



NOTE

Anatomy

Computed tomography examination and mitochondrial DNA analysis of Japanese wolf skull covered with skin

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ABSTRACT. A *Canis* skull, right half of the mandible and part of the left half of the mandible were subjected to three-dimensional (3D) computed tomography (CT) observation and mitochondrial DNA (mtDNA) analysis in order to determine whether the specimens belonged to the extinct Japanese wolf, *Canis lupus hodophilax* (Temminck, 1839). Osteometric analysis of the skull and right half of the mandible revealed that the material (JW275) was indeed typical of the Japanese wolf. Sequence analysis of a 600-bp mtDNA region revealed that the JW275 belonged to haplotype Group B, which is characterized by an 8-bp deletion in the mtDNA control region. The findings of this study suggest that 3D CT analysis is well suited to examining fragile and valuable biological samples, as it removes the need for destructive sampling.

KEY WORDS: *Canis lupus hodophilax*, CT, Japanese wolf, mitochondrial DNA, skull

J. Vet. Med. Sci.

79(1): 14–17, 2017

doi: 10.1292/jvms.16-0429

Received: 21 August 2016

Accepted: 4 October 2016

Published online in J-STAGE:
15 October 2016

The Japanese wolf (*Canis lupus hodophilax*, (Temminck, 1893)) is an extinct subspecies of gray wolf that inhabited the islands of Kyushu, Shikoku and Honshu in Japan. The Ezo wolf (*C. lupus hattai* Kishida, 1931), which inhabited the Japanese island of Hokkaido, is also eradicated. Since the last known Japanese wolf was captured in Higashi-Yoshino Village in Nara Prefecture in 1905, there have been no verified sightings or evidence of the Japanese wolf in Japan. In terms of complete specimens, three stuffed specimens are currently held by academic institutions in Japan (National Museum of Nature and Science, the University of Tokyo and Wakayama Prefectural Museum of Nature History), and one specimen is held at the National Museum of Natural History in Leiden, The Netherlands [4, 5, 9, 11, 13]. Given the extreme scarcity of Japanese wolf biological specimens (bones, skin, etc.), relatively few taxonomic and genetic studies have been conducted on this subspecies to date [6–8].

Osteological analysis is considered to be useful for distinguishing between the bones of Japanese wolves and those of domesticated dog breeds that have been collected at archeological sites. For example, the skull of the Japanese wolf has several characteristic features, including (1) a skull length of 200 to 240 mm, (2) snout gently curving and (3) well-developed crista sagittalis externa [1, 2, 10, 12]. In addition, compared with Asian and European wolves and domestic dogs, Japanese wolf populations were also characterized by having several unique nucleotide substitutions in the mitochondrial DNA (mtDNA) control region [6, 8].

We recently had the opportunity to examine a relatively large *Canis* skull covered with skin. The skull (designated as JW275 and held as private collection, Okayama Prefecture) was recovered from an old house constructed in the Edo period in Shimane Prefecture; however, the detailed history of the skull is not known. Since the presence of skin on the skull meant that we were unable to measure the osteological features of the skull directly, we employed computed tomography (CT) and mtDNA analysis to determine whether the skull belonged to a Japanese wolf. The primary advantages of these methods are that they do not involve destructive sampling of the material (in the case of the CT scan), and only an extremely small quantity of biological material (in the case of the genetic analysis), which are important considerations given the potential rarity of the specimen.

Figure 1A shows the dorsal view of the skull. With the exception of the rostrum, the skull was almost entirely covered by bare skin (i.e., without pelage). The skin covered and supported the right half of the mandible, however, the left half of the mandible could not directly be observed. Figure 2A is a photograph showing a left-lateral view of the skull.

To clarify the osteological features of the skull, CT examinations were conducted using a CT scanner (Aquilion LD, Toshiba, Tokyo, Japan) with the following scan parameters (accelerate voltage: 120 kV; currents: 150 effective mAs; and slice thickness: 0.5 mm). The skull specimen was serially sectioned using the CT scanner, and a 3D image was reconstructed using an image processing workstation (Virtual Place Fujin, AZE, Tokyo, Japan). A 3D CT image of the dorsal view of the skull is shown in Fig. 1B.

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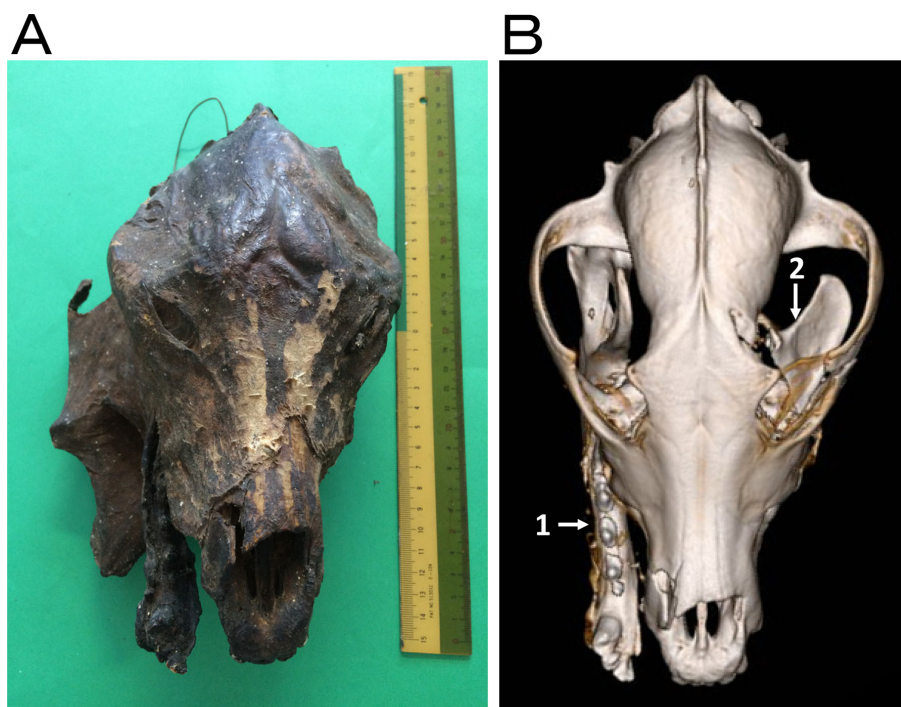


Fig. 1. Dorsal view of Japanese wolf skull (JW275). A: Photograph showing dorsal view of gross external morphology of the skull covered with skin, with right half of the mandible displaced to the right and covered by skin. B: Three-dimensional (3D) CT image of a dorsal view of the skull specimen. The 3D-CT image shows the skull, right half of mandible (arrow 1) and part of the left mandible (Proc. coronoideus, arrow 2).

The CT image of the skull shows several features characteristic of the Japanese wolf, such as (1) a relatively well developed anterior border of the pterygoid fossa and (2) a relatively short viscerocranium length. These morphological characters are consistent with the unique features of the Japanese wolf reported previously [1, 2, 10, 12]. The right half of the mandible (arrow with 1 in Fig. 1B) and a broken piece of the left half of the mandible (arrow with 2 in Fig. 1B), which were both covered by skin, are observed in the CT image.

Figure 2B is a 3D CT image of a right-lateral view of the skull. The image clearly shows features considered to be characteristic of the Japanese wolf, such as (1) a gentle arch to the snout (arrow 3) and (2) a well-developed sagittal crest (arrow 4). A relatively large molar (M1) in the right half of the mandible (arrow 5) and the broken left half of the mandible (arrow 2) are observed in the CT image (Fig. 2B).

Osteometric measurements of the cranium and the right half of the mandible are shown in Table 1. Although considerable care was taken with the measurements in the CT image, some important skull landmarks could not be accurately measured. However, four cranial landmarks and two mandibular landmarks of the JW275 were compared with representative skulls of nine Japanese wolves and an Akita dog. The maximum skull length of the JW275 was larger than those of the JW240, the JW257 and Wakayama, but smaller than those of the JW229 and the JW239. The mandibular length of the JW275 was larger than those of the JW240 and the JW258, but smaller than those of the JW237 and the JW239. The measurements obtained for the JW275 skull revealed that it was a middle Japanese wolf and that it was morphologically distinct from an Akita dog (Table 1).

The tissue for DNA analysis was obtained from occipital condyle, as described by Ishiguro *et al.* [6]. The purified DNA samples were used to amplify a ca. 600-bp sequence of the mtDNA control region using primers mit44, mit57, mit134, mit135 and mit123 as described previously [6, 7]. The PCR products were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.), and the obtained sequences were compared with sequences determined for Japanese wolves in a previous study [7]. Except for two sequence substitutions at positions (T180C and A475G), the 590-bp mtDNA sequence obtained from the JW275 skull was identical to the sequence obtained from another skull, JW237 (Accession No. AB480738), which belonged to the mitochondrial Group B haplotype [7]. Thus, the mtDNA analysis confirmed that the JW275 skull was indeed that of a Japanese wolf.

The JW275 skull is also unique in that it is almost entirely covered with skin, which meant that the skull could not be identified to species by direct osteometric measurements. Another Japanese wolf skull that was partially covered with skin (JW255) was found in Yamanashi Prefecture [6]. Since the skin on the latter skull covered the rostral area, CT imaging analysis was performed on that skull to analyze the nasal structure [3]. In another study, CT imaging analysis was used to compare several Japanese wolf skulls with Akita dog skulls [2]. In combination with this study, these previous studies [2, 3] demonstrated that CT imaging is useful for examining the morphological characteristics of Japanese wolf bones, especially when the bones of the skull are obscured by other tissues, such as skin. In addition, mtDNA analysis is also useful for distinguishing Japanese wolf specimens from those of

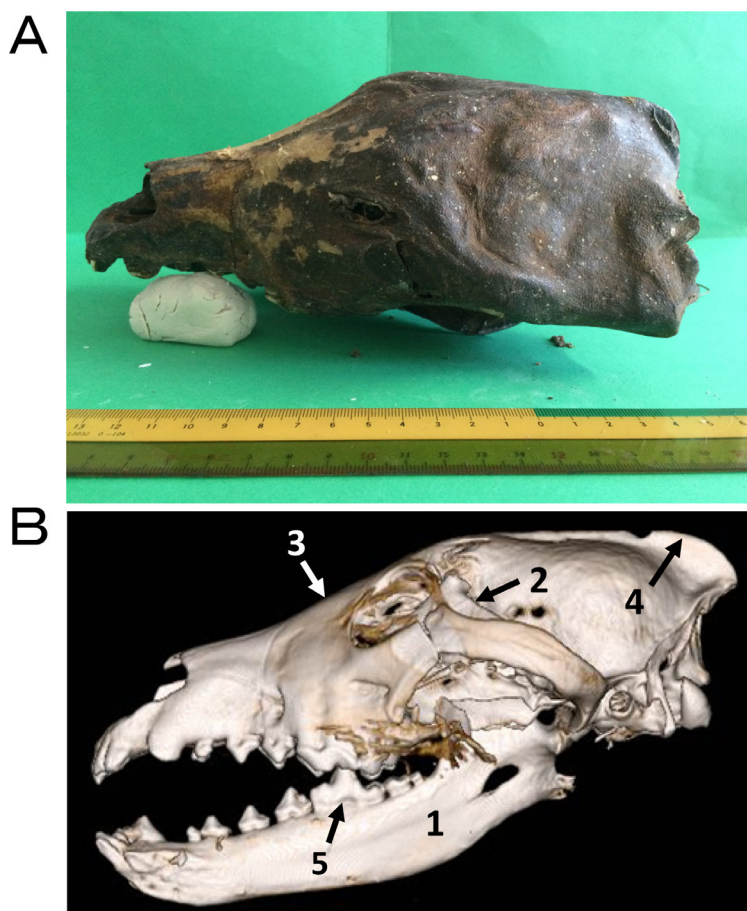


Fig. 2. Left lateral view of Japanese wolf skull (JW275). A: Photograph showing the skull specimen covered with skin. B: Three-dimensional (3D) CT image of the skull examined from the left side. The 3D-CT image shows the cranium, right half of the mandible (1) and part of the left mandible (arrow 2). 3: snout; 4: crista sagittalis externa; 5: M1 in the right mandible.

Table 1. Morphometric measurements of skull and mandibular features of skull JW275, skulls from representative Japanese wolves and the skull of an Akita dog

Site and measurements (mm)	Japanese wolf (<i>C. l. hodophilax</i>) ^{a)}										Reference	
	JW275	JW229	JW237	JW239	JW240	JW255	JW257	JW258	JW259	Japanese wolf		Akita dog
Recovery site (Prefecture)	Shimane	Kouchi	Kanagawa	Kanagawa	Kumamoto	Yamanashi	Hiroshima	Nagano	Ehime	Wakayama	ND	
Skull (item ^{b)})	(points ^{c)})											
Max. skull L.	pr-i	225.4	235.8	-	228	218.8	-	222	-	-	219.2	201.9
Condylbasal L.	pr-o.c	-	216.6	-	-	204.6	-	-	-	-	205.2	194
Zygomatic B.	zy-zy	126.8	128.5	-	127.8	129.6	126.3	-	-	117.7	123.4	119.3
Palatal L.	pr-sta	104.6	-	-	107.5	103.5	10.79	112	105.8	95.6	108.9	101.4
Mandible (item)	(points)											
Mandibular L.	id-cpost	165.7	-	172.2	166.9	160.8	-	-	161.1	-	-	-
Ramus H.		66	-	-	-	-	-	-	-	-	67.2	59.5
mtDNA haploype groups		B	A	B	A	A	A	A	A	A	ND	NA

-, not measured; ND, not detected; NA, not adapted. a) The measured values for several Japanese wolf specimens are quoted from Reference 6. b) L, Length; B, Breadth; H, Height. c) pr-i, prosthion-inion; pr-o.c, pro-occipital condyles; zy-zy, zygion-zygion; pr-sta, prosthion-staphylion; id-cpost, infradetales-condylion posterior.

domesticated dogs.

The findings of this study therefore showed that the JW275 skull was indeed typical of a Japanese wolf belonging to mitochondrial haplotype Group B, which is characterized by an 8-bp deletion in the control region of mtDNA [7]. The Japanese wolf JW275 was found in Shimane prefecture, suggesting that Japanese wolves belonging to mitochondrial haplotype Group

B distributed in relative large area of west and east parts of Honshu island [7]. In addition, the middle size, good condition and presence of skin on the JW275 skull makes this discovery a particularly important find among the Japanese wolf skulls collected to date.

ACKNOWLEDGMENT. The work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 23405040) from the Ministry of Education, Science, Sports and Culture, Japan.

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