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Identification of Species and Sex of Korean Roe Deer (*Capreolus pygargus tianschanicus*) Using *SRY* and *CYTB* Genes

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Abstract: The nucleotide sequences of a male-specific marker *sex determining region Y* (*SRY*) gene and a mitochondrial *cytochrome B* (*CYTB*) gene were characterized and analyzed to establish a molecular method for identification of species and sex of Korean roe deer (*Capreolus pygargus tianschanicus*). Similarity search result of *SRY* sequences showed very similar result to those reported in Moose (*Alces alces*) and Reindeer (*Rangifer tarandus*), both of which had 95.9% similarity in identity. *CYTB genes* were very similar to those reported in Siberian roe deer (*C. pygargus pygargus*) which had 98.6% similarity and not to European roe deer (*C. capreolus*), suggesting that the DNA samples tested were of Siberian roe deer lineage. Polymerase chain reaction (PCR)-based sex typing successfully discriminated between carcasses of male and female roe deer. Males had *SRY* band on agarose gels and females did not. The result of this molecular sex typing provided similar information with that obtained by genital organ observation. Therefore, this molecular method using male specific marker *SRY* and mitochondrial *CYTB* genes would be very useful for identification of the species and sex of the carcass remains of roe deer.

Key words: species identification, sex determination, roe deer, *SRY*, *CYTB*

The asymmetric evolution of sex chromosomes is one of the most interesting biological processes. In particular, in mammals which have two distinct sex chromosomes X and Y, male development depends actually on the presence of a small gene *sex determining region Y* (*SRY*) which induces the differentiation of testis during embryogenesis (Sinclair et al., 1990; Koopman et al., 1991; Angelo and Moreira, 2002). Several molecular methods for sex determination have been documented, including chromosome analysis, immunological antigen detection, sex chromosome-linked enzymatic activity determination, and Y chromosome-specific probe hybridization (Gardner and Edwards, 1968; Anderson, 1987; Quilter et al., 2002; Cao et al., 2005). PCR-based assays are often more useful for the carcasses because they provide sensitive, rapid, and reliable results than those of other *in vitro* approaches such as the immunological test for H-Y antigen, karyotyping and Barr-body scanning of inactivated X chromosome, and cytogenetic test for Y chromosome specific gene using hybridization. To date, PCR-based sex typing has been used for tissues from living animals, carcasses, embryonic cells, archaeological remains, and even feces (Dreesen et al., 1995; Shea 1999; Yamauchi et al., 2000; Lee et al., 2004).

The roe deer (*Capreolus*, Artiodactyla, Cervidae) has two different species: *C. caprelous* and *C. pygargus*. The former is of smaller European origin distributed in Western Europe and the latter is of larger Siberian origin distributed in Asia and Eastern Europe (Groves and Grubb, 1987; Grubb, 1993; Randi et al., 1997). The Korean roe deer, *C. pygargus tianschanicus*, is widely distributed in Korea particularly in Jeju Island. In 1980s, roe deer population declined due to various reasons such as loss of habitat, illegal hunting and others. At present, killing of roe deer is prohibited in any season and for any reason under the wild animal protection law. This increased roe deer population in the last decades. But now Korean roe deer often brings social problems such as crop damage and traffic accidents in Jeju Island. Data on

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the sex ratio of the post-mortem wildlife could serve as useful information for understanding the structure and dynamics of animal population necessary for planning wildlife management under the ecological and social conditions in the province (Takahashi et al., 1998; Yamamoto et al., 2002). However, there is no established method for sex determination of the uncoated carcasses after hunting and accidents or bone remains of animals that died naturally. Hence, this study was focused on developing a molecular method for PCR based sex determination using male specific gene *SRY* on Y chromosome for various specimens of Korean roe deer.

METERIALS AND METHODS

Sample collection and total DNA extraction

Fifteen tissue samples (kidney, muscle, and skin) were obtained from the Conservation Genome Resource Bank for Korean Wildlife (CGRB) and from Animal Specimen Room of Cheju National University (CNU) with or without sex information. Thirteen individual samples (antler, bone, hair, muscle, and skin) were additionally collected from carcass remains died of unknown causes in Mt. Halla (Table 1). All tissues were used for total DNA extraction using the sucrose-proteinase K method (Birren et al., 1997) with slight modification. The isolated DNA samples were further purified using phenol-chloroform treatment, recovered by ethanol precipitation method, and then used as a template for PCR amplification.

PCR amplification and PCR-mediated sex typing

PCR amplification and subsequent DNA sequencing were carried out to determine the complete sequences of the *SRY* and the mitochondrial *CYTB* genes. In addition, duplex PCR reaction was taken to type the sex of roe deer using amplification patterns of the partial fragments of *SRY* and *CYTB* genes. For amplification of the *SRY* gene, two sets of primers were developed using the sequences reported from Sika deer (GenBank Accession No. AB046700): dSRYF-dSRYR for amplifying the complete coding region and dSRYiF-dSRYiR for amplifying the inner fragment. On the other hand, complete region of *CYTB* gene was amplified with the primers ML103-MH104 previously described by Chikuni et al. (1995). The primers dCYiF-dCYiR for amplifying the inner fragment of *CYTB* were designed after DNA sequencing. The sequences of each primer are listed in Table 2. PCR amplification was carried out in 25-μl volume, containing distilled water, 2.5 μl of 10X reaction buffer, 200 μM of dNTPs, 2.0 units of *Taq* DNA polymerase (Promega, USA), 10 pmol of each primer, 1.5 mM $MgCl_2$, and about 10-30 ng of template DNA. However, in Duplex PCR, the primers were used as 4 pmol of dCYiF/R primer and 6 pmol of dSRY1F/R primer instead of the primer concentrations above. The PCR cycling conditions of the above primers were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles each of denaturation at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The final extension was 72°C for 10 min. The PCR products were separated on 2.0% agarose gels containing ethidium bromide and observed under a UV illuminator, and each band was purified using a QIAEX II Gel Extraction Kit (Qiagen, USA).

DNA sequencing and sequence analysis

The purified PCR products were cloned and subsequently sequenced to verify the sequences. The PCR product was inserted into a plasmid vector using the TOPO™ TA Cloning Kit (Invitrogen, USA). Nucleotide sequencing was carried out using the DYEnamic ET Dye Terminator Kit (Amersham Biosciences, USA). The nucleotide sequences obtained in this study were deposited in GenBank database under accession numbers EF100132 (*SRY*) and EF139148-55 (*CYTB*). They were subsequently compared against the sequence information in GenBank database using BLAST similarity search for confirmation of each gene sequence.

RESULTS AND DISCUSSION

The sequences of *SRY* and *CYTB* genes were determined to identify the species and sex of various carcass remains of roe deer. Roe deer *SRY* sequences were determined from three males in this study and its BLAST search result showed that they were very similar to those from Moose (*A. alces*) and Reindeer (*R. tarandus*), both of which had 95.9% similarity. Particularly, the length of complete coding region of water deer *SRY* was 687-bp, putatively encoding 228 amino acids, which was 3-bp shorter than others like Cervidae including that of Sika deer used for primer design.

On the other hand, the result of similarity search for *CYTB* sequences in this study showed they were very similar to those of Siberian roe deer (*C. pygargus pygargus*, GenBank Accessions AY070227 and AJ000025) with 98.5%, similarity. The sequence homology of *CYTB* to those of European roe deer (*C. capreolus*) showed below 96%

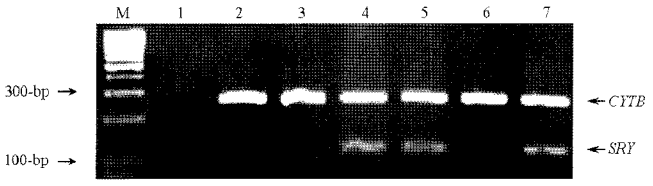

Fig. 1. PCR amplification patterns of roe deer DNA. Lane 1, negative control; lanes 2 and 3, female roe deer DNA; lanes 4 and 5, male roe deer DNA; lanes 6 and 7, nested PCR products from female and male roe deer DNA, respectively. M is the 100-bp DNA ladder.

Table 1. Source of sample and sex information of roe deer used in this study

Specimen ID	Sample source	Phenotype sex[a]	PCR sex typing	
			PCR	Nested-PCR
cgrb644	kidney	male	male	-
cgrb647	muscle	male	male	-
cgrb1838	muscle	male	male	-
cgrb2180	muscle	unknown	female	-
cgrb2216	skin	unknown	male	-
cgrb2341	muscle	unknown	female	-
cgrb2679	muscle	female	female	-
cgrb3221	muscle	female	female	-
cgrb3422	muscle	male	male	-
CNURD1	muslce	male	female	-
CNURD2	muslce	male	male	-
CNURD3	hair	female	female	-
CHURD4	liver	male	male	-
CNURD5	muscle	unknown	male	-
CNURD6	muscle	unknown	female	-
JHRD1	hair	male	female	-
JHRD2	hair	unknown	male	-
JHRD5	hair	unknown	male	-
JHRD6	skin	unknown	-	female
JHRD8	antler	unknown	-	male
JHRD9	bone	unknown	-	-[b]
JHRD10	bone	unknown	-	female
JHRD13	skin	unknown	-	female
JHRD14	antler	unknown	-	male
JHRD16	skin	unknown	-	male
JHRD17	antler	unknown	-	male
JHRD20	bone	unknown	-	female
JHRD23	bone	unknown	-	female

[a]Pehontypic sex information was provided by collectors or obtained by observation for genital organs.
[b]PCR sex typing was actually failed.

similarity, suggesting remarkably that the sequences are members of Siberian roe deer and not that of European roe deer. This result also indicated that the *CYTB* sequence analysis would be useful for identifying the species from roe deer carcasses, which also supported the findings of Koh et al. (2000) that Korean roe deer should be considered as a subspecies, *C. pygargus tianschanicus* of Siberian roe deer (*C. pygargus*), inferred from the mitochondrial phylogeny. However, systematic relationship among the populations of Siberian roe deer can not be described due to limited number of sequences used for comparison in this study. The sequences from more expanded populations are necessary to analyze the systematic relationship and to clarify the subspecies classification of Siberian roe deer.

The duplex PCR tests using male-specific *SRY* and internal positive control *CYTB* were applied to identify the sex of carcass remains. The amplified products classified two distinct migration patterns: those from males produced two different bands for *SRY* and *CYTB*, and those from females produced only a single band for *CYTB* on agarose gels (Fig. 1). Specifically, in sex typing of DNA samples isolated from roe deer bones estimated to die at least two months prior to the experiment, several DNA samples did not directly produce any visible PCR products on agarose gels. In these cases the nested PCR approach was employed for further analysis. As the PCR templates, primary PCR products amplified with the primer sets, dSRYF/R and ML103/MH104 were used. Finally, although one of DNA samples from the bone actually failed to amplify even by nested-PCR, the others successfully typed their sex (Table 1). When using PCR tests for sex determination in sex-known DNA samples, the results were accurate with their phenotypic features. The results for sex-unknown DNA samples provided molecular gender information of the carcass remains except for just one DNA sample, JHRD9.

The PCR-based sex identification methods were recently documented in various cervids including Japanese sika deer

Table 2. List of the primers used in this study

Gene	Primer name	Nucleotide sequence (5'->3')	Product size	Reference
SRY complete region	dSRYF	ATGTTCAGAGTATTGAACGAT	687-bp	AB046700[a]
	dSRYR	TCAATATTGAAAATAAGCGCAAG		
SRY inner fragment[b]	dSRYiF	GCTGGGGTATGAGTGGAAAA	126-bp	This study
	dSRYiR	GTTTTCCGACGAGGTCGATA		
CYTB complete region	ML103	GACTAATGATATGAAAAACCATCGTTG	1,245-bp	Chikuni et al. (1995)
	MH104	TTGTTCTTCATCTCTGGTTTACAAGAC		
CYTB inner fragment[b]	dCYiF	TTGCAGCACTTGCCATAGTC	234-bp	This study
	dCYiR	GAGGGGGTGTGTTAAGTGGA		

[a]GenBank Accession No. unpublished.
[b]used for sex typing by direct PCR and nested PCR procedure.

(*Cervus nippon*) (Takahashi et al., 1998; Wilson and White, 1998; Yamauchi et al., 2000; Cao et al., 2005; Pfeiffer and Brenig, 2005). This study, however, is the first of a kind to describe a molecular method for the roe deer, as the test successfully identified the sex of DNA samples isolated from various carcass remains of roe deer. In addition, this study proved to identify the species of Korean roe deer using sequence analysis of mitochondrial *CYTB* gene. The results provided similar information with that of An et al. (2006), who reported a species identification method using partial *CYTB* sequences for supplying forensic evidence on illegally hunted carcasses of various mammals and birds including Korean roe deer. Consequently, these molecular approaches would be useful for identification of the species and sex of roe deer.

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