume containing 4 μL DNA template, 1X PCR buffer (iNtRON Inc., Seongnam, Korea), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.1 μM of each primer, 2.5 μg bovine serum albumin (Promega Inc., Madison, WI, USA), and 1.25U *i-Star Taq* polymerase (iNtRON Inc.). PCR amplification was performed in a PTC-100 Thermal Cycler (MJ Research, Inc., Watertown, MA, USA) as follows: initial denaturation for 3 minutes at 94°C, followed by 45 cycles (at 94°C for 60 second, 46°C for 45 second, and 72°C for 60 second) and a final extension for 3 minutes at 72°C. The PCR products were resolved through electrophoresis on 2% agarose gel, stained by ethidium bromide, and visualized under an ultraviolet illuminator. Of the 64 PCR samples, we selected 18 from the captive and wild populations, respectively, for sequencing (Table 1). Thirty-six PCR products were puri-

Fig. 1. Map of Gangwon province, South Korea. Baekdoo-daegan mountain range is located in Gangwon province, the main distribution range of Korean goral (*Naemorhedus caudatus*). Soraksan National Park is isolated from other areas by local highways and human settlements. The national park, including Mt. Sorak, consists of four different areas, namely Injae, Gosung, Yangyang, and Sokcho.

fied using a QIAquick Gel Purification Kit (QIAGEN Inc., Valencia, CA, USA). Purified PCR products were sequenced using the forward primer in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA). We only used 310 out of 337 bp for our sequence analyses.

Data analysis

In total, we used 36 sequences for data analysis (Table 1). The phylogenetic relationships between the haplotypes of gorals and serows were reconstructed via the neighbor-joining method (Saitou & Nei, 1987) using the Kimura 2-parameter model (Kimura, 1980). As outgroup, corresponding mitochondrial cytochrome *b* gene sequences of one goat (accession no. D84201) and one sheep (accession no. D84205) were chosen to root the phylogenetic tree (Douzery & Randi, 1997). Confidence in the estimated relationship was determined using the bootstrap approach (Felsenstein, 1985) obtained through 1,000 replicates using the same model as mentioned above. Bootstrap analyses and phylogenetic reconstruction were conducted using MEGA version 3.1 (Kumar *et al*., 2004). In addition, a minimum-spanning network was created combining goral haplotypes using TCS software (Clement *et al*., 2000). To determine goral genetic diversity, three haplotype sequences were aligned and edited using BIOEDIT ver-

Table 2. Summarized statistics for cytochrome *b* variation in Korean goral populations (*Naemorhedus caudatus*)

Statistics	Population			
	Non-soraksan	Soraksan	Unknown	Total
N	10	17	9	36
A	1	2	2	3
h	0	0.221	0.222	0.160
p_s	0	0.300	0.600	0.400
π	0	0.071	0.072	0.053
Tajima's *D*	NA	–0.491 (*P*>0.05)	–1.088 (*P*>0.05)	–1.284 (*P*>0.05)
Fu and Li's *D*	NA	0.677 (*P*>0.05)	–1.190 (*P*>0.05)	–0.800 (*P*>0.05)

N, fecal sample size; *A*, number of haplotypes in each population; *h*, haplotype diversity; p_s, sequence divergence (%); π, nucleotide diversity (%); NA, not available.

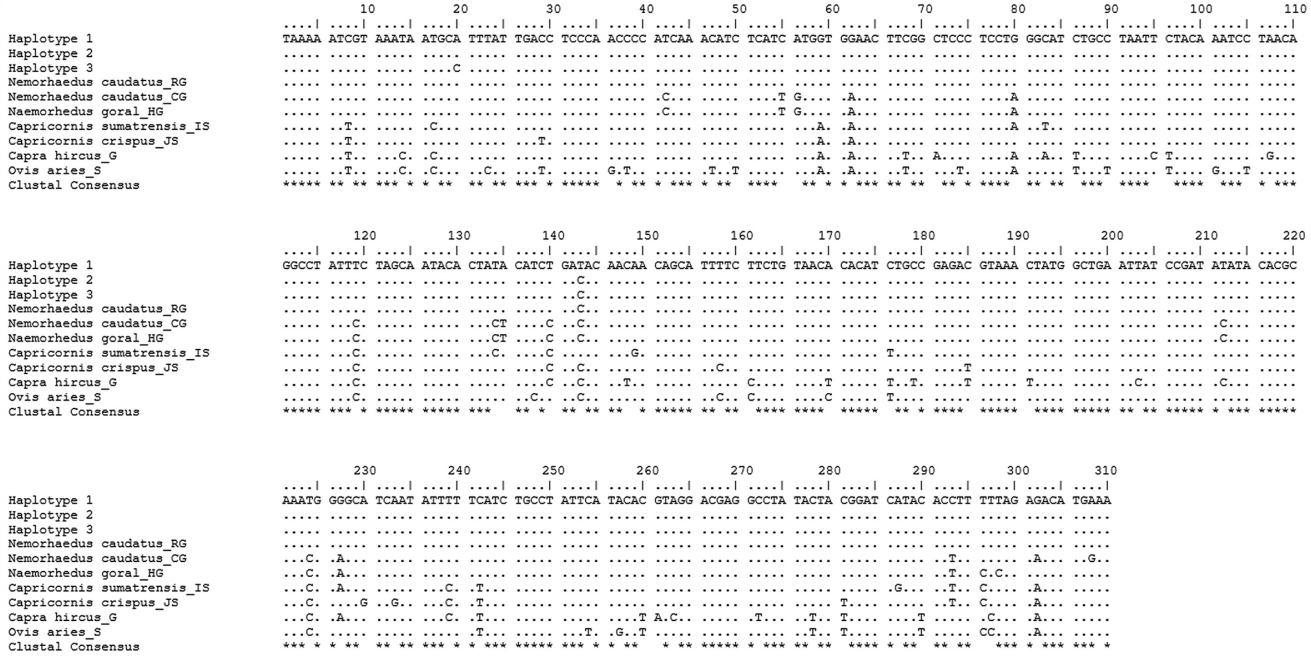

Fig. 2. Alignment of partial sequences of mitochondrial cytochrome *b* gene of Korean goral (*Naemorhedus caudatus*) and closely related species. Dots represent same base as those of haplotype 1. Two polymorphic sites were found in Korean goral.

sion 7.0.5.3 (http://www. mbio.ncsu.edu/BioEdit/bioedit.html; Hall, 1999). Sequences that varied by at least one nucleotide were considered different haplotypes. Haplotype and nucleotide diversity were calculated according to Nei (1987), and the neutral molecular evolution hypothesis was tested according to Tajima (1989) and Fu and Li (1993) using DNASP version 4.10.9 (http://www.ub.es/dnasp; Rozas *et al*., 2003).

Results

Three goral haplotypes defined by only two polymorphic sites were found (Table 2). The polymorphic sites were a synonymous transversion (Ala, GCA/GCC) and a synonymous transition (Asp, GAC/GAT), and no insertion or deletion existed (Fig. 2). The haplotype (H) frequencies of H1, H2, and H3 were 0.056, 0.917, and 0.028, respectively, in the total Korean goral population. Among the three haplotypes, H2 was found in the non-Soraksan (1), Soraksan (0.882), and unknown (0.889) populations, but

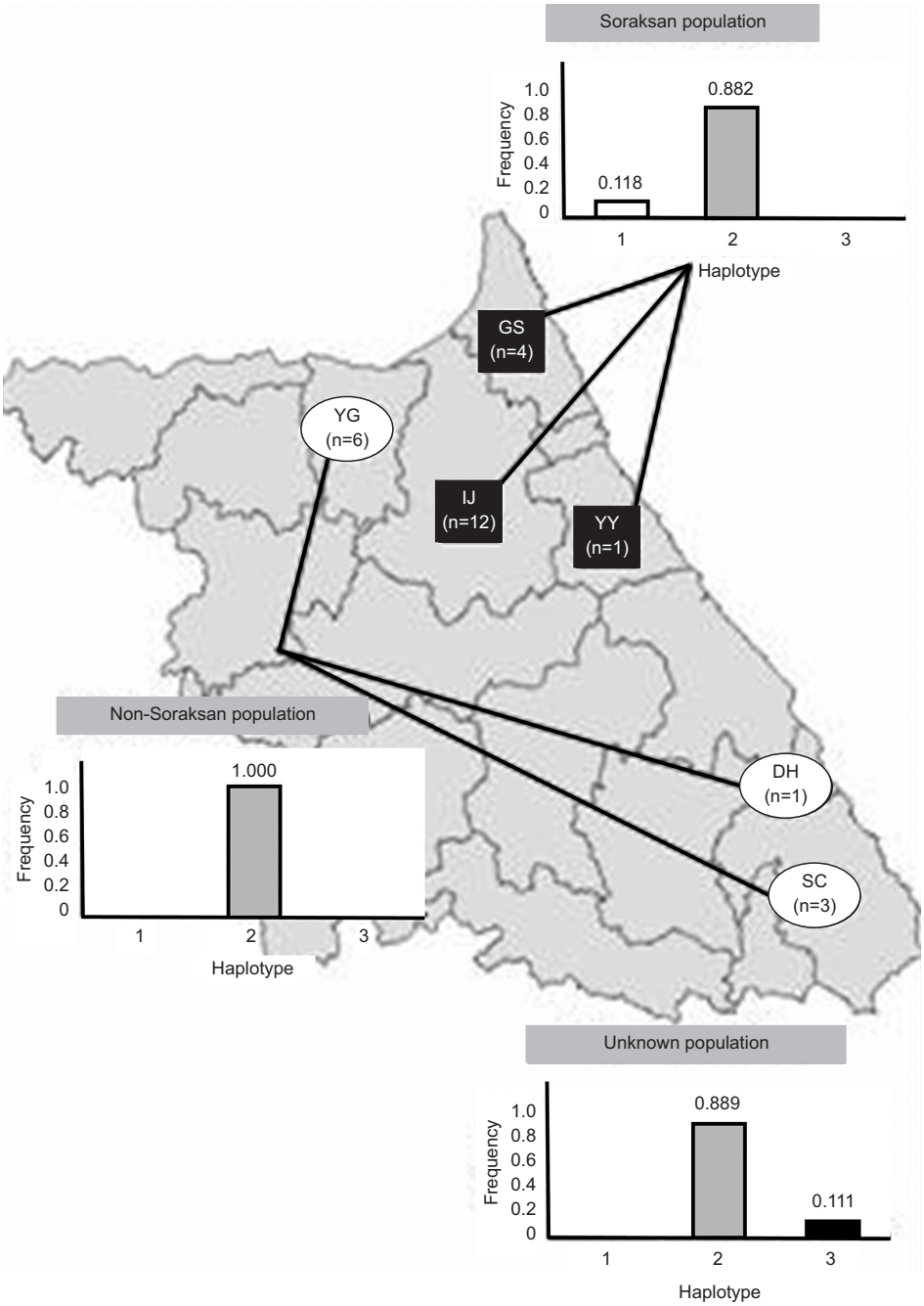

Fig. 3. Haplotype frequencies of partial mitochondrial cytochrome *b* gene in Gangwon province, South Korea. Non-Soraksan population samples were collected from Yanggu (YG), Donghae (DH), and Samcheok (SC), and Soraksan population samples were collected from Injae (IJ), Gosung (GS), and Yangyang (YY). Three allele frequencies were found for each population. No information from samples was obtained from unknown population. n, sample size.

H1 was restricted to the Soraksan population (0.118), and H3 only occurred in the unknown population (0.111; Fig. 3). The frequency of H2 was similar in the non-Soraksan (1) and Soraksan (0.882) populations, excluding the unknown population (Fig. 3). I also tested the neutral evolution hypothesis for the Korean goral, and neither Tajima's *D* value nor Fu and Li's *D* value showed strong selective sweeps in this species (Table 2).

The consensus neighbor-joining tree was reconstructed with the three haplotypes and other reference sequences and rooted by two outgroup species, such as goat and sheep (Fig. 4). The three haplotypes formed a separate cluster from those of Chinese and Himalayan gorals but not from the Russian goral (Fig. 4). The latter showed one of the three Korean goral haplotypes (H2). Furthermore,

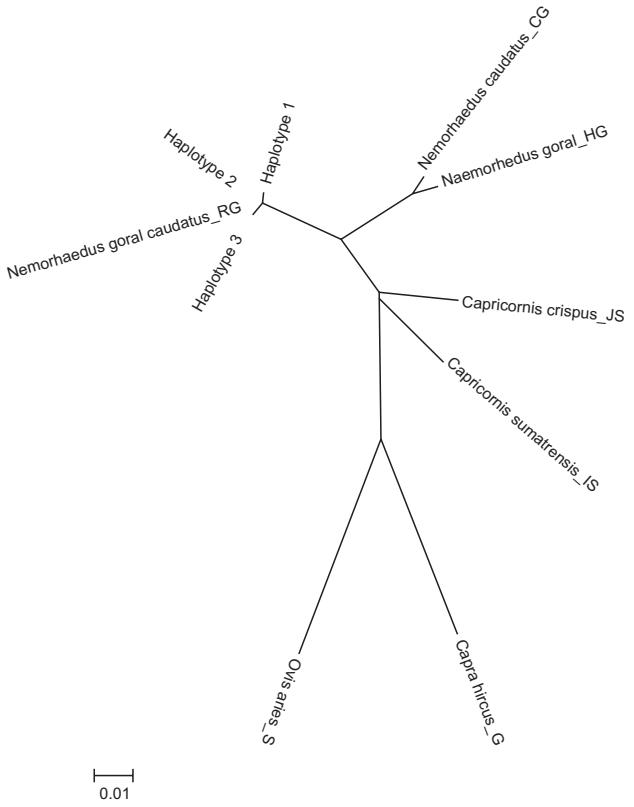

Fig. 4. Consensus neighbor-joining tree of mitochondrial cytochrome *b* haplotypes of Korean goral (*Naemorhedus caudatus*) and closely related species. Tree was computed using Kimura-2-parameter distance matrix of haplotypes and was rooted using goat (*Capra hircus* D84201) and sheep (*Ovis aries* D84205) sequences as outgroups. Haplotypes 1, 2, and 3 were obtained from Korean goral. *Nemorhaedus caudatus* RG, Russian goral; *Nemorhaedus caudatus* U17861, Chinese goral; *Naemorhedus goral* HG, Himalayan goral; *Capricornis crispus* D32191, Japanese serow; *C. sumatraensis* DQ459334, Indian serow.

TCS generated a 95% parsimony haplotype network for the Korean, Chinese, and Himalayan goral haplotypes (Fig. 5). The network showed a clearly separated distribution pattern between the Korean goral and Chinese and Himalayan gorals (Fig. 5). The haplotype diversity was 0.160 in the total population and ranged from 0 in the non-Soraksan population to 0.221 in the Soraksan population (Table 2). The nucleotide diversity was 0.053% in the total population and differed slightly between the non-Soraksan (0%) and Soraksan (0.071%) populations (Table 2).

Discussion

The Korean long-tailed goral is a species in the tribe Rupicaprini, subfamily Caprinae, and family Bovidae (An, 2006). Within the tribe Rupicaprini, gorals and serows are classified as *Naemorhedus* and *Capricornis*, respectively (Mead, 1989; Nowak, 1999; von Dolan, 1963). In contrast, Groves and Grubb (1985) suggested that serows should also be classified as *Naemorhedus* (Corbet & Hill, 1992; Grubb, 1993). Few studies have hitherto reported genetic differences between the two genera (An, 2006; Min *et al*., 2004). The present study also showed similarities in the phylogenetic status of the two genera based on the neighbor-joining tree (Fig. 4) and maximum spanning network (Fig. 5). The Korean goral should be classified as a genus distinct from Capricornis. In addition, the Korean and Russian gorals have the same haplotype and clustered within the same clade (Fig. 4). However, the Chinese goral (*N. caudatus*) has relatively higher genetic variation than the Korean and Russian gorals do and therefore clustered with the Himalayan goral (*N. goral*; Fig. 4). This result agreed with those of previous studies (An, 2006; Min *et al*., 2004). For the reasonable solution of this taxonomical issue, more samples of Chinese and Russian gorals in eastern China and Russia adjacent to Korean habitats are necessary.

Based on the partial mitochondrial cytochrome *b* gene sequences (310 bp), only three haplotypes were found for the Korean goral (Table 2). The sequence divergence between these haplotypes was 0.4% for the total population (Table 2). Min *et al*. (2004) found only two haplotypes (Korean goral types a and b) in partial cytochrome *b* sequences (646 bp) of the species, with a 0.16% sequence divergence. According to a more recent study on the Korean goral (An, 2006), seven haplotypes (*A*) of complete mitochondrial cytochrome *b* gene sequences were identified (*N. caudatus* KG-a, b, c, d, e, f, and g), but the sequence divergence (p_s) ranged from 0 to 0.4%. Furthermore, the haplotype (h=0.323) and nucleotide (π=0.053%) diversity of the species according to the present results were low (Table 2). In contrast, other ungulate species, such as moose (*Alces alces*; 403 bp; A=8; p_s=0.2-1.8%;

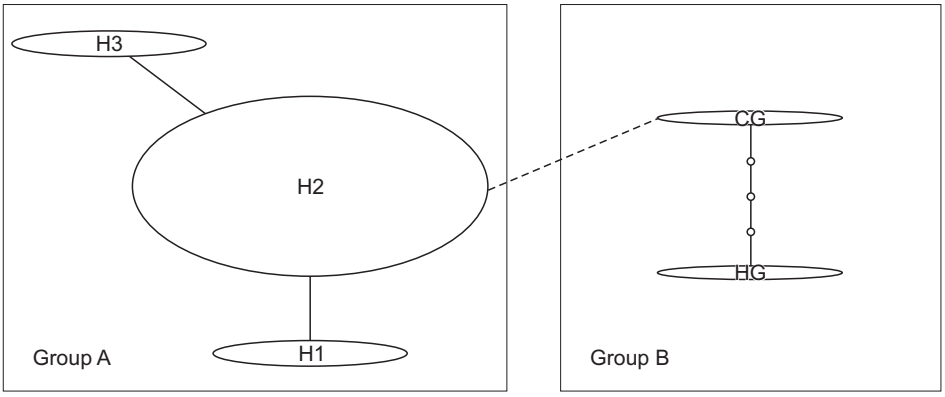

Fig. 5. A 95% parsimony network of five goral haplotypes obtained via our sequence and previous sequence data. Each haplotype is represented by an oval, the size of which is proportional to haplotype frequency. Group A involved 27 Korean goral haplotypes (H1, H2, and H3) and one Russian goral haplotype (H2). Group B, separate from Group A, included Chinese and Himalayan goral haplotypes (CG and HG, respectively). Two groups differed with a large sequence variation. Each branch connecting haplotypes represents single mutation event. ---, 15 substitutions between H2 and CG; ◦, unsampled haplotypes.

h=0.56-0.60; π=0-0.3%; Hundertmark *et al*., 2002), western red deer (*Cervus elaphus*; complete 1,140 bp; *A*=21; p_s=1.86±0.22%; *h*=0.98±0.02; Ludt *et al*., 2004), and eastern red deer (*C. elaphus*; complete 1,140 bp; *A*=13; p_s=1.27±0.020%; *h*=0.98±0.03; Ludt *et al*., 2004), showed relatively high genetic diversity in their mitochondrial cytochrome *b* gene sequences.

The population structure of the long-tailed goral has been rarely studied despite it being one of the most endangered species in South Korea. Yang (2002) reported that the total population consisted of many small, fragmented populations. Choi and Park (2004; 2005) indicated that Korean goral populations are isolated by local highways or forest trails in one of its main habitats. Using invasive sampling based on 12 microsatellite loci (n=68 gorals), Choi *et al*. (2015) recently reported that the goral population in the lower northeastern regions of South Korea was distinct from the upper northeastern population. The genetic integrity and individual identification of goral in Soraksan National Park were studied using non-invasive sampling based on the mitochondrial D-loop region (n=38 fecal samples) and nine microsatellite loci (Jang *et al*., 2020). This study is the first to report results of the phylogenic relationship and genetic diversity of the Korean goral compared with those of other species using non-invasive fecal samples. In particular, non-invasive molecular genetic studies using fecal samples could provide more information to support the conservation of South Korean goral populations.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

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