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Author(s): Naotaka Ishiguro, Yasuo Inoshima, Tokuma Yanai, Motoki Sasaki, Akira Matsui, Hiroki Kikuchi, Masashi Maruyama, Hitomi Hongo, Yuri E. Vostretsov, Viatcheslav Gasilin, Pavel A. Kosintsev, Chen Quanjia, and Wang Chunxue

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

Naotaka Ishiguro[1]*, Yasuo Inoshima[1], Tokuma Yanai[2], Motoki Sasaki[3], Akira Matsui[4], Hiroki Kikuchi[5], Masashi Maruyama[6], Hitomi Hongo[7], Yuri E. Vostretsov[8], Viatcheslav Gasilin[9], Pavel A. Kosintsev[9], Chen Quanjia[10], and Wang Chunxue[10]

[1]*Laboratory of Food and Environmental Hygiene and* [2]*Laboratory of Veterinary Pathology, Veterinary Medicine, Faculty of Applied and Biological Sciences, Gifu University, Gifu 501-1193, Japan*
[3]*Laboratory of Veterinary Anatomy, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan*
[4]*Nara National Culture Properties Research Institute, Nara 630-8577, Japan*
[5]*Institute for Research in Humanities, Kyoto University, Yoshidahonmachi, Sakyo-ku, Kyoto 606-8501, Japan*
[6]*Department of Maritime Civilizations, School of Marine and Technology, Tokai University, Shizuoka 424-8610, Japan*
[7]*Department of Advanced Sciences, Graduate University for Advanced Studies, Hayama 240-0193, Kanagawa, Japan*
[8]*Laboratory of Human Paleoecology, Russian Academy of Science, Far Eastern Division, Institute of History, Archaeology and Ethnology, 89, Pushkinskaya, St. Vladivostok, 690600, Russia*
[9]*Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences, Yekaterinburg, 620144, Russia*
[10]*Research Center of Chinese Frontier Archaeology of Jilin University, Changchun, 130012, China*

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

* Corresponding author. Tel. : +81-58-293-2864;
Fax : +81-58-293-2864;
E-mail: ishiguna@gifu-u.ac.jp
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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 postglenoidal 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 mDNA 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-

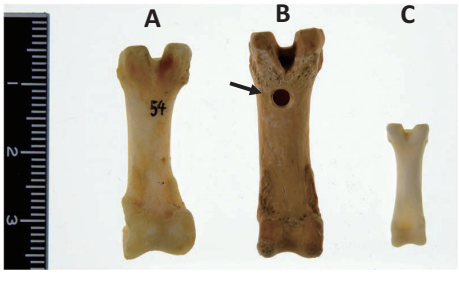

Fig. 1. Size comparison of first phalanges: Korean wolf (*C. lupus chanco*, male) **(A)**, Japanese wolf JW274 **(B)** and a mid-sized Japanese dog (female) **(C)**. The number and location of left or right first phalanx of JW274 were not morphologically estimated. The hole in the sample JW274 resulted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

Table 1. Characteristics of Japanese wolf and dog specimens and variation of the mtDNA control region.

Dog or Wolf	Group	Sample No.	Isolation site (Prefecture)	Sample	Period	Accession No.	Amplification mtDNA(bp)	Nucleotide positions[a)] 33	34	39	66	68	69	70	71	72	73	74	75	78	97	139	155	171	172	196	344	385	456	475	482	569	Reference
Dog		Shiba 1	Hiroshima	Blood	Modern	D83627	980	A	C	C	T	T	C	C	C	C	T	C	C	–	A	C	T	A	T	G	T	G	C	A	G	C	Okumura et al., 1996
Dog		Akita-b	Japan	Blood	Modern	AB007381	673	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	A	C	T	T	·	·	T	Tsuda et al., 1997
		S-Husky102	Osaka	Blood	Modern	D83637	972	G	A	T	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	A	C	·	T	·	·	T	Okumura et al., 1996
		Kishu25	Osaka	Blood	Modern	D83611	980	G	·	·	C	–	–	–	–	–	–	–	–	C	·	·	C	G	C	A	C	·	T	·	·	T	Okumura et al., 1996
Japanese wolf	A	JW229	Kouchi	Mandible (R)	Edo	AB480736	598	G	·	·	C	·	·	·	·	·	·	·	·	C	G	·	C	G	C	A	C	·	T	G	–	T	Abe, 2001
		JW239	Kanagawa	Mandible (R)	Edo-Meiji	AB480737	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	A	C	T	T	·	–	T	Ishiguro et al., 2009
		JW240	Kumamoto	Mandible (R)	Muromachi-Edo	AB480739	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	·	C	·	T	G	–	T	Kitamura et al., 1999
		JW255	Yamanashi	Mandible (R)	Edo-Meiji	AB480740	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	·	C	A	C	T	T	·	–	T	Endo et al., 2004
		JW257	Hiroshima	Mandible (R)	Edo-Meiji	AB480741	598	G	·	·	C	·	·	·	·	·	·	·	·	C	G	·	C	G	C	A	C	·	T	G	–	T	Yoneda, 1997
		JW258	Nagano	Mandible (R)	Edo-Meiji	AB480742	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	T	C	G	C	A	C	T	T	·	–	T	Ishiguro et al., 2009
		JW259	Ehime	Mandible (L)	Edo-Meiji	AB500700	583	G	·	·	C	·	·	·	·	·	·	·	·	C	G	·	C	G	C	A	C	T	T	G	–	T	Obara, 1990
		JW261	Gunnma	Mandible (L)	Edo-Meiji	LC064091	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	A	C	T	T	·	–	T	Komiya et al., 2011
		JW262	Fukushima	Mandible (R)	Jomon	LC064092	198	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	A	/	/	/	/	/	/	This study
		JW269	Nagano	Mandible (L)	Edo-Meiji	LC064093	598	G	·	·	C	·	·	·	·	·	·	·	·	C	·	·	C	G	C	·	C	T	·	G	–	·	This study
	B	JW237	Kanagawa	Mandible (L)	Edo-Meiji	AB480738	590	G	·	·	C	–	–	–	–	–	–	–	–	C	·	·	C	G	C	A	C	·	T	·	–	T	Ishiguro et al., 2009
		JW263	Kyoto	Mandible (R)	Muromachi-Edo	LC064094	590	G	·	·	C	–	–	–	–	–	–	–	–	C	·	·	C	G	C	A	C	·	T	·	–	T	Ishiguro, 2015
		JW271	Iwate	Cranium	Edo-Meiji	LC064095	590	G	·	·	C	–	–	–	–	–	–	–	–	C	·	·	C	G	C	A	C	·	T	·	–	T	This study
		JW274	Nagano	First phalanx	Yayoi-Kofun	LC064096	590	G	·	·	C	–	–	–	–	–	–	–	–	C	·	·	C	G	C	A	C	·	T	G	–	T	This study

a) Nucleotide position 1 corresponds to base position 33 in the whole dog mtDNA control region described by Okumura et al. (1996). Dots indicate nucleotide identity with the Shiba 1 haplotype. A dash represents a deleted nucleotide. A slash indicates that corresponding data point was not determined. Accession No. LC064091 to LC064096 were obtained from DDBJ/EMBL/GenBank database in this study.

Table 2. Information of the ancient continental *Canis* specimens examined for this mtDNA analysis.

Country	District or site	Year	Wolf/Dog	Sample	Sample No.	Periods
Russia	Vladivostok	2012	Wolf/Dog	Mandible	7	1000–3000 B.C.
			Wolf/Dog	Ulna	7	1000–3000 B.C.
			Wolf/Dog	Radius	3	1000–3000 B.C.
			Wolf/Dog	Humerus	2	1000–3000 B.C.
			Wolf/Dog	Skull	2	1000–3000 B.C.
			Wolf/Dog	Axis	1	1000–3000 B.C.
			Wolf/Dog	Talus	1	1000–3000 B.C.
				Sub-Total	23	
	Ural (Ekaterinburg)	2013	Wolf	Mandible	37	2600 B.C.–1800 A.C.
			Dog	Mandible	24	1800 B.C.–1800 A.C.
				Sub-Total	61	
China	Xicha site	2014	Wolf/Dog	Mandible	4	3000 B.C.
			Wolf/Dog	Coxa	2	3000 B.C.
			Wolf/Dog	Cranium	1	3000 B.C.
			Wolf/Dog	Radius	1	3000 B.C.
			Wolf/Dog	Ulna	1	3000 B.C.
			Wolf/Dog	Tibia	1	3000 B.C.
	Haminmangha	2014	Wolf/Dog	Mandible	1	5500 B.C.
			Wolf/Dog	Cranium	1	5500 B.C.
	Yanjialiang site	2014	Wolf/Dog	Mandible	4	800 B.C.
			Wolf/Dog	Femur	2	800 B.C.
			Wolf/Dog	Humerus	1	800 B.C.
			Wolf/Dog	Radius	1	800 B.C.
			Wolf/Dog	Tibia	1	800 B.C.
			Wolf/Dog	Cranium	1	800 B.C.
	Houtaomuga	2014	Wolf/Dog	Mandible	3	4900 B.C.
			Wof/Dog	Ulna	2	4900 B.C.
			Wolf/Dog	Humerus	1	4900 B.C.
			Wolf/Dog	Femur	1	4900 B.C.
				Sub-Total	29	
				Total	113	

bp amplified with the mit3 and mit52 primers; part B: 316-bp amplified with the mit123 and mit138 primers; part C: 277-bp amplified with the mit136 and mit123 primers) (Ishiguro et al., 2009) using the following thermal conditions: initial denaturation and Ampli-Taq Gold activation at 95°C for 5 min, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by 50 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s. If the part A region could not be amplified with the mit3 and mit52 primers, a short mtDNA control region (198-bp) was amplified with the mit63 and mit52 primers (Ishiguro et al., 2009). The product was checked via electrophoresis through a 1.5% agarose gel, and a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) was used to remove the primers.

The corresponding primers, a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and a DNA sequencer were used to directly sequence the purified PCR products. The mtDNA 600-bp sequences were determined combining sequences from each set of three DNA fragments—parts A, B, and C (Ishiguro et al., 2009).

DNA isolation and sequencing for the ancient *Canis* specimens collected from China and Russia

To determine whether any *Canis* species represented by ancient samples from foreign countries (Russia and China) were related to Japanese wolf, we obtained bone powder samples from 113 ancient, continental *Canis* specimens (84 from Russia and 29 from China) and with the procedure used for the Japanese wolf samples, we extracted DNA from these continental samples. A segment of the A region (360-bp) of the mtDNA control region was amplified first with primers mit3 and mit52 and sequenced with these same primers. If mtDNA sequences similar to those of Japanese wolf were found in part A region, parts B and C were amplified independently

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), Labradol 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 Retriver (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

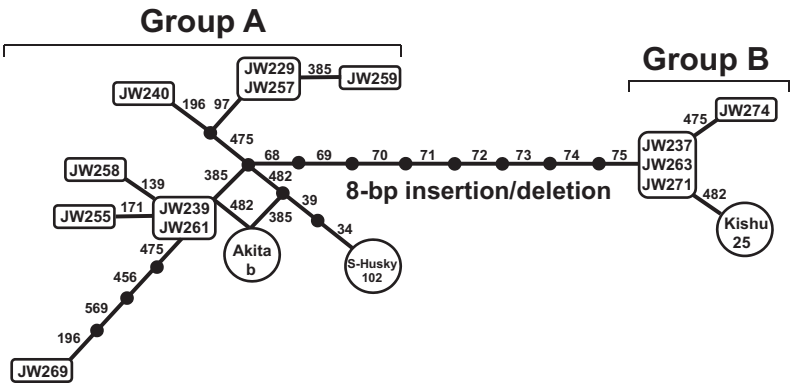

Fig. 2. Parsimonious network constructed with 16 mtDNA haplotypes representing 13 Japanese wolves and three dogs. Haplotype abbreviations, nucleotide positions, and substitutions are given in Table 1. Nine rectangles and three circles indicate mtDNA haplotypes from Japanese wolves and dogs, respectively.

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 to 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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