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Mitochondrial DNA Analysis of Human Skeletal Remains Obtained from the Old Tomb of Suubaru: Genetic Characteristics of the Westernmost Island Japan

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Abstract Yonaguni-jima is the westernmost island in Japan. Because of the geographical position of this island, it is considered to have played an important role as a route of migration from the southern part of Asia to the Japanese archipelagos. In order to investigate the genetic structure of the ancient Yonaguni people and to assess their genetic relationship with other East Asian populations at a molecular level, we analyzed the hypervariable regions (HVR) 1 and 2 of the mitochondrial DNA (mtDNA) from 16 tooth samples excavated from the old tomb of Suubaru, located on the north coast of the island. This tomb, which belongs to the early modern to modern periods, contained 32 graves. The distribution of mtDNA haplotypes among the skeletal remains obtained from the cemetery indicated the existence of several different maternal lineages. The mtDNA sequences can be tentatively classified under specific haplogroups on the basis of mutations in the HVR1 and HVR2 regions. The frequencies of these haplogroups were compared with those of haplogroups present in Asian populations. The fact that the haplogroup M7a was found at a high frequency and that dominant haplogroups of Northeast Asian populations were found among the Suubaru people indicates that the Okinawa mainland had an enormous influence on the formation of the modern people in Sakishima Islands.

Key words : Ancient DNA, Mitochondria, Yonaguni-jima, Old tomb of Suubaru, Population genetics

Introduction

The origin of the genetic diversity in the Japanese population remains controversial, although multidisciplinary approach is being used to address this issue. The DNA analysis of ancient human remains is useful in this regard because it provides information on the genetic characteristics of a society that existed at a specific time in the past (Alzualde et al. 2006; Wang et al. 2007). DNA analysis can shed light on the origin of the genetic composition of the present population (Maca-Mayer et al. 2005; Casas et al. 2006).

Yonaguni-jima is the westernmost island in Japan located at the end of the Ryukyu Archipelago, 125 km from the east coast of Taiwan and 520 km from the main island of Okinawa (Figure 1). Because of the geographical position of Yonaguni-jima, this island is considered to have played an important role as a route of migration from Taiwan and Southeast Asia to the Ryukyu and Japanese archipelagos. Archaeological excavations have been conducted at more than 20 sites on the island; the skeletal remains excavated were from sites ranged from the Neolithic period to the modern period, and many of these were built after the 14th century.

The archaeological evidence suggests that until the 12th century, the Sakishima area, which comprised the Miyako and Yaeyama island groups, formed a cultural sphere different from the main island of Okinawa. This culture was not

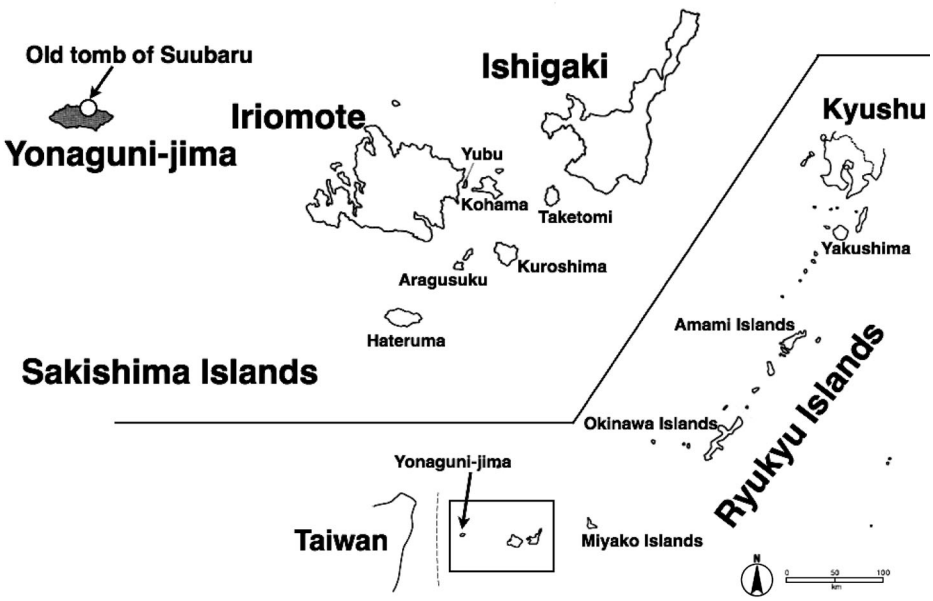

Fig. 1. Map of Ryukyu Archipelago and Taiwan.
Map showing the location of Yonaguni-jima and the old tomb of Suubaru.

influenced by the Japanese mainland (Jomon and Yayoi cultures), and the evidence obtained from existent remains indicated that the Sakishima area had more in common with the southern regions of Asia. Perhaps the seas lying between the main islands of Okinawa and Miyako formed the boundaries for the extension of the Japanese culture toward the south (Takamiya, 2005).

In the 12th century, the hunter-gatherer lifestyle began to change with the advent of an agricultural society in all parts of Ryukyu Islands; thus, the cultural differentiation was eliminated. The relationship between the hunter-gatherers who inhabited this region in ancient times and between the farmers who arrived later on has been particularly interesting; however, little is known about their biological relationships. This is because none of the human remains that have been excavated from the Yaeyama islands belong to the Neolithic period (Asato and Doi, 1999).

Genetic analysis of ancient human remains is the most effective biological approach for determining the relationships between these people. In order to obtain more information on the genetic characteristics of this westernmost island of Japan, we are currently analyzing DNA extracted from human remains that were excavated from cemeteries belonging to the early modern and modern periods.

Materials and Methods

Archaeological site and specimens

The human skeletal remains used for the analysis were obtained from the old tomb of Suubaru, which is located toward the north coast of Yonaguni-jima (Figure 1). As a part of the airport expansion project that was undertaken in Okinawa Prefecture in 2004–2005, the site was fully excavated by the government to obtain any items of cultural significance that may have been buried. At least 77 skeletons were excavated from 32 graves. On the basis of the funerary objects procured, it was concluded that the tomb belonged to the early modern to modern period.

The skeletons excavated generally had a lower face and long head—a characteristic similar to that observed in the modern Okinawa population (Figure 2). The average height of the skeletons was estimated at 157.5 cm for males and 144.6

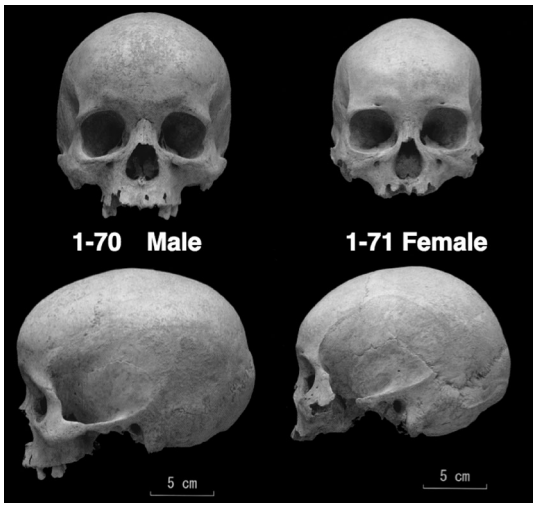

Fig. 2. Frontal and lateral views of the adult male and female skulls excavated from the old tomb of Suubaru.

cm for females, indicating that the ancient humans were fairly short in stature. Further, periostitis was observed in the lower limb of many of the skeletons; this indicated that the people had been exposed to severe environmental conditions (Doi, 2007).

A total of 16 remains obtained from 12 graves were selected for the DNA analysis. Of these samples, 3 were collected from a single skeleton in order to verify the authenticity of the retrieved DNA. Almost all the samples were collected from the surface of the tomb chamber.

Authentication and prevention of contamination

During analysis of ancient DNA samples, it is necessary to exclude false positive results that can arise because of postmortem damage and contamination with more recent DNA samples (Cooper and Poinar, 2000; Bandelt, 2005). During each step of sample preparation, all possible precautions were taken to prevent contamination of the DNA samples with more recent samples. Separate laboratory rooms were used for extraction, amplification, and post-polymerase chain reaction (PCR) analysis of the ancient DNA. The samples were kept pure by regular treatment with DNA-OFF (TaKaRa Co.) and ultraviolet (UV) light. All metallic materials used were sterilized in an oven at 210°C for at least 6 h. Other rigorous authentication methods that have been described previously were employed throughout the DNA-based analyses (Shinoda et al., 2006). Tooth sample preparation, DNA extraction, and PCR amplification were performed in a laboratory room that was reserved for analysis of the ancient DNA.

DNA Extraction and purification

The tooth samples were dipped in a DNA-OFF solution for 5 min to eliminate contamination, rinsed several times with DNase-/RNase-free distilled water, and air dried. When the samples were completely dry, they were pulverized in a mill (Multi-beads shocker MB400U; Yasui Kikai, Osaka, Japan).

DNA was extracted in 2 steps by using a DNA extraction kit (Mo Bio Co.). The pulverized tooth powder (0.3 g) was placed in a 15-ml conical tube and demineralized in 5 ml of 0.5 M ethylenediamminetetraacetic acid (EDTA). The samples were rotated and incubated at 37°C for 12–15 h. After digestion with proteinase K (0.5 mg/ml), the resultant pellet was used for DNA extraction. The eluted DNA (approximately 50 μl) was amplified by PCR, without prior processing.

Amplification and sequencing of HVR1 and HVR2

In all the samples, segments of hypervariable region (HVR) 1 (nucleotide positions 16121–16238 and 16209–16402, as per the revised Cambridge reference sequence; Andrews et al., 1999) and HVR 2 (nucleotide positions 128–267) were sequenced.

Aliquots (2 μl) of the extracts were used as templates for PCR. Amplifications were carried out in a reaction mixture (total volume, 25 μl) containing 1 unit of Taq DNA polymerase (HotStarTaq^{TM} DNA polymerase; Qiagen), 0.1 μM of each primer, and 100 μM of deoxyribonucloside triphosphates (dNTPs) in 1×PCR buffer provid-

ed by the manufacturer. The PCR conditions were as follows: incubation at 95°C for 15 min; followed by 40 cycles of heat treatment at 94°C for 20 s; 50–56°C for 20 s; and 72°C for 15 s; and final extension at 72°C for 1 min.

The following primers were used to amplify HVR1 and HVR2.

L16120 5′-TTACTGCCAGCCACCATGAA-3′
H16239 5′-TGGCTTTGGAGTTGCAGTTG-3′
L16208 5′- CCCCATGCTTACAAGCAAG-3′
H16403 5′-TTGATTTCACGGAGGATGGTG-3′
L127 5′-AGCACCCTATGTCGCAGTAT-3′
H268 5′-GTTATGATGTCTGTGTGG-3′

The PCR products were subjected to agarose gel electrophoresis on a 1.5% gel and were recovered by using a QIAEX II agarose gel extraction kit (Qiagen, Germany). Aliquots of the samples were prepared for sequencing, on a BigDye cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), which was performed using forward and reverse primers. The primers that were used in the PCR amplification were also used in the sequencing reaction. Sequencing was performed in both directions so as to enable identification of polymorphisms or ambiguous bases using a single primer. The sequencing reactions were performed on a DNA Sequencer (ABI model no., 3130) equipped with SeqEd software.

Data analysis

The nucleotide diversity and the mean number of pairwise differences between the mitochondrial D-loop sequences were computed using the Arlequin software package version 3.0 (Excoffier et al., 2005), considering Tamura and Nei distances and a gamma parameter value of 0.26 (Mayer et al., 1999). The differences (Raymond and Rousset, 1995) between the population of the old tomb of Suubaru and the other populations were also computed using the Arlequin software. Neighbor-joining (NJ) trees were constructed on the basis of the pairwise Fst values by using the Mega 3.0 program (Kumar et al., 2004) in order to study the relationships between the populations. The haplogroup status was tentatively assigned on the basis of a search for an HVR1 motif specific to a haplogroup and by matching or almost matching the values with the mitochondrial mtDNA haplotypes in the global database. The haplogroup status was further characterized on the basis of other specific mutations in the HVR2 motif.

Results and discussion

In this study, we assessed the mtDNA variations in samples obtained from the old tomb of Suubaru. MtDNA was extracted from the teeth of each skeleton, and sequencing was successfully performed for 15 of the 16 samples. DNA was independently extracted from 3 teeth of same individual (tomb 15) in order to assess the reproducibility and authenticity of the results. As shown in table 1, the HVR1 sequences determined for these 3 samples completely coincided. Thus, the authenticity of the results was confirmed.

In general, hot and humid conditions are unfavorable to the preservation of DNA in human skeletal remains; hence, the possibility of finding well-preserved DNA in a tropical region such as Okinawa is low. However, our success rate (94%) in this regard was reasonably higher than the usual success rate associated with obtaining other ancient materials, i.e., 50–70%. This can be attributed to the fact that the bones excavated in the present study were well preserved and that these samples were relatively recent when compared with ancient DNA.

The fact that we identified 7 different haplotypes among the skeletal remains of 13 individuals (corresponding to a haplotype diversity of 53.8%) indicates that the genetic characteristics of the Suubaru population were relatively diverse (Table 1). Identical haplotypes were observed for 6 individuals, and these haplotypes were classified into 2 groups; the remaining 7 individuals had unique haplotypes. Since mtDNA is maternally inherited, the finding that some of the studied individuals shared the same haplotype suggests the possibility that they were maternally related. This would not be very surprising, since

Table 1. Changes in the nucleotides of mtDNA and the haplogroups observed in the old tomb of Suubaru.

Tomb No.	Sample No.	HVR1 Sequence (+16000)	HVR2 sequence	Haplogroup
	1	209, 223, 324	CRS	M7a
1	2	209, 223, 324, 344	CRS	M7a
	14	223, 298, 327	193, 195, 249d	C
2	692	209, 223, 324	CRS	M7a
6	151	223, 290, 319	N.D.	A4
9	128	223, 278, 362, 384	CRS	G2
11	164	232A, 304, 311, 344	N.D.	F1b1a
14	172	209, 223, 324	150	M7a
	856	223, 269, 278, 362	200, 260	
15	857	223, 269, 278, 362	N.D.	G2a2b1
	858	223, 269, 278, 362	N.D.	
23	261	N.D.	N.D.	N.D.
25	803	209, 223, 324, 344	N.D.	M7a
33	579	209, 223, 324, 344	N.D.	M7a
36	611	209, 223, 324	CRS	M7a
38	649	216, 223, 362	N.D.	D/G

Table 2. Diversity indices of the mtDNA HVR1 haplogroup for the skeletal remains obtained from the old tomb of Suubaru and those of other populations.

Site	n	Gene diversity	Nucleotide diversity	Mean number of pairwise differences
Suubaru	13	1.00+/−0.030	0.020+/−0.012	3.897+/−2.089
Jomon (Kanto)	67	0.91+/−0.024	0.018+/−0.010	3.429+/−1.775
Yayoi (Kuma-Nishioda)	31	0.85+/−0.051	0.012+/−0.007	2.241+/−1.268
Aboriginal Taiwanese	28	1.00+/−0.010	0.022+/−0.012	4.161+/−2.133

The references for the population are as follows:
Kanto Jomon: Shinoda and Kanai (1999), Shinoda (2003)
Yayoi: Shinoda (2004)
Aboriginal Taiwanese: Tajima et al. (2003)

the burial ground was used by a rather small group of individuals. The above results suggest that these cemeteries were used over a long period and that the studied individuals were related across generations.

Genetic variation within the populations was determined by calculating the sequence diversity, nucleotide diversity, and the mean number of pairwise differences (Table 2). These factors were also calculated for the ancient Japanese and contemporary aboriginal Taiwanese populations. The Suubaru population exhibited values of 0.02 for nucleotide diversity and 3.89 for the mean number of pairwise differences, which were higher than the values obtained in the case of the other Japanese ancient populations (Table 2) and lower than those obtained in the case of the aboriginal Taiwanese population. This result indicates the existence of a relatively high degree of genetic variation among the Suubaru people.

The haplogroups of the Suubaru population were classified on the basis of variations in the HVR1 and HVR2 sequences of mtDNA. It was difficult to define the haplogroups determined for the skeletal remains obtained from tomb 38 because they contained unusual motifs. Nevertheless, these samples were classified under haplogroup D or G because of the presence of mutations at sites 16223 and 16362. Finally, 6 haplogroups were detected in the ancient samples, haplogroup M7a being the most prevalent. The changes observed in the nucleotides in the D-loop regions of the mtDNA and the haplogroups of each individual are shown in Table 1.

The mtDNA haplogroup distribution often exhibits spatially-varying frequency patterns

Table 3. Estimated frequencies of the mtDNA haplogroups among regional populations.

Haplogroup	Northern Chinese (n=125)	Korea (n=694)	Mainland Japanese (n=1312)	Ryukyuans (n=326)	Taiwan Aborigine (n=640)	Taiwan Han Chinese (n=221)	Southern Chinese (n=78)	Suubaru old tomb (n=13)
D4	35.2	28.8	32.6	34.0	1.5	14.0	14.1	19 (D/G)
D5	6.4	3.5	4.8	2.7	4.8	6.3	5.1	
G	5.6	5.8	6.9	1.6	0	0	1.3	
M7a	0	3.4	7.5	24.2	0	0.5	0	45
M7b	2.4	3.4	4.8	4.7	9	12.0	7.7	0
M7c	2.4	3.7	0.8	0.5	9	4.3	2.6	0
M8	6.4	2.4	1.4	0	0	0	2.6	0
M9E	3.2	3.2	0	2.1	11.4	0	0	0
M10	3.2	4.7	1.3	0	0.4	0	2.6	0
CZ	1.6	3	1.8	0.3	0	0	0	9
A	4.0	7.8	6.9	8	0	0.5	0	8
B4	9.6	4.8	7.7	11.7	17.1	24.5	25.6	0
B5	1.6	4.7	4.3	2.4	5.9	6.7	1.3	0
F	7.2	4.9	5.3	2.1	26.7	19.2	23.1	0
N9a	3.2	3.9	4.6	0.3	1.2	0	1.3	0
N9b	0	0.2	2.1	4.3	0	0	0	0
Y	1.6	2.2	0.4	0.5	1.4	1	0	0
R	1.6	0	0.1	0	2.9	7.7	2.6	0
Other	4.8	9.8	6.7	0.5	8.7	3.3	10.1	0

The references for the population are as follows:
Northern Chinese: Yao et al. (2002)
Korean: Lee et al. (2006)
Mainland Japanese: Tanaka et al. (2004)
Ryukyu: Umetsu et al. (2005)
Taiwan aborigines: Trejaut et al. (2005)
Taiwan Han Chinese: Horai et al. (1996), Tsai et al. (2001)
Southern Chinese: Yao et al. (2002)

(Forster 2004). To determine the genetic characteristics of the Suubaru population, we compared their mtDNA data with that of populations in geographically related areas. The data are listed in Table 3. Haplogroups A, C, D, G, Y, and Z occur frequently in the Northeast Asian populations, whereas haplogroups B and F are predominant in Southeast Asians; haplogroups C, Y, or Z have rarely been found in Southeast Asian populations (Kivisild et al. 2002).

All the haplogroups known to occur in the Southeast and Northeast Asian populations, i.e., C, D, G, A, and F were detected in the Suubaru population. However, most of the haplogroups that were found at the Suubaru site are dominant in Northeast Asian populations. Therefore, it can be assumed that the buried people studied here did not mainly originate from Southeast Asia and Taiwan but rather from Northeast Asia, and they may have migrated into the study area via Japanese mainland and Okinawa Island.

The results of our phylogenetic analysis based on the Fst values show that the Suubaru population shares a relationship with mainland Okinawa (Figure 3). Archaeological findings indicate that travel and exchange of goods between the Japanese mainland and Ryukyu Archipelago began in about the 12th century (Asato, 1996). Further, some findings have suggested that a large number of immigrants migrated to Okinawa from mainland Japan and further to the Sakishima area. These immigrants are assumed to be the direct descendants of the Okinawa population that immigrated after the Gusuku period. Moreover, the fact that haplogroup D4, which is dominant in mainland Japan, was absent among the haplotypes determined in the present study indicates that people who emigrated from Okinawa Island after the Gusuku period were probably native to this island. Archaeological evidence suggests that

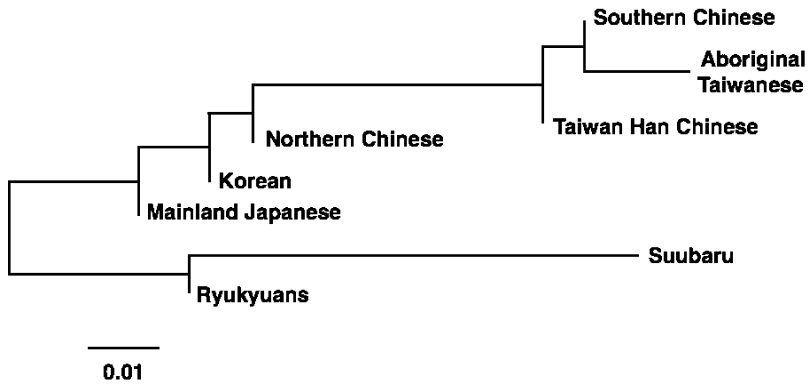

Fig. 3. Neighbor-joining tree based on the Fst values determined for 8 populations.

the people who first settled in Yonaguni-jima might have emigrated from Taiwan and the Philippines. However, in this study, we could not establish a genetic relationship between the aboriginal Taiwanese and the Suubaru people. Thus, it appears that the influx of emigrants from the Okinawa mainland to Sakishima area had large influence at the Gusuku period.

It is therefore possible that the similarities in the haplogroup composition between the Suubaru population and the contemporary population of the main island of Okinawa is the result of the arrival of immigrants, who almost replaced the indigenous population and brought along with them a new haplogroup distribution.

Determination of the kinship among the numerous individuals buried at a single burial site may provide extremely valuable insights into the social structure during ancient times. Furthermore, obtaining DNA data from people inhabiting a single region over a prolonged time period may enable estimation of the movement of groups and the population dynamics in the region.

Our study of the old tomb of Suubaru unraveled the genetic characteristics of the westernmost island of Japan after the Gusuku period. However these conclusions must be regarded as tentative since they are based on small sample sizes, even though the analysis have been highly efficient. Thus, we believe that further experiments to obtain more detailed data on the human skeletal remains from the Ryukyu Archipelago are important. We hope to pursue this matter in future studies.

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