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Reconstructing the colonization history of lost wolf lineages by the analysis of the mitochondrial genome

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ABSTRACT

The grey wolves (*Canis lupus*) originally inhabited major parts of the Northern hemisphere, but many local populations became extinct. Two lineages of wolves in Japan, namely, Japanese or Honshu (*C. l. hodophilax*) and Ezo or Hokkaido (*C. l. hattai*) wolves, rapidly went extinct between 100 and 120 years ago. Here we analyse the complete mitochondrial genome sequences from ancient specimens and reconstruct the colonization history of the two extinct subspecies. We show a unique status of Japanese wolves in wolf phylogeny, suggesting their long time separation from other grey wolf populations. Japanese wolves appeared to have colonized the Japanese archipelago in the Late Pleistocene (ca. 25,000–125,000 years ago). By contrast, Ezo wolves, which are clearly separated from Japanese wolves in phylogeny, are likely to have arrived at Japan relatively recently (<14,000 years ago). Interestingly, their colonization history to Japan tallies well with the dynamics of wolf populations in Europe and America during the last several millennia. Our analyses suggest that at least several thousands of wolves once inhabited in the Japanese archipelago. Our analyses also show that an enigmatic clade of domestic dogs is likely to have originated from rare admixture events between male dogs and female Japanese wolves.

1. Introduction

The grey wolves (*Canis lupus*) originally inhabited major parts of the Northern hemisphere, and are sometimes referred as the most widely distributed wild terrestrial mammal (Aggarwal et al., 2007; Ellegren et al., 1996). Up to 37 subspecies are recognized (Wilson and Reeders, 2005) though their taxonomy has long been the subject of debate. However, eliminations of populations have been reported in many areas of their previous range (Ellegren et al., 1996; Wilson and Reeders, 2005). Wolves in Japan, which became extinct approximately 100 years ago, are one of the most mysterious subspecies among them. Due to the rapidity of the decline, little information is known about their ecology and behaviour in their natural habitat. The number of bone specimens is limited, and less than 10 stuffed specimens are currently available (Ishiguro et al., 2009, 2010). Therefore, the taxonomic status of wolves in Japan has long been controversial (Ishiguro et al., 2009).

Two lineages of wolves are known from Japan (Fig. 1). Japanese or Honshu wolves (*C. l. hodophilax*) once inhabited central and southern parts of Japan. Because of the smaller body size than continental grey wolves and some distinguishing osteological characters, they are sometimes treated as an independent species, *C. hodophilax* (Imaizumi, 1970). Another subspecies, Ezo or Hokkaido wolves (*C. l. hattai*) were only found in the northern island of Hokkaido and neighbouring small islands. As documented in other places (e.g., Berger et al., 2001), wolves in Japan must have played an important role in the ecosystem as large terrestrial predators. Their population size at that time is unknown, but skeletal remains found at some archaeological sites (Ishiguro et al., 2009) indicate that Japanese wolves had a wide distribution since the Jomon Period (10,000 to 250 BC). Wolves are mentioned in classical Japanese literature (Walker, 2005); they were familiar animals to local people, in particular, farmers and were sometimes worshipped by them as a god. Conflict between local people and wolves appeared to be rare until around 300 years ago, when rabies was first described in Japan (Walker, 2005). Japan opened up to foreign trade in 1854 after hundreds of years of isolation and adopted many Western customs, including livestock farming. Hunting and poisoning of wolves over the next several decades, along with severe weather in some years, led to a drastic decline in the number of wolves; Ezo wolves went extinct between 1880 and 1890, followed by Japanese wolves in the 1900s (Ishiguro et al., 2009, 2010; Walker, 2005).

Advances in molecular genetics have enabled the study of DNA from ancient samples, such as museum specimens and bones from

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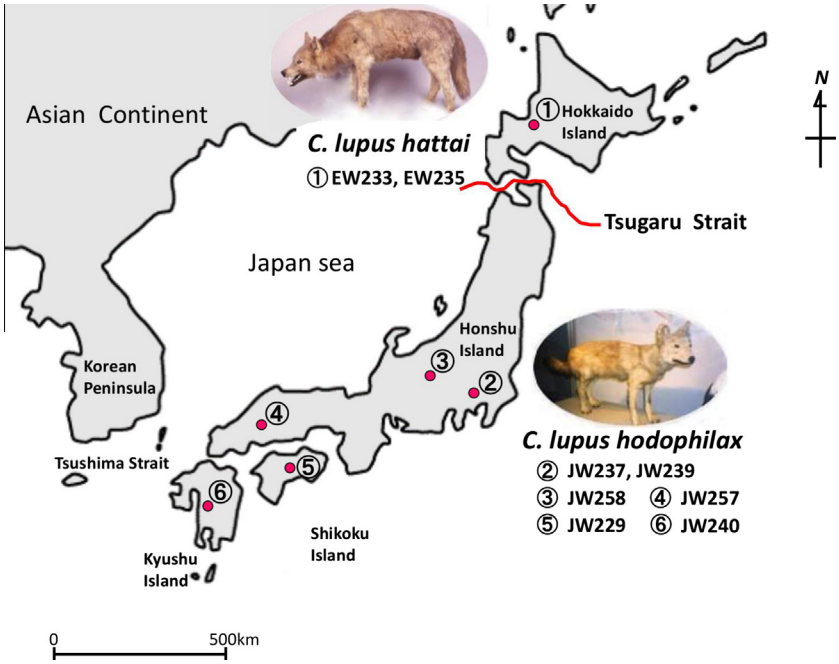

Fig. 1. Sampling sites and distribution of Japanese wolves (*Canis lupus hodophilax*) and Ezo wolves (*C. l. hattai*).

archaeological sites (Pääbo et al., 2004). In the present study, we sequenced the complete mitochondrial genome (16,562–16,731 bp) of six Japanese wolves and two Ezo wolves from ancient specimens and compared them with published complete mitochondrial DNA (mtDNA) sequences of wolves. Extracting and sequencing the ancient mitochondiral genome are easier than the nuclear genome mainly because of higher copy numbers in each cell. The total number of ancient mitochondrial genome sequences has increased rapidly over the last decade (Ho and Gilbert, 2010). The use of mtDNA has several advantages including maternal inheritance, absence of recombination, and high substitution rate. Complete mtDNA sequences have a potential for more reliable phylogenetic reconstruction than short fragments of mtDNA. In particular, analyses of complete mtDNA sequences including ancient DNA samples have been shown to be useful for phylogenetic reconstruction within a species or between closely related species (e.g., Gilbert et al., 2008; Lindqvist et al., 2010; Thalmann et al., 2013). The objectives of the present study are to clarify the phylogenetic status of Japanese wolves and Ezo wolves and to reconstruct the history of their colonization of the Japanese archipelago.

2. Materials and methods

2.1. Complete mtDNA sequence generation

DNA was extracted from bones stored in several museums or personal collections throughout Japan (Table 1) according to the methods described by Okumura et al. (1999) and Ishiguro et al. (2009). The criteria for the authenticity of ancient DNA (Hofreiter et al., 2001) were applied, except that we use only a single extract from each specimen to keep valuable specimens as intact as possible, and that we did not send samples to a second laboratory to reproduce the results. The outer layers of bone were removed by scraping with a sterile razor blade. Bone powder (0.1 to 0.3 g) was obtained from the specimens by using an electric drill, suspended in 10 ml of 0.5 M ethylenediamine tetraacetic acid (EDTA) at pH 7.0, and rotated for decalcification. After centrifugation, the pellet of bone powder was collected and repeatedly decalcified by washes with 10 ml of 0.5 M EDTA until the supernatant was clear. The bone powder sample was then treated for 24 h with 5 ml of 0.5 M EDTA with proteinase K (300 μg/ml) and N-lauryl sarcosine (0.5%). The samples were centrifuged at 3000 rpm for 10 min, and the supernatant contain in the ancient DNA was extracted twice with phenol, once with chloroform:phenol (1:1), and once with chloroform to remove protein. The supernatant was concentrated by using a Centricon 30 spin column (Amicon, Beverly, MA, USA) and was washed with distilled water. The extracted DNA samples (about 1–3 μl) were used directly for PCR. Precautions were taken to prevent contamination with DNA from modern dogs, and all ancient DNA was handled in a biohazard hood with a ventilation system independent from the PCR products and samples. A centrifuge rotor was used for only the ancient dog samples, and all experiments with modern dog samples were made in a different room.

Complete mitochondrial genome sequences were determined using simplex PCR and Sanger sequencing method. Based on published mitochondrial genome sequences of dogs (Kim et al., 1998), primers were designed to generate overlapping fragments. Primers used in the present study are illustrated in Supplementary Fig. A1 and listed in Table A1. PCR was carried out in a 50 μl reaction volume containing about 1 μl ancient DNA extracts using Ampli-Taq Gold polymerase (Applied Biosystems, Foster City, CA, USA). The thermal conditions were as follows: initial denaturation and Ampli-Taq Gold activation at 95 °C for 5 min, annealing at 55 °C for 30 min and extension at 72 °C for 30 min, followed by 45 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. Amplification products were checked by gel electrophoresis on a 1.5% agarose gel and primers were removed using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Purified DNA fragments were sequenced directly with the corresponding primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). At every occasion of PCR of ancient DNA samples, reagents without bone powder were used as negative controls to check for contamination. Sequences were determined based on multiple PCR products using different pairs of primers when the sequencing outcome was