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Mitochondrial DNA analysis of the skeletal remains excavated from the old Tokijin tomb, located in the Nakijin village of Okinawa Island

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Abstract In order to investigate the genetic structure of the ancient Okinawa people at a molecular level, we analyzed the mitochondrial DNA (mtDNA) haplogroups of skeletal remains excavated from two of the old tombs of the Tokijin site, which is located at the center of Okinawa Island. These tombs, which date back to the 17th to 19th century, contained the skeletal remains of 102 individuals. Twenty-four samples were analyzed in this study. The mtDNA obtained from the tombs were tentatively classified under specific haplogroups using multiplex amplified product-length polymorphism (APLP) analysis. Twenty samples were successfully analyzed, and haplogroups were assigned. The results indicated the existence of several different maternal lineages. The frequencies of the haplogroups were compared with those of the modern Japanese population. Although a high frequency of haplogroup M7a is characteristic of the contemporary Ryukyu population, only one sample belonged to this haplogroup. Owing to the kinships among these skeletons, the haplogroup frequencies may be different from the modern population. From the comparison of the haplogroups, it is possible that the people of the Tokijin community were closely related.
Key words: Ancient DNA, Mitochondria, haplogroup, APLP analysis, Okinawa, modern period

Introduction

The elucidation of the formation of the people of the Ryukyu archipelago, the so called Ryukyuans, plays an important role in the clarification of the origins and formation of the Japanese. Therefore, in the field of physical anthropological research in Japan, many studies have been conducted using human bones excavated from the Ryukyu archipelago. As a result, it has been established that the modern Ryukyuans are a group that strongly influenced the Jomon people, together with the Ainu of Hokkaido (Hanihara, 1991). However, the genetic and morphological characteristics of the modern Ryukyuans are different from those of the Ainu, and it is clear that they have their own formation history (Dodo *et al.*, 1998; Jinam *et al.*, 2015).

Recently, archaeological data have pointed out that the southern Kyushu people of medieval times contributed to the formation of the modern Ryukyuan people (Asato, 1996; Shinzato, 2018). There is some archaeological evidence that indicates that a wave of immigrants moved to Okinawa from southern Kyushu. However, an analysis of the anthropological studies has not been conducted from such a point of view. The verification will be a future issue.

Advances in molecular biology over the last 30 years have enabled the analysis of DNA

extracted from ancient bone samples, making it possible to obtain lineage information with significantly higher probabilities of accuracy. Ancient DNA analysis could therefore potentially help to resolve the questions surrounding the formation of the recent Ryukyu population and verify the immigrant hypothesis.

In order to obtain information about the genetic characteristics of the population of Okinawa Island, we have continued to conduct a DNA analysis of human bones that have been excavated from several ruins of the Ryukyu archipelago (Shinoda and Doi, 2008; Shinoda *et al.*, 2012; Shinoda *et al.*, 2013). In this study, we analyzed DNA extracted from human remains that were excavated from the old Tokijin tombs. This ruin is located at the center of Okinawa Island, where almost no excavation research has been done. Therefore, DNA analysis of the human bones excavated from this tomb is important to know the genetic characteristics of the community after medieval times.

Materials and methods

Archaeological sites and specimens

The human skeletal remains analyzed in this study were excavated from the ancient Tokijin tombs in the Nakijin village of Okinawa Island (Figure 1). A lot of old tombs had formed on the coast of various parts of the village. The ancient Tokijin tombs are located in the hillside of the south cliff of the limestone crest that extends from east to west. However, there is no historical evidence that shows the origin of these old tombs.

The site was excavated during 2013 to 2015 by the education committee of Nakijin village.

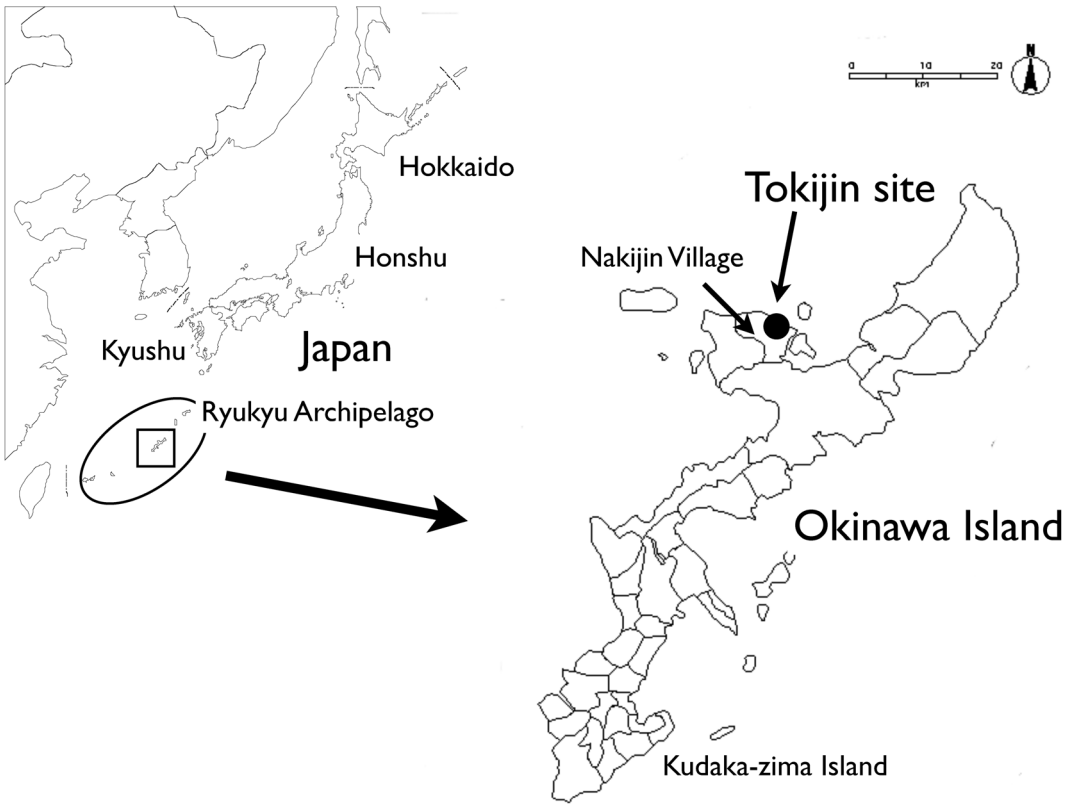

Fig. 1. Geographic distribution of the Ryukyu archipelago. Map showing the location of the Nakiji village and the old Tokijin tomb.

The dates of interment for these tombs are estimated to range approximately from the 17th to 19th century by carbon-14 dating. At this time, the people of this village disposed of a dead body by exposing it to the elements. After the dead bodies became skeletons, they gathered the bones and stored them in miniature sanctuaries. Among the four tombs, a survey of the 2 tombs (ST3 and ST4) on the east side was conducted.

Both tombs contained several miniature sanctuaries, and each of these contained human bones from more than one person. ST3 had a front width of 2.7 m, a height of 1.3 m, and a depth of 1.9 to 2.2 m. There were 21 miniature sanctuaries and five wooden coffins. Among them, the skeletal remains from 9 of the miniature sanctuaries were investigated. ST4 had a front width of 2.7 m, a height of 1.4 m, and a length of 2.2 m. There were 34 miniature sanctuaries and four wooden coffins; skeletal remains from 16 of the miniature sanctuaries were investigated.

The human bones of 102 individuals were excavated at this site. In tomb ST3, 49 bodies, including 11 males, 16 females, 1 sex unknown, 1 young, 3 juveniles, 9 infants, and 8 babies, were buried. In tomb ST4, 53 bodies, including 22 males, 13 females, 2 young, 4 juveniles, 10 infants, and 2 babies, were buried. Among these bones, juvenile human bones accounted for 39% of the total, which was higher than the 30% average for the ruins of the Ryukyu archipelago. The characteristics of the skulls were similar to those of the modern Ryukyu population (Doi, 2017).

In this study, 24 well-preserved adult bone samples from both tombs were selected for DNA analysis. It has been suggested that tooth enamel forms a natural barrier to exogenous DNA contamination; furthermore, the DNA recovered from teeth appears to lack most of the inhibitors of the enzymatic amplification of ancient DNA (Woodward *et al.*, 1994). Therefore, in most cases, teeth samples were used in the present analysis. When teeth samples were not available, metatarsal bones were used. A list of all of the samples used in this study is presented in Table 1.

Table 1. Sample used for DNA exreaction

No.	Tomb	No. of miniature sanctuary	No. of bone	Sex	Portion
1	ST3	1	46		Maxilla, Right, M3
2		2	45		Maxilla, Right, M3
3		3	44		Maxilla, Right, M2
4		4	62		Maxilla, Right, M2
5		5	65	Female	Mandible, Right, M2
6		6	73		Maxilla, Left, M1
7		7	74		Maxilla, Left, M2
8		8	96-1	Female	Mandibel, Left, M1
9		12	60	Female	Maxilla, Left, M2
10	ST4	1	1	Female	Mandible, Right, M2
11		3	11		Metatarsal
12		5	15		Maxilla, Right, M3
13		6	13		Mandible, Right, M1
14		7	14		Maxilla, Left, Premolar2
15		8	24		Maxilla, Left, M3
16		9	105	Male	Maxilla, Right, M1
17		12	25		Maxilla, Right, M1
18		12	35-1		Mandible, Right, M2
19		12	36-1		Maxilla, Left, M1
20		12	40		Maxilla, Right, Premolar2
21		13	118		Maxilla, Left, M3
22		14	119		Maxilla, Right, Premolar2
23		15	120		Maxilla, Left, M2
24		19	111		Mandible, Right, M3

*Sex was judged from morphologocal feature.

Methods

First, exact replicas of each tooth were prepared for other morphological studies. When ancient DNA is analyzed, it is necessary to exclude false-positive results that stem from contamination with contemporary DNA (e.g., Sampietro *et al.*, 2006). To prevent contamination from post-excavation handling, the tooth samples were rinsed with DNA-decontamination agents and then washed thoroughly with distilled water before drying. In order to ensure the accuracy and reliability of the results, rigorous authentication methods were employed throughout the DNA-based analyses, as described elsewhere (Shinoda *et al.*, 2006).

DNA analyses were performed at the Yamanashi University, which are dedicated to ancient DNA analysis. The samples were encased in silicone rubber (Provil novo Heraeus Kulzer GmbH, Hanau, Germany). The tip of the root of the tooth sample was removed by a horizontal cut using a cutting disk, and the dentin around cavities and dental pulp were powdered and removed through the root tip by using a dental drill as described by Gilbert *et al.* (2003). The powdered samples were decalcified with 0.5 M EDTA (pH 8.0) (Invitrogen, Carlsbad, CA, U.S.A.) at room temperature overnight, and then, the EDTA buffer was replaced by fresh buffer, and samples were decalcified for a further 48 h. Decalcified samples were lysed in 500 μl of Fast Lyse (Genetic ID, Fairfield, IA, U.S.A.) with 30 μl of 20 mg/ml Proteinase K (Invitrogen) at 60°C for 4 h. DNA was extracted from the lysate using a FAST ID DNA Extraction Kit (Genetic ID) in accordance with the technical manual.

In this study, mtDNA SNPs that define the haplogroup diagnostic sites found in Southeast Asian and East Asian populations were detected using the two-step APLP method (Umetsu *et al.*, 2001, 2005). This method has been applied for ancient DNA analyses and has yielded convincing results (e.g., Adachi *et al.*, 2009; Shinoda *et al.*, 2006).

In the first step, 26 SNPs in the coding region and a 9-bp repeat variation in the non-coding cytochrome oxidase II/tRNALys intergenic region were analyzed by the multiplex APLP method. If a result was obtained from this experiment, we proceeded to the next step. The second step of the analysis used inosine-flapped primers and was specifically designed for the detailed haplogrouping of degraded mtDNA (Kakuda *et al.*, 2016). The polymorphic sites examined in this study have been shown to cover most of the haplogroup-defining mutations in East Asian mtDNA. The components of the PCR reaction mixture, the thermal conditions, and the methods for the separation and detection of PCR products were the same as those described by Adachi *et al.* (2009).

In some cases, segments of hypervariable region 1 (HVR1; nucleotide positions 16120–16367; HVR2; nucleotide positions 127–257) of mtDNAs, as per the revised Cambridge reference sequence (rCRSs ; Andrews *et al.*, 1999), were sequenced for the detaild analysis. Primers used to amplify the hypervariable regions and PCR conditions were the same as those used by Adachi *et al.* 2009.

Results and Discussion

In the present analysis, the number of samples for which DNA sequences could be determined was small; therefore, unfortunately, we could not make major conclusions on the basis of these results. However, this is the first report of genetic data from the central part of Okinawa Island from a recent time period; it may be possible to deduce the genetic characteristics of the region. Table 2 shows the results from genotyping the coding regions by the APLP methods. Due to the poor quality of the mtDNA extracted from some of the ancient material, it was not possible to amplify all of the samples. Among the 24 individuals considered in this study, ancient DNA data were successfully extracted from 20 of the samples.

A serious problem that is faced when working with degraded templates typical of ancient DNA

Table 2. Result of the analysis

Site	Samples	Results APLP analysis	HV2 sequence (127–257)	HV1 sequence (16120–16367)	Result
ST 3	1-46	D5a (752T, C5178A, 5301G)			D5a
	2-45	B5 (8281–8289 del, 8584A)	T131C (B5b2a), T204C (B5b2a1), A263G	T16140C (B5), A16182–16183C, T16189C, C16234T (B5b2), T16243C (B5b), C16291T (B5b2a1a)	B5b2a1a
	3-44	B5 (8281–8289 del, 8584A)			B5*
	4-62	N.D.			N.D.
	5-65	B5 (8281–8289 del, 8584A)	T131C (B5b2a), T204C (B5b2a1), A263G	T16140C (B5), A16182–16183C, T16189C, C16234T (B5b2), T16243C (B5b), C16291T (B5b2a1a)	B5b2a1a
	6-73	M7b1a1a1 (6455T, 6680C, 10345C)			M7b1a1a1
	7-74	B4 (not a, b, c) (8281–8289 del, T16217C)			B4*
	8-96-1	D5a (752T, C5178A, 5301G)			D5a
	12-60	B4 (not a, b, c) (8281–8289 del, T16217C)	A200G (B4f), T213C	N.D.	B4f?
ST 4	1-1	B5 (8281–8289 del, G8584A)	T131C (B5b2a), T204C (B5b2a1), A263G	T16140C (B5), A16182–16183C, T16189C, C16234T (B5b2), T16243C (B5b), C16291T (B5b2a1a)	B5b2a1a
	3-11	D4 (not a, b, e, g, h, o) (G3010A, C5178A)			D4 (not a, b, e, g, h, o)
	45-15	M7* (not M7a, M7b1a, M7c1) (6455T)			M7*
	46-13	D5a (C752T, C5178A, A5301G)	CRS	A16182–16183C, T16189C, T16362C (D)	D5a
	47-14	D4 (not a, b, e, g, h, o) (G3010A, C5178A)			D4 (not a, b, e, g, h, o)
	48-24	D4e (G3010A, C5178A, C11215T)	CRS	C16223T, T16362C (D)	D4e
	49-105	D4 (not a, b, e, g, h, o) (G3010A, C5178A)	191.1A (D4c1a), C194T (D4c1a), T199C (D4c1a), T204C, G207A (D4c1a)	T16223C (D4c1)	D4c1a
	12-25	N.D.			N.D.
	12-35-1	M9a (4491A, A14308C)			M9a
	12-36-1	M7b1a1a1 (6455T, 6680C, 10345C)			M7b1a1a1
	12-40	M7a1 (6455T, 2772T, 14364A)			M7a1
	13-118-4	D4 (not a, b, e, g, h, o) (G3010A, C5178A)	191.1A (D4c1a), C194T (D4c1a), T199C (D4c1a), G207A (D4c1a)	T16223C (D4c1), C16245T (D4c), T16362C (D)	D4c1a
	14-119	N.D.			N.D.
	15-120	B5 (8281–8289 del, 8584A)			B5*
	19-111	N.D.			N.D.

All polymorphic sites are numbered according to the revised Cambridge reference sequence (Andrews *et al.*, 1999).
N.D. denotes "not determined".
* indicates that it could not any more classifications.

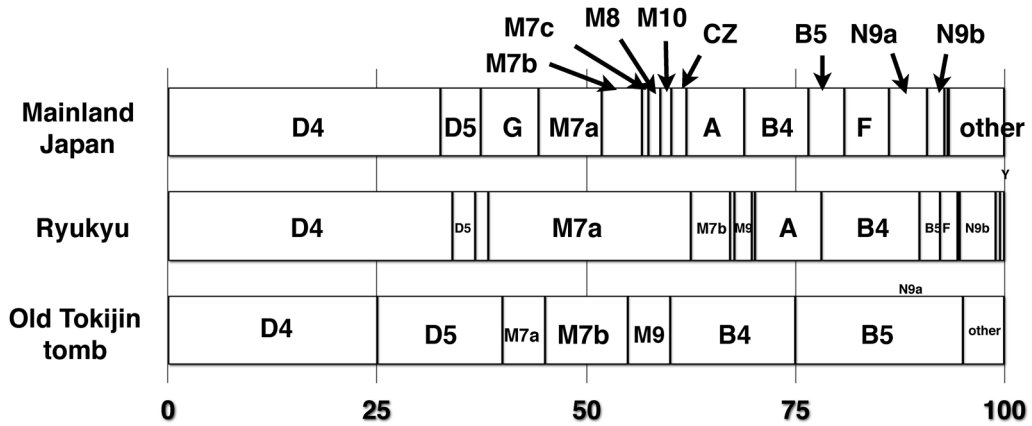

Fig. 2. Estimated frequencies of the mtDNA haplogroups in regional populations.
References:
Ryukyu: Umetsu *et al.* (2005).
Mainland Japanese: Tanaka *et al.* (2004).

is contamination of the samples, even when all reasonable safeguards are taken. In these samples, we were able to successfully retrieve different variants from the first and second step of the APLP analysis, and both results matched. As both of the APLP analyses were carried out independently, the consistency of the results proves the certainty of the experiment.

Hot and humid conditions are generally unfavorable for the preservation of DNA in human skeletal remains. However, the success rate of this study was 83.3%. Past studies have shown that the success rate for the DNA analysis of ancient human remains excavated from the Ryukyu archipelago is 10%–50%, at best, even when well-preserved samples were used (e.g., Shinoda, 2012); accordingly, our results suggest that the preservation conditions for the DNA in these samples are relatively good.

Phylogenetic analysis, based on coding region information, clustered the observed haplotypes into 12 distinct major haplogroups and subhaplogroups. The mtDNA haplogroups showed geographic specificity within Asia (e.g., Kivisild *et al.*, 2002; Li *et al.*, 2007). All mtDNA lineages of this group were assigned to the previously defined haplogroups for East Asians. Figure 2 shows the comparison of the haplogroup frequencies between the Tokijin site, modern Ryukyuans, and Japanese. Although the haplogroups found for the DNA at the Tokijin site are popular in contemporary Japanese, the haplogroup frequencies were quite different from other modern Japanese.

The most distinct feature of the modern Ryukyu population is the higher frequency of the D4 and M7a haplogroups compared to other populations. It is noteworthy that only one M7a haplogroup, which is characteristic of about one-third of modern Ryukyuans, was found at this site. Both of these two tombs are considered to be family graves, because it seems that the buried people had a blood relationship with each other. It is likely that the kinship caused the differences in the haplogroup frequencies compared to other Japanese populations.

Combinations of the same haplogroup were observed among the same tomb or between the two tombs. Haplogroups D5a, B5b2a1a, and M7b1a1a1 were common to both tombs. A pair with haplogroup D4c1a appeared in tomb ST4. Half of the individuals were the same type as the others. As mtDNA is inherited from the maternal lineage, individuals with the same haplogroup may be related by maternal relatives. This finding also provides proof for a genetic continuity

between these two tombs. From the comparison of the haplogroups of these two tombs, it is possible that the people of the Tokijin community were closely related and probably married each other. As ST3 and ST4 are adjacent to each other, there is the possibility that the kinship ties between the two tombs were especially close.

On the other hand, it is also important to note that several kinds of haplogroups were found from both tombs. If the buried people were connected by maternity, there would not be so many types of haplogroups in the same tomb. Perhaps only one or two haplogroups would have been occupied. From this result, it seems reasonable to suppose that these tombs consisted of a paternal line of members. Of course, this will not be obvious unless we examine the Y chromosome DNA of the buried people.

It has been indicated that an intensive analysis of particular archaeological sites using molecular biology methods could shed light on the blood relationships of ancient communities and the relative structure. Though tantalizing, these conclusions must be regarded as tentative, as they are based on small sample sizes and limited analyses. Recently, even with ancient human bones, analysis of the nuclear genome has become possible using next generation sequencing. By analyzing these samples with this method, more precise relationships in this community will be revealed. The present experiment showed the possibility that sufficient amounts of DNA are retained in human skeleton samples. We believe, therefore, that it is important to continue the experiments to obtain more detailed data from this site. We hope to pursue this matter in future studies.

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