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Japanese Wolves are Genetically Divided into Two Groups Based on an 8-Nucleotide Insertion/Deletion Within the mtDNA Control Region

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The mitochondrial DNA (mtDNA) control region (198- to 598-bp) of four ancient *Canis* specimens (two *Canis* mandibles, a cranium, and a first phalanx) was examined, and each specimen was genetically identified as Japanese wolf. Two unique nucleotide substitutions, the 78-C insertion and the 482-G deletion, both of which are specific for Japanese wolf, were observed in each sample. Based on the mtDNA sequences analyzed, these four specimens and 10 additional Japanese wolf samples could be classified into two groups— Group A (10 samples) and Group B (4 samples)—which contain or lack an 8-bp insertion/deletion (indel), respectively. Interestingly, three dogs (Akita-b, Kishu 25, and S-husky 102) that each contained Japanese wolf-specific features were also classified into Group A or B based on the 8-bp indel. To determine the origin or ancestor of the Japanese wolf, mtDNA control regions of ancient continental *Canis* specimens were examined; 84 specimens were from Russia, and 29 were from China. However, none of these 113 specimens contained Japanese wolf-specific sequences. Moreover, none of 426 Japanese modern hunting dogs examined contained these Japanese wolf-specific mtDNA sequences. The mtDNA control region sequences of Groups A and B appeared to be unique to grey wolf and dog populations.

Key words: *Canis lupus hodophilax*, *Canis lupus familiaris*, genetic variation, Japanese wolf, mitochondrial DNA, phylogeography

INTRODUCTION

Two extinct subspecies of wolf (Japanese wolf and Ezo wolf) once inhabited Japan. The Japanese wolf (*Canis lupus hodophilax*, Temminck, 1893) inhabited the Kyushu, Shikoku

and Honshu Islands, and the last Japanese wolf was captured at Higashi-Yoshino village in Nara Prefecture in 1905. The Ezo wolf (*Canis lupus hattai*, Kishida, 1931) inhabited Hokkaido Island; the extermination of Ezo wolf occurred during the Meiji period, but the exact timing is not clearly documented. Four stuffed Japanese wolf specimens are distributed among and maintained by three Japanese academic institutes (National Museum of Nature and Science, The University of Tokyo and Wakayama Prefectural Museum of Natural History) and one museum (National Museum of

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Natural History, Leiden) in The Netherlands (Imaizumi, 1970a, 1970b; Miyamoto, 1991; Naora, 1965; Obara, 2002). Two stuffed Ezo wolf specimens are maintained at the Botanic Garden (Hokkaido University Natural History Museum) of the Field Science Center for the Northern Biosphere at Hokkaido University (Kishida, 1931). Because the number of stuffed specimens and bone specimens representing these two extinct wolf populations is small, genetic analysis has been limited to the investigation of the taxonomic and phylogenetic relationships among Japanese and Ezo wolves.

Archaeologists and biologists have investigated some Japanese wolf bone specimens (Imaizumi, 1970a, 1970b; Naora, 1965). Osteological comparisons with Asian and European wolves (e.g.: *Canis lupus chanco* or *Canis lupus lupus*) and dogs (*Canis lupus familiaris*) indicate that Japanese wolves had 1) skull lengths of 205.0 to 226.0 mm, 2) an auditory meatus that developed externally to the post-glenoidal foramen, 3) a relatively well developed anterior boarder of the pterygoid fossa, and 4) an M1 in mandible that is relatively large (Abe, 2001; Endo et al., 1997; Endo et al., 1999; Miyamoto and Maki, 1983). These osteological analyses have been very useful for definitively identifying of Japanese wolf specimens among archaeological specimens (Shigehara and Hongo, 2000).

Recently, Ishiguro et al. (2009) reported the results of about 600-bp of sequences from the mitochondrial DNA (mtDNA) D-loop control region for eight Japanese wolf specimens; these sequences are closely related to each other, and cluster into a single lineage that is separated from Eurasian wolf lineages (Ishiguro et al., 2009). These mtDNA control region sequences from Japanese wolf specimens differ from those of Ezo wolf, and they are unique among corresponding sequences from wolf populations throughout the world (Ishiguro et al., 2010). Among all mtDNA sequences recorded from modern dogs, mtDNA sequences related to Japanese wolf sequences have been found in only three dogs Akita-b, Kishu 25, and Siberian Husky (S-Husky 102). This lineage of mtDNA sequences in three dogs is classified as group F among the canine mtDNA groups (Savolainen et al., 2002; Matsumura et al., 2014). Nevertheless, the origin or historical lineage of Japanese wolf among wolf populations and the distribution of Japanese wolf-specific mtDNA in ancient Eurasian wolves and modern dogs remain controversial.

Here, we examined ancient *Canis* bones from Japan, Russia, and China; we then amplified and sequenced mtDNA from bone powder to determine the relationships between these *Canis* specimens and those of Japanese wolf. Additionally, to determine whether some Japanese dogs harbor Japanese wolf-specific mtDNA, we examined mtDNA sequences from modern hunting dogs in several prefectures in Japan. Two types of mtDNA sequences lineages, those with or without an eight-nucleotide sequence, were identified in Japanese wolf populations. However, no *Canis* samples sharing Japanese wolf-specific mtDNA sequences were found in ancient samples from Russia or China or from modern Japanese hunting dogs.

MATERIALS AND METHODS

Ancient *Canis* specimens examined

Two large *Canis* mandible samples (JW262 and JW269) and a

Canis cranium (JW271) that were previously determined to be Japanese wolf specimens were used in this study (Table 1). A large-sized first phalanx (JW274) isolated from the Tengu-iwakage site in Nagano prefecture was also examined (Fig. 1, Table 1). Although the location or number of first phalanx is unknown, it is larger than homologous specimens from middle-sized Japanese native dogs or from one subspecies of continental wolf, *C. lupus chanco* (Fig. 1).

We used 84 *Canis* specimens (23 bones from Vladivostok and 61 bones from Ural) isolated from Russia for this study (Table 2). We also used the 23 *Canis* specimens stocked in Laboratory of Human Paleoecology, Russian Academy of Science, Far Eastern Division, Institute of History, Archaeology and Ethnology in Vladivostok and the 61 *Canis* specimens stocked in Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences in Ekaterinburg. Morphological distinction between ancient bones from wolf and those from dog were definitive, but most *Canis* specimens could not be definitively identified as wolf versus dog. We also used 29 ancient *Canis* specimens isolated from four archaeological sites (Xicha site, Haminmangha, Yajialiang site and Houtaomuga) maintained by Jilin University in China (Table 2).

Modern hunting dogs examined

Blood samples were obtained from 426 hunting dogs representing 18 prefectures in Japan for this study. The dog breeds and locations of the hunting dogs are listed in Table 3.

Extraction, PCR amplification, and direct sequencing of mtDNA from Japanese wolf samples

DNA was extracted from four bones specimens from Japanese wolf as described by Ishiguro et al. (2009). An electric drill was used to obtain bone powder (0.1 to 0.3 g) from each specimen; the powder was then suspended in 10 ml of 0.5 M ethylenediamine tetraacetic acid (EDTA) pH 7.0 and decalcified on a rotator. A pellet of bone powder was generated from each sample and subjected to repeated decalcification in 10 ml of 0.5 M EDTA until each sample produced a clear supernatant. The bone powder sample was treated for 48 hr with 5 ml of 0.5 M EDTA that contained proteinase K (300 µg/ml) and N-lauryl sarcosine (0.5%). The samples were then centrifuged at 3,000 rpm for 15 min, and the supernatant containing DNA was extracted with phenol and chloroform. Each sample was concentrated with an Amicon Ultra Centrifugal Filter 30K (Merck Millipore Ltd, Tullagreen, Germany) and washed with distilled water. These purified samples (about 1.5 µl each) were used directly for PCR. The precautions described by Okumura et al. (1996) were used to prevent contamination by modern DNA.

To determine the sequence of the 600-bp region from the mtDNA control region of these four Japanese wolf specimens, we independently amplified three mtDNA control regions (part A: 360-

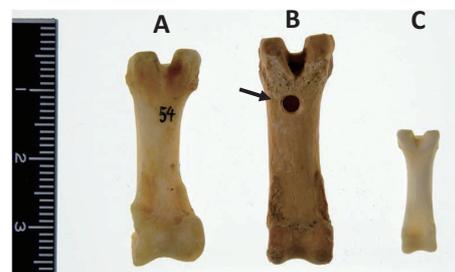


Fig. 1. Size comparison of first phalanges: Korean wolf (*C. lupus chanco*, male) (A), Japanese wolf JW274 (B) and a mid-sized Japanese dog (female) (C). The number and location of left or right first phalanx of JW274 were not morphologically estimated. The hole in the sample JW274 resulted from the drilling use to collect the bone powder.

Table 3. Information about the Japanese hunting dogs examined for this mtDNA analysis.

Year	No. of Samples	Sampling prefecture (No. of Samples)	Dog breed (No. of Samples)
2009	13	Kagawa (7), Kouchi (6)	English Setter (2), Pointer (1), Beagle (1), Mix (9)
2010	119	Shizuoka (32), Nagano (21), Mie (39), Gifu (20), Niigata (7)	Beagle (15), Kishu (9), Setter (5), Kai (4), Pointer (3), Shiba (2), Hokkaido (2), English Setter (1), G-S-Pointer (1), Walker Hound (1), Plott Hound (1), Plott (1), Mix (74)
2011	146	Aichi (15), Kagoshima (99), Nagasaki (19), Niigata (13)	Beagle (35), Hound (14), Setter (10), Brittany Spaniel (8), Plott Hound (7), American Beagle (4), Kai (4), Pointer (3), Shikoku (1), Mix (60)
2012	112	Miyagi (8), Yamagata (20), Iwate (16), Aomori (26), Miyazaki (1), Akita (11), Unknown (30)	Setter (42), Beagle (14), Pointer (13), B.Spaniel (8), Labrador Retriever (8), F.B.Spaniel (4), Kishu (2), Hokkaido (2), Shikoku (2), Cocker Spaniel (1), French Britany Spaniel (1), Shiba (1), Mix (14)
2013	36	Hokkaido (Hakodate, Obihiro: 36)	Hokkaido (10), Kishu (7), Plott Hournd (4), Kai (3), Labadol Retrivers (3), Mix (9)
Total	426		

with the appropriate primers; QIAquick PCR Purification kit (Qiagen) was used to purify the PCR products, which were then sequenced and assembled to determine the sequence of the entire 600-bp mtDNA control region of the respective ancient continental sample.

Isolation and sequencing of DNA from modern hunting dogs

A DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, U S A) was used to extract DNA from 426 blood samples representing hunting dogs. To identify Japanese wolf-specific mtDNA sequences, we used primers mit3 and mit52 to amplify part A (360-bp) of the mtDNA D-loop region. PCR products representing part A were purified with QIAquick PCR Purification Kits (Qiagen) and used for DNA sequencing. After these part A sequences were compared to the corresponding Japanese wolf sequences, parts B and C were amplified with the appropriate primers; the PCR products were purified- and sequenced; the sequences then were assembled to determine the sequence of the 600-bp mtDNA region in each of the 426 dogs.

Alignment of DNA sequences and phylogenetic analysis

Genetyx-MAC Version 10 software (Software, Development, Tokyo, Japan) was used to align approximately 600-bp of sequence from each of the four Japanese wolf specimens with haplotypes representing 10 Japanese wolves and three dogs (Ishiguro et al., 2009). We used the split decomposition method (Dopazo et al., 1993) to perform a parsimony network analysis with the haplotypes from Japanese wolves and related dogs.

RESULTS

mtDNA analysis of Japanese wolves

Previously, archaeologists determined that two mandibles (JW262 and JW269) and one cranium (JW271) were ancient Japanese wolf specimens because they had unique morphological characters of Japanese wolf. The osteometrical size of a fourth sample, JW274 (first phalanx), was larger than that of *C. lupus chanco* or mid-sized Japanese dog (Fig. 1); therefore, we conducted an mtDNA analysis of these four Japanese wolf specimens. We isolated mtDNA from each of the four Japanese wolf specimens, and mtDNA D-loop sequences (590- or 598-bp) were amplified from three of the specimens; however, we could amplify a fragment of only 198-bp from the specimen JW262. The 598-bp of sequence from JW261 were identical to that of JW239, while the 590-bp sequences from JW263 and JW271 were

identical to that of JW237. The 590–598-bp sequences from 8 Japanese wolves (JW229, JW230, JW240, JW255, JW257, JW258, JW259 and JW237) reported in a previous study (Ishiguro et al., 2009), the 598-bp from JW261 (Komiya et al., 2011) and the 590-bp sequences from JW263 (Ishiguro, 2015) are listed in Table 1. The part A sequences from JW262 were not successfully amplified with primers mit3 and mit52, but we amplified 198-bp from part A with primers mit63 and mit52. The 198-bp mtDNA sequence within part A from JW262 was completely to that of JW239. The JW262 specimen was isolated from the Tengu-iwakage site in Nagano prefecture in Jomon period, and amplification of mtDNA generally depends on the degree of preservation of ancient specimen JW262.

Table 1 shows the four Japanese wolf haplotypes aligned with haplotypes from a representative Shiba1 dog, 10 other Japanese wolf specimens, and three dogs that are known to be related to Japanese wolf (Ishiguro et al., 2009; Tsuda et al., 1997). Based on these alignments, the four mtDNA sequences determined here could be classified into two groups Group A (JW262 and JW269) or Group B (JW271 and JW274) based on the presence or absence, respectively, of a 8-nucleotide sequence (TCCCCTCC) that represents position 68 to 75 positions in Group B. This 8-nucleotide sequence was also absent from the Japanese wolf specimen JW237 and a modern dog, Kishu 25. Each of these 14 Japanese wolf sequences, including the four Japanese wolf sequences determine here, (Table 1) exhibited a pair of features, the insertion of a C at position 78 (78-C insertion) and the deletion of a G at position 482 (482-G deletion) in mtDNA control region, that is specific for Japanese wolf specimens (Table 1). Interestingly, the 78-C insertion was also observed in three dog haplotypes (Akita-b, S-Husky 102 and Kishu 25) related with Japanese wolf, but the 482-G deletion was not detected.

To examine the genetic relationships between each Japanese wolf group (A and B) and dogs, a parsimony network was constructed using the mtDNA haplotypes listed in Table 1. The mtDNA 598-bp sequence from the Group A specimens (JW239 and JW261) was identical to that of Akita-b, and the 590-bp sequence from the Group B specimens (JW237, JW263 and JW271) was identical to that of

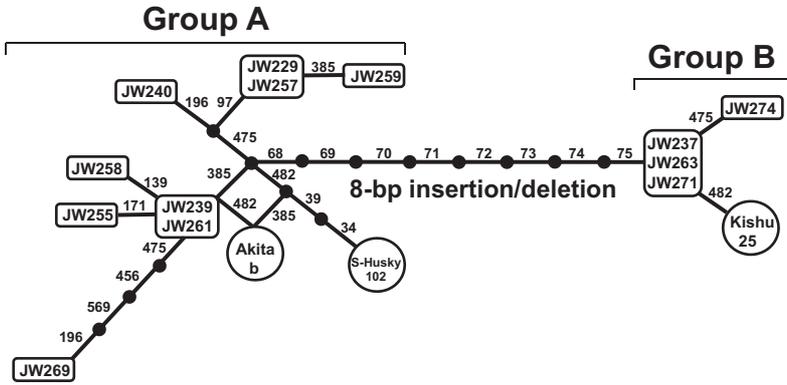


Fig. 2. Parsimonious network constructed with 16 mtDNA haplotypes representing 13 Japanese wolves and three dogs. Haplotype abbreviations, nucleotide positions, and substitutions are given in Table 1. Nine rectangles and three circles indicate mtDNA haplotypes from Japanese wolves and dogs, respectively.

Kishu 25 (Fig. 2). The Group A haplotype was evident in samples representing nine separate prefectures distributed throughout the Honshu, Shikoku, and Kyushu Islands; the Group B haplotype was evident in samples from four prefectures in the middle and eastern areas of the Honshu Islands (Table 1).

mtDNA analysis in ancient specimens from foreign countries

Features of the 113 continental *Canis* specimens (84 from two Institutes in Russia and 29 from Jilin University in China) are listed in Table 2. To determine whether the Japanese wolf was related to or originated from any ancient wolf population represented by these 113 specimens, we amplified 360-bp of mtDNA control sequences in part A from each of the 113 samples; we then sequenced the amplification products and compared these sequences with the 14 Japanese wolf haplotypes. Although the 360-bp sequences were all amplified and sequenced from the 113 samples, none of them showed any of the 17 substitutions (substitutions at positions 33, 66, 68, 69, 70, 71, 72, 73, 74, 75, 78, 97, 139, 155, 171, 172, and 196) that are characteristic of Japanese wolf specimens.

mtDNA analysis in modern hunting dogs in Japan

Japanese wolf-specific mtDNA have been found in three modern dogs previously; therefore, dogs that have Japanese wolf-specific mtDNA substitutions may be currently living in modern Japan. Therefore, we amplified a 360-bp region of mtDNA (part A) from 426 hunting dogs, which represented 18 prefectures. We then sequenced these amplifications products and compared the sequences with the Japanese wolf haplotypes. However, none of the modern dogs had any Japanese wolf-specific substitutions.

DISCUSSION

In this study, the mtDNA control regions of four *Canis* specimens were analyzed, and each specimen was genetically identified as Japanese wolf. Each of these mtDNA sequences (598-bp or 590-bp) was closely related to each other, as well as to 10 previously reported mtDNA sequences from Japanese wolves specimens (Ishiguro et al., 2009);

however, a smaller 198-bp fragment was amplified from JW262, probably because this specimen was less well preserved than the others. Notably, two Japanese wolf-specific nucleotide substitutions, a unique 78-C insertion and a 482-G deletion, were evident in each of the four Japanese wolf specimens. Therefore, these two nucleotide substitutions are useful markers for distinguishing Japanese wolf specimens from specimens representing other subspecies of grey wolf or dogs (Ishiguro et al., 2009). Interestingly the 8-bp indels were found among the 14 Japanese wolf mtDNA haplotypes, and we used it to divide these samples into two groups—Group A (10 samples) or B (4 samples)—based on the 8-bp indel. The four mtDNA sequences examined in this study seemed to demonstrate that the 8-bp indel was a common polymorphism among

Japanese wolves and was specific to Japanese wolves (Table 1) because this 8-bp indel was not found in any of the continental grey wolf or dog samples examined. The Group B haplotype was detected in Japanese wolf specimens from middle and eastern areas of Honshu Island; this finding indicated that Japanese wolves with the Group B haplotypes were widely distributed in eastern areas of ancient Japan.

In addition to the cranium and mandibles morphologically identified as Japanese wolf specimens, a large first phalanx was also genetically identified as a Japanese wolf bone in this study, which indicated that any isometrically large-sized bone specimens should be examined genetically to determine whether they are Japanese wolf specimens. On the other hand, about 600-bp sequences constructed from A, B and C regions, except for a truncated 198-bp fragment, were not detected from a mandible JW262 which was isolated from Tengu-iwakage site in Jomon period. Amplification efficiency of mtDNA from ancient bones is generally dependent of the quality or preservation conditions of ancient bones. The 198-bp sequences in part A region are easily amplified from ancient and less-conserved specimens to distinguish Japanese wolf from continental wolves and dogs. The 78-C insertion or an 8-bp indel uniquely found in Japanese wolf sequences were not detected in 198-bp in part A region amplified from ancient dog samples isolated from archaeological sites (Okumura et al., 1999).

To identify potential ancestors of the Japanese wolf, we analyzed 113 ancient *Canis* specimens from continental Russia and China to determine whether they contained any Japanese wolf-specific mtDNA sequences. None of these samples had any mtDNA sequences that indicated that the Japanese wolf was closely related. Generally, a nucleotide substitution (the 78-C insertion) or an 8-bp indel within the part A region are used to distinguish Japanese wolf or Japanese wolf-related haplotypes from continental grey wolf haplotypes. The ancient *Canis* specimens from Russia examined in this study were excavated from an archaeological site representing 3000 BC; moreover, the *Canis* specimens in China have been isolated from a Houtaomuga archaeological site representing 4900 BC. The number of ancient *Canis* specimens from Russia and China examined in this study was relatively small; nevertheless, these results

may indicate that the ancestor or subspecies that gave rise to Japanese wolves may have been eradicated from Eurasian wolves by about 5000 BC. Based on recent comprehensive analysis of mitochondrial genomes from ancient wolves in Japan (Matsumura et al., 2014), the Japanese wolf subspecies appears to have separated from other grey wolf populations a very long time ago and to have colonized the Japanese archipelago in the Late Pleistocene (ca. 25,000–125,000 years ago). If this putative divergence time for Japanese wolf populations is correct, it may be difficult to recover an ancient *Canis* specimen in Eurasia that represent a population of wolves that were closely related or ancestral to the Japanese wolf. Furthermore, the finding that an 8-bp indel was only observed in Japanese wolves and three dogs in Japan may suggest that this deletion occurred within Japanese wolf populations after Japanese wolves have separated from continental wolves.

Only three dogs (Akita-B, Kishu 25, S-husky 102) have mtDNA sequences similar to Japanese wolf sequences, and these are classified into the distinctive clade F (Savolainen et al., 2002). Their mtDNA sequences without 8-bp indel region were closely related to each other and grouped in a single lineage (Ishiguro et al., 2009; Matsumura et al., 2014). Although the origin of clade F is not obscure, this result indicated that these three dogs are maternally descended from an ancestor of Japanese wolves. However, none of the 426 Japanese hunting dogs examined in this study included sequences representing Japanese wolf-specific mtDNA. It is likely that these three dogs originated from rare crossbreed events between dogs and Japanese wolves. Interesting questions about the timing and location of these crossbreed events remain, and the Japanese wolf continues to be a mysterious animal.

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