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A Genetic Study of 2,000-Year-Old Human Remains From Japan Using Mitochondrial DNA Sequences

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ABSTRACT We present nucleotide sequence data for mitochondrial DNA extracted from ancient human skeletons of the Yayoi era (ca. 2,000 BP) excavated from the Takuta-Nishibun site in northern Kyushu of Japan. Nucleotide sequence diversity showed that the Yayoi people of the Takuta-Nishibun site were not a genetically homogeneous population. This site shows a diversity in the burial style. Phylogenetic analysis indicated a statistically significant correlation between burial style and the genetic background of the Takuta-Nishibun individuals, and revealed no discrete clustering patterns for the Yayoi individuals, for early modern Ainu, or for the Jomon people.

When ancient DNA analysis was initiated, cloning of the DNAs extracted from organic materials was the only technique available for amplifying DNAs (Higuchi et al., 1984; Johnson et al., 1985; Pääbo, 1985; Doran et al., 1986). The use of the polymerase chain reaction (PCR) now allows amplification of a relatively small quantity of crude ancient DNA to a quantity sufficient for analysis without molecular cloning (Saiki et al., 1985, 1988). Successful amplification of DNA from various extinct species has been reported (Rollo et al., 1988; Golenberg et al., 1990; DeSalle et al., 1992; Cano et al., 1993). For ancient human remains, soft tissue from mummies was initially used as the study material (Pääbo et al., 1988; Pääbo, 1989), but more recently DNA extracted from hard tissues including bones and teeth has been amplified (Hagelberg et al., 1989; Horai et al., 1989, 1991; Lawlor et al., 1991; Kurosaki et al., 1993; Stone and Stoneking, 1993).

In this study we analyzed mitochondrial DNAs (mtDNAs) from the skeletons of ancient human individuals known as the Yayoi people, excavated from the Takuta-Nishibun site in northern Kyushu of Japan. Judging from excavated earthenware, this site is estimated to be about 2,000 years old (Mori, 1966), corresponding to the Japanese Yayoi era. The morphological traits of the Yayoi people in northern Kyushu are considerably different from those of the earlier Jomon people (Naito, 1981; Dodo and Ishida, 1988; Nakahashi and Nagai, 1989; and references therein). Therefore, it has been proposed that the Yayoi people in northern Kyushu might have been migrants from the Asian continent to the Japanese islands (Kanaseki, 1966). On the basis of this migration theory,

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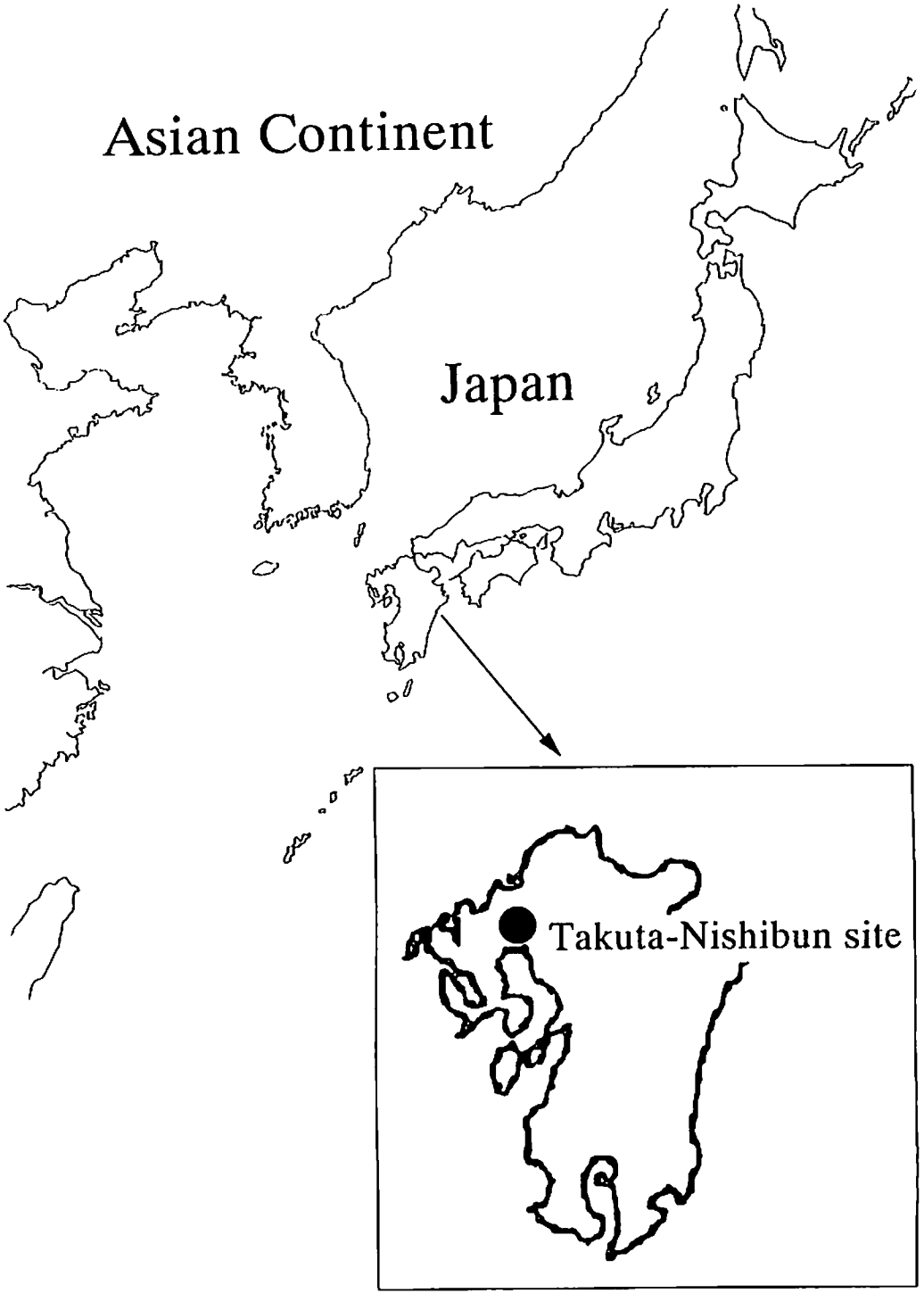

Fig. 1. Map of the Takuta-Nishibun shell mound site, located in northern Kyushu, the westernmost main island of Japan.

Hanihara (1991) developed a dual-structure model for the evolution of modern Japanese.

Here we describe a preliminary analysis of the genetic background of the Yayoi people by constructing phylogenetic trees of ancient and modern humans based on nucleotide sequences of their mitochondrial DNA. We also examine the correlation between burial style and genetic relationships at the Takuta-Nishibun site.

MATERIALS AND METHODS

Sites

The Takuta-Nishibun Shell Mound Site is located in Saga Prefecture, northern Kyushu (Fig. 1). This site is dated to the 1st century BC–1st century AD, corresponding to the middle Yayoi era of Japan. One hundred and twelve skeletons were excavated at the Takuta-Nishibun site, of which 37 individuals were buried in separate earthenware jar-coffins (*kamekan*). In contrast, 75 skeletons were interred using another style, direct burial in earth, called *dokoubo*. Of these, remains of 35 individuals were used for DNA analysis (9 and 26 from *kamekan* and *dokoubo*, respectively).

Extraction and purification of ancient DNAs

Extraction and purification of ancient DNAs were based on the method of Kurosaki et al. (1993) with some modifications: reduction of the number of times of ethanol precipitation in order to simplify the procedures, and use of a UV irradiator (Spectrolinker XL-1500, Spectronics Corp, Westbury, NY) to inactivate possible DNAs from other origins. Bones and teeth were used as materials. However, most of the ancient DNAs were extracted from tooth roots, because the quality of DNA extracted from teeth was usually higher than that of DNA from bones (see Results and Discussion of this paper; cf. Table 2 of Kurosaki et al., (1993). These hard tissues were physically powdered (after freezing in liquid nitrogen for 3 min in the case of tooth samples), suspended in 10 ml of 0.5 M EDTA, and then extracted twice with a half volume of water-saturated phenol and chloroform/isoamyl alcohol (24:1). The aqueous phase was removed and precipitated with 2 vol of cold ethanol at −20°C. The precipitate was redissolved in 2 ml of Milli-Q (Millipore Corp, Bedford, MA) water, 0.5 ml of 10 M lithium chloride was added, and the solution was kept on ice for 30 min. After centrifugation, the supernatant was removed and precipitated with 2 vol of cold ethanol. The precipitate was dissolved in 0.1 ml of Milli-Q water, and this extract was used as a template for PCR. All the procedures were performed on a clean bench, using disposable and ultraviolet (UV)-irradiated instruments (tubes and pipettes). Twenty-six modern non-Ainu Japanese from the main islands were also examined using DNA extracted from peripheral blood lymphocytes.

DNA amplification by PCR

Two regions in the D-loop of mitochondrial DNA were analyzed after DNA amplification by the PCR technique using two primer sets, MT1 and MT3. PCR primers for MT1, 5′-CCCCATGCTTACAAGCAAG-3′ and 5′-ATTGATTTCACGGAGGATGG-3′, were from Horai et al. (1991), corresponding to positions 16190–16208 and 16422–16403 of Anderson et al. (1981), respectively. PCR primers for MT3, 5′-GCGAGACGCTGGAGCCGGAG-3′, and 5′-TGGCCAGAAGCGGGGGAGGG-3′, were after Kurosaki et al. (1993), corresponding to positions 90–109 and 326–307 of Anderson et al. (1981), respectively. PCR amplification was performed in a 40-μl reaction volume containing 67 mM Tris–HCl (pH 8.8), 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 160 g/ml gelatin, 200 μM each dATP, dCTP, dTTP, and dGTP, and 25 pmole each of the 5′ and 3′ oligonucleotide PCR primers. For ancient DNAs, 2–5 units of *Taq* DNA polymerase were added according to their quality (purity), and 30 PCR cycles were carried out as follows: denaturation at 94°C for 1 min, annealing at 45°C or 55°C (45°C for ancient DNAs and 55°C for modern DNA) for 1 min, and extension at 72°C for 2 min. The final cycle ended with an additional extension of 5 min at 72°C.

In the case of ancient DNA, as it was usually very difficult to obtain enough DNA by a single round of amplification, secondary amplification was also carried out. This was done by removing a 1-μl aliquot of the first PCR amplification mixture and adding it to

TABLE 1. Nucleotide sequences of Yayoi people mtDNAs[1]

Sample no.	Material	Burial style	MT1 region																				
			16231	16232	16244	16245	16249	16251	16255	16258.1	16266	16290	16291	16296	16297	16298	16304	16309	16311	16319	16324	16325	16362
			T	C	G	C	T	C	G	–	C	C	C	C	T	T	T	A	T	G	T	T	T
002	T	K	·	·	A	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
005	T	K	·	·	·	T	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·
012	T	K																					
014	T	K	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
016	T	D																					
017	T	D																					
020	T	D																					
021	T	K	·	·	·	T	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
023	T	K	·	·	A	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
030	T	D	·	·	·	T	·	·	A	–	·	·	·	·	·	·	·	·	·	·	·	·	C
036	B	K																					
040	B	D																					
041	T	D	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
043	T	D																					
046	T	D																					
047	T	K																					
051	B&T	D																					
052	T	K																					
058	T	D																					
062	T	D																					
063	T	D	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·
064	T	D	·	·	A	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
071	B	D																					
075	T	D	·	·	A	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
076	T	D	·	·	·	·	·	T	·	–	·	·	·	·	·	·	·	·	C	·	·	·	·
078	T	D	·	·	·	T	·	T	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·
082	T	D																					
088	B	D																					
090	T	D	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
093	B	D																					
101	T	D																					
105	T	D																					
107	T	D																					
109	B	D																					
111	B	D	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·

(Table continues across to facing page)

a fresh PCR reaction mixture. The secondary PCR was performed under the same condition as that for the first round of amplification except for annealing temperature at 55°C instead of 45°C. To verify the reliability of the results obtained, we repeated the experiments in addition to negative control experiments without template DNAs and compared the raw data obtained in each case. To exclude the possibility of contamination, the DNA was extracted separately; PCR amplifications were also performed separately.

Direct sequencing of mtDNA

In the case of the modern Japanese samples, template DNA for direct sequencing were prepared by dialysis using membrane filters (pore size 0.025 μm, Millipore Ltd.). In the case of the ancient DNAs, template DNAs for direct sequencing were recovered as a single band by 5% polyacrylamide gel electrophoresis (PAGE). Primers used for direct sequencing were 5′-GCAAGTACAGCAATCAACCC-3′ and 5′-[M13]GGAGGATGGTGGTCAAGGGA-3′ for the forward and reverse sequences in the MT1 region, respectively, and 5′-[M13]CCGGAGCACCCTATGTCGCA-3′ and 5′-CGGGGGAGGGGGGGTTTGGT-3′ for the forward and reverse sequences in the MT3 region, respectively. [M13] represents 5′-GTTTTCCCAGTCACGAC-3′ of the M13 phage DNA. In our previous study we designed [M13]-attached sequences as primers for PCR, where the [M13] sequence was used as a primer for the sequen-

TABLE 1. Nucleotide sequences of Yayoi people mtDNAs[1] (Continued)

			MT3 region																	
Sample no.	Material	Burial style	146 T	150 C	152 T	191.1 –	194 C	195 T	198 C	199 T	202 A	207 G	214 A	215 A	235 A	239 T	243 A	247 G	248 A	263 A
002	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
005	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
012	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
014	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
016	T	D	·	·	·	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
017	T	D	·	·	·	A	T	·	·	C	G	A	·	·	·	·	·	·	·	G
020	T	D	·	·	C	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
021	T	K	·	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
023	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
030	T	D	·	·	C	–	·	C	·	·	·	·	·	·	·	·	·	A	·	·
036	B	K	·	·	·	–	·	·	T	·	·	·	·	G	·	·	·	·	·	G
040	B	D																		
041	T	D	·	·	C	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
043	T	D	·	·	·	–	·	·	·	·	·	·	·	·	·	·	G	·	·	G
046	T	D	·	·	C	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
047	T	K	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
051	B&T	D																		
052	T	K	C	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
058																				
062																				
063	T	D	C	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
064	T	D	·	·	·	–	·	·	T	·	·	·	·	G	·	·	·	·	·	G
071	B	D	·	·	·	–	·	·	T	·	·	·	·	G	·	·	·	·	·	G
075	T	D	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
076	T	D	·	·	·	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
078	T	D	·	·	·	–	·	C	·	·	·	·	·	·	·	·	·	·	C	G
082	T	D	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
088	B	D																		
090	T	D	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
093	B	D																		
101	T	D																		
105	T	D																		
107	T	D	·	·	·	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
109	B	D																		
111	B	D	·	·	·	–	·	·	·	·	·	·	G	·	·	·	·	·	·	G

[1] Dots represent identical nucleotides with those of Anderson et al. (1981). Hyphens represent gaps. K and D represent *kamekan* and *dokoubo*, respectively. B and T represent bone and teeth specimen, respectively. No sequence samples were unsuccessful in PCR amplification. The notation of Anderson et al. (1981) is used for numbering of bases.

tial direct sequencing. But that sequence has no such significance in this study.

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using primers end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeling mixture included sequencing buffer (16 mM Tris–HCl, pH 9.0, 80 mM KCl, 0.16% Triton X-100), 1 unit of *Taq* DNA polymerase, an end-labeled sequencing primer at 2 pmole, and a fractionated PCR product at 0.2 pmole in a final volume of 20 μl. The termination reaction was then performed by adding 4 μl of the labeling mixture to 2 μl of each of the four termination mixes (180 μM ddGTP, 500 μM ddCTP, 1 mM ddATP or 1.2 mM ddTTP along with 60 μM 7-deaza-2′-dGTP and each 30 μM of dCTP, dATP and dTTP). The cycling conditions were 15 cycles of 30 sec at 95°C, 30 sec at 55°C, and 1 min at 70°C followed by as many cycles of 30 sec at 95°C and 1 min at 70°C. The chain termination products were heated to 95°C for 5 min immediately before loading them onto an 8% polyacrylamide gel containing 7 M urea.

Phylogenetic analysis

For phylogenetic analysis of ancient Japanese, nucleotide sequences of 2 Yayoi and 5 Jomon individuals determined by Kurosaki et al. (1993) and Horai et al. (1991), respectively, were used in addition to the present data. Besides the data for modern Japanese

TABLE 2. Nucleotide sequences of modern non-Ainu Japanese mtDNA[1]

	MT1 region																				
Sample no.	16231 T	16232 C	16244 G	16245 C	16249 T	16251 C	16255 G	16258.1 –	16266 C	16290 C	16291 C	16296 C	16297 T	16298 T	16304 T	16309 A	16311 T	16319 G	16324 T	16325 T	16362 T
JPN 1	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 2	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 3	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 4	C	·	·	·	·	·	·	–	T	·	·	·	·	·	·	·	·	·	·	·	·
JPN 5	·	·	·	·	·	·	·	–	·	·	·	·	·	·	C	·	·	·	·	·	·
JPN 6	·	·	·	·	·	·	·	–	·	·	T	·	·	·	·	·	·	·	·	·	C
JPN 7	·	·	·	·	·	·	·	–	·	·	·	·	C	C	·	·	·	·	·	·	·
JPN 8	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 9	·	·	·	T	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 10	·	·	·	·	·	·	·	–	·	·	·	·	·	·	C	·	·	·	·	·	·
JPN 11	·	·	·	·	·	·	·	–	·	·	·	·	·	·	C	·	·	·	·	·	·
JPN 12	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	G	·	·	·	·	C
JPN 13	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	C	C
JPN 14	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 15	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 16	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·
JPN 17	·	·	·	·	C	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 18	·	·	·	·	·	·	·	–	·	T	·	T	·	·	·	·	C	A	·	·	·
JPN 19	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 20	·	·	·	·	·	·	·	–	·	T	·	·	·	·	·	·	·	A	·	·	·
JPN 21	C	·	·	·	·	·	·	–	T	·	·	·	·	·	·	·	·	·	·	·	·
JPN 22	·	A	·	·	C	·	·	–	·	·	·	·	·	·	C	·	C	·	·	·	·
JPN 23	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	C
JPN 24	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	C	·	·
JPN 25	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	C	·	·	·	C
JPN 26	·	·	·	·	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·

(*Table continues across to facing page*)

obtained in this study, available sequence data from 163 modern individuals including 6 early modern Ainus were also used (Anderson et al., 1981; Horai et al., 1991; Vigilant et al., 1991). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987) and the maximum parsimony method (Fitch, 1977). Both tree-making methods are known to reconstruct correct phylogenetic trees with high probabilities, when closely related sequences are compared (e.g., Saitou and Imanishi, 1989; Kuhner and Felsenstein, 1994). Kimura's (1980) two-parameter method was used to estimate number of nucleotide substitutions. Programs NJBOOT2 and TREEVIEW (kindly provided by Dr. K. Tamura) were used for the neighbor-joining method. The nucleotide sequence of the common chimpanzee (Foran et al., 1988) was used as an outgroup to determine roots of the trees. We also computed the nucleotide diversity of each population using a computer program developed by one of us (N.S.). The nucleotide diversity is the average number of nucleotide difference per site between two randomly sampled sequence from a population, and is equivalent to heterozygosity at the nucleotide level under a randomly mating population (Nei, 1987).

RESULTS AND DISCUSSION

It is known that impurities are usually present in DNAs extracted from ancient remains and that these inhibit PCR amplification (Hagelberg et al., 1989; Pääbo et al., 1989; Kurosaki et al., 1993). Using 41 samples from 35 individuals (13 bones and 28 teeth), we were able to amplify DNAs of 14 individuals (1 bone and 13 teeth) for the MT1 region and 26 individuals (3 bones and 23 teeth) for the MT3 region. DNA amplification was better for the MT3 region than for the MT1 region under the conditions employed. For both the MT1 and MT3 regions, the ancient DNA was amplified under identical conditions, although each of the PCR primers for the MT3 region had a higher

TABLE 2. Nucleotide sequences of modern non-Ainu Japanese mtDNA[1] (Continued)

	MT3 region																		
Sample no.	146 T	150 C	152 T	191.1 –	194 C	195 T	198 C	199 T	202 A	207 G	214 A	215 A	235 A	239 T	243 A	247 G	248 A	263 A
JPN 1	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 2	·	T	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 3	·	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 4	C	·	·	–	·	·	·	·	·	A	·	·	·	·	·	·	·	G
JPN 5	·	T	·	–	·	·	·	·	·	·	·	·	·	·	·	·	–	G
JPN 6	·	·	·	–	T	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 7	·	T	·	–	·	·	·	C	·	·	·	·	·	·	·	·	·	G
JPN 8	C	T	·	–	·	C	·	·	·	·	·	·	·	C	·	·	·	G
JPN 9	·	·	·	A	T	·	·	C	·	A	·	·	·	·	·	·	·	G
JPN 10	C	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	–	G
JPN 11	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	–	G
JPN 12	·	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 13	·	T	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 14	·	·	·	–	T	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 15	·	T	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 16	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 17	·	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 18	·	·	·	–	·	·	·	·	·	·	·	·	G	·	·	·	·	G
JPN 19	·	·	·	–	·	C	·	·	·	·	·	·	·	·	·	·	·	G
JPN 20	·	·	C	–	·	·	·	·	·	·	·	·	G	·	·	·	·	G
JPN 21	C	·	·	–	·	·	·	·	·	A	·	·	·	·	·	·	·	G
JPN 22	·	·	C	–	·	·	·	·	·	·	·	·	·	·	·	·	–	G
JPN 23	·	T	·	–	T	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 24	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G
JPN 25	·	·	·	–	·	·	T	·	·	·	·	G	·	·	·	·	·	G
JPN 26	·	·	·	–	·	·	·	·	·	·	·	·	·	·	·	·	·	G

[1] The notation of Anderson et al. (1981) is used for numbering of bases. Dots represent identical nucleotides with those of Anderson et al. (1981). Hyphens represent gaps.

TABLE 3. Nucleotide diversity of four populations

	Sample size	Nucleotide diversity	Reference
Yayoi people	14	0.013 ± 0.002	Present study
Modern non-Ainu Japanese	26	0.016 ± 0.002	Present study
Papua New Guinean	11	0.019 ± 0.002	Vigilant et al. (1991)
African (!Kung, Western Pygmy, Eastern Pygmy)	22	0.034 ± 0.003	Vigilant et al. (1991)

Tm value than that for the MT1 region. The wider difference between the annealing temperature employed and that expected might have been the reason for the more successful amplification of the MT3 region. The lengths of the DNAs amplified were 233 bp and 237 bp for the MT1 and MT3 regions, respectively. However, for phylogenetic analysis, we used 154 and 141 nucleotides with high reliability at the MT1 and MT3 regions, respectively (Tables 1 and 2).

Table 3 shows the nucleotide diversities of three modern human populations and the ancient Yayoi population of Japan, which were obtained using 295-bp nucleotide sequences for the MT1 and MT3 regions, the longest nucleotides currently available. An African population showing a nucleotide diversity of 0.034 was the most diverse among the four populations, while the Takuta-Nishibun Yayoi people showed a value of 0.013. This value is similar to that of modern non-Ainu Japanese (0.016) and a little smaller than that of Papua New Guineans (0.019). There was no significant difference in nucleotide diversity between the ancient Yayoi population and modern non-Ainu Japanese, indicating that the Yayoi people from the Takuta-Nishibun site were not a genetically homogeneous population.

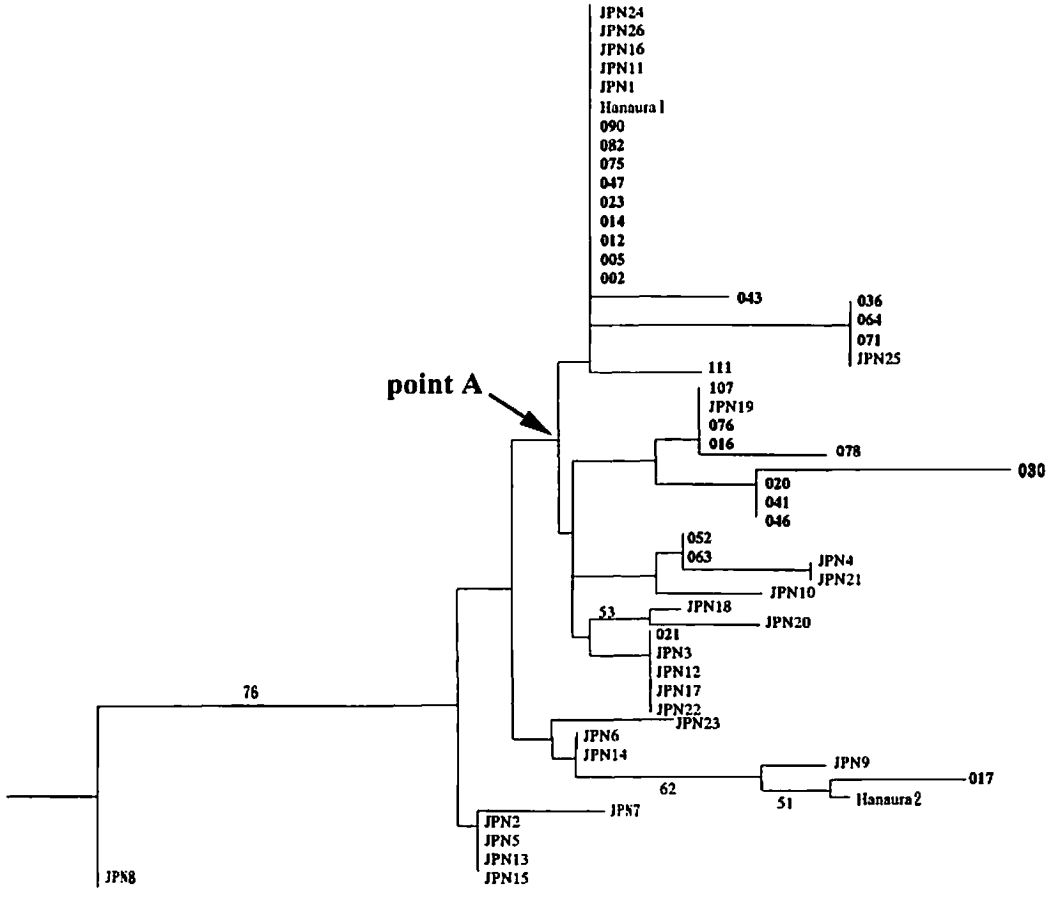

Fig. 2. Phylogenetic tree constructed using nucleotide sequences of the MT3 region for modern non-Ainu Japanese and the Yayoi people including individuals buried at the Takuta-Nishibun and Hanaura sites. Bootstrap resampling was employed 1,000 times, and the resulting bootstrap probabilities of more than 0.5 (50%) are shown on the corresponding branches. Common chimpanzee was used as an outgroup and the individuals at the Hanaura site are from Foran et al. (1988) and Kurosaki et al. (1993), respectively.

Figure 2 shows a phylogenetic tree obtained using the neighbor-joining method for modern Japanese and the Yayoi individuals including two Yayoi specimens obtained at the Hanaura site reported previously by Kurosaki et al. (1993). The Hanaura site is located about 6 km north of the Takuta-Nishibun site. For this analysis, nucleotide sequences of the MT3 region were available. The Takuta-Nishibun Yayoi individuals are included in the cluster diverging at point A in this figure except for one sample, Takuta 017. This sample (an adult female) is clustered with one of the two Hanaura samples, Hanaura 2 (SJ5 in the original paper; a juvenile female), while the other Hanaura sample, Hanaura 1 (SJ4 in the original paper; an adult female) is clustered together with all of the remaining 25 Takuta-Nishibun individuals. Hanaura 1 and 2 were buried in separate earthenware *kamekan* jar-coffins side by side halfway up a hill, and were ornamented with about 20 cone-shell bracelets on their arms. On the basis of these burial states, archaeologists considered that they were members of the same family, who had ruled over the area as shaman or leader. However, the possibility of parent-and-child kinship, or kinship on the maternal side between Hanaura 1 and 2 was ruled out using

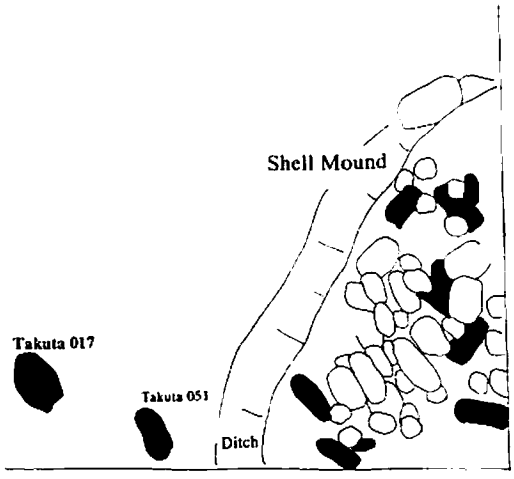

Fig. 3. Map of burial locations at the Takuta-Nishibun site. Clear and solid ellipses represent *kamekan* and *dokoubo*, respectively. Only the remains examined in the present study are indicated.

short-nucleotide tandem repeats (STR or microsatellites) and mitochondrial DNA as genetic markers (Kurosaki et al., 1993). Takuta 017 was buried far from the other remains, at a location considered by archaeologists to have been a dwelling site (Fig. 3). Difference of burial location between Takuta 017 and the others might reflect the genetic difference between them. We expect to extract more correlations between social and genetic difference in the future.

Using nonmetrical cranial traits, it has been shown that a strong resemblance exists between the indigenous Jomon people and modern Ainu, suggesting that the Ainu are descendants of the Jomon people (Dodo and Ishida, 1990; and references therein). Based on the restriction-enzyme analysis of mitochondrial DNAs, Horai and Matsunaga (1986) divided the modern Japanese into two groups, Groups I and II. Horai et al. (1991) recently showed by direct-sequencing of mtDNAs that the Jomon people and Ainu belong to Group II. Divergence time between Groups I and II was estimated at 125,000 years ago (Horai and Matsunaga, 1986). Because the Jomon era started only 12,000 years ago (e.g., Koyama, 1979), such a large divergence time of mitochondrial DNA groups indicates the existence of a considerable genetic polymorphism in the ancestral population of the Jomon people. The phylogenetic tree shown in Figure 4 was constructed by including some other data sets. Identical nucleotide sequences were found among modern Japanese, the Jomon and Yayoi peoples, and Europeans (see Fig. 3 of Horai et al. (1991) and Fig. 4 of this paper). This is not surprising, however, because it has been shown to be rare even for individuals of related geographic origin to form discrete clusters in phylogenetic trees constructed using mitochondrial DNA data (Cann et al., 1987; Vigilant et al., 1991).

The Takuta-Nishibun Yayoi site has two features that are markedly different from contemporary sites in northern Kyushu (Matsushita et al., 1984). One is the notable diversity of morphological traits shown by the human remains. On the basis of craniometrical characteristics, the skeletons excavated at the Takuta-Nishibun site can be divided into three types. The first type is characterized by higher stature, a higher and narrower facial contour, higher orbital openings, a much shallower nasal root, and remarkably flat glabella and superciliary arches, which are typical of Yayoi individuals excavated in northern Kyushu who are considered to have been migrants or their descendants. The second type has features opposite those of the first type; a shorter stature, a relatively low and wide facial contour, rather square orbital openings, a markedly depressed nasal root, and prominent glabella and superciliary arches, which are known to be characteristic of the indigenous Jomon people. The third type is intermediate between the first and second types. Another remarkable feature is the diversity of burial style. At the Takuta-Nishibun site, two burial styles, *kamekan* and *dokoubo*, were found, as mentioned under Materials and Methods, whereas most of the human remains unearthed from contemporary sites in northern Kyushu were buried in *kamekan*. Unfortunately, because of the small number of human remains with full skulls, it was very difficult to search for statistical correlations between these morphological traits and burial style (Matsushita et al., 1984).

For the same reason, it was also impossible to examine the correlation between mito-

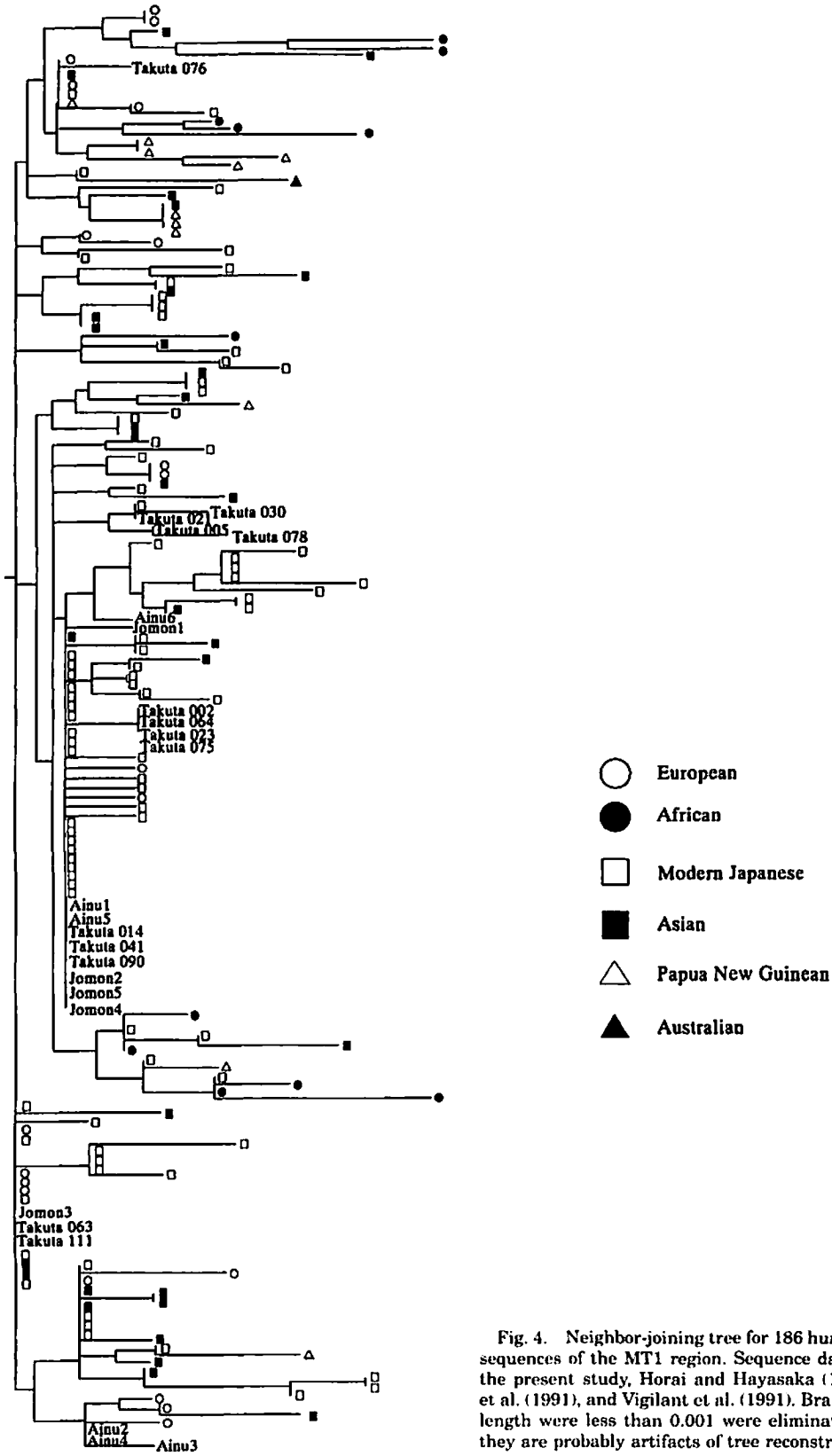

Fig. 4. Neighbor-joining tree for 186 human mtDNA sequences of the MT1 region. Sequence data are from the present study, Horai and Hayasaka (1990), Horai et al. (1991), and Vigilant et al. (1991). Branches whose length were less than 0.001 were eliminated, because they are probably artifacts of tree reconstruction.

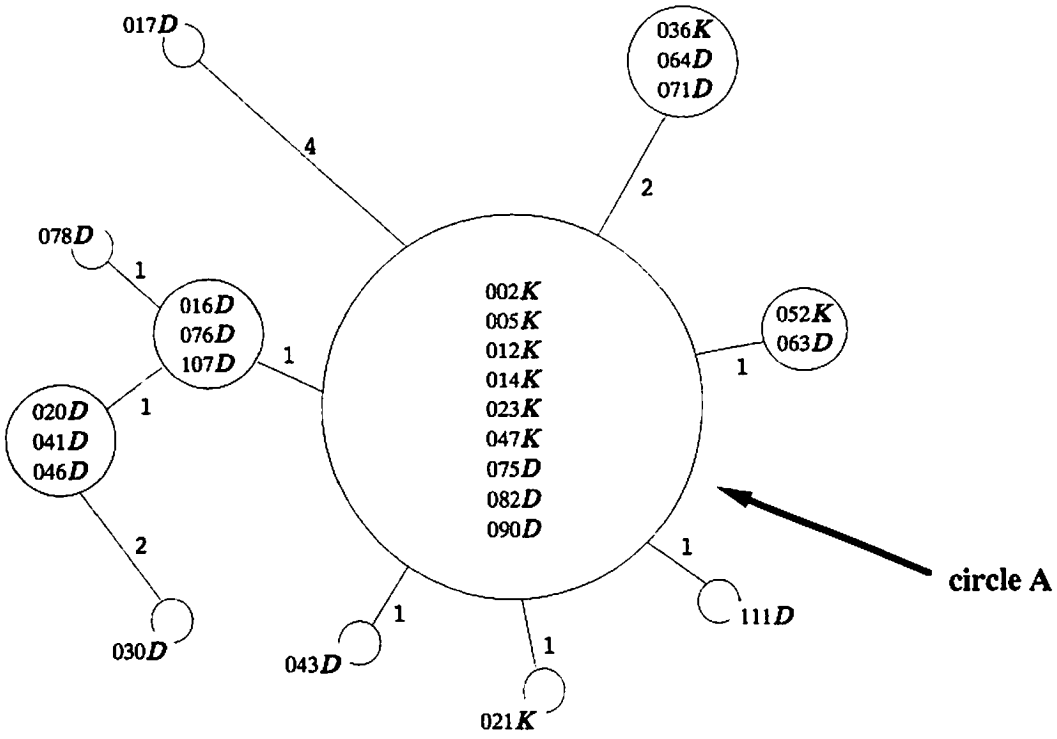

Fig. 5. Unrooted maximum-parsimony tree constructed using nucleotide sequences of the MT3 region. Diameter of each circle is proportional to the number of individuals with an identical nucleotide sequence. Length of each line between circles is proportional to genetic distance. K and D indicate the individuals buried in *kamekan* and *dokoubo*, respectively.

chondrial DNA type and morphological traits. However, we were able to examine the correlation between burial style and mitochondrial DNA type in Takuta-Nishibun individuals. Figure 5 shows an unrooted tree constructed using the maximum parsimony method based on 11 mtDNA types of nucleotide sequences obtained from 26 individuals. The topology of this tree is identical to that of the same 26 individuals constructed using the neighbor-joining method shown in Figure 2. The diameter of each circle in Fig. 5 was proportional to the number of individuals showing an identical nucleotide sequence, while the length of each line between the circles is proportional to the estimated numbers of nucleotide change. The largest circle had 9 individuals, 6 of which were excavated from *kamekan* (designated K in Fig. 5).

To investigate the possibility of correlation between burial style and genetic relatedness, we computed the probability of observing this distribution using Fisher's exact test for independence in a 2 × 2 table (see Table 4). The exact probability was 0.028, thus allowing rejection of the null hypothesis (no correlation) at the 5% level. This indicates two possibilities about the correlation between burial style and mitochondrial DNA type. One is that if the two burial styles, *kamekan* and *dokoubo*, were used at the

TABLE 4. A 2 × 2 table for correlation between burial style and genetic relations

	Individuals in circle A[1]	Other individuals[1]	Total
Kamekan	6	3	9
Dokoubo	3	14	17
Total:	9	17	26

[1]See Fig. 5.

same period, the Takuta-Nishibun people might have been buried in consideration of their genetic background (probably kinship). The other possibility is that if these two burial styles were used in different periods, the genetic constitution of the population might have been somewhat different between the periods designated by these two burial styles. This might be caused by an inflow of people with a different genetic background, together with a different culture, into the earlier Takuta-Nishibun population.

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