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Cross-Species Amplification of Bovidae Microsatellites and Low Diversity of the Endangered Korean Goral

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The Korean goral (*Nemorhaedus caudatus*) is an endangered species of wild goat. The conservation and management of this species could benefit from a better understanding of its genetic diversity and structure. Cross-species amplification of 34 Bovidae microsatellite loci was tested on a panel of 6 Korean gorals and 10 domestic goats. After polymerase chain reaction (PCR) optimization, 29 (85.3%) microsatellite loci amplified successfully for the Korean gorals and 27 (79.4%) for the domestic goats. Of the amplified products, 16 (55.2%) were polymorphic in the Korean goral and 22 (81.5%) in domestic goats. Nei's unbiased mean heterozygosity and mean allele number per locus were, respectively, 0.356 and 2.6 in the Korean goral and 0.636 and 4.8 in domestic goats. Low genetic diversity in the Korean gorals observed in this preliminary microsatellite survey suggests an urgent need for further detailed study of genetic diversity in Korean goral populations and a population management strategy based on these studies. Current results of cross-species amplification of domestic Bovidae microsatellites could be employed for the necessary population genetic studies on the Korean goral and other endangered Caprinae species.

The Korean goral (*Nemorhaedus caudatus*) is an endangered species of wild goat (Ministry of Environment of Korea 1998). The goral population has been reduced dramatically by habitat destruction and overexploitation. As a result, the current goral habitat in South Korea is restricted and fragmented. The Korean goral population in South Korea is presumed to be less than 250 (Cultural Properties Administration of Korea 1999), placing it in the International Union for Conservation of Nature and Natural Resources (IUCN) endangered category (IUCN 1996). At present, commercial trade of this species is banned among countries signatory to the Convention on International Trade in Endangered Species (CITES) (Hutton and Dickson 2000). Conservation of the Korean goral is a high priority of the Korean government, as indicated by its designation as Natural Monument animal species number 217. Unfortunately, systematic study for long-term management and conservation of this species is still insufficient. Recent molecular phylogenetic study using mitochondrial DNA (mtDNA) analysis revealed that the Korean goral population is genetically close to the population of the Primorsky region in Russia, but may be distinct from central and southern Chinese populations (Min et al. 2004).

To develop strategies for proper conservation and effective long-term management of an endangered species, detailed information on the present genetic status of the species is important. Polymorphic genetic markers could be of benefit for the conservation and population management of endangered species (Maudet et al. 2001). Microsatellites, short tandem repeated DNA sequences, are desirable in population and conservation studies because they show highly polymorphic codominant inheritance and abundant genomic distribution. They could be amplified via the polymerase chain reaction (PCR) using noninvasive sampling, which is important for the study of endangered species. However, novel microsatellites often have to be isolated before they can be utilized for each species, which generally demands considerable time and high cost. Recently many studies have demonstrated cross-species amplification of Bovidae microsatellites in the Caprinae, allowing possible population studies on species for which microsatellites are not yet developed (Li et al. 2002; Obexer et al. 2003; Saitbekova et al. 1999). This approach is advantageous to the study of endangered species, which might have reduced genetic variation (Maudet et al. 2001). Here we used microsatellites designed for population study of domestic

Bovidae (cattle, sheep, and goat) to survey the Korean goral. We then compared the genetic variability of the Korean goral with that in domestic goats.

In Korea the domestic goat (*Capra hircus*) is one of the most common species of Caprinae. It is also the closest species to the Korean goral. Because the domestic goat has been traditionally grazed in mountain areas in South Korea, the habitat and food resources of the domestic goat can overlap with those of goral. Thus discrimination of goral from the domestic goat might be an important issue, especially for demographic study of the endangered goral population using unidentifiable samples such as feces, hair, or corpora. In addition, because the level of heterozygosity in a larger sample of domestic goats has been surveyed in a previous report (Kim et al. 2002), the level of heterozygosity from this study's much smaller sample size could be compared with the previous report in order to presume reliability of cross-species amplification.

In this study, our objectives are to (1) screen usable polymorphic markers for genetic study of the Korean goral from Bovidae (cattle, sheep, and goat) microsatellites, (2) evaluate current genetic diversity of the endangered Korean goral, and (3) compare genetic diversity of the endangered Korean goral with that of other Caprinae species.

Materials and Methods

Specimens and DNA Isolation

Tissue samples from six Korean gorals were collected from different regions of South Korea. Four of the samples were from wild animals from two provinces (one from Gyeongsangbuk-do and other three from Gangwon-do, where most of the Korean goral populations are assumed to exist), killed either by accident or by poaching. The two other samples were from carcasses from Everland Zoo in Yongin, South Korea. The zoo-kept samples are thought to derive from Gangwon-do. As the Korean goral is designated as a Natural Monument species, all processes involved in obtaining goral samples strictly followed the guidelines set by the Korean government. Fresh blood was taken from 10 domestic goats. Each individual was from a distinct geographical area and was chosen at random, without consideration of the relationship between animals. Genomic DNA was extracted according to the standard protocols (Maniatis et al. 1982) and using a QIAamp DNA mini kit (QIAGEN, Valencia, CA).

Microsatellite Primers Tested

A total of 34 microsatellite primers, polymorphic in their respective species, were selected in this study (30 bovine [*Bos taurus*], 2 ovine [*Ovis aries*], and 2 caprine [*Capra hircus*]). Of these, bovine microsatellites are recommended by the International Society for Animal Genetics (ISAG) for the genetic diversity in cattle (http://www.projects.roslin.ac.uk/cdiv/markers.html). All primers in this study worked well in the species from which they were isolated. Each primer set was analyzed against a panel of 6 Korean gorals and 10 domestic goats (Table 1).

PCR Conditions

The PCR reaction was carried out in a total volume of 12.5 μl using 25 ng of genomic DNA, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 4 pmol of each primer, and 0.5 unit of *Taq* polymerase (Takara Panvera, Madison, WI). The PCR amplification was conducted in a T-gradient thermal cycler (Biometra, Germany) using an initial denaturation for 3 min at 94°C, 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at the optimized temperatures (Table 1), and an extension for 1 min at 72°C followed by a final extension of 10 min at 72°C. Two different PCR reactions were carried out. Initially bovine microsatellites were tested at an annealing temperature of 55°C. If a clear PCR product was not found on 1.7% agarose gel in 0.5 × TBE buffer, PCR was repeated by fluctuating the annealing temperature by a series of ±3°C, allowing a maximum range of ±12°C. Ovine and caprine microsatellites were tested using the touchdown method, conducted with a 1°C reduction at each cycle from 60°C to 53°C, followed by 27 cycles at an annealing temperature of 53°C.

Three microliters of the optimized PCR product were mixed with an equal volume of formamide loading dye and heated to 95°C for 2 min. Four microliters were separated on a 6% polyacrylamide gel and silver stained according to manufacturer's standard protocols (Promega, Madison, WI). The exact allele size was determined by direct comparison with agreed marker genotypes of reference animals distributed by the ISAG for standardization of allele assignments across different studies.

Data Analysis

Allele frequency, number of alleles per locus, and observed heterozygosity (H_o) and expected heterozygosity (H_e) from Hardy-Weinberg assumptions for each locus were computed using the software package GENETIX (Belkhir et al. 2000). Genetic diversity in a population was estimated using average heterozygosity across all loci, which was corrected for small sample size (Nei 1978). For the Hardy-Weinberg equilibrium estimation, we followed the probability test approach (Guo and Thompson 1992) using the program GENEPOP (Raymond and Rousset 1995).

Results and Discussion

Of the 34 microsatellite primer pairs tested, 29 (85.3%) produced a clear product following amplification of samples for the Korean goral and 27 (79.4%) for domestic goats (Table 1). Of these amplified products, 16 (55.2%) were polymorphic in the Korean goral and 22 (81.5%) were variable in domestic goats. Of the seven primer pairs that failed to amplify DNA from the gorals or goats examined, four (HEL5, TGLA227, ETH185, ILSTS006) did not amplify

Table 1. Characteristics of Bovidae microsatellite markers amplified in the test panel of Korean goral and goat and observed (H_o) and expected (H_e) heterozygosity values for each microsatellite in each species

			Nemorhaedus caudatus ($n = 6$)			*Capra hircus* ($n = 10$)			
Locus	Origin	Annealing temp. (°C)	Size range (no. of alleles)	H_o	H_e	Size range (no. of alleles)	H_o	H_e	Reference for primer sequence pair
CSSM66	Bovine	64	184–194 (3)	0.667	0.611	178–212 (7)	0.400	0.805	Barendse et al. 1994
BM1818	Bovine	55	262 (1)	—	—	254–264 (5)	0.200	0.745	Bishop et al. 1994
BM2113	Bovine	55	136–138 (2)	0.500	0.375	130–156 (7)	0.800	0.785	Bishop et al. 1994
ILSTS005	Bovine	55	194–212 (6)	0.667	0.778	176–190 (5)	0.700	0.545	Brezinsky et al. 1993
TGLA53	Bovine	52	143–153 (5)	0.667	0.681	137–147 (4)	0.400	0.715	Georges and Massey 1992
TGLA122	Bovine	61	144–162 (5)	0.500	0.667	134–148 (5)	0.700	0.595	Georges and Massey 1992
TGLA126	Bovine	55	257–273 (6)	0.667	0.694	—[a]	—	—	Georges and Massey 1992
HEL1	Bovine	58	110 (1)	—	—	125–137 (6)	0.600	0.710	Kaukinen and Varvio 1993
HEL13	Bovine	52	188–198 (3)	0.500	0.486	—[a]	—	—	Kaukinen and Varvio 1993
MM12	Bovine	58	95–123 (4)	0.167	0.653	89–115 (6)	0.500	0.800	Mommens et al. 1994
SPS115	Bovine	61	242–244 (2)	0.500	0.486	242–256 (3)	0.400	0.445	Moore and Byrne 1993
CSRM60	Bovine	64	81 (1)	—	—	79–89 (5)	0.700	0.740	Moore and Byrne 1994
ETH10	Bovine	64	209–223 (4)	0.500	0.694	203–209 (3)	0.400	0.405	Solinas-Toldo et al. 1993
ETH225	Bovine	61	155–159 (3)	0.667	0.569	135–161 (7)	0.600	0.660	Steffen et al. 1993
INRA005	Bovine	55	131 (1)	—	—	137–145 (4)	0.300	0.565	Vaiman et al. 1992
INRA023	Bovine	58	205–213 (4)	0.333	0.625	197–215 (7)	0.600	0.830	Vaiman et al. 1994
INRA032	Bovine	55	140–156 (3)	0.333	0.292	132–144 (5)	0.600	0.665	Vaiman et al. 1994
INRA035	Bovine	55	112–116 (2)	0.167	0.153	110–114 (2)	1.000	0.500	Vaiman et al. 1994
INRA037	Bovine	55	132 (1)	—	—	132–142 (5)	0.700	0.660	Vaiman et al. 1994
INRA063	Bovine	55	175–181 (4)	0.667	0.653	161–171 (4)	0.600	0.720	Vaiman et al. 1994
BM1824	Bovine	61	171 (1)	—	—	171 (1)	—	—	Bishop et al. 1994
HEL9	Bovine	49	—[a]	—	—	101–102 (2)[b]	—	—	Kaukinen and Varvio 1993
ETH3	Bovine	64	101 (1)	—	—	—[a]	—	—	Solinas-Toldo et al. 1993
ETH152	Bovine	58	195 (1)	—	—	201 (1)	—	—	Steffen et al. 1993
HAUT24	Bovine	55	120 (1)	—	—	121–122 (2)[b]	—	—	Thieven et al. 1997
HAUT27	Bovine	55	108–109 (2)[b]	—	—	108–109 (2)[b]	—	—	Thieven et al. 1997
McMA47	Ovine	60→53[c]	117 (1)	—	—	131–145 (5)	0.300	0.680	Beh et al. 2000
McMA49	Ovine	60→53[c]	187–197 (4)	0.667	0.722	143–159 (8)	1.000	0.820	Beh et al. 2000
CRSP21	Caprine	60→53[c]	134 (1)	—	—	137–143 (4)	0.300	0.570	Yeh et al. 1997
CRSP26	Caprine	60→53[c]	134 (1)	—	—	128–144 (6)	0.600	0.550	Yeh et al. 1997
Mean			2.6[d]	0.292	0.356	4.8[d]	0.517	0.636	

[a] Signifies no amplification.

[b] Marker revealed polymorphisms only by size difference of one nucleotide in the test panel, which is excluded from the data analysis.

[c] PCR was conducted with the touchdown method.

[d] Mean number of alleles across loci.

DNA of either species, two (HEL13, ETH3) produced specific PCR product only in gorals, and one (HEL9) produced specific PCR product only in domestic goats.

Amplification from three primer pairs (HAUT27 for both goral and goat, and HEL9 and HAUT24 for goat) revealed polymorphisms in the test panel with a size difference of only one nucleotide. These primer sets were excluded from further genetic analysis because they might show homoplasy due to imperfect/compound microsatellites or single-base deletion/insertion.

Primer pairs showing the largest number of alleles were ILSTS005 and TGLA126 in the Korean goral (six alleles in both primer pairs) and McMA49 in domestic goats (eight alleles). The levels of observed and expected heterozygosity varied depending on microsatellite markers as well as species applied (Table 1). ILSTS005 showed the highest level of heterozygosity in Korean gorals ($H_e = 0.778$) and INRA023 in domestic goats ($H_e = 0.830$).

Most of the successful primer pairs showed similar or smaller allele sizes compared with the species from which they were developed, and similar results from cross-species amplification have been reported in previous studies (Primmer et al. 1996; Rubinsztein et al. 1995; van Hooft et al. 1999). Some primers, such as TGLA126 and MCMA49, showed quite different amplification products among the species examined (Table 1). In particular, TGLA126 primers showed larger allele sizes in the Korean goral (with the size range of 257–273 bp) compared with domestic cattle (with the known size range of about 117–131 bp), and these primers failed to amplify the DNA of domestic goats. These primers could therefore be employed for identification of the Korean goral among other domestic Bovidae species because differentiating goral from domestic goats is sometimes hard by corpora, hair, or feces found in the wild.

For the majority of the primer pairs showing polymorphisms in both species, larger numbers of alleles, as well

as higher levels of heterozygosity, were observed in the domestic goat than in the Korean goral (Table 1). The average heterozygosity and mean number of alleles per marker were 0.356 and 2.6 in the Korean goral and 0.636 and 4.8 in domestic goats, respectively. The heterozygosity value of the domestic goats in this study is within the range of the heterozygosity of goats reported in previous studies—for example, 0.38–0.67 in northeast Asian domestic goats (Kim et al. 2002), 0.43–0.60 in southeast Asian goat populations (Barker et al. 2001), and 0.44–0.69 in wild goats (Maudet et al. 2001). Although different markers and small samples have been used for diversity studies of the subfamily Caprinae, the values make it possible for us to assume the present status of genetic variability of the Korean goral. The Korean goral revealed the lowest level of heterozygosity among the Caprinae subfamilies examined, which included severely bottlenecked Alpine ibex (*Capra ibex ibex*) and endangered Spanish ibex (*Capra pyrenaica*) (Maudet et al. 2001).

The actual heterozygosity of the Korean goral was lower than expected under Hardy-Weinberg equilibrium ($H_o = 0.292$, $H_e = 0.356$), and statistically significant departures reflect the deviation in the direction of heterozygote deficit ($P < .01$). Although a possible null allele might influence the finding, this is not unexpected considering an assumed population bottleneck due to habitat fragmentation and inbreeding due to small population size.

To our knowledge, this is the first report of genetic diversity in the endangered Korean goral. Low genetic diversity in Korean gorals observed in this preliminary microsatellite survey suggests an urgent need for population management and conservation of this species. Because loss of genetic diversity in endangered species is often associated with inbreeding and a reduction in reproductive fitness (Reed and Frankham 2001), efforts to increase the genetic diversity of the endangered Korean goral should be considered as a high priority for conservation of this species. Since the decline in genetic diversity in Korean gorals could be related to habitat fragmentation, corridors among fragmented habitats or even relocation of individuals should be considered. However, detailed studies on the level of genetic diversity in each fragmented population are necessary to identify the most suitable management prescriptions for the fragmented populations. In this regard, the cross-species amplified microsatellite markers in this study would be greatly useful for providing data on genetic diversity among the fragmented populations, especially if they are used in connection with noninvasive genetic sampling techniques. We are currently working on this possible application.

When one conducts cross-species amplification it is important to remember that there is possible microsatellite ascertainment bias, and that this bias might somehow effect the interpretation of results. Because the results are obtained using microsatellites developed in a different species, the probability of occurrence of a null allele, as well as homoplasy, will be much higher than in the case of testing in the species from which they were isolated. Both null alleles and homoplasy will produce bias in estimating the genetic structure of a population. Future studies likely will investigate the influence of null alleles and homoplasy in cross-species amplified microsatellites and their applicability in other species.

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