Successful Molecular Cloning and Nucleotide Sequence Determination of Partial Amelogenin (AMELX) Exon DNA Fragment Recovered from a Mounted Taxidermic Pelt Specimen Tentatively Identified as an Extinct Wolf Species, Canis lupus hodophilax Temminck, the Japanese Wolf and Stocked at School of Agriculture and Life Sciences, the University of Tokyo

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Abstract. The Japanese wolf, Canis lupus hodophilax Temminck, which putatively became extinct decades ago, remains a taxonomical or phylogenetic enigma. In Japan, 3 mounted taxidermic pelt specimens believed to represent C. lupus hodophilax have been stocked at the University of Tokyo, the National Science Museum and Wakayama University, respectively. Using an improved method developed by our group for the extraction and the PCR amplification of ancient DNA from preserved mammalian pelts, a 351-nucleotide partial genomic DNA stretch of the X-linked gene coding for amelogenin (AMELX) was successfully cloned from a skin specimen derived from the mounted female taxidermic pelt specimen of the University of Tokyo, and its nucleotide sequence was determined. Two allelic lupine AMELX sequences, i.e., the J-type (accession no. AB080688, submitted to DDBJ on 1/Mar./2002) and the R-type (accession no. AB080689, submitted to DDBJ on 1/Mar./2002) were identified. Both the J-type and the R-type alleles differed considerably at the nucleotide as well as the amino acid level from the corresponding AMELX region shared by the dog and the Mongolian wolf. Although the presently discovered lupine variants of the AMELX partial sequence are of great interest from the point of view of taxonomical identity of the Japanese wolf, our preliminary results (unpublished) strongly indicate that polymorphism in the nucleotide as well as the amino acid sequence is likely to exist in this particular region of AMELX among different breeds of domestic dogs, Canis familiaris. Further molecular analysis of the intraspecific as well as the
interspecific variations in the AMELX DNA will be needed to gain clear insight into the taxonomical and phylogenetic positions of the Japanese wolf.

**Key words:** Japanese wolf, Amelogenin, Ancient DNA, Mongolian wolf, Dog


The Japanese wolf, *Canis lupus hodophilax* Temminck, has generally been considered to be an extinct species, and neither the taxonomical nor the phylogenetic position of this species is clearly understood; numerous controversies have been evoked among researchers regarding the problem [1–3]. In Japan, 3 mounted taxidermic pelt specimens believed to represent *C. lupus hodophilax* are stocked at the University of Tokyo, the National Science Museum and Wakayama University, respectively.

Recent advances in the methodology of extraction and ultra-micro analysis of ancient DNA recovered from biological specimens preserved either naturally or artificially have made it possible to analyze at the molecular level the taxonomical identities and the phylogenetic relationships of species, races or breeds that became extinct long ago [4–7].

In our previous report, we described an improved method [8] for the extraction of DNA from preserved pelt specimens of mammals based on the method originally described by Pääbo [6]. Using this improved method [8], we were able to recover genomic DNA from a small piece of skin derived from the mounted female taxidermic pelt specimen stocked at the University of Tokyo, and to determine the sequence of a PCR-amplified and cloned DNA stretch of 351 nucleotides from the coding region of the X-linked amelogenin gene (AMELX).

**Materials and Methods**

**Lupine dermal specimen**

A piece of dermal specimen, approximately 10 mm² in size, was excised from the lower abdominal region with a pair of dry surgical scissors on 26th May 2000, from the mounted female taxidermic pelt specimen which had tentatively been identified as an extinct wolf species, *Canis lupus hodophilax* Temminck, the Japanese wolf, and has been stocked at the Graduate School of Agriculture and Life Sciences, the University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo, Japan since 1881. The collected dermal specimen was wrapped with a piece of aluminium foil and had been stored in a plastic container at room temperature until use.

**Extraction of DNA**

A strip approximately 1 mm × 3 mm in size was taken from the excised lupine dermal specimen and used for each experiment. The modified Pääbo’s method [4–6] for the extraction and PCR amplification of ancient DNA, as described by Asano *et al.* [8] was essentially followed. The specimen strip was carefully shaven, cut into small pieces, added to 1 ml of acetone and gently shaken at room temperature (RT; approx. 25 C) overnight for defatting [8]. Acetone was then removed and the dermal fragments were passed through an ethanol (EtOH) series, soaking consecutively for 30 min in each EtOH solution, using approximately 1 ml of 100%, 70%, 50% EtOH. After the specimens were washed with 1 ml of MilliQ water for 30 min, they were transferred to 500 µl of 10 µM Tris buffer (pH 7.5) containing 0.1M CaCl₂ (TC buffer) and added with collagenase (final conc. 2.5 µg/µl).

After incubation at 37 C for 2 h, the specimens were collected, washed briefly with 50mM EDTA solution containing SDS (0.5%) and 50mM dithiothreitol (DTT) (ESD solution) and then subjected to proteinase K (final conc. 1 µg/µl) digestion at 50 C for 20 h in 410 µl of the ESD solution. If the digestion was incomplete, fresh proteinase K (1 µg/µl) was added and the mixture was incubated for an additional 2 h at 60 C. The digest was then extracted with 580 µl of phenol with gentle shaking for 2 h at RT under light-proof conditions. After centrifugation at 15,000 rpm for 20 min at RT, the aqueous phase was collected. The remaining phenol phase was re-extracted with 100 µl of MilliQ water and the recovered aqueous phase was combined with the aqueous phase collected...
previously. The pooled aqueous phase was extracted with an equal volume of PCI (phenol:chloroform:isoamyl alcohol=25:24:1) mixture and subsequently with an equal volume of CI (chloroform : isoamyl alcohol=24:1) mixture with gentle shaking for 1 h each. After centrifugation as described before, the aqueous phase was collected and added with 1/10 vol. of 3 M sodium acetate (pH 5.2) and 2.5 vol. EtOH. The precipitated DNA was rinsed with 70% EtOH, dried, taken up in the TE (10 mM Tris (pH 7.4), 1 mM EDTA) buffer and stored at 4°C.

**PCR analysis**

The primer pair described by Asano et al. [8] designed from a region of AMEL (both X-linked and Y-linked) exon DNA highly conserved among species was used for the PCR amplification of the lupine X-linked AMELX partial coding sequence. The nucleotide sequence of each primer is as follows: forward primer, 5’-ATGGGTGGATGGCTGCACCA-3’ and reverse primer, 5’-GCTTGGTCTTGTCTGTGTTGCT-3’. Thirty-five amplification cycles (denaturation at 94°C for 1 min, annealing at 67.5°C for 1 min and elongation at 72°C for 1 min) were performed after 5 min of the preparatory denaturation reaction at 94°C, and PCR was terminated by a 7-min elongation reaction at 72°C. A DNA thermal cycler (Model 2400; Perkin Elmer, CT, USA) was used for the purpose. The PCR products were subjected to electrophoresis on 2% agarose gels in TAE (40 mM Tris (pH7.7), 5 mM sodium acetate, 1 mM EDTA) buffer at 100 V for 40 min and stained with ethidium bromide. The extraction of PCR-amplified DNA fragments from the gels was done using a QIAquick Gel Extraction Kit (QIAGEN, CA, USA).

**Cloning and determination of nucleotide sequence of PCR products**

The PCR-amplified AMELX DNA fragments were cloned into pGEM-T vector (Promega, Madison, WI, USA). Single white colonies were picked up, and more than 10 independent clones were used for the nucleotide sequence analysis. Sequencing reactions were performed using DSQ2000L (Shimadzu, Kyoto, Japan) according to the recommended protocols provided by the manufacturer.

**Results and Discussion**

We successfully cloned and determined the sequence of a 351-nucleotide partial genomic DNA stretch coding for amelogenin (AMELX), from a dermal specimen derived from the mounted female taxidermic pelt specimen widely held to represent the controversial Japanese wolf species, C. lupus hodophilax as described in Materials and Methods. The gene coding for amelogenin (AMEL) is sexually dimorphic (X-linked AMELX and Y-linked AMELY) in many animal species and was first described by Nakahori et al. in humans [9]; the dimorphism of AMEL was used for the determination of the sexes of ancient DNA recovered from human mummies [10].

Two putatively allelic lupine AMELX sequences which we tentatively named the J-type (accession no. AB080688, submitted to DDBJ on 1/Mar./2002) and the R-type (accession no. AB080689, submitted to DDBJ on 1/Mar./2002) were identified (Fig. 1A and B). The J-type and R-type differ in 9 nucleotide positions (Fig. 1A), and the deduced amino acid sequence yielded variations in 5 amino acid residues (Fig. 1B). Alignment of the newly-identified lupine AMELX partial sequences to those of the dog (Canis familiaris; Labrador retriever) and the Mongolian wolf (Canis lupus), both of which have a completely identical sequence in this region, as previously described by Asano et al. [8], revealed that both the J-type and the R-type alleles of the AMELX partial sequence of C. lupus hodophilax exhibited considerable variations compared with the corresponding sequence shared by the dog and the Mongolian wolf at the nucleotide (Fig. 2A) as well as the amino acid (Fig. 2B) level.

Although the presently discovered lupine variants of the AMELX partial sequence are of great interest from the point of view of the taxonomical identity of the Japanese wolf, our preliminary results (unpublished) strongly indicate that polymorphisms of the nucleotide as well as the amino acid sequence might exist in this particular region of AMELX, depending upon the different breeds of domestic dogs, Canis familiaris.

Extensive sequence polymorphisms have been detected in the mtDNA D-loop region among domestic dog breeds as well as in wolves [11], and the analysis of nucleotide substitutions in this region provided no solid evidence for a difference in the specificity for the substitution between the
two species [11]. Previously reported molecular genetic data showed that although extant canids are very closely related and probably diverged from a common ancestor about 10 million years ago, domestic dogs exhibit remarkable diversity in mitochondrial and nuclear genes [12]. It has also been suggested that domestic dogs have originated from or interbred with wolves throughout their history at different times and in different places [12].

No definitive conclusion, therefore, could be drawn from the present data regarding the taxonomical authenticity of the tentative identification of the specimen stocked at the University of Tokyo as *C. lupus hodophilax*. Further molecular analysis of the intraspecific as well as the interspecific variations of the *AMELX* DNA will be needed to gain clear insights into the taxonomical or phylogenetic positions of the Japanese wolf. It will also enable us eventually to answer the long-standing questions on the ancestry of dogs and wolves in Japan [13–15].

Our present work will be an important stepping stone toward the goal.
Fig. 2. Alignment of the 2 allelic forms of *AMELX* partial sequences of the Japanese wolf, *C. lupus hodophilax* with the corresponding sequence that is shared [8] by the domestic dog (*Canis familiaris*; Labrador retriever) and the Mongolian wolf (*Canis lupus*). The alignment of *JWOLF/J/AMELX* vs *DOG/MWOLF/AMELX* revealed differences in 22 nucleotide positions and 11 amino acid positions. The *JWOLF/R/AMELX* vs *DOG/MWOLF/AMELX* alignment revealed differences in 26 nucleotide positions and in 12 amino acid positions.
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References