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Mitochondrial DNA Analysis of the Japanese Wolf (*Canis lupus hodophilax* Temminck, 1839) and Comparison with Representative Wolf and Domestic Dog Haplotypes

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Mitochondrial DNA (mtDNA) D-loop control region sequences ranging in length from 583 to 598 bp were determined for eight Japanese wolf specimens (*Canis lupus hodophilax* Temminck, 1839) collected from several sites and compared with 105 haplotypes from the domestic dog (*C. lupus familiaris*) and continental grey wolf (*C. lupus lupus*). Also, a 197-bp mtDNA sequence was amplified from archaeological wolf specimens and two continental wolf specimens (*C. lupus chanco*) as reference sequences for analysis. The mtDNA haplotypes from the eight Japanese wolf specimens were closely related to each other and grouped in a single lineage with an 88% bootstrap value in a neighbor-joining analysis. The results provide valuable information for understanding the taxonomic and phylogenetic relationships of the Japanese wolf, which have long been controversial.

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

INTRODUCTION

The Japanese wolf (*Canis lupus hodophilax* Temminck, 1893) has generally been considered to be extinct, with the last specimens recorded at Higashi-Yoshino village in Nara Prefecture in 1905. Stuffed animal specimens believed to be *C. lupus hodophilax* are stored at three academic institutes in Japan (Naora, 1965; Imaizumi, 1970a, 1970b; Miyamoto and Maki, 1983; Miyamoto, 1991; Tachi et al., 2002): the National Museum of Nature and Science, The University of Tokyo, and Wakayama University. In addition, a stuffed taxonomic type specimen of *C. lupus hodophilax* is stored in the Siebold Collection (Ph. F. von Siebold, 1796–1866) at the National Museum of Natural History, Leiden, Netherlands (Obara, 2002). Skeletal remains of the Japanese wolf have been found in archaeological sites dating from the Jomon Period (10,000 to 250 B.C) (Miyao et al., 1980; Naora, 1965; Shigehara and Hongo, 2000). Based on these findings, the Japanese wolf is thought to have had a wide distribution and to have been native to three of the main islands of Japan (Kyushu, Shikoku and Honshu), but not Hokkaido Island. However, the number of bone specimens tentatively identified as the Japanese wolf is limited, and only the three stuffed specimens in Japan and the one stuffed specimen in the Netherlands provide intact specimens for identification and analysis.

A number of osteological characters of the Japanese wolf have been investigated in order to distinguish it from Japanese native dogs (*Canis lupus familiaris*) (Naora, 1965; Imaizumi, 1970a, 1970b). The distinguishing characteristics (or combination thereof) of the skull of the Japanese wolf include 206.4 to 226.0 mm in total length, a well-developed auditory meatus external to the postglenoidal foramen, and an emarginated anterior border of the pterygoid fossa. In the mandible, M1 is relatively larger in the Japanese wolf than in any other canid species (Miyamoto and Maki, 1983). Similarly, skulls of a wild canine called Yama-Inu in local museums or personal collections in several districts of Honshu Island have been identified (Obara and Nakamura, 1992). The Yama-Inu has occasionally been confused with the Japanese wolf because of the osteological similarities between the two. Use of morphological characters alone is not sufficient to distinguish the Japanese wolf from large domesticated dogs such as the Akita breed. Thus, the taxonomic and phylogenetic relationships of the Japanese wolf are not clearly understood. Several questions regarding the relationships between the Japanese wolf and the Yama-Inu, and cross breeding between the Japanese wolf and native dogs, remain controversial.

Advances in DNA techniques in the field of ancient biology can now provide direct information on the phylogenetic relationships of extinct species of *Canis* (Okumura et al., 1999; Leonard et al., 2002). This information can be used in conjunction with simultaneously accumulated mitochondrial DNA (mtDNA) data from modern dogs and wolves (Okumura et al., 1996; Takahashi et al., 2002; Vila et al., 1997; Tsuda et al., 1997). DNA analysis provides an opportunity to improve the understanding of the taxonomic and phylogenetic relationships of the Japanese wolf.

In this study, we made osteometrical measurements on Japanese wolf specimens from the University Museum,

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National and local museums, and personal and high school collections. In addition, we amplified mtDNA from the bones of ancient Japanese wolf specimens using PCR, and used sequences from the mtDNA D-loop control region to analyze the phylogenetic relationship among the Japanese wolf, modern Japanese dogs, and the continental wolf.

MATERIALS AND METHODS

Japanese wolf samples and morphological measurements

Nine ancient wolf specimens (Nos. 242–250) and two wolf specimens (*Canis lupus chanco*) (Wolf251 and Wolf252) in the Hasebe Collection at the University of Tokyo Museum were used in this study (Table 1). The Hasebe Collection holds the skeletons of dogs and wolves collected by Dr. Kotondo Hasebe from Japan and neighboring islands from before to after the Second World War (Shigehara, 1986). The nine ancient specimens are from archaeological sites of six prehistoric periods as described by Shigehara (1986). The two wolf specimens (Wolf251 and Wolf252) are thought to be *C. lupus chanco*, but the collection sites and the dating to period are not exact. Eight Japanese wolf specimens (JW229, JW237, JW239, JW240, JW255, JW257, JW258 and JW259) from distinct sites and periods that were stored in several museums or personal collections were also used in this study (Table 1). Fourteen major features of the cranium and mandible of the bone specimens were measured to the nearest 0.1 mm with a Vernier caliper (Saito, 1963; Shigehara, 1986).

DNA extraction

DNA was extracted from the nine ancient specimens, the two *C. lupus chanco* specimens in the Hasebe Collection, and the eight Japanese wolf specimens (Table 1), according to methods described by Okumura et al. (1999). 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 hr 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 3,000 rpm for 10 min, and the supernatant containing 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) and was washed with distilled water. The extracted DNA samples (about 50 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, as described by Okumura et al. (1999).

PCR and direct sequencing of mtDNA

A portion of the mtDNA D-loop control region of approximately 600 base pairs (bp) was amplified by PCR using primers mit3 and mit123, as described by Takahashi et al. (2002). If the control region sequences could not be amplified, amplifications were carried out in three parts using newly designed primers, as follows: A (amplification length, 360 bp), mit3 and mitH52; B (316 bp), mit123 and mit138 (5'-AGATAAGTTAGAGTTAGTGC); and C (277 bp), mit136 (5'-GGACATCTCGATGGACTAATG) and mit123. The 600-bp sequence was reconstructed by connecting parts A, B, and C (Okumura et al., 1999; Takahashi et al., 2002). PCR was carried out using the following thermal conditions: initial denaturation and AmpliTaq Gold activation at 95°C for 10 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, 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 1 min. When few or no PCR products were produced for parts A, B, and C from ancient specimens, a semi-nested PCR strategy was used. Semi-nested PCR amplification of only part A was carried out in which 1 μl of the first-round PCR product was amplified for 30 cycles with primers mitH52 and mitL63, as described by Okumura et al. (1999). The product was checked by electrophoresis on a 1.5% agarose gel, and the primers were removed by using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The purified DNA fragments were sequenced directly on a DNA sequencer with the corresponding primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Table 1. Characteristics of the Japanese wolf and ancient wolf specimens, and mtDNA amplification outcomes.

Sample No.	Sample or Archaeological site (Prefecture, Country)	Museum or preservation site	Specimen No. or remarks	Bone part	Period[a]	Amplification mtDNA(bp)	Reference
242	Kuzu (Tochigi)	Hasebe Collection[b]		Mandible (R)	None	–	Shigehara (1986)
243		Hasebe Collection[b]		Tibia	None	–	Shigehara (1986)
244		Hasebe Collection[b]		Talus	None	–	Shigehara (1986)
245	Sakawa (Kochi)	Hasebe Collection[b]		Mandible (L)	Initial Jomon	–	Shigehara (1986)
246		Hasebe Collection[b]		Mandible (R)	Initial Jomon	–	Shigehara (1986)
247	Monzen (Iwate)	Hasebe Collection[b]		Mandible (R)	Middle-late Jomon	–	Shigehara (1986)
248	Shijimizika (Shizuoka)	Hasebe Collection[b]		Mandible (R)	Late Jomon	–	Shigehara (1986)
249	Obora (Iwate)	Hasebe Collection[b]		Mandible (R)	Final Jomon	–	Shigehara (1986)
250	Sugita (Kanagawa)	Hasebe Collection[b]		Mandible (L)	Middle-Final Jomon	197[c]	Shigehara (1986)
Wolf251	Wolf (None)	Hasebe Collection[b]	Loop	Mandible (R)	None	598	Shigehara (1986)
Wolf252	Wolf (Korea)	Hasebe Collection[b]	Nukute	Mandible (R)	None	597	Shigehara (1986)
JW229	Japanese Wolf (Kochi)	Kataoka Personal Collection		Mandible (R)	Edo	598	Abe and Iwata (2001)
JW237	Japanese Wolf (Kanagawa)	National Museum of Nature and Science	12919	Mandible (L)	Edo-Meiji	590	
JW239	Japanese Wolf (Kanagawa)[d]	National Museum of Nature and Science		Mandible (R)	Edo-Meiji	598	
JW240	Japanese Wolf (Kumamoto)	Kumamoto City Museum		Tibia (R)	Muromachi-Edo	598	Kitamura et al. (1999)
JW255	Japanese Wolf (Yamanashi)	Yamanashi Prefectural Museum		Cranium(R)	Edo-Meiji	598	Endo et al. (2004)
JW257	Japanese Wolf (Hiroshima)	Akiohota-cho Education Board		Mandible (R)	Edo-Meiji	598	Yoneda (1997)
JW258	Japanese Wolf (Nagano)	Ueda High School		Mandible (R)	Edo-Meiji	598	
JW259	Japanese Wolf (Ehime)	Ehime Prefectural Museum		Mandible (L)	Edo-Meiji	583	Obara (1990)

[a] Periods of the archaeological samples are designated according to Shigehara (1986).
[b] The Hasebe Collection is stored in the Museum of The University of Tokyo.
[c] The mtDNA sequence from specimen No. 250 was identical to accession no. D83617.
[d] Ueno personal collection.

Alignment of DNA sequences and phylogenetic analysis

The mtDNA D-loop control region sequences from two wolf samples (*C. lupus chanco*) and seven Japanese wolf samples were aligned with 78 dog mtDNA haplotypes (Okumura et al., 1996; Takahashi et al., 2002) and 25 representative wolf mtDNA haplotypes (Koop and Crockford, 2000; Tsuda et al., 1997; Vila et al., 1997) by using GENETYX-MAC software Version 10 (Software Development, Tokyo, Japan). Phylogenetic trees were constructed with the neighbor-joining (NJ) and the maximum parsimony (MP) methods implemented in MEGA4. Confidence levels for each node in the phylogenic tree were estimated from analysis of 500 bootstrap replicates.

RESULTS

Morphological characters of the Japanese wolf

The osteometrical measurements of the cranium and mandible of all 15 Japanese wolf specimens, two *C. lupus chanco* specimens, and one domestic dog (Akita breed) are shown in Table 2. Although the examinations were carried out as thoroughly as possible, several important morphological characters could not be measured. Among the mandibles of the ancient Japanese wolf specimens in the Hasebe Collection, the Kuzu sample (No. 240; not identified as male or female) is conspicuously the largest. Six ancient specimens from the Sakawa, Monzen, Shijimizuka, Obora, and Sugita sites in the Hasebe Collection had damaged mandibles, and as a result, several measurements could not be obtained. The two *C. lupus chanco* specimens were well preserved, and 14 measured parameters were compared to those from eight Japanese wolf specimens. Wolf251 was a male, with osteometrical measurements larger than those of Wolf252 and the Japanese wolf specimens. The osteometrical measurements of the eight Japanese wolf specimens were compared to those of males of a representative large native dog, the Japanese Akita (Table 2). JW229 was the largest among the Japanese wolf specimens and was larger than Wolf252. Three features measured on the cranium (Max. cranial L., Condylobasal L., and Snout B) and three features on the mandible (Mandibular L. id-goc, Mandibular L. id-cpost., and

Table 2. Measured values (millimeters) of cranial and mandibular characters in Japanese wolves, ancient Japanese wolves, and a representative domestic dog specimen (Akita breed).

Measurement		Hasebe collection (Ancient Japanese wolf)								Japanese wolf (*C.l.hodophilax*)								Reference	
		Kuzu	Sakawa	Monzen	Shijimizuka	Obora	Sugita	251	252	JW229	JW237	JW239	JW240	JW255	JW257	JW258	JW259	Wakayama Univ. Japanese wolf	Akita dog
Item	points sex	?	?	?	?	?	?	Female	?	?	?	?	?	?	?	?	?	?	Male
Cranium																			
Max.cranial L.	pr-i	–[a]	–	–	–	–	–	274.0	218.3	235.8	–	228	218.8	–	222	–	–	219.2	201.9
Condylobasal L.	pr-	–	–	–	–	–	–	243.8	200.7	216.6	–	–	204.6	–	–	–	–	205.2	194.0
Zygomatic B.	zy-zy	–	–	–	–	–	–	149.2	111.9	128.5	–	127.8	129.6	126.3	–	–	117.7	123.4	119.3
Smout B.		–	–	–	–	–	–	50.7	36.3	46.7	–	43.6	40.2	–	41	39.8	41.3	40.3	38.2
Nasal curve D.		–	–	–	–	–	–	6.7	4.4	7.6	–	4.1	–	–	–	5.0	–	–	9.5
Palatal L.	pr-sta	–	–	–	–	–	–	129.0	105.8	–	–	107.5	103.5	107.9	112	105.8	95.6	108.9	101.4
Max. palatal B.		–	–	–	–	–	–	83.7	71.3	75.1	–	75.1	70.9	71.1	–	67.3	–	72.2	69.0
Mandible																			
Mandibular L.	id-goc	191.0	164.8	–	–	149.9	–	195.5	155.9	173.1	169.1	168.3	–	–	161	–	–	–	151.8
	id-cpost	–	–	–	–	–	–	–	–	–	172.2	169.7	160.8	–	–	161.1	–	–	–
Mandibular L.	id-c.mid.	194.0	162.4	–	–	148.9	154.6	196.0	156.5	171.9	170.4	166.9	–	–	–	159.6	–	160.6	151.5
Ramus B.	minimum	49.4	40.0	–	–	35.6	–	49.4	35.6	39.4	41.3	41.5	–	–	–	33.9	–	–	37.1
Body H. (2)	M1	34.0	–	30.8	26.5	28.9	27.8	36.7	28.5	28.3	29	30.6	–	–	–	27.7	–	–	24.4
Body Th. (M1)	M1	13.9	12.9	11.7	12.4	13.6	12.4	15.6	12.0	13.4	11.8	13	–	–	–	11.7	–	–	10.6
Masseter fossa D.		9.2	9.0	–	–	8.5	–	11.5	7.0	10.0	9.7	9.8	–	–	–	8.6	–	–	7.5

[a] –, not measured.

Table 3. Variation in the fragment of the mitochondrial DNA control region sequenced in this study.

Sample (Haplotype)	Nucleotide positions[a)]																																					
	33	66	68	69	70	71	72	73	74	75	78	97	139	155	156	169	171	172	176	180	183	187	194	196	344	359	385	392	411	456	460	475	482	489	499	503	547	569
Dog (Shiba.1:D83627)	A	T	T	C	C	C	C	T	C	C	–	A	C	T	T	T	A	T	C	T	A	A	T	G	T	T	C	G	A	C	T	A	G	C	C	C	A	C
Dog (Kishu.25:D83611)	G	C	–	–	–	–	–	–	–	–	C	·	·	C	·	·	G	C	·	·	·	·	·	A	C	·	·	·	·	T	·	·	·	·	·	·	·	T
JW229 (AB480736)	G	C	·	·	·	·	·	·	·	·	C	G	·	C	·	·	G	C	·	·	·	·	·	A	C	·	·	·	·	T	·	G	–	·	·	·	·	T
JW237 (AB480738)	G	C	–	–	–	–	–	–	–	–	C	·	·	C	·	·	G	C	·	·	·	·	·	A	C	·	·	·	·	T	·	·	–	·	·	·	·	T
JW239 (AB480737)	G	C	·	·	·	·	·	·	·	·	C	·	·	C	·	·	G	C	·	·	·	·	·	A	C	·	T	·	·	T	·	·	–	·	·	·	·	T
JW240 (AB480739)	G	C	·	·	·	·	·	·	·	·	C	·	·	C	·	·	G	C	·	·	·	·	·	·	C	·	·	·	·	T	·	G	–	·	·	·	·	T
JW255 (AB480740)	G	C	·	·	·	·	·	·	·	·	C	·	·	C	·	·	·	C	·	·	·	·	·	A	C	·	T	·	·	T	·	·	–	·	·	·	·	T
JW257 (AB480741)	G	C	·	·	·	·	·	·	·	·	C	G	·	C	·	·	G	C	·	·	·	·	·	A	C	·	·	·	·	T	·	G	–	·	·	·	·	T
JW258 (AB480742)	G	C	·	·	·	·	·	·	·	·	C	·	T	C	·	·	G	C	·	·	·	·	·	A	C	·	T	·	·	T	·	·	–	·	·	·	·	T
JW259 (AB500700)	G	C	·	·	·	·	·	·	·	·	C	G	·	C	·	·	G	C	·	·	·	·	·	A	C	·	T	·	·	T	·	G	–	·	·	·	·	T
Wolf251 (AB480743)	·	·	·	T	·	·	·	·	·	·	–	G	·	·	·	·	·	·	·	·	·	G	·	A	C	C	·	·	G	T	·	·	·	T	T	·	G	T
Wolf252 (AB480744)	·	·	·	T	·	·	·	·	·	·	–	G	·	·	·	·	·	·	·	·	·	G	·	A	C	·	·	·	·	T	·	·	–	·	T	·	·	T
Wolf (AB007372)	·	·	·	·	·	·	·	·	·	·	C	·	T	C	·	C	·	·	·	C	·	·	C	·	C	·	·	A	·	T	A	·	–	·	·	T	G	T
Wolf (AB007373)	·	·	·	T	·	·	·	·	·	·	–	·	·	·	C	·	·	·	T	·	G	·	·	A	C	C	·	·	·	T	·	·	–	·	T	·	·	T

[a] Nucleotide position 1 corresponds to base position 33 described by Okumura et al (1996). Dots indicate nucleotide identity with the Shiba 1 haplotype.

Body Th.M1) were markedly larger in the Japanese wolf than in the Akita dog, suggesting that Japanese wolf bones are morphologically different from Akita breed dog bones.

mtDNA analysis

Ancient DNA was extracted from nine ancient wolf, two wolf, and eight Japanese wolf specimens. D-loop control region sequences 590–598 bp long were amplified from the two wolf specimens and seven of the eight Japanese wolf specimens, except that only a truncated 583-bp fragment was obtained from specimen JW259. No mtDNA could be amplified from the nine ancient wolf specimens, except that a 197-bp fragment (part A) was amplified from bone from specimen No. 250. The successful amplification of ancient DNA appears to be correlated with the degree of preservation of the bone and the period of origin of the ancient specimens.

The 197-bp mtDNA fragment (part A) sequenced from one ancient wolf specimen showed complete identity with that of domestic dog Pug.45 (D83617: Okumura et al., 1996), which is obviously different from that of the Japanese wolf. The 583- to 598-bp mtDNA sequences from six of the Japanese wolf specimens were different from those from the two wolf specimens and were different from each other, except that the 598-bp mtDNA sequence of JW229 was identical to that of JW257. Table 3 shows the Japanese wolf haplotypes aligned with representative domestic dog (Shiba 1 and Kishu 25: Okumura et al., 1996) and wolf (AB007372 and AB007373: Tsuda et al., 1997) haplotypes. A comparison of dog, wolf, and Japanese wolf mtDNA sequences revealed that an insertion of nucleotide C at position 78 (78-C insertion) and a deletion of nucleotide G at position 482 (482-G deletion) in the mtDNA D-loop control region are specific to the eight Japanese wolf specimens (Table 3). In addition, the 590-bp mtDNA sequence from specimen JW237 possessed the same unique 8-bp deletion found in the dog Kishu 25 (Okumura et al., 1996). The 583- to 598-bp mtDNA sequences in the eight Japanese wolf specimens were more closely related to each other than to those from the wolf (Wolf251 and Wolf252). No identical mtDNA sequences were found in the DDBJ/EMBL/GenBank database. Therefore, we deposited the eight novel Japanese wolf and two novel wolf mtDNA sequences in the DDBJ/EMBL/GenBank database (Accession nos. AB480736–AB480744 and AB500700).

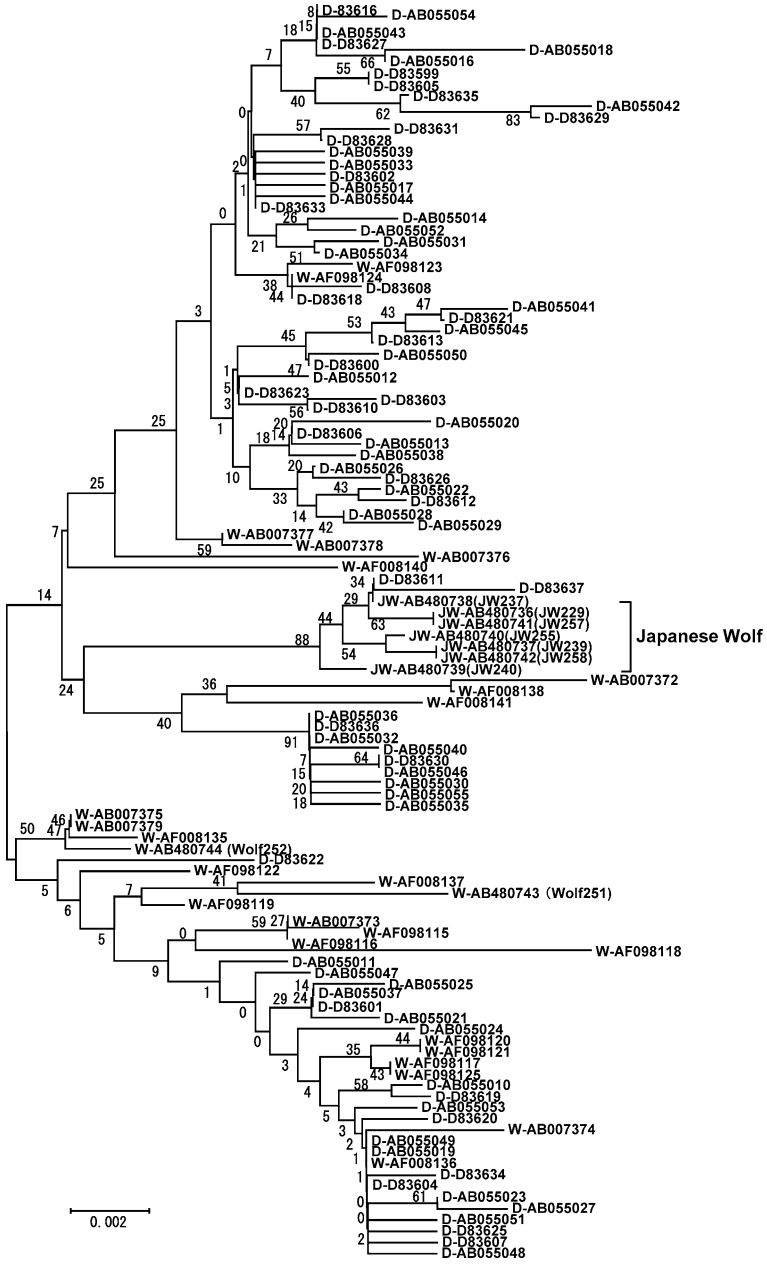

Fig. 1. Neighbor-joining (NJ) phylogenetic tree based on a 590- to 598-bp mtDNA control region fragment and including nine novel haplotypes detected in this study, 78 dog haplotypes (Okumura et al., 1996; Takahashi et al., 2002), and 25 representative wolf haplotypes (Koop and Crockford, 2000; Tsuda et al., 1997; Vila et al., 1997). Bootstrap resampling was performed 500 times, and bootstrap values in percent are shown near corresponding nodes. Accession numbers: Japanese wolf haplotypes, AB480736–AB480742; dog haplotypes, D83599–D83608, D83610–D83616, D83618–D83621, D83623, D83625–D83637, AB055010–AB055014, AB055016–AB055055; wolf haplotypes, AB480743–AB480744, AB007372–AB007379, AF008135–AF008138, AF008140, AF008141, AF098120, AF098115–AF098119, AF098121–AF098125. The scale at lower left indicates genetic distance in substitutions per site.

Phylogenetic analysis

To determine phylogenetic relationships, a NJ tree was constructed with the novel mtDNA D-loop control region haplotypes from the Japanese wolf (n=7; JW229, JW237, JW239, JW240, JW255, JW257 and JW258) and wolf (n=2; Wolf251 and Wolf252) along with mtDNA haplotypes from the domestic dog (n=78; Okumura et al., 1996; Takahashi et al., 2002) and representative wolf species (n=27; Tsuda et al., 1997; Vila et al., 1996). The truncated mtDNA sequence from JW259 was excluded from this phylogenetic analysis. A previous phylogenetic analysis of mtDNA haplotypes did not clearly distinguish the wolf lineage from the domestic dog lineage (Fig. 1 in Vila et al., 1997). However, in the present study, the mtDNA haplo-

types from the seven Japanese wolf sequences formed a distinct cluster with a bootstrap value of 88% in the NJ tree, suggesting that Japanese wolf haplotypes are genetically grouped in a single lineage. Two dog haplotypes (Kishu 25 [D83611] and Siberian husky [S-Husky] 102 [D83637]) (Okumura et al., 1996) were included in the Japanese wolf lineage. The Kishu 25 specimen possessed the unique 78-C insertion in the mtDNA sequence. The two mtDNA sequences from Wolf251 and Wolf252 were located in the major wolf cluster in the NJ tree. The single lineage of Japanese wolf mtDNA sequences was confirmed by the MP analysis (data not shown).

DISCUSSION

This is the first study to investigate mtDNA sequences and the phylogenetic relationships of the Japanese wolf, although there is a report regarding the isolation and characterization of a partial amelogenin exon (AMELX) fragment from the Japanese wolf (Tachi et al., 2002). The mtDNA haplotypes (590- to 598-bp sequences) from the seven Japanese wolf specimens were closely related to each other and grouped in a single lineage in the NJ tree. The genetic characterization of the Japanese wolf has been controversial for a long time. Among the arguments regarding taxonomic status, the Japanese wolf is thought to be a native wolf different from the continental grey wolf (*C. lupus lupus*); alternatively, it is thought to be endemic to Japan or the result of cross breeding with dogs. The mtDNA analysis in our study indicates that it is reasonable to classify the Japanese wolf as a subspecies of *C. lupus*. Two nucleotide substitutions, the unique 78-C insertion and the 482-G deletion, are specific to the Japanese wolf sequences. The 78-C insertion is sometimes found in the wolf group (*C. lupus lupus*) but is rarely found in dogs. The 482-G deletion is frequently found in the wolf group but rarely in dogs. These two nucleotide substitutions are very useful markers for distinguishing the Japanese wolf from the continental grey wolf and domestic dog.

In this study, we planned to examine all nine archaeological wolf specimens in the Hasebe Collection. Unfortunately, the mtDNA amplification was not successful for these specimens, with the exception of one 197-bp sequence (A) from specimen No. 250 from the Sugita site. However, analysis of this sample suggests that it is domestic dog, because the 197-bp sequence is identical to a portion of the domestic dog Pug.45 sequence, but without the unique 78-C insertion. Since many archaeological wolf specimens are damaged or found as parts of skeletons, it is possible that skeletal remains of domestic dogs were among the archaeological specimens.

After analysis of mtDNA from more than 600 dog specimens, the unique 8-bp deletion found in the Japanese wolf specimen JW237 has so far been observed in only one domestic dog specimen, Kishu 25 (Okumura et al., 1996; Takahashi et al., 2002). On the other hand, the JW237 sequence also possesses the unique 78-C insertion and 482-G deletion, suggesting that this haplotype belongs to the Japanese wolf lineage. Although it is not clear whether the 8-bp deletion originated in the wolf or domestic dog lineages, the possibility of crossbreeding between the Japanese wolf and native dogs is worth noting.

To specifically investigate nucleotide diversity within local populations of the Japanese wolf, eight Japanese wolf specimens were examined from the Kanto (W237, JW239, JW255, and JW258) and Chugoku (JW257) regions of Honshu Island, and from Kyushu (JW240) and Shikoku (JW229 and JW259) Islands. In the mtDNA D-loop control region of approximately 600 bp, the nucleotide diversity was limited to a few nucleotide substitutions (Table 3). These results may have implications for determining the ancestral origins of the Japanese wolf. It appears that the Japanese wolf inhabited several areas on Kyushu, Shikoku, and Honshu Islands, but not Hokkaido Island, before the Jomon Period, since skeletal remains have been excavated from archaeological sites of the Jomon Period (Miyao et al., 1980; Naora, 1965; Shigehara and Hongo, 2000). Since the Japanese wolf has been not found on Hokkaido Island, the ancestor of this species may have migrated from the Asian continent to Japan via the Korean Peninsula. The limited number of nucleotide substitutions in the mtDNA D-loop control region suggests a bottleneck event (Table 3). Opportunities for the ancestors of the Japanese wolf to migrate to Japan were likely limited, and the founder population is likely to have been small. Alternatively, the bottleneck may have already occurred before the Jomon Period. We know that the ancestor of the Japanese wolf migrated to Japan in prehistoric times, but the precise timing and pathway of migration remain unknown.

There are several reports on the morphological characters of the Japanese wolf, especially with regard to distinguishing Japanese wolf specimens from large domestic dogs (Abe, 2001; Endo et al., 1997, 1999; Imaizumi, 1970a, 1970b; Obara, 1990, 2002). The Japanese wolf has sometimes been called Yama-Inu, and bone specimens of this animal are stored as a kind of treasure in personal or local governmental collections (Obara and Nakamura, 1992). We morphologically identified the eight Japanese wolf specimens used in the DNA analysis of this study as *C. lupus hodophilax*. Cranial and mandibular measurements show sizes intermediate to those of the continental grey wolf (*C. lupus chanco*) and large-sized Japanese native dogs such as the Akita breed (Table 2). The skeletons of the Japanese wolf are morphologically smaller than those of the continental grey wolf, and the reduction in size may be an island-isolation effect. However, from the comparison of Japanese and continental wolf measures, it is difficult to estimate the direct pressures leading to the size reduction. In the mtDNA analysis, a domestic dog, S-Husky 102, was found to belong to the Japanese wolf lineage. However, with the exception of S-Husky 102, almost all the S-Husky dog specimens possessed mtDNA haplotypes different from those of the Japanese wolf. The discovery of an mtDNA haplotype in the S-Husky dog breed related to the Japanese wolf haplotype indicates that the ancestor of the Japanese wolf lived on the Asian continent and that its genetic makeup may have been influenced by specific domestic dog populations or breeds. To further examine the genetic relationships among the Japanese wolf, the continental grey wolf, and domestic dog populations, larger mtDNA fragments need to be sequenced. A project to sequence the whole mtDNA genome of the Japanese wolf is now in progress.

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