

Ancient DNA Analyses of Human Skeletal Remains from the Gusuku Period in the Ryukyu Islands, Japan

Ken-ichi Shinoda¹, Tsuneo Kakuda¹ and Naomi Doi²

¹Department of Anthropology, National Museum of Nature and Science,
4–1–1, Amakubo, Tsukuba City, Ibaraki Prefecture 305–0005, Japan

²Department of Anatomy, Faculty of Medicine, University of the Ryukyus,
Uehara 207, Nishihara, Okinawa Prefecture 203–0216, Japan

Abstract Identifying genetic characteristics of the Gusuku period is indispensable to clarifying the colonizing process of the Ryukyu Islands and formation of the modern Ryukyu population. However, no genetic information on this period has hitherto been reported. In the present study, we analyzed ancient DNA recovered from Gusuku skeletons excavated from Okinawa Island (Nakandariyama site) and Hateruma Island (Moubaru site). Sequence polymorphisms in hypervariable region 1 (HVR1) and 24 single nucleotide polymorphisms in the coding region of mitochondrial DNA (mtDNA) were analyzed in eight tooth samples from Nakandariyama and seven samples from Moubaru. We assigned 12 mtDNAs (six from both sites) to relevant haplogroups. Haplogroups M7a, M7b, D4, A, and B were observed in these individuals, with M7a and D4 predominant in both archaeological sites, indicating characteristics of the contemporary Okinawan population. The geographic distribution of haplogroups M7a and D4 at both sites seems to support the claim inferred from the archaeological evidence for a relationship between the Okinawa and Sakishima Islands during the Gusuku period. The fact that all these haplogroups were observed with relatively high frequencies in the late Kaizuka, Gusuku, and contemporary Ryukyu populations implies that formation of the extinct population from the Ryukyu Islands might result from a population expansion after the late Kaizuka period.

Key words: Ancient DNA, Mitochondria, Gusuku period, Okinawa, Population genetics

Introduction

Due to its geographical position and distinction as the largest of the Ryukyu Islands, Okinawa may have been an important place along the migration route from southern Asia to the Japanese archipelago. The first human skeletal evidence on Okinawa dates to the Paleolithic period (Matsu'ura, 1982). Human genetic diversity on this island has largely been shaped by migration processes occurring after the initial Paleolithic occupations. This was followed by the spread of farming, which probably entailed population movements from Kyushu Island.

Much is already known about the main demographic transitions revealed by studies of genetic diversity in extant populations (Koganebuchi

et al., 2012; Matsukusa *et al.*, 2010) and research into historical, cultural, and classic anthropological sources from the Ryukyu Islands (e.g., Fukumine *et al.*, 2006; Takamiya, 2005; Asato, 1996; Tagaya and Ikeda, 1976). However, the consistency in the information from these myriad sources is still debated.

Because of the Ryukyu Islands' complex history and prehistory, it is not surprising that they have often attracted the interest of population geneticists (e.g., Jinam *et al.*, 2012). Nevertheless, we still know very little about the genetic structure of their human populations. To answer questions regarding the genotypes of ancient populations, genetic analysis of skeletons excavated from archaeological sites is the most straightforward approach. Ancient DNA analyti-

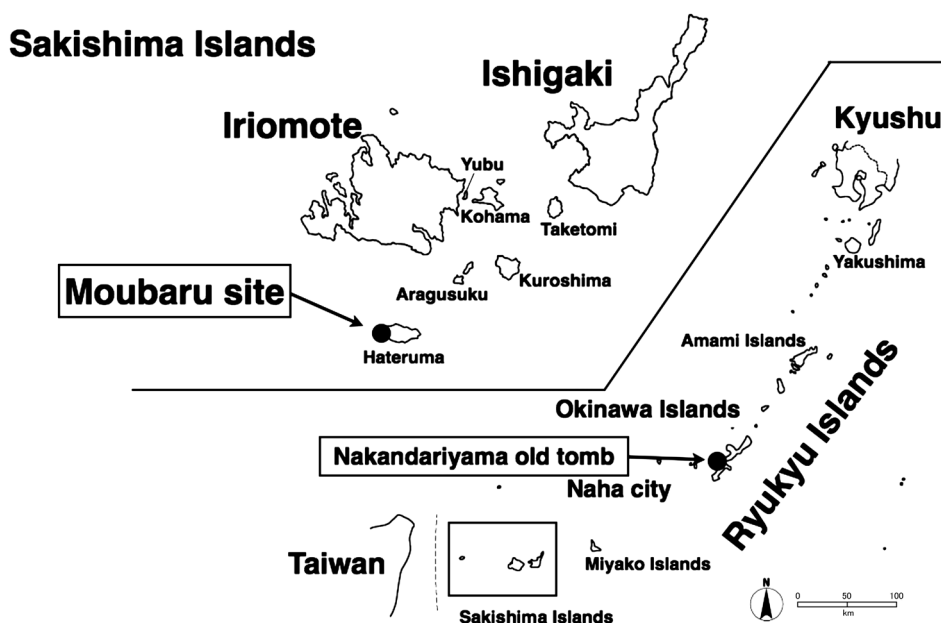


Fig. 1. Geographic distribution of the Ryukyu archipelago. Map showing location of the Nakandariyama old tomb and the Moubaru site.

cal methods developed significantly in the last two decades directly compare ancestral populations to their present-day counterparts. They permit direct reconstruction of the genetic history of populations through time, providing a temporal dimension of the study of past human populations.

Our previous investigation of the genetic structure of humans from the Shell Midden (Kaizuka) period on Okinawa provides proof of genetic continuity between the ancient and contemporary populations of this island from at least the late Kaizuka period (Shinoda *et al.*, 2012). However, no genetic evidence has been reported from the intermediate Gusuku period (thirteenth–sixteenth centuries) mainly because sufficient skeletal remains for ancient DNA analysis are lacking.

During this period, agricultural production of rice, wheat, and millet was developed and overseas trading of these goods began. All areas of the Ryukyu Islands formed a common culture during this time (Asato, 1996). In order to understand the history of these islands, it is important

to clarify the human genetic characteristics of this period. The present study aims to explain the genetic composition of the population from the Gusuku period, and address their genetic relationship (at a molecular level) with other Ryukyu groups from different cultural periods.

Materials and Methods

Archaeological sites and specimens

In this analysis, we used human skeletal remains excavated from two archaeological sites on mainland Okinawa (Nakandariyama site) and Hateruma Island (Moubaru site), the two southernmost and inhabited islands of Japan. The approximate locations of these sites are shown in Figure 1.

Nakandariyama is a cliff burial site located in the city of Naha. It was excavated in 2003 by the Center for Buried Cultural Properties (Okinawa Prefecture) and comprises at least 47 individuals. These consisted of 15 males, 10 females, 15 juveniles, and 7 individuals of indeterminate gender, ranging from infants to mature adults.

These skeletons generally display a prolonged upper jaw, which is characteristic of medieval humans but different from former Kaizuka people (Doi, 2005).

Since no cemetery artifacts remain, the chronology of this site was determined based on the design of burial goods. All tombs except No. 7 belong to the early modern period. In contrast, the age of tomb No. 7 is estimated to be in the Gusuku period. In order to determine the genetic characteristics of the Gusuku population from mainland Okinawa, eight well-preserved samples excavated from tomb No. 7 were selected for DNA analysis.

A sediment production project in the 1980s identified the Moubaru site, located in the Misyuku village in the northwest part of Hateruma Island. By counting skulls and limb bones, the minimum number of individuals was estimated at 29 (13 adult males, 10 adult females, 6 immature individuals) (Doi, 1998). According to ^{14}C dating of bone samples, the remains belong to the Gusuku period (Dr. Yoneda, personal communication). Seven well-preserved samples were selected for DNA analysis. A list of all samples used in this study is presented in Table 1.

Authentication methods and extraction of DNA

DNA analyses were performed at the National Museum of Nature and Science, which is dedicated to ancient DNA analysis. In the present study, we employed standard precautions to avoid contamination, e.g., separation of pre- and post-PCR experimental areas, use of disposable laboratory wares and filter-plugged pipette tips, treatment with DNA contamination removal solution (DNA Away; Molecular Bio Products, San Diego, CA, USA), UV irradiation of equipment and benches, and negative extraction and PCR controls (Shinoda *et al.*, 2006).

Extraction and purification of DNA and sequence analysis were mainly performed by T.K. and K.S. The multiplex amplified product-length polymorphism (APLP) analysis (Umetsu *et al.*, 2005) was conducted in different laboratories as described in the genotyping section.

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 powder from each sample using a commercial DNA extraction kit (Qiagen, DNA Investigator kit, Germany) after Proteinase K digestion. In the case of the Moubaru sample, the tooth root was cut off and used for ^{14}C dating and stable isotope analysis.

Amplification and sequencing of HVR1

Segments of HVRs (nucleotide positions 16121-16238, HVR1-1; 16209-16291, HVR1-2; and 160289-16366; HVR1-3) of mtDNAs, as per the revised Cambridge reference sequence (Andrews *et al.*, 1999), were sequenced in all samples.

Aliquots (2 μl) of the extracts were used as templates for PCR. Amplifications were carried out in a reaction mixture (total volume, 15 μl) containing 1 unit of Taq DNA polymerase (Hot-StarTaqTM DNA polymerase; Qiagen, Germany), 0.1 M of each primer and 100 mM of deoxyribonucleoside triphosphates in 1 $\times$ 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, HVR1-3:

L16120 5'-TTACTGCCAGCCACCATGAA-3'
 H16239 5'-TGGCTTTGGAGTTGCAGTTG-3'
 L16208 5'-CCCCATGCTTACAAGCAAG-3'
 H16312 5'-ACTATGTACTGTAAAGGGTG-3'
 L16269 5'-CTAGGATACCAACAAACCTA-3'
 H16367 5'-ATCTGAGGGGGGTTCATCCAT-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 Big-

Table 1. Sample used for DNA extraction and the result of the analysis.

No.	Code Name	Sample	HV1-1 (16121- 16238) (16,000+)	HV1-2 (16209- 16291) (16,000+)	HV1-3 (16289- 16366) (16,000+)	856 (D4d1b) (16,000+)	3010 (D4)	3644 (D4h)	4048 (M7b)	4386 (M7a)	4820 (B4b)	5147 (N9b)	5178 (D)	5231 (N9a)	5964 (D4e1)	6689 (D4b1b)	8272 (B)
<i>Nakandariyama site</i> (Okinawa Island)																	
1	7-6	Maxilla, Left, M3	N.D.	N.D.	N.D.	N.D.	G	T	A	T	G	G	C	G	T	N.D.	not delete
2	7-13(2)	Mandible, Right, M3	N.D.	N.D.	N.D.	N.D.	G	T	G	C	G	G	C	G	T	N.D.	not delete
3	7-21(2)	Maxilla, Right, M2	N.D.	223	N.D.	A	A	T	G	T	G	G	A	G	T	N.D.	not delete
4	32	Maxilla, Left, M2	N.D.	N.D.	362	N.D.	A	T	G	T	N.D.	N.D.	A	N.D.	T	N.D.	not delete
5	35	Maxilla, Right, M2	N.D.	N.D.	N.D.	N.D.	G	T	G	T	N.D.	G	C	G	T	N.D.	not delete
6	41	Mandible, Left, M2	N.D.	N.D.	N.D.	N.D.	G	T	G	T	N.D.	G	C	G	T	N.D.	not delete
7	56-1	Mandible, Right, M1	N.D.	N.D.	N.D.	N.D.	G	T	G	C	G	G	C	G	T	N.D.	not delete
8	no name	Maxilla, Right, M3	N.D.	217,249	N.D.	A	G	T	G	T	G	G	C	G	T	N.D.	delete
<i>Moubarri site</i> (Hateruma Island)																	
1	1	Maxilla, Left, M3	N.D.	N.D.	N.D.	N.D.	G	T	G	T	G	G	C	G	T	N.D.	not delete
2	2-2	Maxilla, Right, M3	N.D.	N.D.	CRS	N.D.	A	T	G	T	N.D.	G	A	N.D.	N.D.	N.D.	not delete
3	4	Maxilla, Right, M3	N.D.	N.D.	CRS	N.D.	A	T	G	T	N.D.	G	N.D.	G	N.D.	N.D.	not delete
4	9-1	Mandible, Left, M3	N.D.	N.D.	N.D.	N.D.	A	T	G	N.D.	N.D.	N.D.	N.D.	G	N.D.	N.D.	not delete
5	10	Maxilla, Left, M1	N.D.	N.D.	N.D.	N.D.	G	T	G	C	G	G	C	G	N.D.	N.D.	not delete
6	11-1	Maxilla, Right, M3	N.D.	N.D.	290,311,319	N.D.	G	T	G	N.D.	N.D.	G	C	G	T	N.D.	not delete
7	11-2	Maxilla, Right, M3	N.D.	N.D.	N.D.	N.D.	G	T	G	C	G	G	C	N.D.	T	N.D.	not delete
<i>Nakandariyama site</i> (Okinawa Island)																	
1	7-6	Maxilla, Left, M3	G	N.D.	C	T	G	N.D.	N.D.	N.D.	T	G	G	A	A	M7b	M7b
2	7-13(2)	Mandible, Right, M3	G	T	C	T	G	N.D.	T	N.D.	T	G	G	A	A	M7a	M7a
3	7-21(2)	Maxilla, Right, M2	G	N.D.	C	T	G	N.D.	T	N.D.	C	G	G	A	A	D4a	D4a
4	32	Maxilla, Left, M2	N.D.	N.D.	N.D.	T	G	N.D.	T	N.D.	T	G	G	A	A	D4	D4
5	35	Maxilla, Right, M2	N.D.	T	C	T	G	N.D.	T	N.D.	T	G	G	A	N.D.	M	M
6	41	Mandible, Left, M2	N.D.	N.D.	C	T	G	N.D.	N.D.	N.D.	T	G	N.D.	A	A	N.D.	N.D.
7	56-1	Mandible, Right, M1	G	N.D.	C	T	G	N.D.	T	N.D.	T	G	N.D.	A	A	M7a	M7a
8	no name	Maxilla, Right, M3	A	N.D.	C	T	G	N.D.	C	N.D.	T	G	G	A	A	B	B4
<i>Moubarri site</i> (Hateruma Island)																	
1	1	Maxilla, Left, M3	G	N.D.	N.D.	T	G	G	T	N.D.	T	G	N.D.	A	A	M	M
2	2-2	Maxilla, Right, M3	N.D.	N.D.	N.D.	T	G	N.D.	N.D.	N.D.	T	G	G	A	A	D4	D4
3	4	Maxilla, Right, M3	N.D.	T	C	T	G	G	N.D.	N.D.	T	G	N.D.	A	A	D4	D4
4	9-1	Mandible, Left, M3	N.D.	N.D.	N.D.	N.D.	G	N.D.	N.D.	N.D.	T	N.D.	N.D.	A	N.D.	D4	D4
5	10	Maxilla, Left, M1	N.D.	T	N.D.	T	G	N.D.	T	N.D.	T	G	G	A	A	M7a	M7a
6	11-1	Maxilla, Right, M3	N.D.	N.D.	N.D.	T	G	N.D.	N.D.	N.D.	T	N.D.	G	A	A	N.D.	A
7	11-2	Maxilla, Right, M3	G	N.D.	C	N.D.	G	G	T	N.D.	T	G	G	A	A	M7a	M7a

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.

Diagnostic polymorphisms of the PCR-Luminex analysis are emphasized by bold italic type.

N.D. denotes "not determined".

Dye cycle sequencing kit Ver.3.1 (Applied Biosystems, Foster City, CA, USA), which was performed using forward and reverse primers. The primers 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 3130) equipped with SeqEd software.

Genotyping of Polymorphisms

To confidently assign mtDNAs to the relevant haplogroups, 24 haplogroup-diagnostic single nucleotide polymorphisms (SNPs) including a nine b.p. repeat variation in the non-coding cytochrome oxidase II/tRNA^{Lys} intergenic region that define major haplogroups found in Japanese and East Asian population were analyzed by multiplex APLP (Umetsu *et al.*, 2005). SNPs that define major haplogroups were detected by use of suspension-array technology (Luminex 100; Luminex) at the laboratory of G&G Science, Fukushima. The methodology used for genotyping and primer sequences have been described in detail elsewhere (Itoh *et al.*, 2005, Shinoda *et al.*, 2012).

Results and Discussion

There are many advantages to using mtDNA. Because it is present in high copy numbers in mammalian cells, mtDNA can most likely be detected even in highly degraded samples, such as the ancient ones studied here. However, hot and humid conditions are generally unfavorable to the preservation of DNA in human skeletal remains, limiting the possibilities for finding well-preserved DNA in subtropical regions such as the Ryukyu Islands (Shinoda *et al.*, 2012). Due to the poor quality of mtDNA extracted from the ancient material, it was not possible to amplify all samples.

Table 1 shows the results of PCR amplification. Suspected false positive results stemming from contamination with contemporary DNA and

other questionable data were omitted from this study. In these samples, 6 out of 45 PCR amplifications were successfully analyzed. In contrast, 12 of the 15 samples were successfully typed in each haplogroup by multiplex SNP typing assays to simultaneously analyze important SNPs within the mtDNA coding region. Two samples that defined macrohaplogroup M could not be identified further to haplogroup.

In the case of multiplex analysis, to minimize the risk of contamination and to raise the efficiency of PCR, very short DNA fragments (average 60–80 bp) were amplified. However, the length of amplified HVR1 portion exceeded 100 bp in this experiment. Therefore, it is presumable that DNA extraction products of both archaeological sites were degenerated and most of their lengths were under 80 bp.

The haplogroup composition of the samples is M7a (33.3%); M7b (8.3%); D4 (41.7%); A (8.3%); and B (8.3%) in total (Table 1). Owing to the small sample size, it is difficult to verify the genetic characteristics by statistical methods, although it is noteworthy that the M7a and D4 haplogroups from both archaeological sites were well-represented. The distribution of mtDNA haplogroups in this period will provide some insight on the human population history of the Ryukyu Islands.

Figure 2 shows the haplogroup frequencies of the Gusuku period (present study), modern Ryukyu, and Taiwanese aboriginal populations. Regardless of their geographical location, the Ryukyu and Taiwanese aboriginal populations differed when compared according to their share of haplogroups. Taiwanese aborigines do not have haplogroup M7a and D4 that dominate the extant Ryukyu and Gusuku populations. Therefore, we assume there was no genetic connection between the Taiwanese and Gusuku populations, who had different origins and population histories overall.

The biased distribution pattern of haplogroup M7a in Japan may have diversified from the ancestral M7 haplogroup, which was introduced to Japan around the Last Glacial Maximum

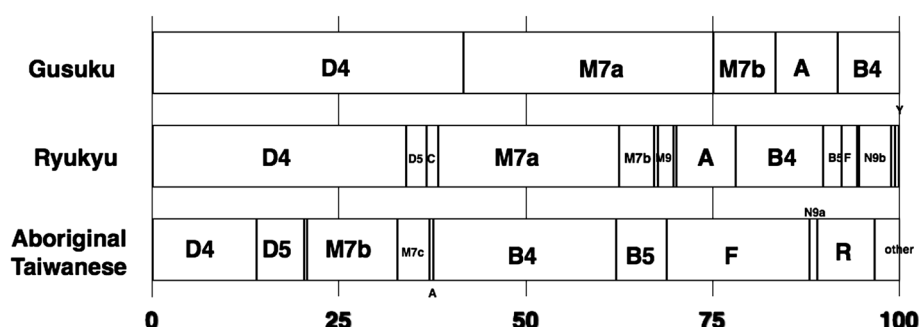


Fig. 2. Estimated frequencies of mtDNA haplogroups in regional populations.

References:

Ryukyu: Umetsu *et al.*, (2005)

Taiwan aborigines: Trejaut *et al.*, (2005)

within the Japanese archipelago (Adachi *et al.*, 2011). The Ryukyu Islands are candidates for the origin of this haplogroup (Shinoda, 2007). On the other hand, haplogroup D4 is observed in northeastern China and Korea as well as mainland Japan (Tanaka *et al.*, 2004) and there is no biased distribution among the mainland Japan. Moreover, this haplogroup apparently occurred at a low frequency in the Jomon population (Shinoda and Kanai, 1999; Adachi *et al.*, 2011). A reasonable theory is that the Yayoi immigrants were predominantly from haplogroup D4.

Archaeological findings indicate that travel and trade between mainland Japan and the Ryukyu archipelago began around the twelfth century. There are also some indications that waves of immigrants moved to Okinawa from mainland Japan and reached the Sakishima area (Asato, 1996). The geographic distribution of haplogroups M7a and D4 in both sites seems to support the claim inferred from the archaeological evidence that a relationship existed between the Okinawa, Sakishima, and Hateruma Islands in the Gusuku period.

This study identified four distinct haplogroups (M7a, M7b, D4, A, B) in the archaeological specimens sampled from the Okinawa and Hateruma Islands, which agree well with the genetic variation of extant Okinawan and mainland Japanese populations (Figure 2). It follows that these mtDNA haplogroups except M7b have

been present in the late Kaizuka population (Shinoda *et al.*, 2012) and that haplogroup D4 most probably reached the Okinawa Islands from mainland Japan at least by the end of the late Kaizuka period.

High frequencies of haplogroups M7a and D4 are characteristic of the contemporary Okinawan population (Tanaka *et al.* 2004). The distribution of these haplogroups among the late Kaizuka, Gusuku, and modern Ryukyu populations suggests that the formation of the extinct Ryukyu Islands population was the result of population expansion.

The number of samples for which DNA haplogroups could be determined was small in the present analysis, so unfortunately, the results provided minimal insight. However, the establishment of genetic characteristics among numerous burial sites in the Gusuku period may provide us with extremely valuable information regarding human migration and population dynamics in the Ryukyu Islands. The preliminary experiment presented here proved that sufficient amounts of DNA are retained in some human skeleton samples, even though the analytical efficiency may be poor. Thus, we believe it is worthwhile to continue the experiments to obtain more detailed data on the human skeletal remains from the Gusuku period site. Moreover, insight on the population history of the Ryukyu Islands could be obtained by comparing ancient mtDNA

sequences collected from the Kaizuka sites that are contemporaneous with the Jomon period in mainland Japan with sequences from the late-Kaizuka, Gusuku, and present-day populations.

Acknowledgments

We want to thank Mr. Katagiri of the Okinawa Prefectural Museum and his colleagues at the Center for Buried Cultural Properties, Okinawa Prefecture, for permitting us to use the skeletal materials and for their valuable advice.

Literature Cited

- 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).
- Doi, N., 2005. Chapter VI. Human skeletal remains. In: Nakandariyama old tomb. *Report of Center for Buried Cultural Properties, Okinawa Prefecture*, Vol. 26: 159–163 (in Japanese).
- Doi, N., 1998. Investigation of human skeletal remains excavated from Hateruma Island. *Interdisciplinary report of Hateruma Island*, 96–113. Okinawa Prefectural Museum (in Japanese).
- Fukumine, T., T. Hanihara, A. Nishime, H. Ishida, 2006. Nonmetric cranial variation of early modern human skeletal remains from Kumejima, Okinawa and the peopling of the Ryukyu Islands. *Anthropological Science*, 114: 141–151.
- 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.
- Jinam, T., N. Nishida, M. Hirai, S. Kawamura, H. Oota, K. Umetsu, R. Kimura, J. Ohashi, A. Tajima, T. Yamamoto, H. Tanabe, S. Mano, Y. Suto, T. Kaname, K. Naritomi, K. Yanagi, N. Niikawa, K. Omoto, and N. Saitou, 2012. The history of human populations in the Japanese Archipelago inferred from Genomewide SNP data with a special reference to the Ainu and the Ryukyuan Populations. *Journal of Human Genetics*, 57: 787–795.
- Koganebuchi, K., T. Katsumura, S. Nakagome, H. Ishida, S. Kawamura, and H. Oota, The Asian Archival DNA Repository Consortium, 2012. Autosomal and Y-chromosomal STR markers reveal a close relationship between Hokkaido Ainu and Ryukyu islanders. *Anthropological Science*, 120: 199–208.
- Matsukusa, H., H. Oota, K. Haneji, T. Toma, S. Kawamura, and H. Ishida, 2010. A Genetic Analysis of the Sakishima Islanders Reveals No Relationship with Taiwan Aborigines but Shared Ancestry with Ainu and Main-island Japanese. *American Journal of Physical Anthropology*, 142: 211–23.
- Matsu'ura, S., 1982. Relative dating of the Minatogawa Man by fluorine analysis. In: *The Minatogawa Man*. Ed. H. Suzuki and K. Hanihara, University of Tokyo Press, Tokyo.
- 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., N. Adachi, S. Guillen, and I. Shimada, 2006. Mitochondrial DNA analysis of ancient Peruvian highlanders. *American Journal of Physical Anthropology*, 131: 98–107.
- Shinoda, K. 2007. *People who became the ancestor of the Japanese*. Tokyo: Nihon Housou Syuppan Kyokai (in Japanese).
- Shinoda, K., T. Kakuda, N. Doi, 2012. Mitochondrial DNA polymorphisms in late Shell midden period skeletal remains excavated from two archaeological sites in Okinawa. *Bulletin of the National Museum of Nature and Science Series D*, 38: 51–61.
- 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*, 14: 1832–1850.
- Treajaut 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-speak-

- ing 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.