Ancient DNA: A New Strategy for Studying Population History

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Introduction

The development of biotechnology has made it possible to reconstruct phylogeny using molecular information of organisms. However, evolutionary relationships between and within species have been inferred almost exclusively from extant material. Ancient genetic data hold the promise of adding direct data about past organisms for studying their evolutionary lineages. To obtain genetic information of past organisms, ancient DNA analysis were initiated more than 10 years ago (eg., Higuchi et al., 1984). The first work with ancient human DNA was the molecular cloning of repetitive sequences extracted from Egyptian mummies (Pääbo, 1985).

Use of Polymerase Chain Reaction (PCR) allows amplification of a small quantity of crude ancient DNA to a quantity sufficient for analyses without molecular cloning (Saiki et al., 1985, 1988). Successful amplification of DNA from various species has been reported (Rollo et al., 1988, 1994; Golenberg et al., 1990; Thomas et al., 1989, 1990; Janczewski et al., 1992; DeSalle et al., 1992; Cano et al., 1993; Poiner et al., 1993; Goloubinoff et al., 1993; Brown et al., 1994; Roy et al., 1994; Höss et al., 1994, 1996a; Hagelberg et al., 1994a; Cooper et al., 1992; Cooper 1994; Cano and Bourucki, 1995). For ancient human remains, Pääbo et al. (1988) reported nucleotide sequences of mtDNA extracted from brain tissue (7,000 years BP). From the early stage of ancient DNA analyses involving human remains, soft tissues, like that found in mummified remains, have

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been most widely used (Del Pozzo and Guardiola 1989; Horai et al., 1991; Richards et al., 1995; Gill et al., 1994; Handt et al., 1996). Recently, Handt et al. (1994) determined nucleotide sequences of mtDNA from tissue samples of a late-Neolithic individual, the "Tyrolean Ice Man."

Hard tissues, such as bones and teeth, are much more abundant than soft tissue remains in archaeological sites, thus providing opportunities for ancient DNA analyses. The first successful DNA amplification extracted from bones was reported by Hagelberg et al. (1989). This report indicated that hard tissues as well as soft tissues were valuable materials for ancient DNA analyses. In the same year, PCR-amplification and a nucleotide sequences of mtDNA were reported of a human bone from a Jomon Period (ca. 7,000 years BP) skeleton from Japan (Horai et al., 1989). Further, the nucleotide sequences of four individuals representing the Jomon Period (3,000-6,000 years BP) have been reported (Horai et al., 1991).

The application of these techniques made it possible to analyze not only phylogeny between ancient and modern people but also to examine aspects of kinship and their intrapopulation structure. By using short-nucleotide tandem repeats and mitochondrial DNA, Kurosaki et al. (1993) identified kinship of two sets of ancient human remains, one each from two different sites in the Japanese Archipelago. Using soft and hard tissues from various ages and locations, additional data have been reported (Lawlor et al., 1991; Stone and Stoneking, 1993; Hagelberg and Clegg, 1993; Hagelberg et al., 1994b; Shinoda and Kunisada, 1994; Merriwether et al., 1994; Hauswirth et al., 1994; Béraud-Colomb et al., 1995; Stone et al., 1996).

In anthropological studies, investigation of a large number of samples is important for understanding the population history. Although ancient DNA has been extracted from various ancient specimens, there are few reports of the mass screening because of small samples of extinct species and the difficulties of analyzing multiple samples at the same time. To reveal genetic structure in ancient groups, we first need to establish a systematic procedure of ancient DNA analyses for the rapid extraction and the efficient amplification for mass screening. An improved procedure for analyzing many samples is described in this paper. Later we first describe ways of preventing contamination by the use of UV-irradiation. Secondly, based on data from several buried situations, the differential effects of preservation are discussed. Finally, we report the results of a correlation between the burial pattern and the genetic background of the individuals using ancient DNA analysis.

Table 1 List of analzyed samples

	age	burial location	materials	numbers	successful amplification	extraction method	Reference
Japanese Archipelago	early modern	humid cave	bone	1	0	phenol/EDTA	this study
			tooth	1	0		
	ca. 600BP	seashore	tooth	3	1	silica-based	this study
	ca. 2,000BP	sandhill	tooth	1	1	phenol/EDTA	this study
	ca. 2,000BP	shell mound	bone	13	3	phenol/EDTA	Oota et al.,
			tooth	28	23		1995
	Jomon	peat bog	bone	1	0	phenol/EDTA	ref. 1
East Eurasian Continent	ca. 2,000BP		tooth	42	24	silica-based	ref. 2
	ca. 3,000BP		tooth	8	1	phenol/EDTA	ref. 3
			bone	2	0		
Malay Peninsula	Neolithic	limestone cave	tooth	4	4	phenol/EDTA	ref. 4
	late-Paleolithic	limestone cave	tooth	1	1	phenol/EDTA	ref. 4

Note.- ref. 1: H. Oota, T. Matsushita and S. Ueda, unpublished data
ref. 2: H. Oota, N. Saitou, T. Matsushita and S. Ueda, unpublished data
ref. 3: H. Oota, N. Saitou, T. Matsushita and S. Ueda, unpublished data
ref. 4: H. Oota, K. Kurosaki, S. Pookajorn, T. Ishida and S. Ueda, unpublished data

MATERIALS AND METHODS

Specimens

The specimens used in this study are given in Table 1. Six samples from three archaeological sites in the Japanese Archipelago are examined. The results are compared to the data of "Yayoi people" from the Takuta-Nishibun site (Oota et al., 1995) and the following unpublished data: one bone from a peat bog in Kyushu, Japan; 52 samples (two bones and 50 teeth) from a site near the Yellow River in China; and five teeth from two limestone caves located in the Malay Peninsula.

Extraction and purification

The EDTA/phenol method (Maniatis et al., 1982, 1989) is the most popular method for ancient DNA analyses, and several variants of the EDTA/phenol method have been proposed (eg., Pääbo et al., 1988; Hagelberg et al., 1989; Hagelberg and Clegg, 1991; Kurosaki et al., 1993; Béraud-Colomb et al., 1996). We have omitted some of the proposed steps so as to simplify the procedure. This dramatically reduces the chances of contamination during the process of extraction. Crushed teeth were suspended in 10 ml of 0.5 M EDTA, and then ex-

tracted twice with a half volume of water-saturated phenol and chloroform/isoamyl alcohol (24:1). The aqueous phase was removed and precipitated with two volumes of cold ethanol at −20°C. The precipitate was redissolved in 2 ml of distilled 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 two volumes of cold ethanol. The precipitate was dissolved in 0.1 ml of distilled water, and this extract was used as a template for PCR.

As silica particles easily trap DNA, it is very convenient to extract very small amounts of DNA such as ancient DNA. A silica-based method was originally developed for application in clinical microbiology (Boom et al., 1990). It was then applied to DNA extraction from Pleistocene bones of the horse family (Höss and Pääbo, 1993). The silica-based method has become more popular than the EDTA/phenol method. However, there is a high risk of contamination by modern DNA in the preparation of the silica particles and the solutions. In order to prevent contamination, the volume of distilled water was reduced from 500 ml to 40 ml, and the weight of silica particles (silicon dioxide; Sigma Chemical Co., St. Louis, Mo.) was reduced from 60.0 g to 1.2 g. This made it possible to perform all the procedures on a clean bench in the preparation of the silica particles and the solutions. We used ULTRASPEC™ WATER (molecular biology grade degassed and sterile pyrogen and RNase free; BIOTECH) to prepare silica particles and all solutions. Fine powdered teeth were suspended in 1 ml of lysis buffer (L6 buffer) containing 10 M guanidinium thiocyanate (GuSCN), 0.1 M Tris-HCl pH6.4, 0.02 M EDTA pH8.0 and 0.3 g Triton X-100, and shaking well by hand. This solution was incubated at 65°C over night with agitation. After centrifugation for 5 min at 5,000 rpm, 0.5 ml of the supernatant was removed to another test tube and 0.5 ml of L6 buffer and a 40-μl silica suspension was added. The mixture was incubated for 15 min at room temperature. After centrifugation for a few seconds, the supernatant was removed and the silica pellet was washed twice with 0.5 ml of washing buffer (L2 buffer) consisting of 10 M GuSCN and 0.1 M Tris-HCl pH 6.4. Subsequently, the silica pellet was washed twice with 0.5 ml of 70 % ethanol and once with acetone. After drying the pellet at 60°C in a heat block, ancient DNA molecules were eluted at 56°C in a 65-μl ULTRASPEC™ water, a 50-μl portion of which was recovered and stored at −20°C.

To exclude possible contamination by modern DNA into L6 and L2 buffers, buffers were preincubated with a 40-μl of silica suspension for three hours before extraction. After centrifugation for 3 min at 3,000 rpm, the supernatant was recovered and stored at room temperature in the dark.

Table 2

Region	Oligo ID	Nucleotide Sequences
MT1	OL-482	5'[-TGTAAAACGACGGCCAGTCC]CCATGCTTACAAGCAAG-3'
	OL-483	5'-CCCCATGCTTACAAGCAAG-3'
	OL-484	5'-ATTGATTTCACGGAGGATGG-3'
	OL-488 (Reverse)	5'[-GTTTTCCCAGTCACGACG]GAGGATGGTGGTCAAGGGA-3'
	OL-489 (Forward)	5'-GCAAGTACAGCAATCAACCC-3'
MT3	OL-452	5'[-TGTAAAACGACGGCCAGTGC]GAGACGCTGGAGCCGGAG-3'
	OL-453	5'-GCGAGACGCTGGAGCCGGAG-3'
	OL-454	5'-TGGCCAGAAGCGGGGGAGGG-3'
	OL-462 (Forward)	5'[-GTTTTCCCAGTCACGACC]CGGAGCACCCTATGTCGCA-3'
	OL-411 (Reverse)	5'-CGGGGGAGGGGGGGTTTGGT-3'
MT4	OL-465	5'[-TGTAAAACGACGGCCAGTAT]AAATACTTGACCACCTGT-3'
	OL-466	5'-ATAAATACTTGACCACCTGT-3'
	OL-467	5'-GGGGACGAGAAGGGATTTGA-3'
	OL-492 (Reverse)	5'-GAGAAGGGATTTGACTGTAA-3'
	OL-493 (Forward)	5'-AGTACATAAAAACCCAATCC-3'

Note.- "Reverse" and "Forward" in parentheses represent primers for a direct sequencing.
[ ] represents sequences of M13 phage DNA.

Amplification

We designed three primer-sets within the D-loop region of mtDNA. The sequences of oligonucleotide are presented in Table 2. The MT1 and MT4 regions are in the hyper-variable region I, and the MT3 region is in the hyper-variable region II of mitochondrial control regions (nomenclature from Vigilant et al., 1989). Of the MT1 and MT3 regions, we observed more efficient amplifications in the MT3 region than in the MT1 region (Oota et al., 1995). However, in the DDBJ/EMBL/GenBank international nucleotide sequence database, much more nucleotide sequence data are registered from the region I than from the region II. In order to compare our data with more sequence data registered in the international nucleotide sequence database, we designed another primer-set, which corresponds to the MT4 region, by using a program for designing primers, OLIGO ver. 4.0-s (TAKARA).

For the EDTA/phenol method, we added 2-5 units of *Taq* DNA polymerase (TOYOBO), and 30 PCR cycles were carried out as follows: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min. When it was not possible to obtain enough DNA by a single round of amplification, a second amplification was carried out. This was done by removing a 1-μl aliquot of the first PCR-amplification mixture and adding it to a fresh PCR reaction mixture. The reaction components were the same as those for the first round of am-

plification and 30 PCR cycles were carried out as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min.

For the silica-based method, hot start PCR was applied by using TaqStart™ Antibody (CLONTECH) or AmpliTaqGold™ (PERKIN ELMER). After mixing the antibody of Taq polymerase with the dilution buffer, we added two units of Taq DNA polymerase (PERKIN ELMER) per reaction, and 40 PCR cycles were carried out as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. A second amplification was sometimes carried out. This was done without Taq antibody and 40 PCR cycles were carried out as follows: denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min.

Amplification products were analyzed by electrophoresis in 8 % acrylamide gels. To verify the reliability of the results obtained, we repeated the experiments along with negative control experiments without template DNA and com-

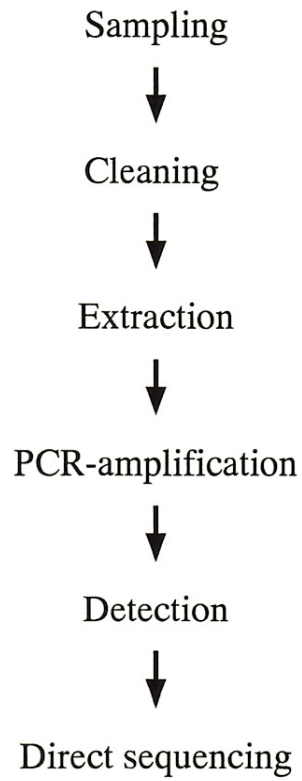

Fig. 1 Flow chart of ancient DNA analyses

pared the raw data obtained in each case. Furthermore, we carried out an extraction control without samples for the silica-based method (eg., Fig. 3b).

RESULTS AND DISCUSSION

Prevention of contamination

There are five steps in ancient DNA analyses: 1) sampling, 2) cleaning, 3) extraction, 4) amplification, and 5) detection (see Fig. 1). In all steps, contamination prevention is of primary importance. For specimens in museums, there is a high risk of contamination due to handling. To avoid contamination and ensure the reproducibility of results, ancient DNA researchers should personally go to the archaeological sites and collect the samples themselves. In the case of teeth, it is better to pull them out from jaws directly. It is not as good for ancient DNA analyses to use isolated teeth.

As ultraviolet rays destroy the helix structure of DNA, it is necessary to exclude contaminant DNA by UV-irradiating all instruments. Figure 2 shows the effect of UV-irradiation to DNA. After 15 ng of modern human DNA were UV-irradiated for 3 min (lane 3), 6 min (lane 6), and 9 min (lane 9), these DNA

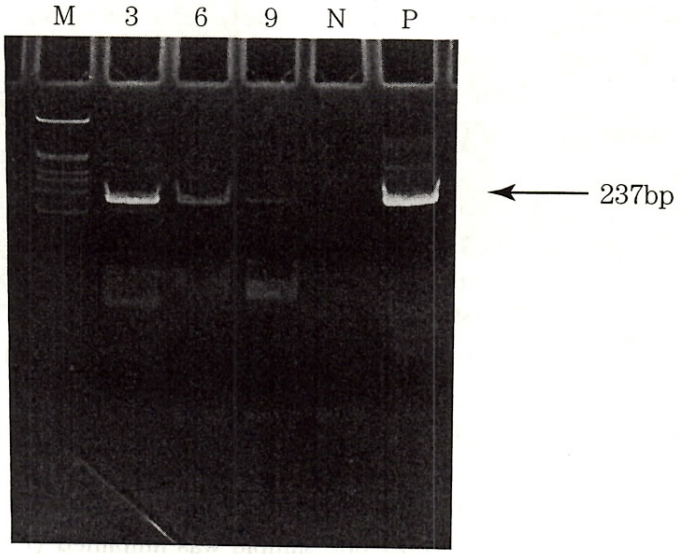

Fig. 2 Effect of UV-irradiation
8% Acrylamide gels showing PCR products of UV-irradiated 15 ng modern human DNA for 3 min (lane 3), 6 min (lane 6) and 9 min (lane 9) as templates. Lanes P and N represent positive control with high molecular weight DNA from a modern human and the negative control without DNA, respectively. Lane M represents a size marker.

molecules were used as templates for PCR. Lanes P and N represent the positive control with high molecular weight DNA and the negative control without template DNA, respectively. The DNA UV-irradiated for 3 min as well as the positive control can be amplified by PCR. However, the predicted bands of 6 or 9 min UV -irradiation showed smaller amounts of PCR product than 3 min irradiation. Given the capacity of ultraviolet rays to destroy the helical structure of DNA, all instruments should clean using UV-irradiation. UV-irradiation should also be used to destroy contaminant DNA on the surface of samples. Teeth are the most suitable materials, because the ultraviolet rays are unable to penetrate the cement and enamel, while leaving endogenous DNA undamaged.

Effects of the state of preservation

To investigate the possible effects of burial conditions on PCR efficiency, six samples from three sites in three different situations—a humid cave, a seashore and a sand hill—were analyzed and compared with 99 samples (Table 1). For a bone sample excavated from a humid cave, two extracts from one bone on different stages of purification were used as templates. Although this individual is the most recent one among 105 samples, there are no bands in the predicted length (Fig. 3a, lanes 1 and 2). We were not able to amplify DNA from the tooth of this individual either (data not shown). For three individuals excavated from the seashore (ca. 600 years BP), the predicted band was detected in lane 2 but strange bands were found in lanes 1 and 3 (Fig. 3b). For one individual, representing the Yayoi period, excavated from a sand hill, two extracts of different concentrations were used as templates for PCR. In lane 1, the band in the predicted length was detected (Fig. 3c).

DNA hydrolysis and oxidation occur in tissues of mummies or skeletons from archaeological sites (Lindahl 1993a, 1993b). Low quantity of ancient DNA is due to the limited chemical stability of DNA (Höss et al., 1996b). Furthermore, impurities are usually present in DNA extracted from ancient organisms and these inhibit the activities of Taq polymerase. Damaged states of DNA and impurities in human remains are affected by the states of their preservation. For the humid cave, DNA hydrolysis and oxidation might have occurred more rapidly than when the remains were buried in soil. Therefore, the DNA fragments from this sample were not able to be amplified though it was not very old. For three individuals dated 600 years BP, DNA from only one sample was amplified (Fig. 3b, lane 2). Samples collected from the seashore were likely exposed to seawater. The extra bands in lanes 1 and 3 might be due to the high concentration of salt found in seawater. Thus, unknown factors often make ancient DNA analyses difficult. For samples taken from hill deposits, we achieved PCR-amplification on one individ-

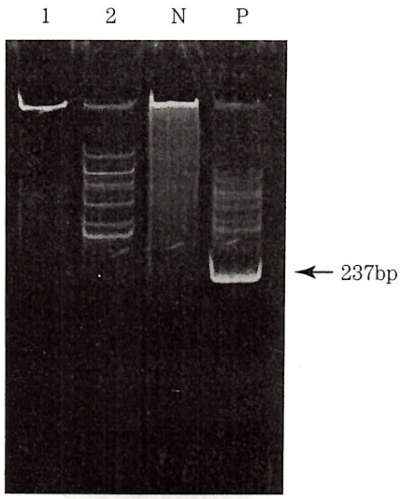

Fig. 3a Results of Polymerase Chain Reaction (PCR) on human remains from three sites found in the different situations 8% Acrylamide gels showing; a. Results of an early modern bone from a humid cave in 3rd-round PCR at the MT3 region. Lane 1 represents PCR using extracts 1/200 diluted out as a template. Lane 2 represents PCR using extracts purified by MicroSpin™ S-300 HR column (Pharmacia Biotech). Lanes 1 and 2 show no bands in the predicted length. Lanes P and N represent the positive control with high molecular weight DNA from a modern human and the negative control without DNA, respectively.

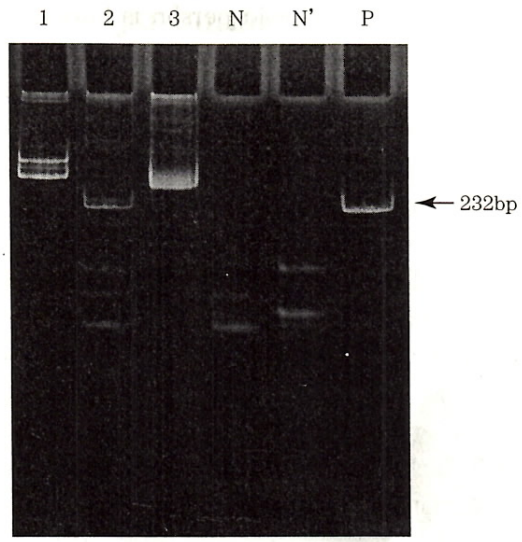

Fig. 3b Results of three individuals (ca. 600 years BP) from a seashore in 2nd-round PCR at the MT4 region. Lanes 1,2 and 3 represent different individuals. Lanes 1 and 3 show extra bands but no bands in the predicted length. Lane 2 shows products amplified by PCR in the predicted length. Lanes N and N' represent extractions by the silica-based method without a sample and the negative control without DNA, respectively. Lane P represents the positive control with high molecular weight DNA from a modern human.

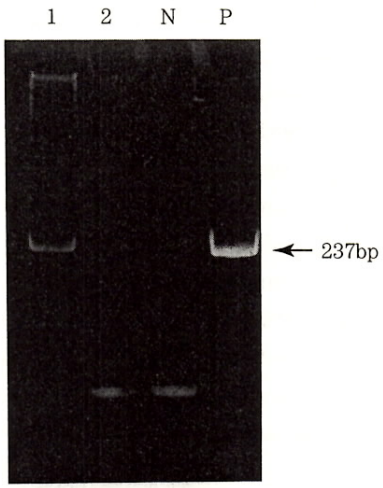

Fig. 3c Results of human remains (ca. 2,000 years BP) from a sand hill in 2nd-round PCR at the MT3 region. Lane 1 represents PCR-products using 1/40000 dilution of extracts as a template. Lane 2 represents PCR-products using 1/200 dilution of extracts as a template. Lanes P and N represent the positive control with high molecular weight DNA from a modern human and the negative control without DNA, respectively.

ual. This particular hill is composed of powdered shells. Therefore, many skeletons excavated from the sand hill deposits are in a good state of preservation morphologically. Four Neolithic individuals and one late-Paleolithic individual from two limestone caves in the middle Malay Peninsula were analyzed and all tested were successfully amplified (see Table 1). Thus, we conclude that alkaline earth is a very suitable pH for the preservation of DNA in buried skeletons. However, in the case of one Jomon individual excavated from a peat bog, which is also alkaline, DNA was not amplified (see Table 1). This indicates that wet conditions are not suitable to the preservation of DNA in human remains, even if they had been buried in the alkaline earth.

Next, we compared the efficiency of PCR-amplification on DNA from two burial patterns. The Takuta-Nishibun site is located in northern Kyushu of the Japanese Archipelago (2,000 years BP). At the Takuta-Nishibun site, two burial patterns, a jar-burial and a pit-burial, are found. A jar-burial indicates a pattern using an earthenware jar-coffin; a pit-burial indicates a dead person is buried directly in the ground. We analyzed nine individuals buried in jar-burials and 26 individuals buried in pit-burials at the Takuta-Nishibun site. All DNA from individuals buried in jars were amplified (100%), whereas DNA from 17 individuals buried in the earth were amplified only 65.4 % (Fig.4a). This result shows that coffins remarkably prevent DNA from degradation.

Finally, we compared the PCR efficiency of DNA extracted from bones and teeth. Thirteen bones and 28 teeth from the Takuta-Nishibun site were analyzed (see Table 1). DNA from three bones (11.5%) were amplified, whereas DNA from 23 teeth (66.7%) were amplified (Fig. 4b). From these results we have speculated that enamel and cement of teeth may protect DNA from hydrolysis and oxidation, as in a "capsule". On the other hand, the DNA in bones that are subjected to ground water, may not be similarly protected from degradation. Our results suggest that teeth are more useful materials than bones for ancient DNA analyses.

Because of the small number of samples, it was difficult to search for statistical correlation between the efficiency of PCR-amplification and the state of preservation. However, our analyses indicate that 1) DNA does not preserve well in remains exposed to water; 2) DNA from skeletons buried in an alkaline earth will be generally well preserved; 3) individuals buried in coffins are better than those buried directly in the ground, and 4) teeth are better than bones as material for ancient DNA analyses.

Application to anthropological and archaeological studies

As mentioned earlier, both jar-burials and pit-burials were found at the

Fig. 4a

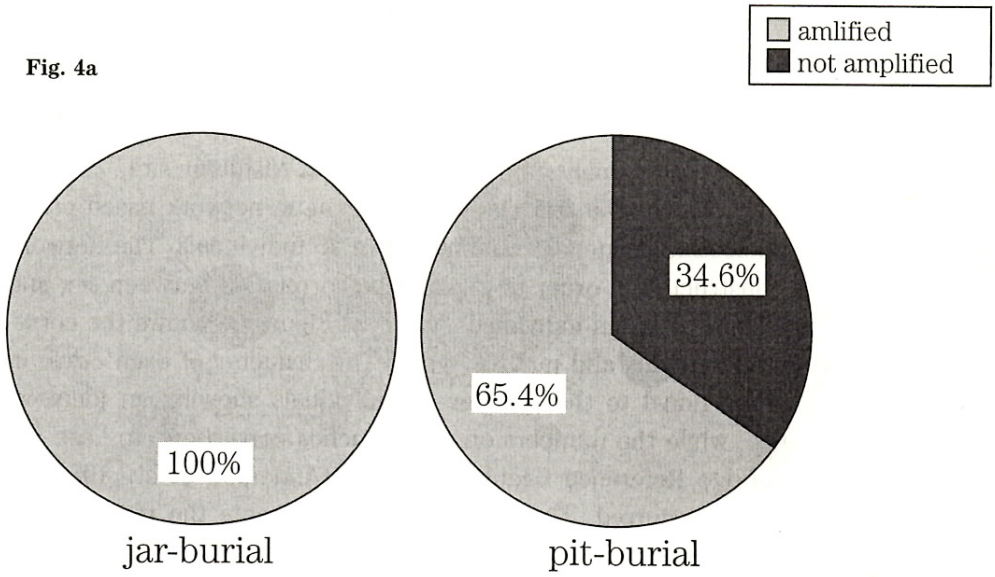

Fig. 4b

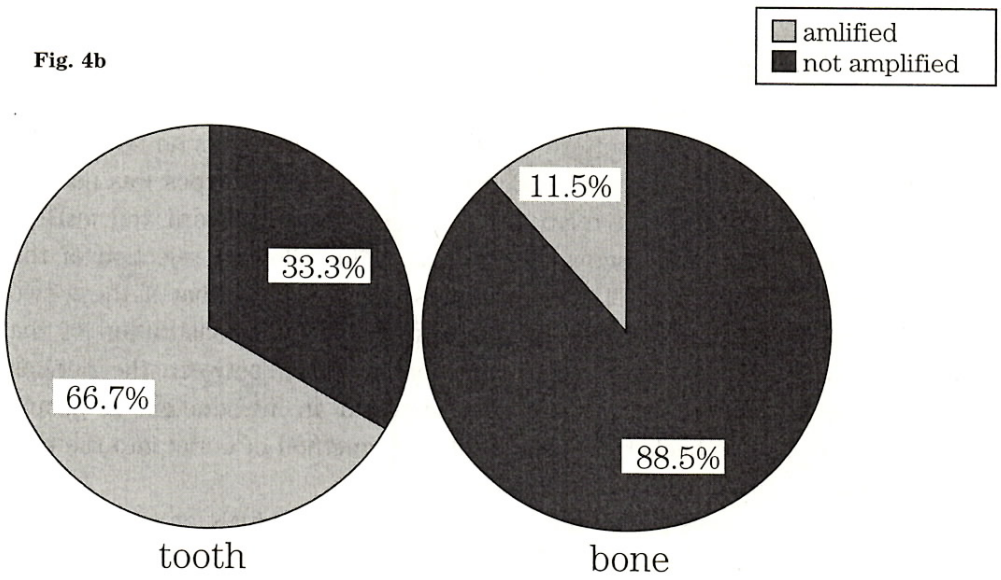

Fig. 4 a. Comparison on efficiency of PCR-amplification
Comparison between a jar-burial and a pit-burial, both containing teeth and bones.
b. Comparison between teeth and bones found in jar-burials and pit-burials.

Takuta-Nishibun site, whereas most of the human remains excavated from contemporary sites in northern Kyushu were buried in jars (Matsushita and Naito, 1989). Another feature of the Takuta-Nishibun site was the notable diversity of morphological traits exhibited by the human remains (Matsushita et al., 1984).

Of thirty-five individuals analyzed from the Takuta-Nishibun site, 26 were successfully amplified. Figures 5a and 5b show the genetic network based on 11 mtDNA types of nucleotide sequences obtained from 26 individuals. The network of Figure 5a was constructed in order to explain the correlation between sex and mtDNA types in Takuta-Nishibun individuals, whereas Figure 5b shows the correlation between burial patterns and mtDNA types. The diameter of each circle in these figures is proportional to the number of individuals showing an identical nucleotide sequence, while the numbers on the branches correspond to base position in the Cambridge Reference Sequence (CRS) (Anderson et al., 1981) at which substitution has occurred. The largest circle represents the major group and consisting of nine individuals. Six females, two males and one unknown individual were included in the major group (Fig. 5a), while six individuals from jar-burials and three individuals from pit burials were included in the major group (Fig. 5b).

To investigate the possibility of relatedness among these individuals, we computed the probability of observing distribution using Fisher's exact test for independence in a 2 x 2 table (Tables 3a and 3b). In the correlation between sex and mtDNA types, the exact probability was 0.195, thus there was no rejection of the null hypothesis. The relationship between sex and mtDNA types was not significant. However, a significant relationship between burial pattern and mtDNA types was found. The exact probability is 0.028, thus allowing rejection of the null hypothesis at the 5% level. This indicates the possibility that if these two burial patterns were used in different periods, the genetic constitution of the people buried there might have been somewhat different between the periods designated by these two burial styles. This suggests an introduction of people with a different genetic background and a different method of burial into the earlier occupation of the Takuta-Nishibun site.

Recently, we achieved PCR-amplification on 24 individuals among 42 individuals excavated from the ground near the Yellow River in China (see Table 1). The phylogenetic analyses including the comparison with Yayoi people are now in progress. It is anticipated that we will obtain important information about the migration of people from the East Eurasian Continent to the Japanese Archipelago. Using this new strategy of ancient DNA analyses will greatly expand and contribute to the study of population history of Japan.

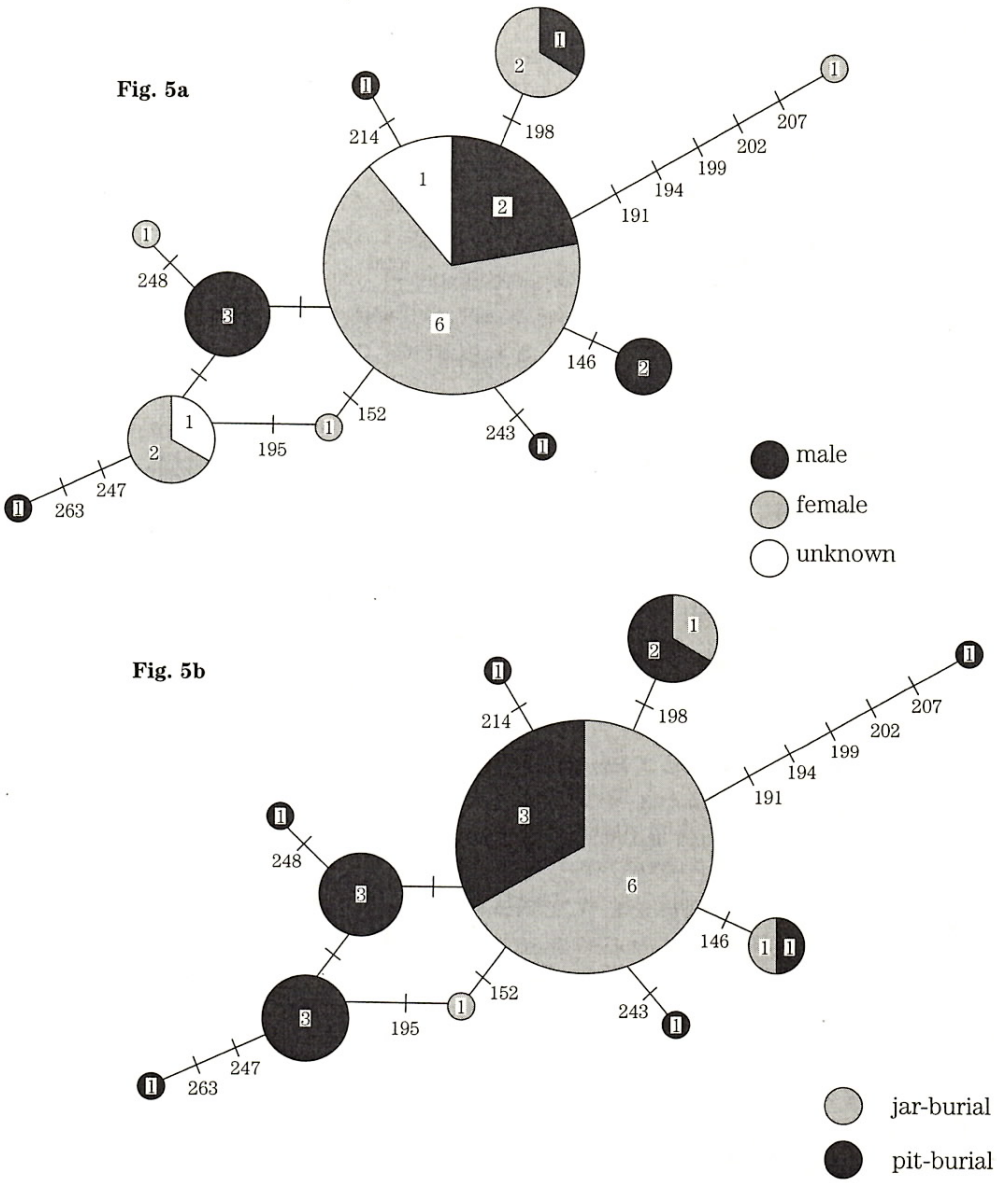

Fig. 5 Network representing the genetic structure of the Takuta-Nishibun site. The network was constructed with nucleotide sequence data of mitochondrial control region by using the method of Bandelt et al. (1995). The diameter of each circle is proportional to the number of individuals with an identical nucleotide sequence. The numbers on the branches correspond to base positions in the CRS (Anderson et al., 1981) at which substitution has occurred. 146:T → C, 152:T → C, 191 : - → A, 194:C → T, 195:T → C, 198:C → T, 199:T → C, 202:A → G, 207:G → A, 214:A → G, 215:A → G, 243:A → G, 247:G → A, 248:A → C, 263:A → G

a. Correlation between sex and mtDNA types

b. Correlation between burial patterns and mtDNA types

Table 3a ratio between male and female

	numbers included in the major group	others	total
male	2	8	10
female	6	7	13
total	8	15	23

Table 3b jar-burial and pit-burial

	numbers included in the major group	others	total
jar	6	3	9
pit	3	14	17
total	9	17	26

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