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Authors: Matsumura, Shuichi, Terai, Yohey, Hongo, Hitomi, and Ishiguro, Naotaka

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Analysis of the Mitochondrial Genomes of Japanese Wolf Specimens in the Siebold Collection, Leiden

Shuichi Matsumura[1]*, Yohey Terai[2], Hitomi Hongo[2], and Naotaka Ishiguro[1,2]

[1]*Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan*
[2]*School of Advanced Sciences, SOKENDAI (Graduate University for Advanced Studies), Hayama 240-0193, Japan*

The taxonomic status of extinct Japanese or Honshu wolves (*Canis lupus hodophilax*) has been disputed since the name *hodophilax* was first proposed by Temminck in 1839 on the basis of specimens stored in Leiden, the Netherlands. Points of controversy include whether the type specimen of *hodophilax* (Jentink c: RMNH.MAM.39181) and the other two specimens from Leiden (Jentink a: RMNH.MAM.39182 and Jentink b: RMNH.MAM.39183) represent different varieties or subspecies of Japanese wolves or not. Two Japanese names, *ookami* and *jamainu*, used to describe wild *Canis* species, further complicate the issue. In this study, the taxonomic status of Japanese wolves was clarified using mitochondrial DNA of the three specimens stored at the Naturalis Biodiversity Center in Leiden, in addition to three Japanese wolf specimens stored at the Museum für Naturkunde in Berlin and five new samples from Japan. The mitochondrial genomes of the type specimen of *hodophilax* (Jentink c) and another sample from Leiden (Jentink b) as well as Berlin specimens were included in the cluster of Japanese wolves distinct from other grey wolves. However, the other sample from Leiden (Jentink a) was identified as a domestic dog. A mitochondrial genome analysis suggested that Japanese wolves could be categorized into two distinct clusters. Studies of nuclear genomes are needed to further clarify the taxonomic status, divergence time, and population genetic structure of Japanese wolves.

Key words: Japanese wolf, *Canis lupus hodophilax*, type specimen, ancient DNA, mitochondrial DNA, phylogenetics

INTRODUCTION

The taxonomic status of wolves in Japan, which became extinct about 100–120 years ago, has long been disputed because a quite limited number of bone specimens and stuffed specimens are available for study (Ishiguro et al., 2009; Ishiguro et al., 2010; Miyamoto, 2001). Generally, two lineages of wolves from Japan are recognized: Japanese or Honshu wolves (*Canis lupus hodophilax* Temminck, 1839) and Ezo or Hokkaido wolves (*Canis lupus hattai* Kishida, 1931). The former inhabited the central and southern islands of Honshu, Shikoku, and Kyushu, whereas the latter was limited to the northern island of Hokkaido and neighboring islands. Recent phylogenetic studies based on mitochondrial genomes have clearly demonstrated that the two lineages are phylogenetically distinct sub-species of grey wolves; Japanese wolves are among the earliest diverged groups within grey wolves (Matsumura et al., 2014), and Ezo wolves are closely related to grey wolves in North America (Ishiguro et al., 2010; Matsumura et al., 2014).

However, there remain unresolved issues about the taxonomy of Japanese wolves in Honshu, Shikoku, and Kyushu. In Honshu, Shikoku, and Kyushu, two different words have historically been used to describe *Canis* species in the wild: *ookami* and *jamainu* (Walker, 2005; Funk, 2015). (These words are sometimes spelled *okami*, *ookame*, or *yamainu*; the names used by Philipp Franz von Siebold (Siebold: 1796–1866) are adopted in this manuscript.) *Ookami* means wolf, whereas the Japanese word *yama-inu* (*jamainu*) literally translates to mountain dog. Therefore, most agree that *ookami* represented Japanese wolves *C. l. hodophilax*, but there is a controversy about what *jamainu* represented. Some believe that *jamainu* refers to free-ranging dogs (i.e., *Canis lupus familiaris*) which breed and range in the mountains, some regard *jamainu* as a synonym of *ookami* (i.e., Japanese wolves), while others maintain that a variety of wolves distinct from Japanese wolves inhabited Honshu, Shikoku and Kyushu until recently (Hiraiwa, 1992; Walker, 2015).

One essential step to clarify the taxonomic status of wolves in Japan is to investigate specimens stored in museums in Europe because the scientific description of wild *Canis* in Japan has been made on the basis of those specimens. In Europe, wolf specimens from Japan are stored in three locations: (1) Naturalis Biodiversity Center (NBC), Leiden, the Netherlands; (2) Museum für Naturkunde, Berlin, Germany; and (3) Natural History Museum, London, United Kingdom. Temminck (1839) first described Japanese wolves as a distinct wolf species, *C. hodophilax*, based on the specimens at Leiden, which Siebold sent from Japan. In Fauna Japonica edited by Siebold, Temminck referred to this new

* Corresponding author. E-mail: matsumur@gifu-u.ac.jp
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species *C. hodophylax* (*hodophilax*) as *jamainu*, and suggested that it is distinct from *ookame* (Walker, 2015). Nehring (1885) examined another specimen, ZMB_Mam_22326 (An 25546), in Berlin, and described it as *Lupus japonicus* or *Canis lupus* var. *japonica*, different from *C. hodophilax*.

NBC at Leiden now registers one skeleton with a skull (Jentink a), two skulls (Jentink b and Jentink c), and one mounted skin as *hodophilax*. According to Jentink (1887), the type specimen of *hodophilax* is the skull "Jentink c" and the skin (Funk, 2015). Peculiarly, the condylobasal length of the skull "Jentink c" (174 mm) is small compared with those of the skull "Jentink b" (206 mm) and other Japanese wolf skulls found in Japan (Saito, 1964; Imaizumi, 1970; Nakamura, 1998). For example, the estimated range of Japanese wolf skull length was 193.1–235.9 mm (P = 99%) based on the measurement of 14 specimens from the Tanzawa population in Honshu (Nakamura, 1998). Imaizumi (1970) concluded that the skull "Jentink a" is a domestic dog based on the position of the external auditory meatus and the form of the anterior border of the mesopterygoid fossa. Based on the size and description in Fauna Japonica, some believe that the skull "Jentink b" is the only Japanese wolf (i.e., *ookame*) specimen at Leiden, and that the skull "Jentink c" is *jamainu*, an undescribed variety of wolves that once lived in Japan.

The objectives of the present study were to clarify the taxonomic status of the specimens registered as *C. hodophilax* at Leiden based on a mitochondrial genome sequence analysis. Specifically, the enigmatic "Jentink c" is expected to be phylogenetically different from "Jentink b" and other Japanese wolf specimens if it represents a distinct variety or a subspecies of Japanese wolves. Specimens of wolves from Japan stored at Museum für Naturkunde in Berlin, Germany, including a "type specimen" of *Lupus japonicus* described by Nehring (1885), and five additional DNA samples collected in Japan by Matsumura et al. (2014) and Ishiguro et al. (2016) were also examined.

MATERIALS AND METHODS

Specimens

To the best of our knowledge, only eight specimens of wolves from Japan are stored in three museums (NBC in Leiden, Museum für Naturkunde in Berlin, and Natural History Museum in London) in Europe. Permission to analyze ancient DNA was obtained for six out of the eight specimens: Jentink a: RMNH.MAM.39182(52995), Jentink b: RMNH.MAM.39183, and Jentink c: RMNH.MAM.39181(52994) in Leiden (Imaizumi, 1970; Miyamoto, 2001; Obara, 2002), and ZMB_Mam_22326 (ZMB22326), ZMB_Mam_42983 (ZMB42983), and ZMB_Mam_48817 (ZMB48817) in Berlin (Miyamoto, 2001; Yagi, 2016). In particular, the ventral nasal concha fragments were kindly provided by NBC in Leiden and the Museum für Naturkunde in Berlin (Table 1). Five Japanese wolf bone samples from personal collections throughout Japan were also examined (JW275, JW286, JW289, JW290, and JW292; Table 1).

Complete mtDNA sequencing

DNA was extracted from bones according to the methods described by Okumura et al. (1999), Ishiguro et al. (2009, 2016), and Matsumura et al. (2014). Bone powder (0.02–0.1 g) was obtained from fragments of the ventral nasal concha of the specimens from the NBC in Leiden and the Museum für Naturkunde in Berlin using a Multi-beads Shocker (Yasui Kikai, Osaka, Japan), and from five Japanese wolf bone samples in Japan using an electric drill. The powder was then suspended in 10 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA) at pH 7.0 and rotated for decalcification. A pellet of bone powder was collected by centrifugation and decalcified by repeated washes with 10 ml of 0.5 M EDTA until the supernatant became 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 containing ancient DNA was extracted twice with phenol, once with chloroform:phenol (1:1), and once with chloroform for protein removal. The supernatant was concentrated using an Amicon Ultra Centrifugal Filter 30K (Merck Millipore Ltd., Tullagreen, Ireland) and washed with distilled water. The extracted DNA samples (about 1–3 μl) were used for direct PCR or next-generation sequencing. Precautions described by Okumura et al. (1999) were taken to prevent contamination with DNA from modern dogs.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and firstly sequenced by Sanger sequencing to obtain a mitochondrial (mt) DNA fragment of 215–223 bp (Ishiguro et al., 2016). Successfully obtained samples were then subjected to next generation sequencing (NGS) to obtain full-length mtDNA genome sequences. Before NGS, DNA samples were repaired using NEBNext FFPE DNA Repair Mix (New England Bio Labs, Ipswich, MA, USA), and DNA libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit (New England Bio Labs) following the manufacturer's instructions, with 11–15 PCR cycles. For each library, 5 Gb of short DNA sequences (paired-end, 125 bp) were determined using the Illumina HiSeq2500 platform (Illumina, Inc., San Diego, CA, USA). Sequence reads were mapped to a reference mtDNA genome (JW229: AB499818.1), and consensus sequences were extracted using CLC Genomics Workbench ver. 11 (https://www.qiagenbioinformatics.com/). The consensus sequences for the control region were manually corrected based on the re-aligned short sequences. A part of the entire mtDNA genome sequences (consensus sequences of the mapped reads), i.e., the sequences of the control regions of JW255 and JW271, have already been published.

Phylogenetic and demographic analyses

In addition to mitochondrial genome sequence data of wolves from central and southern Japan (Honshu, Shikoku, and Kyushu, 13 samples, Table 1), all wolf mitochondrial genome sequences available in GenBank (85 wolf samples except for our data, Supplementary Table S1) were used for analyses. The dataset for the phylogenetic and demographic analyses was created following the methods of Matsumura et al. (2014). The total length of the four concatenated files (tRNA, rRNA, protein-coding regions, and control region) was 15,477 bp. Phylogenetic analyses were performed using MEGAX (Kumar et al., 2018), MrBayes 3.2 (Ronquist et al., 2012), and BEAST v2.5.2 (Bouckaert et al., 2019). Details of the methods for tree reconstruction using the neighbor-joining method (Saitou and Nei, 1987) and Bayesian methods, including parameter settings, were described by Matsumura et al. (2014). A maximum likelihood tree was constructed using MEGAX; the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) was used as a substitution model, and a gamma distribution was assumed to model evolutionary rate differences among sites (four categories). Two coyote (*Canis latrans*) sequences (DQ480510 and DQ480511) were used as outgroups. Eighteen mitochondrial genome sequences from ancient canid samples (Thalmann et al., 2013; Supplementary Table S1) were included to calibrate the ages of nodes in the phylogenetic trees.

RESULTS

Full-length mtDNA genome sequences were newly

Table 1. Characterization of Japanese wolf and dog specimens, and variation of mtDNA control region.

Dog or Wolf	Group	Sample No.	Isolation site (Prefecture)	Sample	Period	Accession No.	Ampification mtDNA(bp)	Nucleotide positions[a)] 1 1 1 1 1 1 1 3 3 4 4 4 5 3 6 6 6 7 7 7 7 7 7 7 9 3 5 6 7 7 8 9 4 8 5 7 8 6 3 6 8 9 0 1 2 3 4 5 8 7 9 5 9 1 2 3 6 4 5 6 5 2 9	Reference
Dog		Shiba 1	Hiroshima	Blood	Modern	D83627	980	A T T C C C C T C C – A C T T A T A G T C C A G C	Okumura et al., 1996
		Jentink a 1887 RMNH.MAM. 39182	NA[b)]	Ventral nasal concha	Edo	LC519904	240	· · · · · · · · · · · · · · C · · T · / / / / / /	This study
Japanese wolf	A	JW229	Kochi	Mandible (R)	Edo	AB499818	mtDNA genome	G C · · · · · · · · C G · C · G C · A C · T G – T	Abe, 2001
		JW239	Kanagawa	Mandible (R)	Edo-Meiji	AB499822	mtDNA genome	G C · · · · · · · · C · · C · G C · A C T T · – T	Ishiguro et al., 2009
		JW240	Kumamoto	Mandible (R)	Muromachi-Edo	AB499823	mtDNA genome	G C · · · · · · · · C · · C · G C · · C · T G – T	Kitamura et al., 1999
		JW255	Yamanashi	Mandible (R)	Edo-Meiji	LC520089	mtDNA genome	G C · · · · · · · · C · · C · · C · A C T T · – T	Endo et al., 2004
		JW257	Hiroshima	Mandible (R)	Edo-Meiji	AB499824	mtDNA genome	G C · · · · · · · · C G · C · G C · A C · T G – T	Yoneda, 1997
		JW258	Nagano	Mandible (R)	Edo-Meiji	AB499825	mtDNA genome	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	Gumma	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 / / / / / /	Ishiguro et al., 2016
		JW269	Nagano	Mandible (L)	Edo-Meiji	LC064093	598	G C · · · · · · · · C · · C G C · · C T · G – ·	Ishiguro et al., 2016
		ZMB22326	NA	Ventral nasal concha	Meiji	LC519905	223	G C · · · · · · · · C · · C · G C · A / / / / / /	This study
	B	JW237	Kanagawa	Mandible (L)	Edo-Meiji	AB499821	mtDNA genome	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	LC520090	mtDNA genome	G C – – – – – – – – C · · C · G C · A C · T · – T	Ishiguro et al., 2016
		JW274	Nagano	First phalanx	Yayoi-Kofun	LC064096	590	G C – – – – – – – – C · · C · G C · A C · T G – T	Ishiguro et al., 2016
		JW275	Shimane	Cranium	Edo-Meiji	LC520091	mtDNA genome	G C – – – – – – – – C · · C · G C A C · T G – T	This study
		JW286	Iwate	Corpus maxillae	Edo-Meiji	LC519906	215	G C – – – – – – – – C · · C · G C · A / / / / / /	This study
		JW289	Iwate	Corpus maxillae	Edo-Meiji	LC519906	215	G C – – – – – – – – C · · C · G C · A / / / / / /	This study
		JW290	Iwate	Ischial tuberosity	Edo-Meiji	LC519906	215	G C – – – – – – – – C · · C · G C · A / / / / / /	This study
		JW292	Nara	Cranium	Meiji	LC520092	mtDNA genome	G C – – – – – – – – C · · C · G C A C · T G – T	This study
		Jentink b 1887 RMNH.MAM. 39183	NA	Ventral nasal concha	Edo	LC520093	mtDNA genome	G C – – – – – – – – C · · C · G C A C · T G – T	This study
		Jentink c 1887 RMNH.MAM. 39181	NA	Ventral nasal concha	Edo	LC520094	mtDNA genome	G C – – – – – – – – C · · C · G C A C · T G – T	This study
		ZMB48817	NA	Ventral nasal concha	Meiji	LC520095	mtDNA genome	G C – – – – – – – – C · · C · G C A C · T G – T	This study

a) Nucleotide position 1 corresponds to the base position 33 described by Okumura et al. (1996) of the whole dog mtDNA control region. Dots indicate identical nucleotides with the Shiba 1 haplotype. Dashes and slashes indicate deleted and undetermined nucleotides, respectively.

b) NA, not applicable.

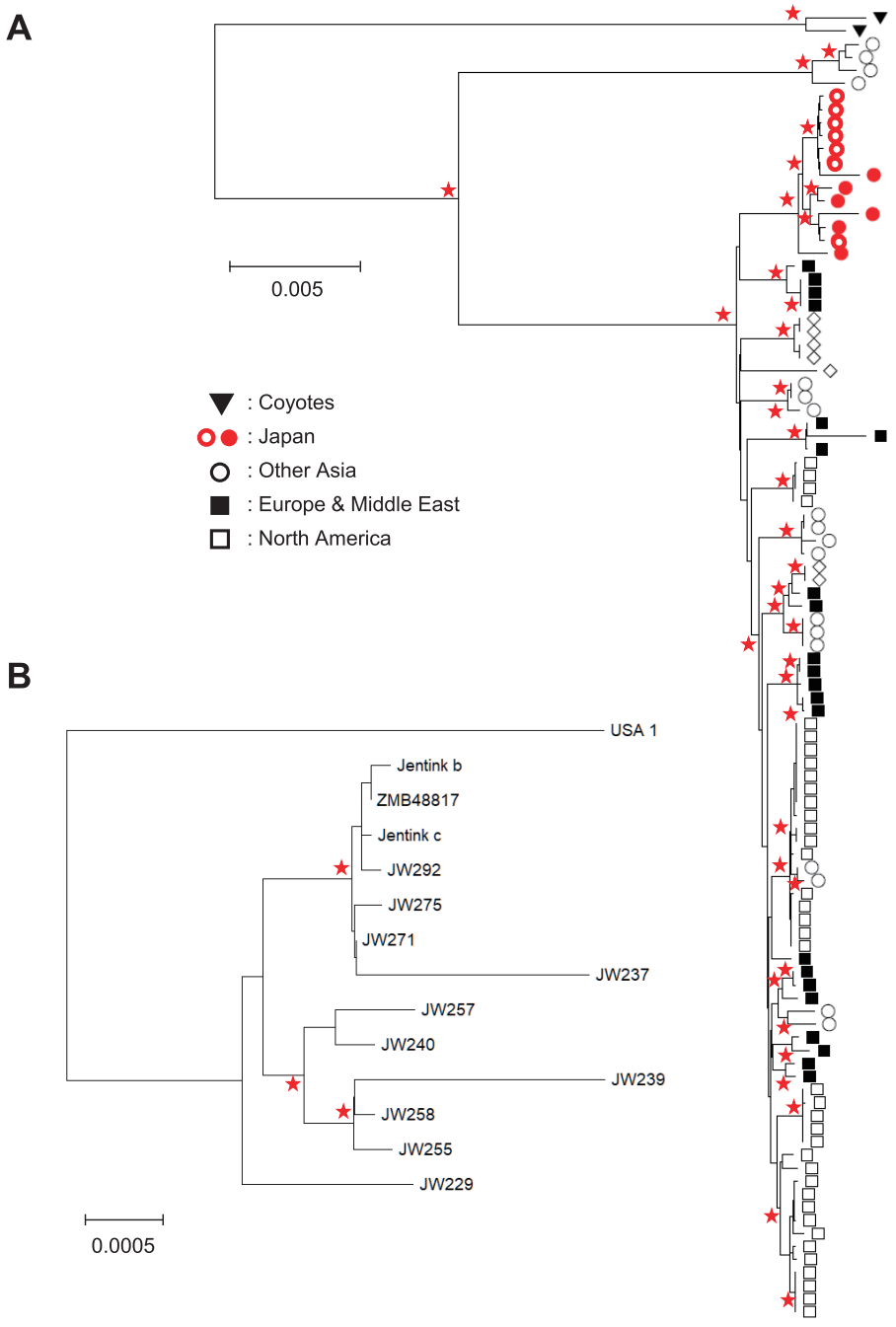

Fig. 1. **(A)** Neighbor-joining (NJ) tree (Saitou and Nei, 1987) of wolf mitochondrial genome sequences, rooted by coyote sequences. Scale bar indicates 0.005 substitutions per site. Among wolves from Japan, previously published sequences (Matsumura et al., 2014) and newly determined sequences are shown by solid and open red circles, respectively. **(B)** NJ tree of mitochondrial genome sequences of Japanese wolves, rooted by a sequence of a grey wolf from the United States. In both trees, red stars indicate bootstrap support values of ≥ 95%.

assembled for ancient DNA samples from seven putative Japanese wolves (JW255, JW271, JW275, JW292, Jentink b, Jentink c, and ZMB48817). In addition, only partial mtDNA control region sequences (215–240 bp) were obtained for five putative Japanese wolves (JW286, JW289, JW290, Jentink a, and ZMB22326), as their DNA was highly degraded. These DNA sequences have been deposited in DDBJ (LC520089–LC520095, LC519904–LC519906). Based on these nucleotide sequences, all ancient DNA samples other than the Jentink a specimen (RMNH.MAM.39182) had nucleotide substitutions specific to Japanese wolves (Table 1). Jentink a appeared to be a domestic dog based on its sequence similarity to a Japanese dog (Shiba-inu breed). No mtDNA sequences were amplified from ZMB42983.

Thirteen full-length mtDNA genome sequences of Japanese wolves were used for phylogenetic analyses. In our previous analysis (Matsumura et al., 2014), all six Japanese wolf samples formed a distinct monophyletic cluster. In this study, the seven newly assembled putative Japanese wolf samples, including the type specimen of *C. l. hodophilax* (Jentink c), formed a distinct cluster with the previous six samples (Fig. 1A, see Supplementary Figure S1 online). The two samples from Leiden (Jentink b and Jentink c) and one sample from Berlin (ZMB48817) were phylogenetically closely related.

Based on their analysis of the control region, Ishiguro et al. (2016) identified two groups of Japanese wolves: one has an 8 bp deletion in the control region (Group B) and the other lacks this deletion (Group A). When this criterion was applied to the 13 full-length mtDNA genome sequences, seven were assigned to Group B. Three samples housed outside Japan (Jentink b, Jentink c, and ZMB48817) were included in Group B. ZMB22326, for which only a partial mtDNA sequence was obtained, was assigned to Group A. Group B shared nine unique substitutions [positions 1552, 1777, 3209, 4754, 8998, 10313, 11141, 12975, and 13944 in JW229 (AB499818)] (Fig. 1B) in addition to the 8 bp deletion (Table 1). Taking the 13 full-length mtDNA genome sequences together with 10 Japanese wolf samples for which only the control region sequences were known, the 8 bp deletion was evaluated in 23 samples (Table 1); 11 and 12 samples were assigned to Group A and B, respectively. Based on the sample locations, no clear geographical structure was detected in the distributions of Groups A and B (Fig. 2).

Based on the 13 complete mtDNA sequences, the estimated divergence time of Japanese wolves from other wolf

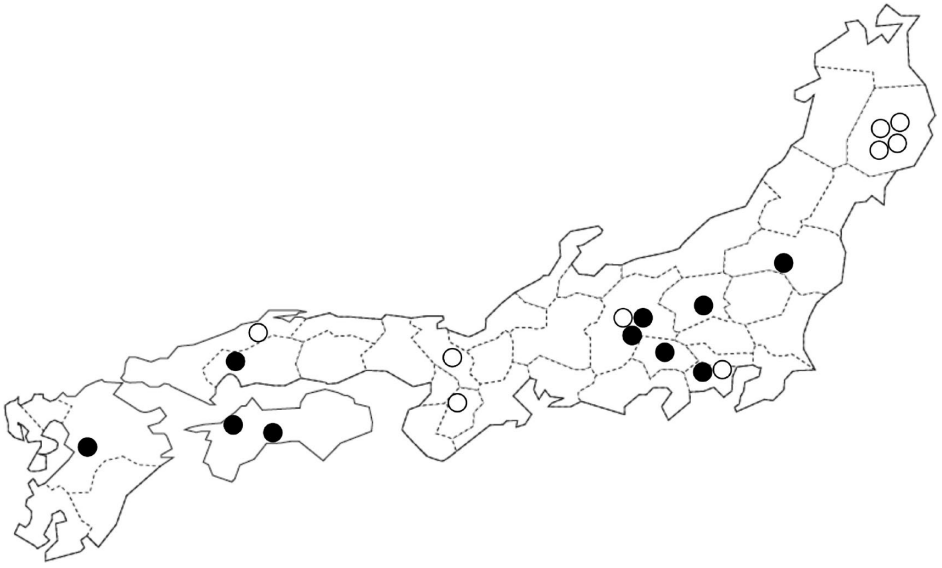

Fig. 2. Geographic distribution of samples assigned to Group A (●) and B (○) based on the absence or presence, respectively, of an 8-bp deletion in the control region.

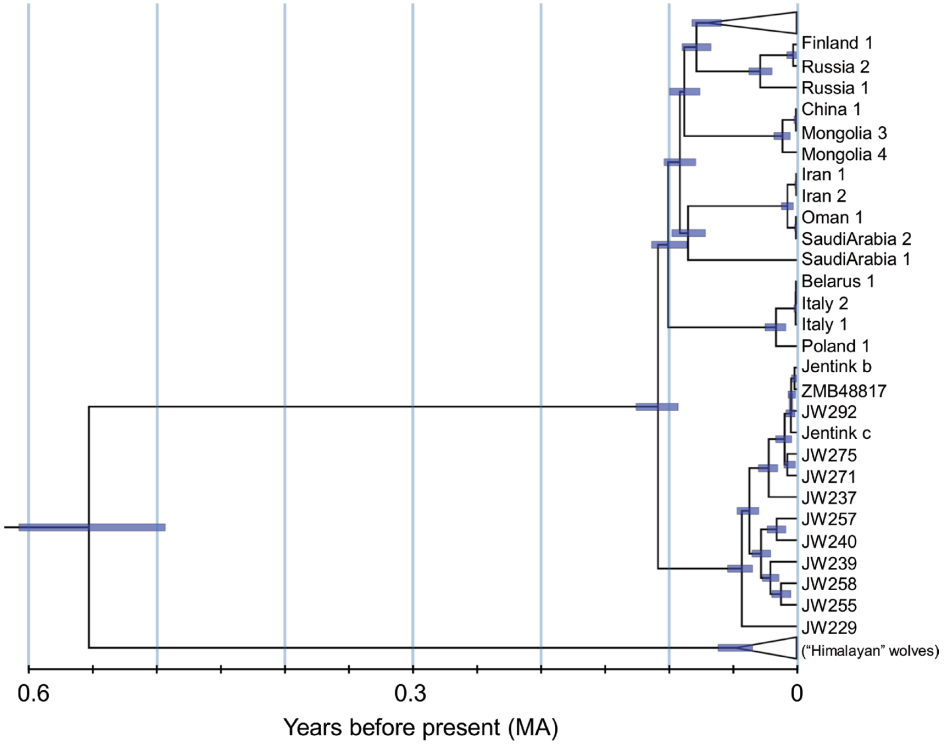

Fig. 3. Age estimates for the Japanese wolf cluster. The phylogenetic tree was inferred by a Bayesian analysis (Bouckaert et al., 2019) of mitochondrial genomes. Blue bars represent 95% highest posterior densities of nodal age estimates.

lineages was 108,000 years ago, assuming that the dog-wolf lineage separated from the coyote lineage 1 million years ago (Fig. 3). The estimated time to the most recent common ancestor (MRCA) of Japanese wolves was 42,000 years ago. However, the estimated divergence time and MRCA were 45,000 and 17,000 years ago, respectively, if the calibration was based on the age of archaeological samples (see Supplementary Figure S2).

DISCUSSION

Our study clearly reveals the phylogenetic status of putative Japanese wolf samples stored in two museums outside of Japan. The mitochondrial genomes of the type specimen of *C. hodophilax* (Jentink c), another sample from Leiden (Jentink b), and one sample from Berlin (ZMB48817) clustered with the mitochondrial genome sequences of other Japanese wolf samples from Japan. Only a portion of the mtDNA control region was successfully sequenced for another sample from Berlin (ZMB22326) as well as the remaining sample from Leiden (Jentink a); the former had several nucleotide substitutions specific to Japanese wolves, but the latter did not. We conclude that Jentink a is likely to be a domestic dog because it had a sequence similar to a Japanese dog, although we are also aware of the limitation involving the first-evolving control region. Imaizumi (1970) concluded that the skull Jentink a at Leiden is a domestic dog based on key morphological features, including the position of the external auditory meatus and the form of the anterior border of the mesopterygoid fossa. Our mtDNA sequence analysis supported this conclusion.

The Jentink c sample has been controversial because it is relatively small, even among Japanese wolves (Saito, 1964; Imaizumi, 1970; Nakamura, 1998). In Japan, the terms *ookami* and *jamainu* have both been used to describe wild canids (Hiraiwa, 1992; Walker, 2005). The two terms translate to wolf and mountain dog, respectively. Unfortunately, there is no consensus regarding the identity of *jamainu*. It is possible that (1) both *ookami* and *jamainu* refer to the Japanese wolf *C. l. hodophilax*, (2) *jamainu* refers to free-ranging domestic dogs *C. l. familiaris*, or (3) *jamainu* is a third canid species that once inhabited Japan. This lack of clarity derived in part from the first description of *C. hodophilax* by Temminck

(1839), which apparently ignored information from Siebold, who sent the specimens from Japan to Leiden (Funk, 2015). In fact, Siebold described in his diary that he collected *ookami* and *jamainu* in Osaka, Japan (Holthius and Sakai, 1970) and stated that the specimen Temminck described as *C. hodophilax* was *jamainu* (Funk, 2015). The stuffed specimen at Leiden is actually labeled *jamainu*. Therefore, those who believe that *jamainu* is a distinct species suspect that Jentink b and Jentink c are *ookami* and *jamainu*, respectively.

Our results clearly show that at least the maternal (mitochondrial) lineages of Jentink c as well as Jentink b are Japanese wolves. The reason why Jentink c is small compared to other Japanese wolves remains unresolved. The difference in size and morphological features (Miyamoto, 2001) might have resulted from hybridization between Japanese wolves and domestic dogs on an isolated archipelago (Naora, 1965). Since only mtDNA was considered in this analysis, further studies of nuclear DNA or entire nuclear genomes may resolve this issue. Such studies will also provide more precise estimates of the divergence time and past population dynamics of Japanese wolves.

Ishiguro et al. (2016) identified two groups of Japanese wolves that can be distinguished by a deletion in the control region. Phylogenetically, Group B (having the deletion) is derived from Group A (lacking the deletion), because the lack of the deletion is shared by all other wolves (85 samples) investigated here. We detected nine additional unique substitutions in the mtDNA genomes of Group B, supporting the distinct phylogenetic status. This suggests that Group B evolved in a geographically isolated area. Surprisingly, no clear geographical structure was detected. Admixture is possible based on the high mobility of wolves after the divergence of the two distinct lineages. Group B samples tend to be relatively recent in terms of age and tend to be from the central and eastern/northern parts of Japan in terms of geographic location. It is possible to hypothesize that the newly emerged Group B is dominant in the central and eastern/northern parts of Japan. Further studies of ancient DNA from Japanese wolves, ideally including samples from several hundred or thousands of years ago, are needed to confirm this hypothesis.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

SM, YT, HH, and NI designed the study, YT and NI conducted experiments, SM and YT analyzed the data, and SM, YT, HH, and NI wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs200019)

Supplementary Table S1. Mitochondrial genome sequence data of wolves included in the analysis.

Supplementary Figure S1. Phylogenetic trees constructed by **(A)** maximum likelihood method and **(B)** Bayesian method, rooted by coyote sequences.

Supplementary Figure S2. Age estimates for the Japanese wolf cluster calibrated with the archaeological samples.

REFERENCES

Abe M (2001) Morphological characterization of cranium between Japanese wolf and Mongolian wolf. Forest Call 8: 22–26 (in Japanese)

Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, et al. (2019) BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS Comput Biol 15: e1006650

Endo H, Sasaki T, Itou T, Koie H, Kimura J (2004) Osteological examination and image analysis of a cranium of the Japanese wolf found at private house in Yamanashi Prefecture. Jpn J Zoo Wildl Med 9: 109–114 (in Japanese with English abstract)

Funk H (2015) A re-examination of C. J. Temminck's sources for his descriptions of the extinct Japanese wolf. Archives of Natural History 42: 51–65

Hasegawa M, Kishino H, Yano T (1985) Dating the human-ape split by a molecular clock of mitochondrial DNA. J Mol Evol 22: 160–174

Hiraiwa Y (1992) Ookami –Sono Seitai to Rekishi–. Tsukiji Shokan, Tokyo (in Japanese)

Holthuis LB, Sakai T (1970) Ph. F. von Siebold and Fauna Japonica – A History of Early Japanese Zoology. (Keigaku Shuppan) Academic Press of Japan, Tokyo

Imaizumi Y (1970) Systematic status of the extinct Japanese wolf, *Canis hodophilax*. 1. Identification of specimens. J Mammal Soc Jpn 5: 27–32 (in Japanese with English abstract)

Ishiguro N (2015) DNA analysis of *Canis* bones isolated from Soukokuji-keidai. Rep Hist Doushisha University 13: 160–162 (in Japanese)

Ishiguro N, Inoshima Y, Shigehara N (2009) Mitochondrial DNA analysis of the Japanese wolf (*Canis lupus hodophilax* Temminck, 1839) and comparison with representative wolf and domestic dog haplotypes. Zool Sci 26: 765–770

Ishiguro N, Inoshima Y, Shigehara N, Ichikawa H, Kato M (2010) Osteological and genetic analysis of the extinct Ezo wolf (*Canis lupus hattai*) from Hokkaido island, Japan. Zool Sci 27: 320–324

Ishiguro N, Inoshima Y, Yanai T, Sasaki M, Matsui A, Kikuchi H, et al. (2016) Japanese wolves are genetically divided into two groups based on an 8-nucleotide insertion/deletion within the mtDNA control region. Zool Sci 33: 44–49

Jentink FA (1887) Catalogue ostéologique des Mammifères (Singes, Carnivores, Ruminants, Pachydermes, Sirènes et Cétacés) Muséum d'Histoire Naturelle des Pays-Bas. Vol 9: 71–73 Leiden

Kitamura N, Obara I, Minami M, Nakamura T (1999) A whole skeleton of a Japanese wolf collected from a cave at Mt. Kyonaojo in Izumi-mura Yatsushiro-gun, Kumamoto prefecture. Bull Kumamoto Pref Mus 11: 35–69 (in Japanese with English

abstract)
Koblmuller S, Vila C, Lorente-Galdos B, Dabad M, Ramirez O, Marques-Bonet T (2016) Whole mitochondrial genomes illuminate ancient intercontinental dispersals of grey wolves (*Canis lupus*). J Biogeogr 43: 1728–1738
Komiya H, Shigehara N, Ishiguro N, Kanai H (2011) A new specimen of Japanese wolf, *Canis lupus hodophilax*, found from Fujioka, Gunma prefecture. Bull Gunma Mus Natu Hist 15: 167–170 (in Japanese with English abstract)
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35: 1547–1549
Matsumura S, Inoshima Y, Ishiguro N (2014) Reconstructing the colonization history of lost wolf lineages by the analysis of the mitochondrial genome. Mol Phylogenet Evol 80: 105–112
Miyamoto F (2001) Morphological studies on 6 specimens of Japanese wolf with their skulls. Bull Fac Ed Wakayama Univ Nat Sci 51: 23–32 (in Japanese with English abstract)
Nakamura K (1998) A biogeographic look on the taxonomy of the Japanese wolves, *Canis lupus hodophilax* Temminck, 1839. Bul Kanagawa Prefect Mus (Nat Sci) 27: 49–60 (in Japanese with English abstract)
Naora N (1965) Nihon san ookami no kenkyu [On Wolves in Japan]. Azekura Shobo, Tokyo (in Japanese)
Nehring A (1885) Uber Dachs, Wolf, Hirsch und Wildschwein Japan's. Sitzungs-Berichte der Gesellschaft Naturforschender Freunde zu Berlin 7: 137–143
Obara I (1990) Skulls of Japanese wolf, *Canis hodophilax*, preserved as old private Atsugi-shi and Kiyokawamura. Kanagawa Prefecture. Nat Hist Rep Kanagawa 11: 53–65 (in Japanese)
Obara I (2002) Notes on the specimens of *Canis hodophilax* and Japanese native dog preserved in Naturalis (National Museum of Natural History, Leiden). Animate 3: 17–24 (in Japanese)
Okumura N, Ishiguro N, Nakano M, Matsui A, Sahara M (1996) Intra- and interbreed genetic variations of mitochondrial DNA major non-coding regions in Japanese native dog breeds (*Canis familiaris*). Anim Genet 27: 397–405
Okumura N, Ishiguro N, Nakano M, Matsui A, Shigehara N, Nishimoto T, et al. (1999) Variations in mitochondrial DNA of dogs isolated from archaeological sites in Japan and neighbouring islands. Anthropol Sci 107: 213–228
Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61: 539–542
Saito H (1964) Nihon no Inu to Ookami [Japanese dogs and wolves]. Sekkasha, Tokyo (in Japanese)
Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
Temminck CJ (1839) Over de kennis en de verbreiding der zoogdieren van Japan. Tijdschrift voor Natuurlike Geschiedenis en Physiologie 5: 273–293
Thalmann OB, Shapiro B, Cui P, Schuenemann VJ, Sawyer SK, Greenfield DL, et al. (2013) Complete mitochondrial genomes of ancient canids suggest a European origin of domestic dogs. Science 342: 871–874
Walker B (2005) The Lost Wolves of Japan. University of Washington Press, Seattle
Yagi H, Inoue Y, Oba I, Morita M (2016) Three specimens of *Canis hodophilax* registered in the Natural History Museum Berlin: skulls, whole body skeleton, and fur skin. Animate 13: 76–81 (in Japanese with English summary)
Yoneda M (1997) Notes on the skeleton specimens of Japanese wolf (*Canis lupus hodophilax*). Rep Hist Kase-cho Geogr Sci 183–196 (in Japanese)

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