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Genetic Analysis of Ancient Bones of Cervidae Animals from Archaeological Site in Jeju, Korea

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Abstract: DNA extracted from ancient bones of Cervidae animals was examined to identify the species and to determine the phylogenetic relationships to those from extant cervids. Abundant ancient bones were excavated from Kumsung archaeological site in Jeju Island, Korea, and were identified as Cervidae animals based on morphological features of their antlers and lower mandibles. Their mitochondrial DNA (mtDNA) control region (CR) was partially sequenced and subsequently compared with those previously reported in database. The results confirmed that the ancient sequences are lineage of Cervidae. On the phylogenetic trees constructed using the sequence diversity of the CR sequences of family Cervidae, the ancient DNA sequences were found on distinct clusters. The ancient sequences were located in the subfamily Capreolinae cluster, and six ancient sequences were closely related to those of extant Korean roe deer in Jeju Island and Korean Peninsula. Consequently, the results of this study suggest that the roe deer inhabited Jeju Island in ancient times. However, there is no evidence for the existence of subfamily Cervinae, including Sika deer, while it has been described in several historical records. The results suggest that this finding could contribute to understanding of the origin and phylogenetic relationships of extant and ancient roe deer on Jeju Island.

Key words: ancient bone, Cervidae, mtDNA, origin, phylogenetic relationship

The ancient remains of several species of animals including human fossils have been discovered in many archaeological sites around the world. The development of molecular biological techniques, such as polymerase chain reaction (PCR) and DNA sequencing, has enabled molecular genetic studies of extinct animals and even fossils. Higuchi et al. (1984) extracted DNA from the tissue of now extinct Quagga (*Equus quagga*), analyzed the sequences of mitochondrial DNA (mtDNA), and consequently found that it was kin to the existing Zebra. In addition, Pääbo (1985) extracted DNA from Egyptian mummies of 2,400 years ago, and succeeded in analyzing the sequences. Afterwards, many evolutionary studies have been conducted on the basis on ancient DNA sequences from various tissues and organs, such as bones, teeth and brains of diverse ancient organisms, including horses, deer, rabbits, pigs, fishes and mammoths, as well as humans (Doran et al., 1986; Merriwether et al., 1996; Faerman et al., 1998; Nabata et al., 2004; Yang et al., 2005). Peculiarly, the mtDNA undergoes maternal inheritance and no genetic recombination takes place, and the base mutation rate is over 10 times higher than the nuclear DNA (Brown et al., 1979; Giles et al., 1980; Wilson et al., 1985; Avise, 1986; Kavar et al., 1999). The base substitution rate of the CR is known to be higher than other regions in the mtDNA (Aquadro and Greenberg, 1982; Cann et al., 1984). For these reasons the mtDNA CR is widely used as a genetic marker not only in the analysis of phylogenetic relationship of extant animals, but also in the molecular genetic analysis of remains of ancient animal bones (Jung et al., 2002; Kim et al., 2005).

Various archaeological sites in Jeju Island (Korea) were found, formed in diverse periods from the Old and New Stone Ages through the Bronze Age to the Iron Age. However, only morphological and osteological studies had been carried out for identification and classification of the excavated animal bones, and the species were not clearly

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identified at the molecular level.

In the present study, DNA was extracted from the animal bones of Cervidae animals excavated at the archaeological site on Jeju Island. We determined partial sequences of the mtDNA CR and compared them with those from extant cervids to identify the species and to analyze the phylogenetic relationship.

MATERIALS AND METHODS

Ancient bones and DNA extraction

The ancient animal bones excavated from Kumsung archaeological site (about A.D. 500 to A.D. 900) were used for this study. All specimens were kindly provided by the Folklore & Natural History Museum Jeju (Jeju, Korea) (Fig. 1, Table 1). Total DNA was extracted from each bone according to the methods of Graham (1978) and Maniatis et al. (1982), with slight modifications. The powdered samples were decalcified by suspension in 50 volumes of 0.5 M EDTA (pH 8.0) and incubated with agitation for 24 h with five fresh changes of EDTA. Total DNA was extracted by incubating 0.5-1 g of bone at 37°C for 24 h in 10 volumes of lysis solution containing 0.5 M EDTA (pH 8.0), 50 μl/ml proteinase K, and 0.5% N-lauroylsarcosine, followed by one extraction with phenol, two extractions with phenol/chloroform (1 : 1), and finally chloroform/isoamyl alcohol (24 : 1) to remove all traces of phenol. The DNA was further purified using cesium chloride/ethidium bromide gradients (Sambrook et al., 1989).

Amplification and purification of the mtDNA control region

Total DNA was used to amplify fragment of the mtDNA CR by PCR. The oligonucleotide primers used in the PCR were designed using internal sequence of mtDNA control region of *Cervus nippon* (Feng et al., 1995; GenBank accession number U12868). Because the maximum size of DNA fragment amplified from ancient bone extracts is usually limited to only a few hundred bases (100-400 bp) (Hagelberg et al., 1991), three primer sets were used to amplify the complete CR.

The primer sequences were as follows: CerD-F (5'-GGA TCC CTC TTC TCG CTC C-3') and CerD-R (5'-CCT ACC ATT ATG GGG ATG CTC A-3'), CerD1-F (5'-TCA CCT AAA ATC GCC CAC TC-3') and CerD1-R (5'-CCA GCT ACA ATT CAT GCT CC-3'), and CerD2-F (5'-GCC CCA TGC TTA TAA GCA TG-3') and CerD2-R (5'-CGG AGC GAG AAG AGG GAT C-3'). To avoid contamination, PCR was performed using AccuPower PCR Premix (Bioneer, Korea). PCR and amplification were performed according to the methods of Jung et al. (2002) with slight modification. PCR-generated DNA was purified by electrophoresis on a low melting point agarose gel matrix in 1 × TAE buffer. After staining the gel with ethidium bromide, the agarose block that contained the DNA was excised under low-wavelength UV light, followed by DNA recover and concentration using the GeneAmp PCR System 9600 Thermal Cycler (Perkin-Elmer, USA), according to the manufacturer's protocol.

Cloning and DNA Sequencing

Purified PCR products were ligated into the vectors with a TOPO TA Cloning Kit (Invitrogen, USA). Transformed plasmid DNA was purified using the Wizard Plus SV Minipreps DNA purification System (Promega, USA) and the sequences were obtained using a Cy5™ AutoCycle™ Sequencing Kit on an ALFexpress DNA sequencer (Pharmacia Biotech, USA). For each PCR product, more than three clones were sequenced. Sequences that were identical in at least two of more than three clones were selected and used for further analysis. All sequences newly determined in this study were submitted to the GenBank database (Table 1).

Sequences analysis

We determined the sequence boundaries of the control region by comparing our data with the published sequences of family Cervidae retrieved from the GenBank database (Table 1) and the sequences were aligned using the CLUSTAL W package (Thompson et al., 1994). Sequence divergence values among species were calculated using the DNADIST program of PHYLIP (Phylogenetic Inference Package) version 3.573c (Felsenstein, 1993) and the number of nucleotide substitutions was estimated by the two-parameter method, assuming a transition/transversion ratio of 2.0 (Kimura, 1980). The phylogenetic trees were constructed by neighbor-joining (Saitou and Nei, 1987) in the PHYLIP package. The strict consensus tree was generated using the SEQBOOT, DNAPARS, and CONSENSUS programs in the PHYLIP package in a sequential method.

RESULTS AND DISCUSSION

Molecular identification of ancient animal bones

To identify species and to analyze phylogenetic relationship with extant animals, the sequences of mtDNA CR were determined from the DNA samples extracted from ancient bones of Cervidae excavated from Kumsung archaeological site on Jeju Island, Korea. This archaeological site is estimated to be formed approximately from A.D. 500 to A.D. 900. The sequences of the mtDNA control region analyzed from the ancient remains were compared for their similarity with the DNA sequences reported in the GenBank database using the BLAST web search program. As a result, our CR sequences showed the highest homology (96-98%) with the species of Siberian roe deer *Capreolus*

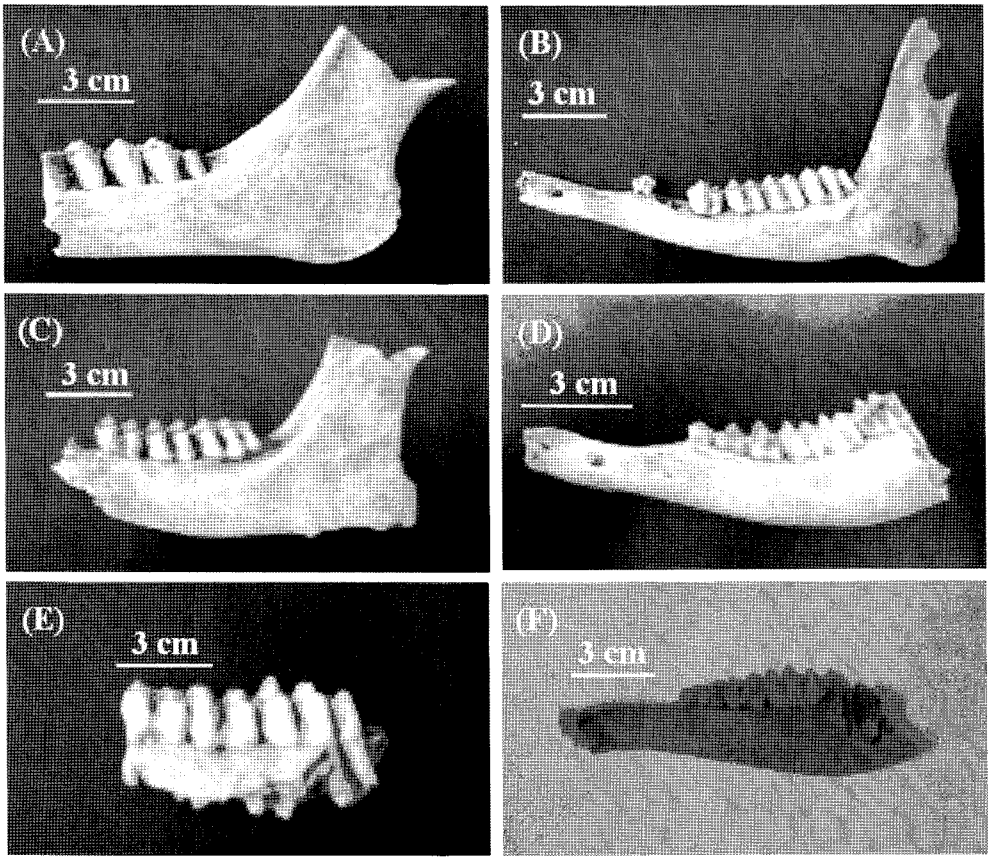

Fig. 1. Photographs of the six ancient remains. (A)-(F) mandibles and tooth from Kumsung archaeological site.

pygargus (the subfamily Capreoleinae). Based on the sequence similarity of move than 95% with extant animals of family Cervidae, genetic distance was calculated among six of our sequences and twelve sequences from extant Cervidae. The degree of genetic distance between the ancient and the extant animals was found to be 1.15% to −11.65%, and the degree of genetic distance between all of family Cervidae and the outgroup (*Bos taurus*) was 3.01% to 19.32%. Calculation of genetic distance among the 6 ancient remains showed that, Korean roe deer (Cpk2) had the lowest distance value of 1.15% to 2.80%, indicating closest relationship, whereas European and Siberian roe deer (Cc12, Cpe15) were farther apart (data not shown). These results agree with Koh et al. (2000) that analyzed the mtDNA control region and cytochrome b sequences of the Korean roe deer and Siberian roe deer.

Phylogentic analyses

For phylogenetic analysis between 6 ancient remains and the 12 existing animals of family Cervidae, a neighbor-joining tree (NJ tree) was constructed by using the NJ method (Saitou and Nei, 1987) (Fig. 2). Then, a consensus tree was constructed for an analysis of parsimony based on the NJ trees of 30 species including 6 ancient remains of family Cervidae and 17 species of the genus Korean, European, and Siberian roe deer under Capreoleinae. As outgroup of each tree, *Bos taurus* and *Alces alces* were included (Fig. 2, 3). The NJ tree included largely 7 subfamilies-Hydropotinae, Capreoleinae, Rangiferinae, Muntiacinae, Cervinae, Alcinae, and Odocoileinae. The ancient animal bones of family Cervidae was shown to have a relationship with Korean roe deer (Cpk2) of the subfamily Capreoleinae. The consensus tree (Fig. 3) included three groups-European roe deer, Korean roe deer; Siberian roe deer, and a relationship was found with roe deer currently inhabiting Mt. Halla of Jeju Island and the Korean Peninsula (Cpk1, Cpk2, Cck1, Cck2) rather than with European and Siberian roe deer. These results suggested that the remains of the ancient Jeju archaeological sites had descended from more than one maternal ancestor. These results are similar with those of the previous study on the osteological and morphological analysis by Shin et al. (1996) and Kim et al. (2002), which made compariors to the existing small deer *Crevus nippon*, deer and, big roe deer.

Record for the mammals of Jeju Island

The documents on the archaeological excavations on the Jeju Island also support the possibility of the existence of the extinct roe deer. The records of the excavated bones of

Table 1. Comparison of the partial sequences of the control region from the Cervidae animal bones and other Cervidae animals

Species (common name)	Abbreviation	Length (bp)	Accession No.	Reference
Cervidae spp.				
Tooth (mandible)-1	ANC-1	438	AF958266	This study
Tooth (mandible)-2	ANC-2	441	AF958268	This study
Tooth (mandible)-3	ANC-3	440	AF958269	This study
Tooth (mandible)-4	ANC-4	441	AF958270	This study
Tooth-5	ANC-5	441	AF958271	This study
Tooth (mandible)-6	ANC-6	444	AF958273	This study
Hydropotinae				
Hydropotes inermis (Chinese water deer)	Hi	439	Y08208	Douzery & Randi (1997)
Muntiacinae				
Muntiacus crinifrons (black muntjac)	Mc	443	AY239042	Li et al. (unpublished)
Muntiacus reevesi (Chinese muntjac)	Mr	443	AF527537	Zhang et al. (2005)
Muntiacus muntjak (muntjac)	Mm	442	AY225986	Shi et al. (unpublished)
Cervinae				
Cervus elaphus canadensis (red deer)	CE	438	AY970666	Lee et al. (unpublished)
Elaphurus davidianus (Pere David's deer)	Ed	442	AF291894	Randi et al. (2001)
Cervus eldi siamensis (brow-antlered deer)	Ces	442	AF291894	Randi et al. (2001)
Cervus dama (fallow deer)	Cd	441	AF291895	Randi et al. (2001)
Axis porcinus (hog deer)	Ap	441	AF291897	Randi et al. (2001)
Odocoileinae				
Odocoileus virginianus (white-tailed deer)	Ov	441	AF421853	Moscarella et al. (2003)
Odocoileus hemionus (black-tailed deer)	Oh	441	AF016952	Polziehn and Strobeck (1998)
Mazama gouazoupira (gray brocket)	Mg	440	Y08570	Douzery & Randi (1997)
Capreoleinae				
Capreolus pygargus (Korean roe deer)	Cpk1	440	AJ311188	Koh and Randi (unpublished)
Capreolus pygargus (Korean roe deer)	Cpk2	440	AJ311189	Koh and Randi (unpublished)
Capreolus capreolus (Korean roe deer)	Cck1	440	DQ323050	This study
Capreolus capreolus (Korean roe deer)	Cck2	441	DQ323051	This study
Capreolus pygargus (eastern roe deer)	Cpe1	441	AY854040	Zhang et al. (2005)
Capreolus pygargus (eastern roe deer)	Cpe2	440	AY854041	Zhang et al. (2005)
Capreolus pygargus (eastern roe deer)	Cpe3	440	AY854043	Zhang et al. (2005)
Capreolus pygargus (eastern roe deer)	Cpe4	440	AY854044	Zhang et al. (2005)
Capreolus pygargus (eastern roe deer)	Cpe5	440	Z70317	Douzery & Randi (1997)
Capreolus capreolus (western roe deer)	Cc1	440	Z70318	Douzery & Randi (1997)
Capreolus capreolus (western roe deer)	Cc2	441	AY625743	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc3	441	AY625744	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc4	441	AY625803	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc5	441	AY625813	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc6	441	AY625816	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc7	441	AY625827	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc8	441	AY625836	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc9	441	AY625854	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc10	441	AY625859	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc11	441	AY625873	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc12	441	AY625874	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc13	441	AY625880	Randi et al. (2004)
Capreolus capreolus (western roe deer)	Cc14	441	AY625887	Randi et al. (2004)
Alcinae				
Alces alces (moose)	Aa	443	U12866	Feng et al. (unpublished)
Rangiferinae				
Rangifer tarandus (caribou)	Rt	441	AF096419	Dueck et al. (unpublished)
Outgroup				
Bos taurus (Cattle)	Bt	441	AY521136	Lai et al. (2006)

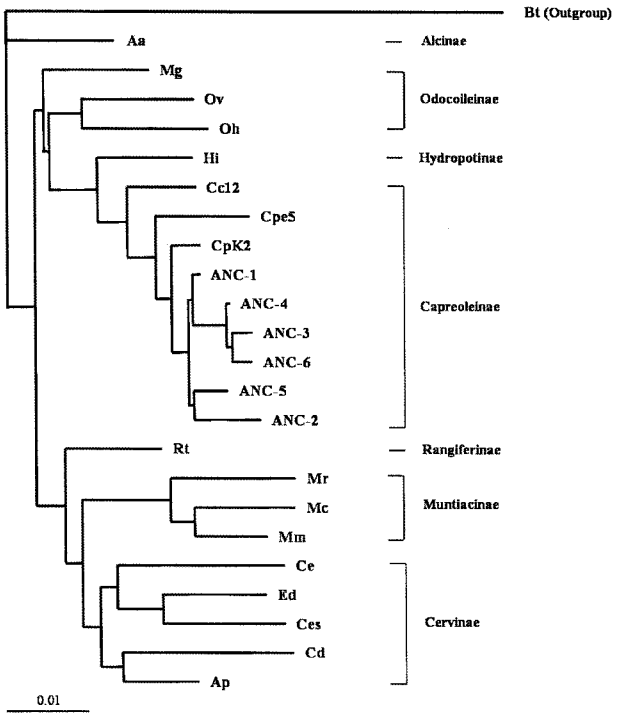

Fig. 2. A neighbor-joining tree constructed from the sequences of 6 Cervidae animal remains and 18 extant Cervidae animals. The tree was generated using genetic distances calculated by Kimura's 2-parameter method. *B. taurus* (AY521136) were used as the outgroup. Species abbreviations are given in Table 1.

brown bear, cow, roe deer, wild boar, and horse from the Jongdali shell mound site I, Konaeli archaeological site, Billemot, and Kimnyungri cave sites also suggest that roe deer existed throughout the period between A.D. 0 and A.D. 1,300 (Jeju-Do, 1989; Shin et al., 1996; Shin, 2001; Kim et al., 2002). Also, some historical documents such as JEJUPUNGTOROK, NAMSAROK, and TAMRAJI support the possibility of the existence of the above mentioned animals through the records of the wild animals that inhabited the Jeju Island (Kim, 1976).

Based on the results of this study as well as the records of archaeological excavations and ancient literature, it is suggested that some groups of roe deer of the maternal line of Mt. Halla and the Korean Peninsular had existed around 1,100-1,500 years ago, and this can be used as the basic material for research on roe deer that lived in Jeju Island during that period. While the animals of Subfamily Cervinae were historically recorded, their existence had not been confirmed. More research should be carried out for the family Cervidae excavated from various archaeological sites. We suggest to modify the assert classification and naming systems for the family Cervidae in Korean peninsula to include various genetic analyses such as nuclear DNA analysis and DNA fingerprinting using the microsatellite DNA.

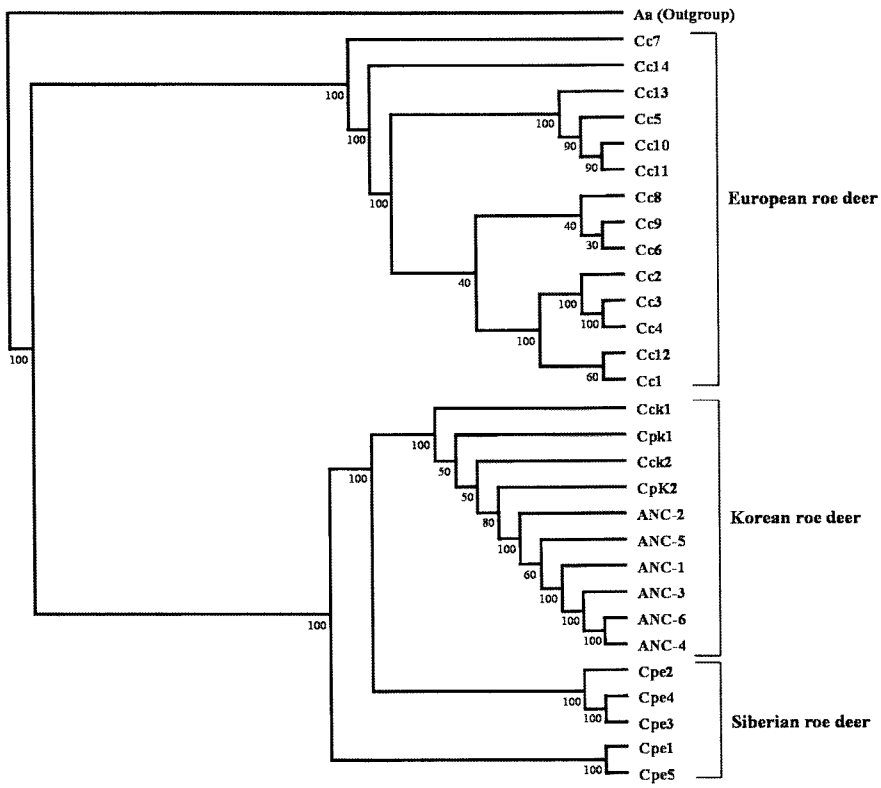

Fig. 3. A strict consensus tree derived from the sequences of the Cervidae animal remains and the extant Capreoleinae. This tree was constructed using the SEQBOOT, DNAPARS, and CONSENSUS programs in the PHYLIP package. Bootstrap values (1,000 replicates) are shown below the nodes on the strict consensus tree. *Alces alces* (U12866) were used as the outgroup taxon.

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