Morphologic and Genetic Evidence for the Kinship of Juvenile Skeletal Specimens from a 2,000 Year-old Double Burial of the Usu-Moshiri Site, Hokkaido, Japan

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Abstract This paper presents a case study of a double burial at the 2,000 year-old Usu-Moshiri Epi-Jomon site in Hokkaido, Japan, using detailed odontometric analysis in conjunction with mitochondrial DNA (mtDNA) analysis in order to more precisely assess their kinship. Consanguinity between the two juveniles found in this burial was suggested by the archaeological context as well as by preliminary morphological studies of the skeletons. Mitochondrial DNA sequences, while consistent with their maternal kinship, did not provide decisive evidence. Supplementing and strengthening the kinship determination, Q-mode correlations between these two individuals with respect to tooth crown measurements and proportions were consistently positive and very high. Statistical analysis of the difference between tooth size correlations in this pair, and those in pairs of related and unrelated individuals from other Japanese populations indicated that they likely had been first-degree relatives. Considering that the two individuals had been approximately seven and eleven years old at the time of their interment side by side, we concluded that they likely had been siblings rather than other first-degree maternal relatives. This study demonstrated that combining odontological analysis with mtDNA testing may be effective for reliable and detailed kinship assessment of ancient skeletal remains.

Keywords: odontological kinship analysis, tooth size proportion, ancient DNA, multiple burial, prehistoric Japan

Introduction

The relationship between individuals from a multiple burial is important for inference about funerary behavior. It is especially important to clarify the relationship between individuals from a double or triple burial because of the possibility that such individuals were consanguineous or had a strong social relationship. Cases of

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double or triple burial are described for the Middle Paleolithic, e.g., Qafzeh 9 and 10 (Bar-Yosef and Vandermeersch, 1993), Upper Paleolithic, e.g., Barma Grande 2, 3 and 4 (Formicola, 1990); Dolní Věstonice 13, 14 and 15 (Klíma, 1988), and, in fact, for several regions and times worldwide. Although its assessment is difficult, the relationship between such individuals represents crucial evidence in reconstructing prehistoric funerary behavior.

Conventionally, kinship analyses of ancient skeletal remains have been performed based on morphologic studies by using the similarity of tooth size proportion and non-metric variation of the skull and teeth (e.g., Spence, 1974; Sjøvold, 1975; Doi et al., 1985, 1986; Alt et al., 1997). Hereditary system of tooth size proportion has been reasonably established (Potter et al., 1976; Townsend and Brown, 1978a, b), and statistically confirmed to reflect the hereditary background of both parents to some extent through studies based on consanguineous modern Japanese (Doi et al., 1986). This method has been applied to specific cases to infer kinship of prehistoric Jomon and protohistoric Kofun people (Hanihara et al., 1983; Doi et al., 1986; Tanaka, 1995; Matsumura and Nishimoto, 1996).

Morphologic methods are still useful, however, they cannot provide decisive information about kinship. DNA analysis, an established method in forensic kinship determination, has been applied successfully to ancient skeletal specimens (e.g., Kurosaki et al., 1993; Oota et al., 1995; Delefosse and Hänni, 1997; Shinoda et al., 1998; Shinoda and Kanai 1999). The objective of the present study is to demonstrate the value of using two independent analytic methods to assess kinship: (1) mitochondrial DNA (mtDNA) testing, and (2) similarity of the tooth size proportion.

Materials and Methods

Skeletal materials

Excavations at the Usu-Moshiri site located on a small island in Usu Bay of Date City, southwestern Hokkaido (Fig. 1), were carried out from 1985 to 1988 under the direction of one of the authors (Y.D.). These excavations disclosed 21 pit-graves of the Final Jomon to Epi-Jomon periods and unearthened skeletal remains of more than 50 individuals associated with many grave goods (Ohshima et al., 1989, 1990). Two double burials were found at the site: one unearthened in 1985 consisted of two juvenile individuals, and the other consisted of two adult females discovered in 1988. The juvenile skeletal specimens were remarkably well preserved, and were named 3A and 3B, respectively. These skeletons were found in squatting position between rocks covered with broken shell layer (Fig. 2). Because these skeletons were situated side by side and maintained most of their anatomical articulations, they were judged to have been buried simultaneously.

In order to date this burial, long bone fragments of each skeleton were submitted for ^{14}C age determination using the accelerator mass spectrometer procedure at NIES-

Figure 1. Map of the Ushu-Moshiri site, located in the southwestern part of Hokkaido, Japan.

TERRA (Tanaka et al., 2000). Details concerning radiocarbon dating of the bone materials are provided elsewhere (Yoneda et al., 2002). The radiocarbon ages for these skeletons were $2,340 \pm 79$ years B.P. for 3A (TERRA-112599a07), and $2,486 \pm 87$ years B.P. for 3B (TERRA-112599a08). These two results were indistinguishable at the probability level of 95% and the weighted average was $2,406 \pm 58$ years B.P. (Ward and Wilson, 1978). Therefore, the two individuals were considered to be contemporaneous, keeping in mind the caveat that radiocarbon ages might be affected by marine reservoir effect (Yoneda et al., 2002).

Preservation and formation stages of the permanent teeth in specimens 3A and 3B are shown in Table 1 and Fig. 3. Formation stages of the teeth are defined by Moorrees et al. (1963). Since the number of remaining mandibular permanent teeth of both specimens was small, the age prediction method proposed by Smith (1991) was difficult to apply in this case. Alternatively, based on the classical chart of Schour and Massler (1941), the ages of these skeletons were estimated to be about 11 years for 3A and 7 years for 3B. These results do not contradict those obtained by using the data of Moorrees et al. (1963).

Sequence analysis of mtDNA

(1) Contamination precautions

No modern DNA-based study was performed previously in the area where these

Figure 2. Plan of the burial of skeletal specimens 3A and 3B. Dotted bones belong to skeletal specimen 3A, and dark shaded bones belong to skeletal specimen 3B. Clear bones are unidentified. (r) indicates the right, and (l) indicates the left side of the skeletal specimen. ①, ②, ③, ④, ⑤, ⑥, and ⑦ denote the left ilium, mandible, left femur, left humerus, left fibula, left tibia, and left ulna, respectively.

experiments were carried out. Standard contamination precautions, such as separation of pre- and post-PCR experimental areas, use of disposable laboratory wares and filter-plunged pipette tips, treatment with DNA contamination removal solution (DNA-OFF™, TaKaRa) and UV ray irradiation to the equipment and benches, negative extraction controls and negative PCR controls, were employed in this study.

To identify and exclude any possible contamination with the modern DNA, mtDNA sequence of the individuals who handled the skeletons at any stage of retrieval and analysis was determined in all regions examined after the completion of ancient DNA analysis.

(2) Extraction and purification of DNA

DNA was extracted from the right upper canine of specimen 3A and the right upper first deciduous molar of specimen 3B. The outer surface of these teeth was removed for about 1 mm in depth using a dental drill (VIVA-MATE III, NAKAN-

Table 1. Preservation and formation stages¹⁾ of the permanent teeth in specimens 3A and 3B

Tooth	3A		3B	
	R	L	R	L
Upper first incisor (UI1)	—	Ac	R _{1/4}	R _{1/4}
Upper second incisor (UI2)	A _{1/2}	—	—	—
Upper canine (UC)	R _{3/4}	—	R _{1/4}	—
Upper first premolar (UP1)	R _{3/4}	R _{3/4}	R _{1/4}	—
Upper second premolar (UP2)	R _{1/2}	R _{1/2}	R _{1/4}	—
Upper first molar (UM1)	Ac	Ac	R _{3/4}	R _{3/4}
Upper second molar (UM2)	R _{1/2}	R _{1/2}	—	—
Lower first incisor (LI1)	—	—	R _{1/2}	R _{1/2}
Lower second incisor (LI2)	—	—	—	—
Lower canine (LC)	—	—	—	—
Lower first premolar (LP1)	R _{3/4}	—	R _{1/4}	—
Lower second premolar (LP2)	—	—	Ri	—
Lower first molar (LM1)	—	—	—	—
Lower second molar (LM2)	—	—	—	—

¹⁾Defined by Moorrees et al. (1963): Ri, Initial root formation; R_{1/4}, Root length 1/4; R_{1/2}, Root length 1/2; R_{3/4}, Root length 3/4; A_{1/2}, Apex 1/2 closed; Ac, Apical closure complete. Note: The minus sign (—) denotes that the tooth was not preserved in the specimen.

Figure 3. Skulls of skeletal specimens 3A and 3B. From the state of tooth formation, the ages of these skeletal specimens were estimated to be about 11 years for 3A and 7 years for 3B.

ISHI INC.). Then these teeth were washed once with DNA contamination removal solution (DNA-OFF™, TaKaRa) and twice with deionized distilled water (ultra PURE™ Distilled Water, GIBCO BRL) and allowed to air dry at 37°C for 2 hrs. Then these teeth were pulverized under liquid nitrogen in a mill (CRYO-PRESS CP-100W, Microtech Nichion). DNA was then extracted from the pulverized sample by using a GENE CLEAN® Kit For Ancient DNA (BIO 101) according to the technical manual.

(3) Amplification of mtDNA

Two point five micro liters of extracts were used as the first PCR templates. Amplifications were carried out in a 25- μ l total reaction volume containing 2.5 units of Taq DNA polymerase (HotStarTaq™ DNA polymerase, QIAGEN), 0.2 μ M of each primer, 200 μ M dNTPs in 1 $\times$ PCR buffer (QIAGEN). Primers used for the first PCR are listed in Table 2. These primers were designed to obtain PCR fragments that contain highly polymorphic positions in previously reported Japanese populations (Horai et al., 1989; Yoshii et al., 1995; Takada and Mukaida, 1996a, b; Sekiguchi et al., 1997; Seo et al., 1998; Nata et al., 1999), and are short enough for easier amplification. Among the nucleotide positions analyzed in the present study, region 16485–75 appeared to be less polymorphic, however, the region contains some informative polymorphic positions, especially position 16519, in Japanese populations (Takada and Mukaida, 1996b).

The first PCR was conditioned as follows: following an initial 15 minutes incubation at 95°C, PCR was run for 34 cycles of 1 min at 94°C, 1 min at 46°C, 1 min at 72°C in a PCR machine (TP240, TaKaRa). After the first PCR, PCR products were purified with a Quickstep™ PCR Purification Kit (Edge Bio Systems).

The second PCR was carried out by using 1- μ l aliquots of purified first PCR products as templates, adding them to 24 μ l of fresh PCR reaction mixture. The constitution of this fresh reaction mixture was identical to that of the first PCR. The condition of the second PCR was the same as that of the first PCR except for the annealing temperature as 50°C instead of 46°C and the number of PCR cycles as 20 instead of 34. In the second PCR, the initial primers that were attached with -21M13 and M13 reverse sequences were used for direct sequencing (Table 2). By attaching -21M13 and M13 reverse sequences to the primers, all the PCR products can be subjected to the direct sequencing using -21M13 and M13 reverse primers only, therefore, error-prone and time-consuming processes of preparing sequence reactions for different regions with different primers were avoided. For cloning and sequencing, primers identical to the initial ones were used.

(4) Sequence analysis

After the second PCR, the PCR products were purified again by using a Quick-

Table 2. Primers used for PCR

Primer	Sequence ¹	Product length (bp)	Reference
Mit 1-L	16190-5'-CCCCATGCTTACAAGCAAG-3'-16208	233	Horai et al. (1989)
Mit 1-H	16422-5'-ATTGATTTACGGAGGATGG-3'-16403		
Mit 2-L	16466-5'-ACTTGGGGGTAGCTAAAGT-3'-16484	199	The present study
Mit 2-H	95-5'-TCTCGCAATGCTATCGCGTG-3'-76		
Mit 3-L	108-5'-AGCACCCCTATGTCGCAGTAT-3'-127	179	The present study
Mit 3-H	286-5'-TGTTATGATGCTCTGTGTGG-3'-268		
Mit 4-L	15980-5'-CCATTAGCACCCAAAGCTA-3'-15998	182	The present study
Mit 4-H	16161-5'-ATGTACTACAGGTGGTCAAAG-3'-16142		
M13-Mit 1-L	-21M13 sequence ² is attached to the 5' end of Mit 1-L		
M13-Mit 1-H	M13 reverse sequence ³ is attached to the 5' end of Mit 1-H	267	Nata et al. (1999)
M13-Mit 2-L	-21M13 sequence is attached to the 5' end of Mit 2-L		
M13-Mit 2-H	M13 reverse sequence is attached to the 5' end of Mit 2-H	233	The present study
M13-Mit 3-L	-21M13 sequence is attached to the 5' end of Mit 3-L		
M13-Mit 3-H	M13 reverse sequence is attached to the 5' end of Mit 3-H	213	The present study
M13-Mit 4-L	-21M13 sequence is attached to the 5' end of Mit 4-L		
M13-Mit 4-H	M13 reverse sequence is attached to the 5' end of Mit 4-H	216	The present study

¹Numbers identify the base at the 5' and 3' ends according to the revised CRS (Andrews et al., 1999).

²-21M13 sequence represents 5'-TGTAACGACGGCCAGT-3'

³M13 reverse sequence represents 5'-AACAGCTATGACCATG-3'

Table 3. Combinations of tooth crown diameters and Q-mode correlation coefficients

Combination of crown diameters ¹	Number of variables	Q-mode correlation coefficients
(1) MD (UI1•UC•UP1•UP2•UM1•LP1)• BL (UP1•UP2•UM1•LP1)	10	0.879
(2) MD (UI1•UC•UP1•UP2•UM1)• BL (UP1•UP2•UM1)	8	0.888
(3) MD (UC•UP1•UP2•UM1•LP1)• BL (UP1•UP2•UM1•LP1)	9	0.888
(4) MD (UC•UP1•UP2•UM1)• BL (UP1•UP2•UM1)	7	0.921
(5) MD (UP1•UP2•UM1•LP1)• BL (UP1•UP2•UM1•LP1)	8	0.854
(6) MD (UP1•UP2•UM1)• BL (UP1•UP2•UM1)	6	0.888
(7) MD (UI1•UC•UP1•UP2•UM1•LP1)	6	0.947

¹MD: mesiodistal diameter, BL: buccolingual diameter, UI1: upper first incisor, UC: upper canine, UP1: upper first premolar, UP2: upper second premolar, UM1: upper first molar, LP1: lower first premolar. All the teeth except UI1 were measured on the right side of each skeletal specimen.

step™ PCR Purification Kit (Edge Bio Systems). As described above, the PCR products obtained with M13 sequences-attached primers (M13-Mit 1~4-L and H) were used for direct sequencing. Sequencing reactions were prepared by using -21M13 and M13 reverse primer and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to the technical manual and analyzed on a 377 DNA Sequencer with SeqEd software (Applied Biosystems Division / Perkin-Elmer). Determined sequences were compared with the revised Cambridge reference sequence (CRS) (Andrews et al., 1999). Both light and heavy strands were analyzed for all samples.

When any kind of ambiguity was observed in the result of direct sequencing, cloning and sequencing was performed. To obtain PCR clones, 20 µl of the purified second PCR products obtained with primers Mit 1~4-L and H were ligated into pBlue-script SK(-) and cloned in XL1-Blue MRF' cells (Stratagene). Plasmid DNA from these clones was prepared by using a FlexiPrep™ Kit (Amersham Pharmacia Biotech) and sequenced as described above.

Similarity of tooth size proportion

(1) Measurement

With the unerupted teeth of specimen 3B removed from the alveoli, permanent tooth crowns were measured to the nearest 0.1mm with a digital sliding caliper (NTD12-15C, Mitsutoyo) according to the method of Fujita (1949). The teeth of both skeletons were not worn and very well preserved for morphological study, a

Table 4. Variable sites in the mitochondrial DNA sequences examined in the present study for skeletal specimens 3A and 3B determined by direct DNA sequencing

No. 16209~16402	16223	16394	No. 16485~75	16519	73	No. 128~267	263
CRS	C	C		T	A		A
3A	T	• ¹		C	G		G
3B	T	N ²		C	G		G

¹Dot indicates that no nucleotide change was observed.

²The letter N indicates 'not determined'.

Note: no nucleotide change was observed in positions 15999 to 16141 both in specimens 3A and 3B.

reliable metric analysis could be performed. Only teeth of the right side were measured except for the left upper first incisor. Mesiodistal diameters of the left upper first incisor and right upper canine, and mesiodistal and buccolingual diameters of the right upper first and second premolars, right upper first molar, and right lower first premolar were measured for the specimens 3A and 3B.

(2) Estimation of the similarity of tooth size proportion

Based on the tooth crown measurements, Q-mode correlation coefficients (Sokal and Sneath, 1963) were calculated between specimens 3A and 3B. The combinations of tooth crown diameters used for calculation of the Q-mode correlation coefficients are shown in Table 3. Before calculation, each crown diameter was standardized by using the means and standard deviations of the sex-combined series of the Jomon data reported by Matsumura (1995).

Results

Sequence analyses of mtDNA

Both negative extraction controls and negative PCR controls consistently showed negative results throughout the study. Moreover, all members who handled the skeletal specimens at any stage of retrieval and analysis were confirmed to be different from the two ancient specimens in term of mtDNA lineage. Consequently, the possibility of contamination with modern DNA was considered to be low.

The two skeletal specimens, 3A and 3B, shared the same sequences except for the region 16209–16402 (Table 4). T-to-C substitution at position 16519 (16519 T → C) and 73 A → G were observed in the region 16485–75, 263 A → G was observed in the region 128–267, and no nucleotide change was recognized in the region 15999–16141. In the region 16209–16402, both 3A and 3B showed 16223 T → C. However, in 3B, the sequence at position 16394 was very ambiguous and could not be determined by direct sequencing (Table 4).

To verify the sequence of 3B in the region 16209–16402, we cloned the amplified

Table 5. The sequence of positions 16209 to 16402 for skeletal specimens 3A and 3B determined by using direct and cloned DNA sequences

	16223	16224	16248	16249	16256	16260	16262	16264	16278	16311	16313	16329	16332	16339	16355	16360	16392	16393	16394	16395
CRS	C	T	C	T	C	C	C	C	C	T	C	G	C	C	C	C	T	C	C	C
direct ¹ 3A	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
direct ¹ 3B	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	N ²	•
3B-1-C1 ³	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-1-C2	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-1-C3	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-1-C4	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-1-C5	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	N	N	N	N
3B-1-C6	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-1-C7	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-1-C8	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-1-C9	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-1-C10	T	•	T	C	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-2-C1	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-2-C2	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-2-C3	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	N	N	N
3B-2-C4	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-2-C5	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	N	N	N	N
3B-2-C6	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
3B-2-C7	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•
consensus ⁴ 3B	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	N	•
direct 3B-E ⁵	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-1-C1 ⁶	T	•	•	•	•	T	T	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-1-C2	T	•	•	•	•	T	T	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-1-C3	T	•	•	•	T	•	•	•	•	•	•	•	A	T	•	•	•	•	•	•
3B-E-1-C4	T	•	•	•	T	•	•	•	T	•	T	•	•	•	•	•	•	•	•	•
3B-E-1-C5	T	•	•	•	•	T	T	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-1-C6	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C1	T	•	•	•	•	•	•	•	•	C	•	•	•	•	T	•	•	•	•	•
3B-E-2-C2	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C3	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C4	T	•	•	•	•	•	•	•	•	•	•	A	•	•	•	•	•	•	•	•
3B-E-2-C5	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C6	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C7	•	C	•	•	•	•	•	T	•	C	•	•	•	•	•	•	•	•	•	•
3B-E-2-C8	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	T	•	•	•	•
3B-E-2-C9	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C10	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
3B-E-2-C11	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
consensus ⁴ 3B-E	T	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•

¹The sequence determined by direct sequencing.²The letter N indicates 'not determined'.³3B-1-Cn: clones derived from the first PCR. 3B-2-Cn: clones derived from the second PCR.⁴The sequence determined using cloned DNA sequences.⁵3B-E denotes that left upper second deciduous molar of specimen 3B was used as a DNA extraction template.⁶3B-E-1-Cn: clones derived from the first PCR. 3B-E-2-Cn: clones derived from the second PCR.

fragments of that region obtained from two independent PCRs and sequenced them. Even after this cloning and sequencing step, the sequence at position 16394 remained unclear and resulted in different sequences (Table 5). In the clones derived from the first PCR (3B-1-C1-C10), 4 clones showed no mutations, while 5 clones showed C → T substitution at this position. And in 1 clone the sequence at this position was still too ambiguous to be determined. In the clones derived from the second PCR (3B-2-C1-C7), 2 clones showed no mutations, 3 clones showed C → T substitution, and 2 clones showed very ambiguous sequence at this position.

Re-analysis of the specimen 3B by using the left upper second deciduous molar as a DNA extraction template was performed. The result was that the DNA sequences determined directly from the amplified products were identical to those of the specimen 3A for all regions examined. Moreover, in the region 16209–16402, no PCR clones from two independent PCRs showed 16394 C → T (Table 5). Therefore, the mtDNA sequences for specimens 3A and 3B were confirmed to be identical for all regions examined.

Similarity of tooth size proportion

In any combinations of the tooth crown diameters examined in this study, Q-mode correlations were consistently positive and very high. As shown in Table 3, the Q-mode correlation coefficients were calculated at 0.879 for the combination (1), 0.888 for the combination (2), 0.888 for the combination (3), 0.921 for the combination (4), 0.854 for the combination (5), 0.888 for the combination (6), and 0.947 for the combination (7). The mean value of these Q-mode correlation coefficients was 0.895.

Discussion

Sequence analysis of mtDNA

In the first analysis, mtDNA sequence of the specimen 3B at position 16394 was very ambiguous. 16394 C → T has never been reported in any previous studies on mtDNA polymorphisms (cf. MITOMAP: a human mitochondrial genome database, Kogelnik et al. 1996) and none of the members could be a possible source of contamination. Therefore, modern DNA contamination was excluded, and this nucleotide change was assumed to reflect degeneration of DNA or heteroplasmy. However, if this mixed sequence was caused by heteroplasmy, we would obtain clear data by cloning and sequencing. In the present study, the sequences of some clones at position 16394 were still too unclear after the cloning step. Moreover, no ambiguity was observed at this position in the re-analysis of the specimen 3B. Therefore, this ambiguity was considered to be caused by the degeneration of DNA.

As shown in the results, both skeletal specimens showed the same sequence in all regions examined. In the region 16209–16402, 16223 T → C was observed. This sequence is relatively rare in modern Japanese population; 1 of 100 individuals (Yoshii

et al., 1995; Seo et al. 1998), 0 of 103 (Takada and Mukaida, 1996a), 2 of 55 (Sekiguchi et al., 1997), and 2 of 152 (Nata et al., 1999). 16519 T → C and 73 A → G were observed in the region 16485–75. This sequence was recognized in 23 of 103 unrelated Japanese (Takada and Mukaida, 1996b). 263 A → G was observed in region 128–267. This sequence was reported in 11 of 55 (Sekiguchi et al., 1997) and 31 of 100 modern Japanese (Seo et al., 1998). No nucleotide change was observed in region 15999–16141. In modern Japanese, this sequence was found in 32 of 55 (Sekiguchi et al., 1997) and 62 of 100 (Seo et al., 1998).

Sekiguchi et al. (1997) and Seo et al. (1998) analyzed the three regions examined in the present study (region 16209–16402, 128–267, and 15999–16141), however, the same sequence as specimens 3A and 3B had not been found in these previous studies ($n = 155$). Consequently, it is apparent that the mtDNA sequence shown in 3A and 3B is distinctive, at least compared with modern Japanese population. The fact that these specimens shared the same distinctive sequence strongly suggests a maternal relationship between these two individuals.

Similarity of tooth size proportion

Since the variances of tooth size measurements in the Jomon population are almost the same as modern Japanese (Matsumura, 1995), the data of modern Japanese can be used to infer the consanguinity between specimens 3A and 3B. As indicated by Q-mode correlation coefficients, the tooth size proportions of the specimens 3A and 3B were very similar. This strength of similarity is relatively rare even in pairs of consanguineous individuals in the modern Japanese population (Doi et al., 1986).

Nevertheless, there is always a risk of stochastic resemblance. Matsumura and Nishimoto (1996) examined 1,378 unrelated pairs of modern Japanese teeth and reported that, with regard to the combinations (4) and (6) shown in Table 3, the rates of stochastic resemblance were 4.3% for (4) and 6.7% for (6) when the Q-mode correlation coefficient of 0.7 was regarded as a criterion of kinship. In the present study, however, since the Q-mode correlation coefficients are about 0.9, the rate of stochastic resemblance must be much lower. Consequently, although the risk of stochastic resemblance cannot be completely excluded, the very high Q-mode correlation coefficients between specimens 3A and 3B strongly suggest the existence of kinship between these two individuals.

The relationship between 3A and 3B inferred from genetic and morphologic analyses

Although some ancient specimens shared the same distinctive mtDNA sequence, it is still possible that the sequence they shared was common around the area they lived in at that time. In this case, validity of the kinship suggested by the mtDNA analysis would be less secure.

Table 6. Comparison of frequencies of the pairs that show high Q-mode correlation coefficients in related and unrelated modern Japanese

(1) Frequencies of the pairs whose Q-mode correlation coefficients exceed 0.7 in related and unrelated modern Japanese			
	number of all pairs	number of pairs whose Q-mode correlation coefficients exceed 0.7	frequency (%)
unrelated	435	11	2.5
first-degree relatives	68	12	17.7**
second-degree relatives	54	4	7.4
third-degree relatives	45	3	6.7
(2) Frequencies of the pairs whose Q-mode correlation coefficients exceed 0.8 in related and unrelated modern Japanese			
	number of all pairs	number of pairs whose Q-mode correlation coefficients exceed 0.8	frequency (%)
unrelated	435	4	0.9
first-degree relatives	68	4	5.9*
second-degree relatives	54	1	1.9
third-degree relatives	45	0	0.0

*, ** Significantly different at the levels of 5% and 1%, respectively, from the unrelated group.

In the present study, however, since the maternal kinship suggested by the mtDNA analysis is strongly corroborated by the result of odontologic kinship analysis, specimens 3A and 3B are very likely to have been maternal relatives. In view of the estimated ages of specimens 3A and 3B, their possible relationship is limited to siblings (first-degree relatives), maternal uncle/aunt — nephew/niece (second-degree relatives), or maternal cousins (third-degree relatives). The degree of relatives can be determined if STR and/or VNTR loci in chromosomal DNA can be analyzed, and these loci were successfully analyzed in some studies on ancient DNA (e.g., Kurosaki et al., 1993; Hummel et al., 1999). Unfortunately, STR and VNTR analyses were not successful in the present study, therefore, the similarity analysis of tooth size proportion is the only possible way to clarify the detailed kinship between specimens 3A and 3B.

As described in the results, the similarity of tooth size proportion between these two specimens was extraordinary high. Therefore, we analyzed the frequencies of the pairs that showed positive and high-valued Q-mode correlation coefficients in related and unrelated modern Japanese using the raw data of Doi et al. (1986). Since the combination (1) shown in Table 3 had the largest number of variables in the present study, we used this combination to compare the frequencies of the pairs that have relatively high Q-mode correlation coefficients between related and unrelated groups.

The frequencies of the pairs whose Q-mode correlation coefficients exceed 0.7 in

related and unrelated modern Japanese are shown in Table 6 (1). When comparing the frequencies of these pairs using Fisher's exact probability test, there were no significant differences between any consanguineous groups at the 5% level. However, there was a significant difference between the unrelated group and the first-degree relatives at the 1% level, whereas no significant difference was found between the unrelated group and the second or third degree relatives at the 5% level. Using the same combination of dental variables, the frequencies of the pairs whose Q-mode correlation coefficients exceed 0.8 were calculated as shown in Table 6 (2). Fisher's exact probability test also revealed no significant differences between any consanguineous groups at the 5% level. However, there was a significant difference between the unrelated group and the first-degree relatives at the 5% level. No significant difference was observed between the unrelated group and the second or third degree relatives. The Q-mode correlation coefficient between the specimens 3A and 3B using the same combination of dental variables is 0.879. Consequently, they are considered to have been first-degree relatives, siblings in this case, rather than other maternal relatives.

Advantage, application and limit of the kinship analyses performed in the present study

In the present study, the validity of the maternal relationship suggested by the mtDNA analysis was strongly supported by the result of odontologic kinship analysis. Reciprocally, the risk of stochastic resemblance, the biggest problem in similarity analysis of tooth size proportion, could be excluded by mtDNA analysis. Therefore, the two methods are complementary. This joint analysis based on morphologic and genetic methods is especially useful for assessing consanguinity between individuals from prehistoric double or triple burials.

In a case where the individuals from such a burial share the same mtDNA sequence but have very different tooth size proportions (e.g., Q-mode correlation coefficient is below zero), those individuals might have been distant maternal relatives or unrelated persons such as a married couple who happened to share the same mtDNA sequence.

In the present study, since the teeth of both skeletal specimens were not worn and very well preserved for morphological study, reliable metric analysis could be performed. When the quality and quantity of the teeth are inappropriate for metric analysis and/or the result of metric analysis are considered to be insufficient to infer the kinship between individuals, non-metric variation of teeth and skulls should be used to corroborate the validity of kinship suggested by DNA analysis.

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