Bull. Natl. Mus. Nat. Sci., Ser. D, 38, pp. 51–61, November 22, 2012

Mitochondrial DNA polymorphisms in late Shell midden period skeletal remains excavated from two archaeological sites in Okinawa

Ken-ichi Shinoda[1], Tsuneo Kakuda[1] and Naomi Doi[2]

[1] Department of Anthropology, National Museum of Nature and Science, 4–1–1 Amakubo, Tsukuba, Ibaraki 305–0005, Japan
[2] Department of Anatomy, Faculty of Medicine, University of the Ryukyus, Uehara 207, Nishihara, Okinawa 203–0216, Japan

Abstract To investigate the genetic structure of individuals from the Shell midden (Kaizuka) period in Okinawa Island, sequence polymorphisms in hypervariable region 1 (HVR1), HVR2, the coding region that defines macrohaplogroup M and N, and 24 single nucleotide polymorphisms in the coding region of mitochondrial DNA were analyzed in 29 tooth samples excavated from an Ufutobaru shell midden site and a Gushikawa Gusuku-Gakeshita site. Suspected false-positive results stemming from contamination with contemporary DNA and other questionable data were not included in this study. Six of the 14 Ufutobaru samples and 9 of the 15 Gusuku-Gakeshita samples were successfully analyzed, and haplogroups were assigned. Genetic variation within a group is inherited from the group's ancestors; therefore, the genetic markers in the contemporary population are expected to be similar to those of its ancestors. High frequencies of haplogroups M7a and D4 are characteristic of the contemporary Okinawa population, and both haplogroups were found among the ancient samples. This finding provides proof of a genetic continuity between the ancient and contemporary populations of Okinawa, at least since the late Shell midden period. In contrast, the existence of haplogroup B4 in these populations hints at their linkage with Taiwanese aborigines.
Key words: Ancient DNA, Mitochondria, late Shell midden (Kaizuka) period, Okinawa, Population genetics

Introduction

The formation of the modern Japanese population is an area of intense study in anthropology, archaeology, and genetics in East Asia. In this context, the Ryukyu Islands attract particular attention owing to their geographical position, which makes them ideal for attempts to reconstruct the population movements of the indigenous Japanese population (e.g., Tagaya and Ikeda, 1976). During the Jomon era, the main island of Okinawa, the largest island of the Ryukyu Islands, was subjected to the cultural influence of Mainland Japan, especially from the southern part of Kyushu Island. While Mainland Japan made the transition from the hunter-gatherer lifestyle of the Jomon society to the agricultural Yayoi period around 800 B.C, the hunter-gatherer lifestyle continued until 12th century in the island of Okinawa. Accordingly, the Neolithic period of this island is referred to as the Shell midden (Kaizuka) period in order to distinguish it from the Jomon period of Mainland Japan. Subsequently, this lifestyle too began to evolve with the advent of the agricultural society, heralding the start of the Gusuku period in all parts of the Ryukyu Islands (Asato, 1996).

To explain the spread of agriculture through the island of Okinawa, 2 contrasting hypotheses have been proposed, namely, the cultural diffu-

sion model and the demic diffusion model. The cultural diffusion model supports the idea that the farmers did not move and that agricultural knowledge was transmitted from the southern part of Kyushu Island to Okinawa due to the movement of technology and ideas. In contrast, the demic diffusion model supports the idea that farmers moved from the southern part of Kyushu Island and spread into Okinawa taking the agricultural technology with them. This model involves gene flow between the hunter-gatherers who inhabited the island of Okinawa at that time and the farmers who arrived at the island (Takamiya, 2005). The extent to which this important cultural transition was mediated by the arrival of new peoples and the degree of hunter-gatherer and early farmer ancestry in modern Okinawa today are not known.

Advances in molecular biology within the last 20 years have enabled the analysis of DNA extracted from ancient bone samples, making it possible to obtain information on lineage with significantly higher probabilities of accuracy. Mitochondrial DNA (mtDNA), which follows a matrilineal pattern of inheritance, has been extensively analyzed in this field (e.g., Adachi *et al.* 2009). Ancient DNA analysis could therefore potentially help to resolve the formation of recent Ryukyu population, as it allows us to directly study the ancient populations that were undergoing these evolutionary processes, rather than their descendent populations.

Although mtDNA have been analyzed in many Jomon populations (e.g., Shinoda and Kanai, 1999) and, the Ryukyu population of the recent period (Shinoda and Doi, 2008), there is a complete absence of mtDNA data in the Shell midden (Kaizuka) period from the island of Okinawa. The aim of this study was to provide the basic genetic data for analyzing the relationships between the hunter-gatherer people and the contemporary population of the Ryukyu Islands. A detailed analysis of the coding and control regions of mtDNA was used to address the question of whether the cultural changes in the island of Okinawa were accompanied by changes in the genetic composition of the population.

Materials and methods

Archaeological sites and specimens

In this analysis, we used human skeletal remains excavated from 2 archaeological sites in mainland Okinawa. The approximate locations of these sites are shown in Figure 1. Since no cemetery inventory remains, the chronology of these sites were determined on the basis of the design of burial goods. The dates of both sites are estimated to range from approximately the 3rd to 8th century, corresponding to the late Shell midden (Kaizuka) period.

The Gushikawa Gusuku-Gakeshita site is a cliff burial site located at Uruma city, excavated in 2004–2007 by researchers from Ryukyu University, and it comprises at least 67 individuals. These individuals consisted of 18 males, 11 females, 19 juveniles, and 19 individuals of undetermined sex, ranging from infants to mature adults (Katagiri *et al.*, 2008). Sixteen well-preserved samples were selected for further DNA analysis.

A sediment production project in the 1970s identified the Ufutobaru site, located at Yomitanson, in the central part of Okinawa Island. One human skull covered with pottery and accumulated human remains, were found during the excavations conducted 1972 and 1989. By counting skull and limb bones, the number of individuals was estimated as at least 18 (12 adult males, 5 adult females, 1 immature individual) (Kobashigawa *et al.* 2008). Of these 18 samples, 14 well-preserved remains were stored in the Okinawa Prefectural Archaeological Center and selected for DNA analysis.

Tooth enamel forms a natural barrier to exogenous DNA contamination; additionally, the DNA recovered from teeth appears to lack most of the inhibitors of the enzymatic amplification of ancient DNA (Woodward *et al.* 1994). Accordingly, tooth samples were used in the present analysis. A list of all the samples used in this study is presented in Table 3.

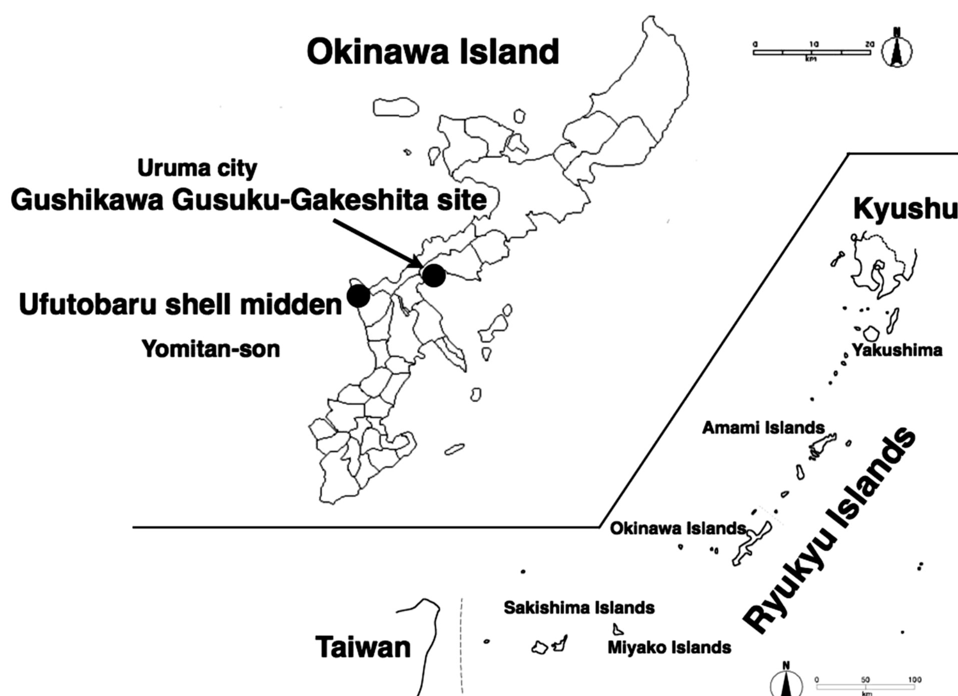

Fig. 1. Geographic distribution of Ryukyu archipelago. Map showing location of the Ufutobaru shell midden and the Gushikawa Gusuku-Gakeshita site.

Authentication methods

When ancient DNA is analyzed, it is necessary to exclude false-positive results that stem from contamination with contemporary DNA (Sampietro *et al.*, 2006). In order to ensure the accuracy and reliability of results, standard contamination precautions, such as separation of pre- and post-PCR experimental areas, use of disposable laboratory ware and filter-plugged pipette tips, treatment with DNA contamination removal solution (DNA-OFFTM; TaKaRa, Otsu, Japan), UV irradiation of equipment and benches, negative extraction controls, and negative PCR controls, were employed in the present study. Other rigorous authentication methods were employed throughout the DNA-based analyses as described elsewhere (Shinoda *et al.*, 2006). Tooth preparation, DNA extraction, and PCR amplification were carried out in a physically separate room, in a laboratory dedicated to the study of ancient DNA.

Extraction and purification of DNA

In order 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. Next, the samples were crushed into powder using a Multi-beads Shocker (Yasui Kikai Corporation). DNA was extracted from 0.5 g of the powder for each sample using a commercial DNA extraction kit (Qiagen, DNA Investigator kit, Germany) after Protainase K digestion.

Amplification and sequencing of HVR1, HVR2, and coding region 10360–10485

In all the samples, segments of HVRs (nucleotide positions 16121–16238, HVR1–1; 16209–16402, HVR1–2; and 128–267; HVR2), and a segment of the coding region (10360–10485) that covers part of the NADH dehydrogenase 3 and tRNA-Arg genes, as per the revised Cambridge reference sequence (Andrews *et al.*, 1999), were

sequenced. This coding region sequence includes sites 10398 and 10400 that are among the defining sites for macrohaplogroups M and N (Quintana-Marci *et al.*, 1999).

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 (HotStarTaqTM DNA polymerase; Qiagen, Germany), 0.1 M of each primer and 100 mM of deoxyribonucloside triphosphates in 1 × PCR buffer provided 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 sec; 50°C–56°C for 20 sec, and 72°C for 15 sec; and final extension at 72°C for 1 min.

The following primers were used to amplify HVR1–1, HVR1–2, HVR2, and the coding region:

L16120 5′-TTACTGCCAGCCACCATGAA-3′
H16239 5′-TGGCTTTGGAGTTGCAGTTG-3′
L16208 5′-CCCCATGCTTACAAGCAAG-3′
H16403 5′-TTGATTTCACGGAGGATGGTG-3′
L127 5′-TAGCACCCTATGTCGCAGTAT-3′
H268 5′-TGTTATGATGTCTGTGTGG-3′
L10360 5′-TCCCCATGCTTACAAGCAAG-3′
H10485 5′-ATTGATTTCACGGAGGATGG-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 Ver.3.1 (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 to enable identification of polymorphisms or ambiguous bases by using a single primer. The sequencing reactions were performed on a DNA Sequencer (ABI model no., 3130) equipped with SeqEd software.

Genotyping of Polymorphisms

Recently, a large number of complete sequences of the human mitochondrial genome have been reported and the haplogroup-defining SNPs have been determined. (e.g., Kong *et al.* 2003, 2006). Moreover, a rapid, economical method for analyzing the mtSNPs has been developed, namely, the multiplex amplified product-length polymorphism technique (Umetsu *et al.* 2001).

In this study, 24-plex PCRs were performed for amplifying mtDNA fragments that contain mtDNA SNPs that define major haplogroups found in Japanese and East Asian population. To minimize the risk of contamination and to raise the efficiency of PCR, the very short DNA fragments (average 60–80 bp) required by this approach. The reaction mixture (25 ml) contained 5 μl of template DNA, 0.2 μmol of each primer, 10 μl of 2.5 × PCR buffer, and 0.625 U of DNA polymerase. The amplification protocol consisted of an initial denaturation at 95°C for 10 min; followed by 30 cycles of denaturation at 94°C for 20 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; with a final extension at 72°C for 7 min. SNPs that define major haplogroups were detected by use of suspension-array technology (Luminex 100; Luminex) with sequence specific oligonucleotide probes (G&G Science, Fukushima). The methodology used for genotyping has been described in detail elsewhere (Itoh *et al.* 2005), and the primers used are shown in Table 1. Probes used for haplotyping are shown in Table 2.

Results

Due to the poor quality of the mtDNA extracted from the ancient material, it was not possible to amplify all samples. Among the 29 individuals considered in this study, ancient DNA data was successfully extracted from 15 samples (9 for the Gushikawa Gusuku-Gakeshita site and 6 for the Ufutobaru site, Table 3). The remaining 14 samples either failed to yield a product on amplification or contained several

Table 1. Promers used for 24-Plex PCR.

Fragment	Target mtSNP	Haplogroup	Primer Sequence: Forward Position	Primer Sequence: Forward Sequence (5′–3′)	Primer Sequence: Reverse Position	Primer Sequence: Reverse Sequence (5′–3′)	Product (bp)
1	856 A>G	D4d1b	822	GATTAACCTTTAGCAATAAACGAAAGTT	885	AAATTGACCAACCCTGGGGTTAGTA	
			811	GAAACAGCAGTGATTAACCTTTAGC	890	GCACGAAATTGACCAACCCTGG	
					894	GCTGGCACGAAATTGACCAACC	
2	3010 G>A	D4	2983	GACCTCGATGTTGGATCAGGACA	3036	CGAACCTTTAATAGCGGCTGCAC	54
3	3644 T>C	D4h	3619	ATTCTAGCCACCTCTAGCCTAGC	3670	CTCACCCTGATCAGAGGATTGAG	52
4	4048 G>A	M7b	4020	CACTACAATCTTCCTAGGAACAACA	4073	GTGTAGAGTTCAGGGGAGAGTG	54
5	4386 T>C	M7a	4359	AGAATCCAAAATTCTCCGTGCCAC	4421	CTTATTTAGCTGACCTTACTTTAGGA	63
6	4820 G>A	B4b	4797	CCCTTTCACTTCTGAGTCCCAG	4846	GATGTCAGAGGGGTGCCTTGG	50
7	5147 G>A	N9b	5121	CTACTCAACTTAAACTCCAGCACCA	5169	AGGTGCGAGATAGTAGTAGGGT	49
8	5178 C>A	D	5155	TACTATCTCGCACCTGAAACAAG	5206	GATGGAATTAAGGGTGTTAGTCATGT	52
9	5231 G>A	N9a	5203	CATCCACCCTCCTCTCCCTAG	5260	CATTTGGGCAAAAAGCCGGTTAG	58
10	5964 T>C	D4e1	5932	CAAACCACAAAGACATTGGAACACT	5992	CCTAGGACTCCAGCTCATGCG	61
11	6689 C>T	D4b1b	6658	GAATAATCTCCCATATTGTAACTTAC	6725	GACCATACCTATGTATCCAAATGG	68
12	8272 C>del.9bp	B	8240	TTTGAAATAGGGCCCGTATTTACC	8306	GTTAGCTTTACAGTGGGCTCTAG	70
13	8701 A>G	N,D4g	8667	ATGACTAATCAAACTAACCTCAAAACAA	8730	TCAGGTTCGTCCTTTAGTGTTGTG	64
14	8793T>C	M10	8768	CAACTAACCTCCTCGGACTCCTG	8819	AGTTGGGTGGTTGGTGTAAATGAG	52
	8794 C>T	A					
15	9296 C>T	D4b2b	9268	CCCTCTCAGCCCTCCTAATGAC	9329	CGTTATGGAGTGGAAGTGAAATCA	62
16	9950 T>C	B5	9923	CTGATACTGGCATTTTGTAGATGTG	9988	GACCCTCATCAATAGATGGAGAC	66
17	10310 G>A	F	10284	CCATGAGCCCTACAAACAACTAAC	10337	TAAGAGGGATGACATAACTATTAGTG	54
18	10397 A>G	D5	10364	GGCCTATGAGTGACTACAAAAAGG	10432	TCATTCGTTTTGTTTAAACTATATACC	69
	10400 C>T	M					
19	13563 A>G	G2	13536	CATATCATACACAAACGCCTGAGC	13591	TCAGGGAGGTAGCGATGAGAGTA	56
20	14979 T>C	D4a	14953	CACTCGAGACGTAAATTATGGCTG	15005	CGCCATTGGCGTGAAGGTAGCG	53
21	15346 G>A	B4c	15319	CCTAGCAACACTCCACCTCCTA	15370	GGGGTTGTTTGATCCCGTTTCG	52
22	15497 G>A	G1	15471	TAACACTATTCTCACCAGACCTCC	15518	GGGTATAATTGTCTGGGTCGC	48
23	15524 A>C	D4b2a	15503	CCAGACAATTATACCCTAGCC	15550	GATGTGGGGAGGGGTGTTTAAGG	48
24	15874 A>G	D4e2	15844	ACCAACTATCTCCCTAATTGAAAACA	15903	TTTATACTACAAGGACAGGCCCATT	60

Table 2. Probe set for haplotyping.

Fragment	Target mtSNP	Haplogroup	Plobe	5'-Seq-3'
1	856 A>G	D4d1b	Sense	AAAGTTTAACTAGGCTATACTA
2	3010 G>A	D4	Antisense	GGCTGCACCATTGGGATGT
3	3644 T>C	D4h	Sense	TAGCCTAGCCGCTTACTCAAT
4	4048 G>A	M7b	Sense	CTAGGAACAACATATAACGCACT
5	4386 T>C	M7a	Antisense	GGTGTGGTAGGTGGCAC
6	4820 G>A	B4b	Sense	TTCTGAGTCCCAGAAGTTACCC
7	5147 G>A	N9b	Sense	CCAGCACCACAACCCT
			Sense	TCCAGCACCACAACCCTA
8	5178 C>A	D	Antisense	GTGTTAGTCATGTTATCTTGTTT
9	5231 G>A	N9a	Sense	TCCCTAGGAGGCCTACCCCC
10	5964 T>C	D4e1	Antisense	CATGCGCCGAATAGTAGGTAT
11	6689 C>T	D4b1b	Antisense	TGGTTCTTTTTTTCCAGAGTAGT
12	8272 C>del.9bp	B	Sense	TATAGCACCTCCTCTAGAGCCCA
			Sense	ATAGCACCTCCTCTAGAGCCCA
13	8701 A>G	N,D4g	Antisense	TAGTGTTGTGTATGGCTATCATTTG
14	8793T>C	M10	Antisense	TGGTGTAAATGAGTGGGGCAG
	8794 C>T	A	Antisense	TGGTGTAAATGAGTAAGGCAGG
15	9296 C>T	D4b2b	Antisense	AAATCACATGGCTAGACCGG
16	9950 T>C	B5	Antisense	CAGAAATAGTCAGACCACATCTA
17	10310 G>A	F	Antisense	ACATAACTATTAGTGGTAGGTTA
			Antisense	ATTAGTGGTAGGTTAGTTGTT
18	10397 A>G	D5	Antisense	ACATAACTATTAGTGGTAGGTTA
			Antisense	ATTAGTGGTAGGTTAGTTGTT
	10400 C>T	M		GGATTAGACTGAGCTGAATTGGTA
19	13563 A>G	G2	Antisense	GATGAGAGTAATAGACAGGGCT
20	14979 T>C	D4a	Antisense	AGGTAGCGGATGGTTCAGC
21	15346 G>A	B4c	Sense	CACCTCCTATTCTTACACGAAAC
22	15497 G>A	G1	Antisense	ATTGTCTGGGTCGCTTAGGAG
23	15524 A>C	D4b2a	Antisense	GTGTTTAAGGGGTCGGCTAG
24	15874 A>G	D4e2	Antisense	AGGCCCATTTGAGCATTTTGTT

ambiguous nucleotide positions during the sequencing reaction, possibly due to misincorporations by the DNA polymerase during PCR. Nine samples failed to amplify any portion of mtDNA, and no DNA was recovered. Collectively, the success rate was 51.7%. Past studies have shown that the success rate of DNA analysis of ancient human remains is 60%–80% at best, even when well-preserved samples are used (e.g., Shinoda, 2004); accordingly, our results suggest that the preservation conditions of DNA in these samples are poor.

We were able to successfully retrieve different variants of the 280-bp HVR1 (16121–16402) sequence. Comparison of the mtDNA sequence enabled the identification of 7 mitochondrial haplotypes in the Gushikawa Gusuku-Gakeshita site and 4 haplotypes in the Ufutobaru site. Table 3 shows the positions at which the sequences of the 2 sites differed from the reference sequence. As seen in Table 3, the base sequence was determined in 141 base pairs of HVR2 (128–267), in which mutations were observed in 6 portions in all. According to analysis of the D-loop sequences, there was no shared haplotype among these sites, which could be attributed to the absence of closely matrilineal relationships.

Of the 29 specimens examined in the coding region (10360–10485), we were unable to obtain PCR products from 16 specimens. Consequently, only 13 specimens were analyzed in this region (Table 3). According to the defining sites for macrohaplogroups M and N, 8 individuals belong to the macrohaplogroup M and 5 individuals belong to macrohaplogroup N.

Fifteen of the 29 samples were successfully typed in each haplogroup by multiplex SNP typing assays to simultaneously analyze important SNPs within the mtDNA coding region (Table 3).

Table 3. Sample used for DNA exreaction and the result of the analysis.

No.	Code Name	Sample	HV1 (16121–16238) (16,000 +)	HV1 (16209–16402) (16,000 +)	HV2 (128-267)	Coding region (10360–10485) (10,000 +)	SNP PCR-Luminex	Hap-logroup
Gusuku-Gakeshita site								
1	85	Maxilla, Right, M3	N.D.	223, 362	152	398, 400	D4	D4
2	121	Maxilla, Right, M3	N.D.	223, 362	152	398, 400	D4	D4
3	237	Mandible, Left, M2	N.D.	N.D.	N.D.	N.D.	M	N.D.
4	270	Mandible, Left, M2	N.D.	217, 311	152, 207	CRS	B4c	B4c
5	273	Maxilla, Right, M3	209, 223	209, 223	152	N.D.	M7a	M7a
6	275	Mandible, Right, M2	N.D.	N.D.	152	N.D.	M7a	M7a
7	329	Maxilla, Left, M3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8	344	Maxilla, Right, M3	N.D.	290, 319, 362	150	CRS	N	A4
9	409	Maxilla, Left, M3	N.D.	217, 311	152	N.D.	*	B4c
10	422	Mandible, Left, M2	N.D.	N.D.	N.D.	N.D.	*	N.D.
11	423	Mandible, Left, M2	N.D.	N.D.	N.D.	398, 400	M	N.D.
12	467	Maxilla, Right, M3	N.D.	N.D.	N.D.	N.D.	*	N.D.
13	622	Maxilla, Right, M3	N.D.	N.D.	N.D.	(398)	*	N.D.
14	671	Mandible, Left, M2	209, 223	209, 223, 324, 325	185	N.D.	*	M7a
15	699	Mandible, Right, M2	209, 223	209, 223, 270, 324	150, 152	398, 400	*	M7a
Ufudobaru shell mound site								
1	93	Mandible, Left, M2	223	223, 298, 325, 327	249d	398, 400	M	C
2	96	Mandible, Left, M2	183,189	N.D.	152	N.D.	B4c	B4c
3	97	Maxilla, Right, PM2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
4	98	Maxilla, Left, M2	223	N.D.	N.D.	398, 400	D4	D4
5	99	Maxilla, Left, M2	182, 183, 189, 217	217, 261	146	CRS	N.D.	B4a
6	100	Mandible, Right, M2	N.D.	N.D.	N.D.	N.D.	*	N.D.
7	109	Mandible, Right, M2	223	N.D.	N.D.	N.D.	D4	D4
8	114	Maxilla, Right, PM2	223	N.D.	N.D.	N.D.	N.D.	N.D.
9	116	Maxilla, Right, M3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10	118	Maxilla, Right, M3	N.D.	N.D.	N.D.	398, 400	M	N.D.
11	119	Maxilla, Right, M3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
12	122	Mandible, Left, M2	N.D.	N.D.	N.D.	398, 400	M	N.D.
13	124	Mandible, Left, M1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
14	125	Maxilla, Left, PM2	(184), 223	223, 290, 319, 362	152	CRS	N	A4

All polymorphic sites are numbered according to the revised Cambridge reference sequence (Andrews *et al.*, 1999).
CRS indicates that the sequence of the segment is identical to the revised
Cambridge reference sequence.
N.D. denotes "not determined".
"d" indicates deletion.
The asterisk means no experiment has done because of the lack of the sampled DNA.

Discussion

A serious problem when working with degraded templates typical of ancient DNA, is contamination of the samples, even when all reasonable safeguards are taken. Multiplexed PCR reactions provide a potential solution to the problem of contamination, because multiple primer pairs amplify different parts of the genome together in a single reaction tube, thereby reducing the chances of contamination dramatically (Endicott *et al.*, 2006). In this study, all of the

mtDNA SNP typing results were concordant with the haplogroup assignments, according to the HVS-I and HVS-II sequence information. Our results therefore also confirmed that multiplex assays are a powerful tool for analyzing highly degraded ancient DNA.

MtDNA haplogroups show geographic specificity within Asia (Kivisild *et al.*, 2002; Li *et al.*, 2007; Soares *et al.*, 2008). Firstly, in order to clarify the genetic characteristics of the modern Ryukyu population, their mtDNA haplogroup frequency data obtained from the published data were compared with those of neighboring modern Japanese and aboriginal Taiwanese populations (Figure 2). As shown in the figure, regardless of their geographical position, the modern Ryukyu and Taiwanese aboriginal populations were not similar when compared according to their share of haplogroups. It may be safely assumed, therefore, that both populations had different origins and population histories as a whole.

The most distinct feature of the modern Ryukyu population is the higher frequencies of the D4 and M7a haplogroups compared with other populations. The Mainland Japanese population shares the same haplogroups with the Ryukyu population: haplogroup D4 is dominant in both groups, although the frequencies of haplogroup M7a is quite different, with a higher frequency in the Ryukyu population (23%) than in the modern Japanese population (7%). Moreover, this haplogroup is absent or scarce in other East and Southeast Asian populations outside Japan.

The M7a haplogroup has been previously suggested to be the "Jomon genotype" (Kivisild *et al.*, 2002; Tanaka *et al.*, 2004), and the clinal pattern from the south to the north in the Japanese archipelago has been interpreted as reflecting a population movement in ancient Japan. It is highly possible that this haplogroup was introduced by a Paleolithic ancestor from Southeast Asia or the southern region of the Asian continent. Considering the biased distribution pattern of the haplogroup M7a in Japan, it may have diversified from the ancestral M7 haplogroup, which was introduced into Japan around the Last Glacial Maxim within the Japanese archipelago (Adachi *et al.* 2011), and the Ryukyu Islands is one of the candidates for the origin of this haplogroup.

The most comprehensive autosomal study of the Japanese population shows a relatively clear difference between the Ryukyu population and Mainland Japanese population (Yamaguchi-Kabata *et al.* 2009). This evidence also suggests a different population history of the Ryukyu Islands compared to Mainland Japan.

The haplogroup composition of the samples of this analysis is M7a (26.7%); D4 (26.7%); B4 (26.7%); A4 (13.3%); and C (6.7%). Owing to the small sample size, it is difficult to verify the

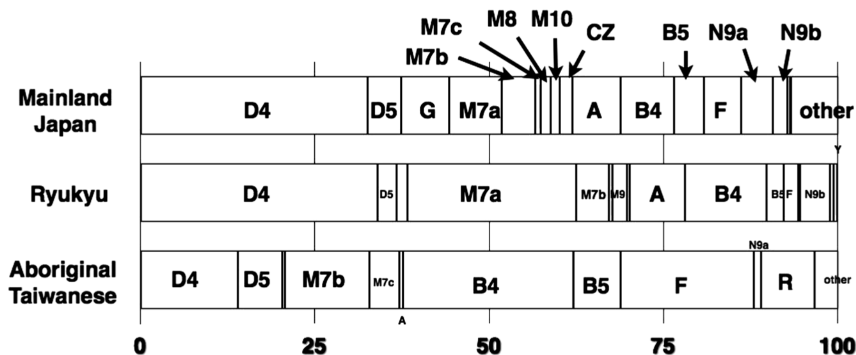

Fig. 2. Estimated frequencies of mtDNA haplogroups in regional populations. Population references are as follows: Mainland Japan; Tanaka *et al.* (2004). Ryukyu; Umetsu *et al.* (2005). Taiwan aborigines; Trejaut *et al.* (2005)

genetic characteristics by statistical methods, although it is noteworthy that M7a and D4 haplogroups were in high proportion. Although the sample size is recognized to be small, all of the dominant haplogroups in the modern Ryukyu population existed in the late Shell midden period. The haplogroup composition of the ancient population is similar to that found in modern populations from the Ryukyu Islands, suggesting a genetic continuity, at least since the late Shell midden period.

The distribution of mtDNA haplogroups in this period will afford some insights into the population history. The M7a haplogroup is dominant only in contemporary Ryukyu populations; in contrast, the frequency of the D4 haplogroup is relatively high in East Asian populations, including in Mainland Japan. We speculate that population expansion southward from Mainland Japan during the ancient period resulted in the admixture of these people with the indigenous Ryukyu population and led to the formation of the basic pattern observed in the modern Ryukyu population.

It is highly possible that the haplogroup D4-dominated profile of the Mainland Japanese mtDNAs was established after the Yayoi period. In fact, immigration of the Yayoi people to northern Kyushu might reflect an increase in the number of people with a haplogroup D4-dominated mtDNA profile in Mainland Japan (Shinoda, 2007). The most plausible explanation for this observation may be that the diffusion of the haplogroup D4 in the island of Okinawa might have occurred after the Yayoi Period. D4-dominated pioneer farming groups carried farming into the island of Okinawa, and therefore the dispersal of agricultural techniques during the late Shell midden (Kaizuka) period took place through a demic diffusion model. To test this hypothesis, we should analyze additional specimens prior to the late Shell midden period from the island of Okinawa.

It seems reasonable to suppose from this analysis that the Ryukyu population shares its maternal ancestry with aboriginal Taiwanese through haplogroup B4. This haplogroup is the most prevalent in the aboriginal Taiwanese of the east coast region (Trejaut *et al.*, 2005), indicating that this haplogroup may have been introduced by the ancient aboriginal Taiwanese. It seems that the ancestral population of coastal East Asia and Island Southeast Asia was enriched by the founder lineages of haplogroup B4, and its northernmost arrival area might be the Ryukyu Islands. This finding indicates that the Southeast input on the ancient Okinawa population affected their genetic structure and that the ancestors of the aboriginal Taiwanese might be the main source of South East Asian mtDNA in the Okinawa.

The number of samples for which DNA sequences could be determined was small in the present analysis; therefore, unfortunately, we could not make major conclusions on the basis of these results. However, if it were possible to collect DNA data of the people inhabiting Ryukyu Islands over a prolonged period, it may be possible to deduce the movement of groups and the population dynamics in the region with greater accuracy. Since hot and humid conditions are unfavorable to the preservation of DNA, the possibilities are low for finding well-preserved DNA in a region like the Ryukyu Islands, which is located in a tropical environment. However, 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 on the human skeletal remains excavated in this region.

Acknowledgments

We wish to thank Mr. Katagiri of the Okinawa Prefectural Archaeological Center and his colleagues for permitting us to use the skeletal materials, and for their valuable advice.

Literature Cited

Adachi, N., K. Shinoda, K. Umetsu and H. Matsumura,

2009. Mitochondrial DNA analysis of Jomon skeletons from the Funadomari site, Hokkaido, and its implication for the origin of Native Americans. *American Journal of Physical Anthropology*, 138: 255–265.

Adachi, N., K. Shinoda, K. Umetsu, T. Kitano, H. Matsumura, R. Fujiyama, J. Sawada and M. Tanaka, 2011. Mitochondrial DNA Analysis of Hokkaido Jomon Skeletons: Remnants of Archaic Maternal Lineages at the Southwestern Edge of Former Beringia. *American Journal of Physical Anthropology*, 146: 346–360.

Andrews, R.M., I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull and N. Howell, 1999. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nature Genetics*, 23: 147.

Asato, S., 1996. The formation of Ryukyu and viewed from archaeology. *Journal of Geography*, 105: 364–371 (In Japanese)

Endicott, P., M. Metspalu, C. Stringer, V. Macaulay, A. Cooper and J.J. Sanchez, 2006. Multiplexed SNP typing of ancient DNA clarifies the origin of Andaman mtDNA haplogroups amongst South Asian tribal populations. *PLoS ONE* 1(1): e81. doi:10.1371/journal.pone.0000081

Itoh, Y., N. Mizuki, T. Shimada, F. Azuma, M. Itakura, K. Kashiwase, E. Kikkawa, J.K. Kulski, M. Satake and H. Inoko, 2005. Highthroughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics*, 57: 717–729.

Katagiri, C., T. Kobashigawa, A. Nishime, N. Doi and M. Yoneda, 2008. Prehistoric cliff burials in the Okinawa Islands: Focused on the findings from excavations at the Gushikawa Gusuku, Uruma City, Okinawa. *Anthropological Science*, 116: 250.

Kivisild, T., H-V. Tolk, J. Parik, Y. Wang, S.S. Papiha, H.J. Bandelt and R. Villems, 2002. The emerging limbs and twigs of the East Asian mtDNA tree. *Molecular Biology and Evolution*, 19: 1737–1751.

Kobashigawa, T., T. Katagiri, C. Tokumine, R. Morimura, M. Oshiro, M. Tengan, N. Doi and M. Yoneda, 2008. Human skeletal remains excavated from the Ufutobaru shell midden site, Tomitan-son, Okinawa: Physical characteristics of the Ufutobaru population. *Anthropological Science*, 116: 252.

Kong, Q.P., Y.G. Yao, C. Sun, H.J. Bandelt, C.L. Zhu and P. Zhang, 2003. Phylogeny of East Asian Mitochondrial DNA Lineages Inferred from Complete Sequences. *American Journal of Human Genetics*, 73: 674–676.

Kong, Q.P., H.J. Bandelt, C. Sun, Y.G. Yao, A. Salas, A. Achilli, C.Y. Wang, L. Zhong, C.L. Zhu, S.F. Wu. A. Torroni and Y.P. Zhang, 2006. Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. *Human Molecular Genetics*, 15: 2076–2086.

Li, H., X. Cal, E.R. Winograd-Cort, B. Wen, X. Cheng, Z. Qin, W. Liu, Y. Liu, S. Pan, J. Qian, C-C. Tan and L. Jin 2007. Mitochondrial DNA diversity and population differentiation on Southern East Asia. *American Journal of Physical Anthropology*, 134: 481–488.

Quintana-Murci, L., O. Semino, H.J. Bandelt, G. Passarino, K. McElreavey and A.S. Santachiara-Benerecetti, 1999. Genetic evidence for an early exit of Homo sapience from Africa through eastern Africa. *Nature Genetics,* 23: 437–441.

Sampietro, M.L., M.T.P. Gilbert, O. Lao, D. Caramelli, M. Lari, J. Bertranpetit and C. Lalueza-Fox, 2006. Tracking down human contamination in ancient human teeth. *Molecular and Biological Evolution*, 23: 1801–1807.

Shinoda, K. and S. Kanai, 1999. Intracemetery genetic analysis at the Nakazuma Jomon site in Japan by mitochondrial DNA sequencing. *Anthropological Science*, 107: 129–140.

Shinoda, K., 2004. Ancient DNA analysis of skeletal samples recovered from the Kuma-Nishioda Yayoi site. *Bulletin of National Museum of Nature and Science, D*, 30: 1–8.

Shinoda, K. 2007. People who became the ancestor of the Japanese. Tokyo: Nihon Housou Syuppan Kyoukai (in Japanese).

Shinoda, K. and N. Doi, 2008. Mitochondrial DNA analysis of human skeletal remains obtained from the old tomb of Suubaru: Genetic characteristics of the westernmost island Japan. *Bulletin of National Museum of Nature and Science, D*, 34, 11–18.

Shinoda, K., N. Adachi, S. Guillen and I. Shimada, 2006 Mitochondrial DNA analysis of ancient Peruvian highlanders. *American Journal of Physical Anthropology*, 131: 98–107.

Soares, P., J.A. Trejaut, J-H. Loo, C. Hill, M. Momina, C-L. Lee, Y-M. Chen, G. Hudjashov, P. Forster, V. Macaulay, D. Bulbeck, S. Oppenheimer, M. Lin and M. Richards, 2008. Climate change and postglacial human dispersals in Southeast Asia. *Molecular Biology and Evolution*, 25: 1209–1218.

Tagaya, A. and J. Ikeda 1976. A multivariate analysis of the cranial measurements of the Ryukyu Islanders (male). *Journal of the Anthropological Society of Nippon,* 84: 204–220.

Takamiya, H., 2005. Prehistory of the Okinawa Islands. Border Inc. Naha, Okinawa. (In Japanese)

Tanaka, M., V.M. Cabrera, A.M. González, J.M. Larruga, T. Takeyasu, N. Fuku, L.J. Guo, R. Hirose, Y. Fujita, M. Kurata, K. Shinoda, K. Umetsu, Y. Yamada, Y. Oshida, Y. Sato, N. Hattori, Y. Mizuno, Y. Arai, N. Hirose, S. Ohta, O. Ogawa, Y. Tanaka, R. Kawamori, M. Shamoto-Nagai, W. Maruyama, H. Shimokata, R. Suzuki and H. Shimodaira, 2004. Mitochondrial genome variation in East Asia and the peopling of

Japan. *Genome Research*, 74: 1832–1850.

Trejaut, J.A., T. Kivisild, J.H. Loo, C.L. Lee, C.L. He, C.J. Hsu, Z.Y. Li and M. Lin, 2005. Traces of archaic mitochondrial lineages persist in Austronesian-speaking Formosan populations. *PLoS Biology*, 3: e247.

Umetsu, K., M. Tanaka, I. Yuasa, N. Adachi, A. Miyoshi, S. Kashimura, K.S. Park, Y.H. Wei, G. Watanabe and M. Osawa, 2005. Multiplex amplified product-length polymorphism analysis of 36 mitochondrial single-nucleotide polymorphisms for haplogrouping of East Asian populations. *Electrophoresis*, 26: 91–98.

Woodward, S.R., M.J. King, N.M. Chiu, M.J. Kuchar and C.W. Griggs, 1994. Amplification of ancient nuclear DNA from teeth and soft tissues. *PCR methods and Applications*, 3: 244–247.

Yamaguchi-Kabata, Y., K. Nakazono, A. Takahashi, S. Saito, N. Hosono, Y. Nakamura and N. Kamatani, 2009. Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effect on population-based association studies. *American Journal of Human Genetics*, 83: 445–456.