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Ancient DNA Analysis of Skeletal Samples Recovered from the Kuma-Nishioda Yayoi Site

Ken-ichi Shinoda

Department of Anthropology, National Science Museum
3–23–1 Hyakunincho, Shinjuku-ku, Tokyo, 169–0073 Japan
E-mail: shinoda@kahaku.go.jp

Abstract Recovery of ancient DNA has become an increasingly important tool in elucidating the origins of ancient populations and their relationships. To assess the genetic affinities of the immigrated Yayoi population, 47 skeletal samples belonging to this group were analyzed by PCR using ancient DNA. The hypervariable segment I (HVSI) portion of the control region in 35 of these individuals was sequenced and the mtDNA haplogroups were determined. The frequency data for haplogroups were used to test the migration hypothesis, which are supported by morphological data. The results suggest that the Yayoi people had migrated to the Japanese archipelago and admixed with the previously inhabiting Jomon people. The frequency distributions of haplogroups in the Jomon and immigrated Yayoi populations differed significantly, suggesting that there exists a genetic discontinuity between these two populations, which supports the hypothesis. However, the frequencies of mtDNA haplotypes in the Yayoi population could be biased by the small sample size, and the true frequencies are likely to be different when more individuals are typed. Further genetic studies are needed to confirm our results.

Key words : Ancient DNA, Mitochondria, Yayoi population, Jomon, Haplogroup

Introduction

The steady progress in the field of analytical techniques since the late 1970s has revolutionized biology. By employing these techniques in the field of physical anthropology, a considerable number of studies have been conducted for analyzing the genetic variations existing among human DNA, which was previously considered to be impossible.

The development of polymerase chain reaction (PCR) method (Saiki *et al.*, 1988) has a special significance in the study of ancient DNA as it can detect and amplify even a single copy of DNA. As ancient materials generally contain DNAs at low concentrations and in degraded conditions, PCR is an ideal tool for amplifying DNA up to quantities at which subsequent molecular biological methods can be performed.

This recently achieved ability to analyze DNA from archaeological remains by PCR provides us with more accurate genetical information than that obtained through the morphological study of bones. In recent years, a few studies have been carried out on the intensive population genetical studies at the archaeological sites (Stone & Stoneking, 1993; Oota *et al.*, 1995; Shinoda & Kanai, 1999) Molecular analyses of ancient bones will become one of the more frequently used methods.

Since 1991, we have focused our attention on the molecular analysis of skeletal remains excavated from the archaeological site at Kuma-Nishioda. These remains belonged to one of the largest Yayoi communities located in northern Kyushu. There are two reasons for selecting the site. The first reason is our desire to examine the contribution of molecular biological methods to the field of archaeology, particularly when subjected to an intensive analysis at a specific site. The result of this work has already been published (Shinoda & Kunisada, 1994). The other reason was to examine the origin of the Japanese people. Considerable disagreement exists regard-

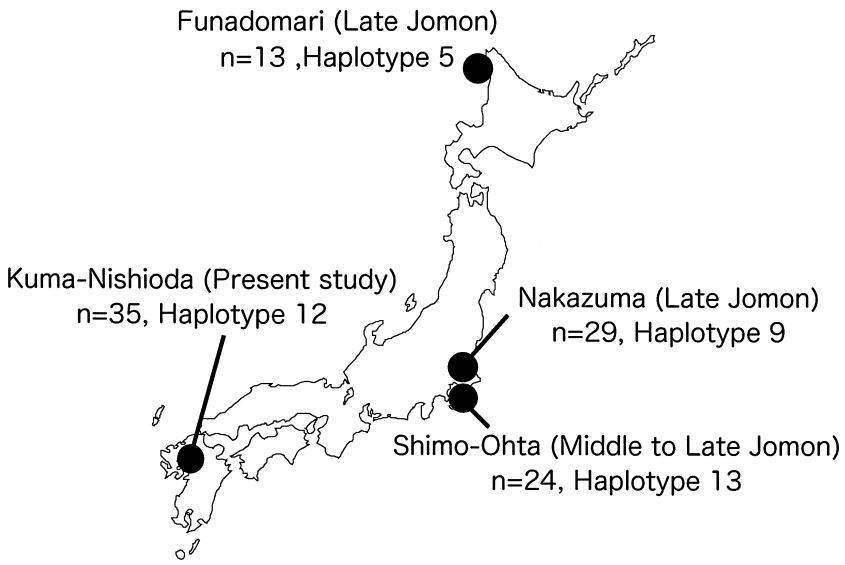

Fig. 1. Geographic location and number of mtDNA haplotypes identified from Kuma-Nishioda Yayoi site and other archaeological sites analyzed in this study.

ing the origin of the Japanese people, particularly during the transition from the Jomon period (1100 B.C.–300 B.C.) to the Yayoi period (300 B.C.–300 A.D.). Two theories, namely, "transformation theory" and "hybridization theory" are more pronounced in this regard. The disagreement between these theories is fundamentally based on the evaluation of the genetic influence of the Yayoi immigrants who migrated from the Korean peninsula to the modern Japan. The Yayoi people who dwelled in northern Kyushu are morphologically dissimilar to the former Jomon people. It is generally agreed that they immigrated to this region during the late Jomon period or the Yayoi period. The archaeological site at Kuma-Nishioda is considered to have been inhabited by one of these immigrant communities. In this study, in order to assess the degree of genetic influence exerted by the Yayoi immigrants, the mitochondrial DNA (mtDNA) haplogroups extracted from these people were compared with those of the Jomon and of the modern Japanese people.

Materials and Methods

Archaeological site

Kuma-Nishioda site located in Fukuoka Prefecture was inhabited by one of the largest Yayoi communities that continued live from the middle to the late Yayoi period (100 B.C.–200 A.D.). The approximate location of the site is shown in Fig. 1. As part of a land development project, the site was fully excavated by the Chikushino Educational Committee between 1983 and 1989. The site comprised 13 distinct parts defined on the basis of geographical location. A residential area and a cemetery were identified. Skeletal remains were excavated from sites 2, 3, 5, 6, 7, 8, 10, and 13. From the site, it appears that large earthenware jars were used as coffins. The skeletal remains were preserved in relatively good condition in those jars that were not damaged.

DNA extraction, PCR amplification, and sequencing

Tooth samples were used in our mtDNA study. It has been argued that teeth form an effective natural barrier to exogenous DNA contamination and that the DNA from teeth appears to lack

Table 1. Sample used for ancient DNA extraction.

Code	Sex	Age	Period	Code	Sex	Age	Period
Site 2				Site 10			
2-123*	Female	Mature	Late middle	10-28*	Male	Adult	Late middle
2-244*	Male	Mature	Late middle	10-46*	Female	Adult	Late middle
2-297	Male	Mature	Early middle	10-55*	Female	Mature	Early middle
2-354*	Male	Mature	Late middle	10-59	Male	Mature	Late middle
2-505	Male	Mature	Late middle	10-71*	Female	Old	Late middle
2-561	Male	Mature	Late middle	10-75*	Female	Adult	Early middle
Site 6				10-78*	Female	Old	Early middle
6-13*	Male	Mature	Late middle	10-87	Female	Mature	Late middle
6-32*	Female	Mature	Late middle	10-88*	Male	Mature	Late middle
6-66*	Female	Old	Late middle	10-99*	Male	Mature	Late middle
6-78*	Female	Mature	Late middle	10-110*	Male	Mature	Late middle
6-83*	Male	Mature	Late middle	10-138*	Male	Adult	Late middle
6-94*	Male	Mature	Late middle	10-173*	Female	Mature	Early late
Site 7				10-175*	Male	Old	Late middle
7-16*	Male	Mature	Late middle	10-180*	Male	Mature	Early late
Site 8				10-210*	Female	Mature	Early late
8-1	Male	Mature	Early late	10-211*	Male	Mature	Late middle
8-4*	Male	Mature	Late middle	10-215*	Female	Mature	Early late
8-9*	Male	Mature	Early late	10-242	Male	Adult	Late middle
8-54*	Male	Old	Late middle	10-244	Female	Mature	Late middle
8-55*	Male	Mature	Early middle	10-245	Male	Old	Late middle
8-63	Male	Adult	Late middle	10-246*	Male	Mature	Late middle
8-70	Male	Mature	Late middle	10-272*	Female	Mature	Late middle
8-78*	Male	Mature	Early late	10-322	Male	Mature	Late middle
8-88*	Male	Mature	Early late	10-326*	Female	Mature	Late middle

Age and sex were judged from the morphological study of excavated bones (Nakahashi, 1993).
Note: * represents the sample that was successfully amplified during DNA analysis.

most of the inhibitors to enzymatic amplification of ancient DNA (Woodward *et al.*, 1994). A well-preserved tooth was extracted from each individual. In order to reduce contamination from previous handling, whole teeth were soaked in DNA contaminant removal solution (TaKaRa DNA-OFF), rinsed with double-distilled water and dried at 37°C for 2 hours. The samples were ground to a fine powder in a press machine. DNA was extracted from 0.3–0.5 g teeth or bone powder according to the method, which is a modification of protocol recommended by the manufacture (GENRCLEAN kit for Ancient DNA; BIO 101 Co.). A more detailed description of the DNA extraction method are detailed in elsewhere (Shinoda 2002). A list of all samples used in this study is presented in Table 1.

Extracted DNA was amplified by PCR. PCR was performed in 50 μl reaction volumes containing 2 units Taq polymerase, 100 mM of each deoxynucleoside triphosphate, 20 pM of each primer, 160 μg/ml of bovine serum albumin (BSA), buffer supplied by the manufacture (TaKaRa) and 5 μl of recovered DNA. The first stage lasted for 40 cycles of amplification consisting of denaturation at 96°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. When the amount amplified was not sufficient, a second PCR was performed for the next direct sequencing step in D-loop amplification. Secondary amplification was carried out by adding 2 μl of the first reaction mixture as the template to a fresh PCR reaction without BSA. Amplification parameters were the same as for the first PCR except that 30 cycles were performed. Sequences of the mtDNA primer used were: primer A 16190-5′-CCCCATGCTTACAAGCAAG-3′-16208; primer B 16422-5′-ATTGATTTCACGGAGGATGG-3′-16403. The numbers identify the base at the 5′ and 3′ ends

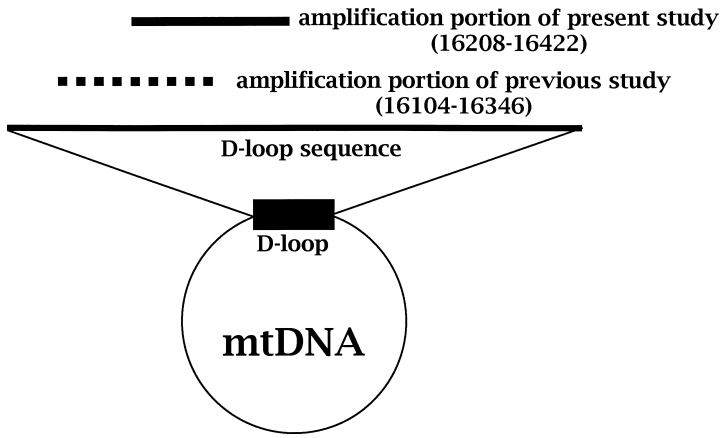

Fig. 2. Analytical portion of mtDNA sequence in this study. Number indicates the mtDNA reference sequence (Anderson *et al.*, 1981).

according to the mtDNA standard numbering (Anderson *et al.*, 1981).

Second PCR products were prepared for sequencing by filtration in a Centricon-100 unit (Amicon). Six microliters for the 50 μl retentate was used for the direct automated DNA sequencing of double stranded DNA on an automated DNA sequencer (Applied Biosystems Inc. model 373A). The total reaction volume was 20 μl, as suggested by the ABI protocol. The same primers used for generating the PCR product were also used in the sequencing reaction. Other reagents were supplied in the Taq DyeDeoxy cycle sequencing Kit (ABI). Sequencing was done in both directions so as to make it possible to check for polymorphisms or ambiguous bases detected with one primer. The sequence of a 181-bp segment of the control region (position 16,218–16,398) was determined.

The amplification portion reported in a previous study (Shinoda & Kunisada, 1994) contained the hypervariable domain box that was proposed by Horai and Hayasaka (1991). However, a later study revealed that this portion had heteroplasmy (Bendall & Sykes, 1995). Therefore, in this study, the amplification portion was changed from that suggested in the previous studies. Fig. 2 shows the relationship between these two portions.

Suspected false positive results stemming from contamination with contemporary DNA (Lawlor *et al.*, 1991) and other questionable data (e.g., Kolman & Tuross, 2000) were excluded. In DNA analysis of ancient samples, however, we must also take into account the possibility that the original sequences may have changed due to the aging of the DNA. Thus, it is not advisable to assume the accuracy of the entire base sequences determined in the investigation under discussion. It should be clearly understood that it is inevitable that such a limitation will occur in analyses of the scarce DNA that remains in ancient samples.

A multiple sequence alignment was made with the CLUSTAL W 1.7 program (Higgins *et al.*, 1991; Thompson *et al.*, 1994). Insertion/deletion (gap) sites were excluded from all analyses.

Results and Discussion

Among the 47 individuals considered in this study, ancient DNA was successfully amplified from 35 samples. Twelve of these samples either failed to yield a product on amplification or produced several ambiguous positions during the sequencing reaction, possibly due to misincorporations by the DNA polymerase during PCR. Therefore, the success rate was approximately 74%, which is high compared with that of other ancient materials. This can be attributed to the

Table 2. Segregating sites in the control region of Kuma-Nishioda mtDNA.

mtDNA standerd number	16217	16223	16245	16257	16260	16266	16275	16278	16284	16290	16298	16304	16311	16327	16380	Haplogroup	n
Consensus sequence	T	C	C	C	C	A	C	C	A	C	T	T	T	C	C		
Type 1	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	?	5
Type 2	·	T	·	A	·	·	·	·	·	·	·	·	·	·	·	N9a	3
Type 3	·	T	·	·	·	·	·	·	·	·	·	·	C	·	·	M10	2
Type 4	·	T	·	·	·	·	·	··			·C	·	·	T	·	C	11
Type 5	·	T	·	·	·	·	·	·	·	T	·	C	·	·	·	?	1
Type 6	C	·	·	·	·	G	·	·	G	·	·	·	·	·	·	B4b	1
Type 7	·	T	·	·	·	·	·	·	·	·	·	·	C	·	·	M8a	3
Type 8	·	T	·	·	·	·	·	T	·	·	·	·	·	·	·	G2	2
Type 9	·	T	·	·	·	·	T	·	·	·	·	·	·	·	·	D5a	3
Type 10	·	T	·	·	T	·	·	·	·	·	·	C	·	·	T	Z	1
Type 11	·	T	·	·	T	·	·	·	·	·	·	·	C	·	T	Z	2
Type 12	·	T	T	·	·	·	·	·	·	·	·	·	·	·	·	D4d	1

A total of 15 segregating sites were observed, shown by the differences from human consensus sequence. The base number of each site in the control region is assigned on the basis of the reference sequence (Anderson *et al.*, 1981). Dots indicate a match with the reference sequence. Haplogroup status and the number of individuals for each sequence type (n) are shown at the right side of each sequence.

Unassigned mtDNAs are marked as "?" for haplogroup status.

bones being preserved in a good condition. A sequence comparison helped identify 12 mitochondrial haplotypes that were defined on the basis of 15 segregating sites. Table 2 shows the positions at which the sequences of the immigrated Yayoi population differed from the reference sequence described by Anderson *et al.* (1981).

Table 3 shows the relationships between mtDNA haplotypes and individuals. As shown in the table, the individuals can be classified into several groups based on their haplotypes. This coincides with the results obtained in our previous study (Shinoda & Kunisada, 1994). Since mtDNA is maternally inherited, the fact that the studied individuals shared the same haplotype suggests the possibility of a maternal relationship. One of the main purposes of studying the specimens from the burial sites was to clarify whether the human remains belonged to unrelated individuals or to members of a single family or limited number of families. A number of individuals were found to share the same haplotype in several instances, which could be due to close matrilineal relationships in this site. Taking into account the fact that this community continued to live for almost 300 years, it is reasonable to assume that close blood relationships existed among its members.

Table 3. Relationships between mtDNA haplotypes and individuals

Haplotype	Individual code number
1	2-123, 6-13, 8-78, 8-88, 10-28
2	8-4, 10-211, 10-215
3	2-354, 10-55
4	2-244, 6-32, 6-94, 7-16, 8-54, 10-71, 10-75, 10-138, 10-175, 10-180, 10-326
5	6-66
6	6-78
7	6-83, 10-110, 10-272
8	8-9, 10-78
9	8-55, 10-46, 10-173
10	10-88
11	10-99, 10-210
12	10-246

A detailed examination of mtDNAs to classify them into haplogroups is essential for identifying spatial frequency patterns (Torroni *et al.*, 2000); therefore, each individual was assigned a haplogroup on the basis of a haplogroup specific D-loop sequence (HVS-I motif) search. In this study, haplotypes refer to distinct DNA sequences defined in D-loop, while haplogroups

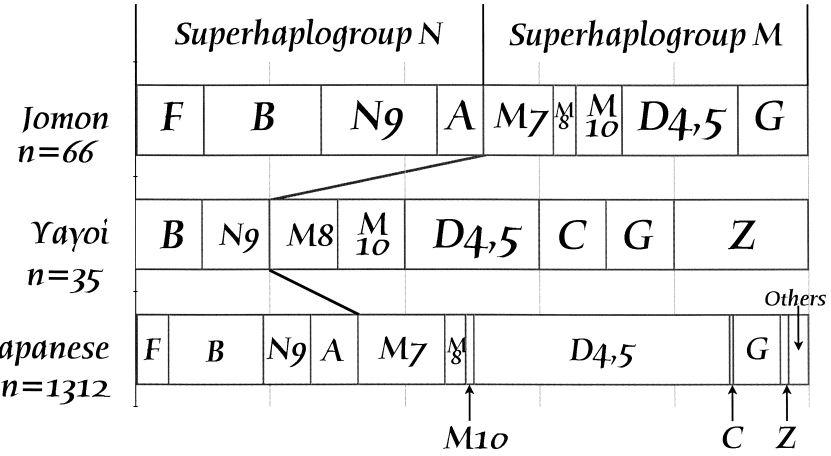

Fig. 3. Frequencies of mitochondrial haplogroups in the ancient and modern Japanese populations.

correspond to major groupings of similar haplotypes. Table 2 also lists the potential haplogroup status of each haplotype. Yao *et al.* (2003) pointed out that there are two prerequisites for interpreting relatively short fragments of ancient East Asian DNA: (1) a reliable basal phylogeny of the major East Asian mtDNA lineages based on D-loop region and coding region information; and (2) a large database of modern East Asian mtDNA D-loop sequences linked with partial coding region sequences or at least single haplogroup-diagnostic sites screened by RFLP typing. To use this information, the haplogroup status of these samples was determined by a haplogroup-specific HVS-I motif search. However, it cannot be denied that the phylogenetic confidence of results based on D-loop sequences has been questioned (Bandelt *et al.*, 2000), mainly due to the frequent occurrence of parallel mutations in independent lineages. In such a case, if the D-loop sequences lack the typical haplogroup-specific motif, they could not be classified unambiguously and were marked as "?" for haplogroups status (Table 2). Eventually, nine out of twelve haplotypes were assigned to this category.

Issues pertaining to population continuity or replacement can be explored using haplogroup frequencies of the different populations. Since genetic variation is inherited from a group's ancestors, frequencies of genetic markers in the Yayoi population are expected to be similar to those of their ancestors; whereas, the Jomon and Yayoi populations with very different frequencies of genetic markers are not likely to be closely related, except in cases of extreme genetic drift or selective forces.

To clarify the genetic characteristics of the Yayoi population, their mtDNA data were compared with those of the Jomon population and the modern Japanese population. The mtDNA haplotypes of Jomon individuals (Fig. 1) and the modern Japanese population (Tanaka *et al.*, 2004) were also classified into haplogroups based on D-loop sequences. The Jomon population data were obtained by Shinoda & kanai (1999), Shinoda (2003), and Adachi *et al.* (2004). In the case of ancient samples, frequency for each haplotype was calculated because all the samples were obtained solely from the group considered to have been related by blood; therefore, it could have been biased. The frequencies of these haplogroups are shown in Fig. 3.

Eurasian mtDNAs belong to two superhaplogroups M and N that originated from single African trunk L3 (Maca-Meyer *et al.*, 2001; Herrnstadt *et al.*, 2002). These superhaplogroups encompass the known Asian-specific haplogroups A, B, F, Y (superhaplogroup N) and C, D, E, G, Z (superhaplogroup M) (Kivisild *et al.*, 2002). In

the superhaplogroup N, the absence of haplogroups F and A in the Yayoi population is somewhat interesting since these are the predominant haplogroups observed in modern Japanese people. On the other hand, these haplogroups apparently occur at a high frequency in the Jomon population. In the superhaplogroup M, the absence of haplogroup M7 in the Yayoi population is interesting because this haplogroup has three branches — M7a, M7b, and M7c. Among these, the highest frequency of M7a is found in the Ryukyuan population. Further, it is common in the mainland Japanese and Ainu populations (Kivisild *et al.*, 2002; Tanaka *et al.*, 2004). The M7 haplogroup found in the Jomon population also belongs to M7a subhaplogroup. It is evident that M7a is one of the prevailing haplogroups in Japan. The absence of haplogroups C and Z in the Jomon population is interesting. Haplogroup C can be considered as a clade with a Northeast Asian radiation, and haplogroup Z extends from west Eurasia to the eastern peninsula of Kamchatka (Schurr *et al.*, 1999). These haplogroups may bring to the Japanese archipelago since the Yayoi period. M8 haplogroup is not detected in the Ryukyuan and Ainu populations. Southeastern China was the primary focus of radiation in this haplogroup. Therefore, it can be safely stated that this haplogroup is also a candidate for the Yayoi-specific haplogroup. The relatively high frequency of the superhaplogroup M in the Yayoi population is also remarkable. It seems reasonable to suppose that the Yayoi immigrants had predominance of the superhaplogroup M.

Unfortunately, a statistically significant difference among these groups could not be detected because of the small number of samples obtained from the Yayoi population. However, as seen in figure 3, the Jomon and Yayoi populations were not similar when compared in light of their share of haplogroups. Therefore, it may safely be assumed that both these populations had different origins.

Conclusions

The distribution of mtDNA haplogroups among the Jomon, Yayoi, and modern Japanese populations suggests that the formation of the Japanese population was not the result of a population expansion. Distinctively different frequencies of mtDNA haplogroups among the Jomon and Yayoi populations indicate significantly different population histories for these groups. However, both populations have contributed to the formation of the modern Japanese population. An eastward population expansion during the Yayoi period resulted in the admixture of these people with the indigenous Jomon people and led to the formation of the basic pattern seen in the modern Japanese people.

In addition, the results of this study revealed the candidate for founder haplogroups in Japan. Further analyses on the distribution of these haplogroups in East Asia will help us find the origin of the Japanese population. In addition, large scale studies on ancient DNA are required to focus on the relationships between the Jomon and Yayoi populations. These will provide useful information that can be used to evaluate the existence and nature of the Yayoi migrations.

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