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Ancient DNA Analysis of Palaeolithic Ryukyu Islanders

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Ishigaki Island is one of the westernmost islands in Japan. Due to its geographical location, it is considered to have played a significant role in the migration route from Southern Asia to the Japanese archipelagos. Recently, human remains were excavated from Shiraho-Saonetabaru Cave, constituting the first physical evidence of human occupation on Ishigaki Island. In order to investigate the genetic makeup of the ancient Ishigaki people and to assess their genetic relationship with other Asian populations at a molecular level, we analysed the single nucleotide polymorphisms of the coding region of mtDNA that defines the haplogroups of these individuals. Because of the poor quality of the DNA extracted from the ancient material, it was not possible to analyse all samples. Among the 10 samples considered in this study, ancient DNA data was successfully extracted from five individuals. MtDNA haplogroups show geographic specificity within Asia; the existence of haplogroup B4e and M7a in this population hints at their linkage with Southeast Asia and the Late Pleistocene Ryukyu Islands.

Introduction

Steady progress in DNA analytical techniques since the late twentieth century has revolutionised the field of physical anthropology. This has meant that instead of relying solely on morphometrical studies of the human skeleton it has been possible to use DNA to analyse genetic variations in the human gene and determine with greater resolution the origins and migration routes of anatomically modern human populations. Conclusions drawn from DNA research have prompted the review of existing views and inspired new theories in human origins research.

While most of the research regarding local population events is based on modern DNA analysis, straightforward retrogressive projection of modern genetic composition and distribution has its inherent limitations. Currently, ancient DNA analysis is the next logical step after obtaining excavated remains from the regional population. Advances in molecular biology techniques in the last 20 years have allowed for the analysis of DNA extracted from ancient bone samples, making it possible to obtain information on lineage, with significantly higher probabilities of accuracy (e.g. Shinoda and Kanai 1999; Maca-Mayer et al. 2005; Melchior et al. 2008).

DNA analysis of ancient materials currently focuses on mitochondrial DNA (mtDNA) owing to its special characteristics such as small size, matrilineal inheritance, high copy number, and fast mutation rate. During the course of human migration across the world, mutations created new types of mtDNA. Further mutation of these types increased the variation. The resultant types are grouped under a single lineage, namely, 'haplogroup'. Relationships within and between haplogroups provide important clues to help reconstruct the history of human migration

(e.g. Kivisild et al. 2002). Currently, DNA-based studies determine the origin of a given population through comparisons of haplogroup compositions with that of neighbouring populations. This approach has led to more detailed data collection, which, in turn, has yielded more sophisticated scenarios regarding human migration.

This approach is restricted not only because of its matrilineal succession, but also by poor preservation, small sample size, and contamination in the case of ancient samples. Further, it depends on successful extraction and analysis of minute quantities of mtDNA. In spite of these difficulties, mtDNA analysis of ancient bones and teeth samples recovered by regionally based archaeological projects offers an effective means of understanding local and/or regional population history and dynamics in Japan (e.g. Adachi et al. 2011).

The genesis of the modern Japanese population is an area of intense study in anthropology, archaeology, and genetics in East Asia. In this context, because of its geographical location, Ishigaki Island is thought to have played a significant role in the migration route from Taiwan and Southeast Asia to the Ryukyu and Japanese archipelagos. According to archaeological evidence, the Sakishima area (Figure 3.1), consisting of the Miyako and Yaeyama island groups, including Isigaki Island, formed a different cultural area from the main island of Okinawa until the twelfth century AD. There was no influence from the mainland of Japan (Jomon and Yayoi cultures), and from archaeological remains, the Sakishima area seems to have a lot in common with the southeastern regions of Asia. Perhaps the sea lying between the main islands of Okinawa and Sakishima was the boundary of the expansion of Japanese culture to the south (Takamiya 2005) and possibly Austronesian expansion from Taiwan towards the north (see Hudson, Chapter 10, this volume).

Due to its complex history, it is not surprising that the Ryukyu Islands have often attracted the interest of population geneticists (e.g. Jinam et al. 2012). Nevertheless, very little is known about the genetic history of their human population. To learn more about the genetic characteristics of the first inhabitants of this westernmost island of Japan, we analysed mtDNA from human remains that were excavated from an archaeological site belonging to the Late Paleolithic period.

Materials and methods

Human skeletal remains from the archaeological site of Shiraho-Saonetabaru Cave on the east coast of Ishigaki Island were analysed for this project (Figure 3.1). As a part of an airport construction project, Shiraho-Saonetabaru Cave was excavated between 2010 and 2013. In total, 10 individuals were sampled for DNA. Eight were directly radiometrically dated using ^{14}C to between 27,000–4000 BP (Table 3.1). For two others the ages were extrapolated from their stratigraphic and chronological relationships with known age samples. The dating indicates that the human remains excavated from Siraho-Saonetabaru Cave belong to the Late Pleistocene to early modern periods (Yoneda 2014). These are the oldest directly radiocarbon dated human skeletal remains from Japan, and these bones constitute the first physical evidence of human occupation on the Ishigaki Island in the Late Pleistocene period.

A study has shown that tooth enamel forms a natural barrier to exogenous DNA contamination – teeth appear to lack most of the inhibitors that prevent the enzymatic amplification of ancient DNA (Woodward et al. 1994). Therefore, tooth samples from the collagen-bone-dated skeletons were the first choice in the present study. When tooth samples were not available, bone samples from other skeletal fragments were used. Results of dating indicated that all the samples were not related to each other.

When ancient DNA is analysed, it is necessary to exclude false-positive results caused by contamination with contemporary DNA (Sampietro et al. 2006). In order to prevent contamination during excavation, the remains were handled using gloves and were not touched with bare hands. Bone samples were wrapped in aluminium foil and stored in a refrigerator at 4°C until DNA extraction. Standard precautions were practised to avoid contamination, such as separation of pre- and post-polymerase chain reaction (PCR) experimental setups, use of disposable laboratory gear and filter-plugged pipette tips, treatment with DNA contamination removal solution (DNA Away; Molecular Bio Products, San Diego, CA, USA), ultraviolet (UV) irradiation of equipment and benches, and negative extraction and PCR controls (Shinoda et al. 2006).

DNA was extracted from the skeletal samples, according to previously published protocols (Adachi et al. 2009). The tooth and bone samples were dipped in DNA contamination removal solution for 15 min, rinsed with DNase-/RNase-free distilled water, and allowed to air-dry. The outer surface of the samples was removed using a dental drill, and the samples were again rinsed with DNase-/RNase-free distilled water and allowed to air-dry under UV irradiation. Then the tooth samples were encased in silicone rubber and the dentin around the *cavitas dentis* and the dental pulp was powdered and extracted through the cut plane of the root tip, as described by Gilbert et al. (2003). The bone samples were pulverised using a mill (Multi-beads shocker; Yasui Kikai, Osaka, Japan).

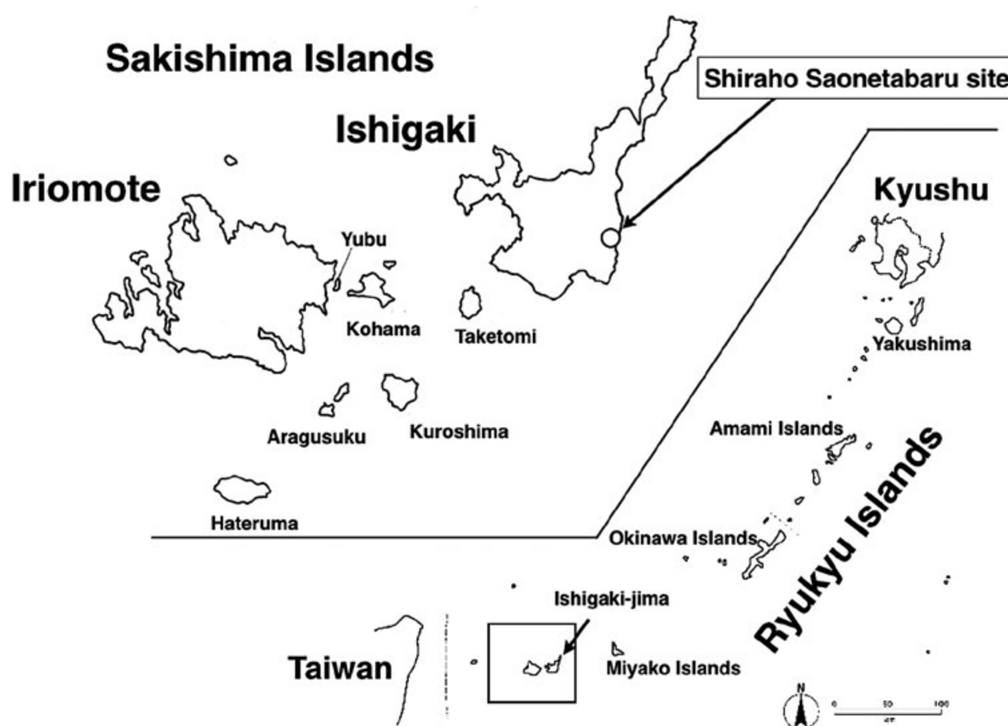

Figure 3.1 The geographic distribution of the islands in the Ryukyu Archipelago, and the location of Shiraho-Saonetabaru on Ishigaki Island.

Source: Ken-ichi Shinoda.

Table 3.1 Radiocarbon dates on collagen and dentine from samples of the Shiraho-Saonetabaru skeletons.

Sample No.	Code	Skeletal Element	C14 date (uncalibrated) BP	C14 datenn (calibrated) BP**	Laboratory codes
1	SRH 12	Right Humerus	20160±108	24558-23997 (95.4%)	MTC-14189
2	SRH 13	Rib	20761±163	25521-24558 (95.4%)	MTC-14196
3	SRH 15	Rib	24556±205	29097-28142 (95.4%)	MTC-14197
4	SRH 94	Right Humerus	16170±60	19767-19341 (95.4%)	PLD-19660
5	SRH 166	Femur	19723±61	24040-23575 (95.4%)	PLD-19658
6	SRH 181	Isolated teeth	16573±51	20238-19842 (95.4%)	PLD-19692
7	SRH 188	Left Humerus	3970±30	4574-4452 (95.4%)	PLD-19659
8	SRH 214	Left Femur	18071±62	22196-21711 (95.4%)	PLD-19657
9	SRH 242	Left Fibula	*20000		
10	SRH 292	Femur	*9000–16000		

* The age of these two samples were assumed through association with bones from the same layers that were chronometrically C14 dated.

**The dates calibrated using OxCal 4.2, IntCal 13.

Source: Ken-ichi Shinoda.

The powdered samples (approximately 0.4 g) were decalcified with 10 ml of 0.5 M EDTA (pH 8.0) at 20°C for three days and lysed in 500 µ l of Fast Lyse (Genetic ID, Fairfield, IA) with 30 µ l of 20 mg/ml Proteinase K at 60°C for 4 h. DNA was extracted from the lysate using a FastDNA™ Extraction kit (Genetic ID), as per the manual. Finally, 100 µ l of DNA extract was obtained from each sample.

Segments of hypervariable regions of mtDNA (HVRs; nucleotide positions 16121–16238, HVR1-1; 16209–16291, HVR1-2; and 160289–16366; HVR1-3) of mtDNA, as per the revised Cambridge reference sequence (Andrews et al. 1999), were sequenced in all samples. Aliquots (2 µ l) of the extracts were used as templates for PCR. Amplifications were carried out in a reaction mixture (total volume, 15 µ l) containing 1 unit of Taq DNA polymerase (HotStarTaqTM DNA polymerase; Qiagen, Germany), 0.1 M of each primer, and 100 mM of deoxyribonucleoside 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 s; 50–56°C for 20 s, and 72°C for 15 s; and final extension at 72°C for 1 min.

The PCR products were separated by agarose gel electrophoresis on a 1.5 per cent 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 (Life Technologies, Foster City, CA, USA), which was performed using forward and reverse primers. The primers used in 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 (ABI).

To confidently assign mtDNA samples to relevant haplogroups, 24 haplogroup-diagnostic single nucleotide polymorphisms (SNPs), including a 9 bp repeat variation in the non-coding cytochrome oxidase II/tRNALys intergenic region, that defines major haplogroups found in Japanese and East Asian populations, were analysed by multiplex APLP (Umetsu et al. 2005). SNPs that defined major haplogroups were detected by using suspension array technology (Luminex 100) at the laboratory of G&G Science, Fukushima. The methodology for genotyping and primer sequences has been described in detail elsewhere (Itoh et al. 2005; Shinoda et al. 2012). Moreover, 26 haplogroup-diagnostic SNPs and a 9 bp repeat variation were analysed by another multiplex APLP, as described previously (Adachi et al. 2009).

Results and discussion

Table 3.2 shows the results of PCR amplification and genotyping of polymorphisms. Both negative extraction and PCR controls consistently showed negative results throughout the study. Because of the poor quality of the mtDNA extracted from the ancient material, it was not possible to amplify all samples. Five samples failed to amplify any portion of mtDNA, and no DNA was recovered. Using multiplex single nucleotide polymorphism (SNP) typing, five of the 10 samples were successfully typed to the smallest named haplogroup they belonged to (Figures 3.2 and 3.3). Thus, three samples were assigned to haplogroup M7a and the other samples to haplogroups B4 and R. In the case where D-loop sequence was revealed, we classified them further based on the mutations observed in these regions. Therefore, we assigned the B4 samples to haplogroup B4e. More detailed data has been previously described by Shinoda and Adachi (2014). Owing to the small sample size, it was difficult to verify genetic characteristics by statistical methods, although the existence of these haplogroups is noteworthy.

Table 3.2 Result of the analysis. N.D. denotes 'not determined'.

No.	Sample	Sequence 16209-16366 (16000+)	SNP PCR-Luminex	APLP analysis	Haplogroup
1	No. 12	217 223 291	B4	N.D.	B4e
2	No. 13	311	R	N.D.	R
3	No. 15	N.D.	N.D.	N.D.	N.D.
4	No. 94	N.D.	N.D.	N.D.	N.D.
5	No. 166	N.D.	N.D.	N.D.	N.D.
6	No. 181	N.D.	N.D.	N.D.	N.D.
7	No. 188	N.D.	N.D.	M7a	M7a
8	No. 214	N.D.	N.D.	N.D.	N.D.
9	No. 242	N.D.	N.D.	M7a	M7a
10	No. 292	N.D.	N.D.	M7a	M7a

Source: Ken-ichi Shinoda.

Figure 3.2 Results of PCR-luminex analysis. Number of each lane shows the sample number. Lane 1 and 2 are positive controls and lane 9 is negative control.

A: 63 base pair amplified products indicates haplogroup M7a.

B: 54 base pair products indicates haplogroup M7a1.

M: size marker.

Source: Ken-ichi Shinoda.

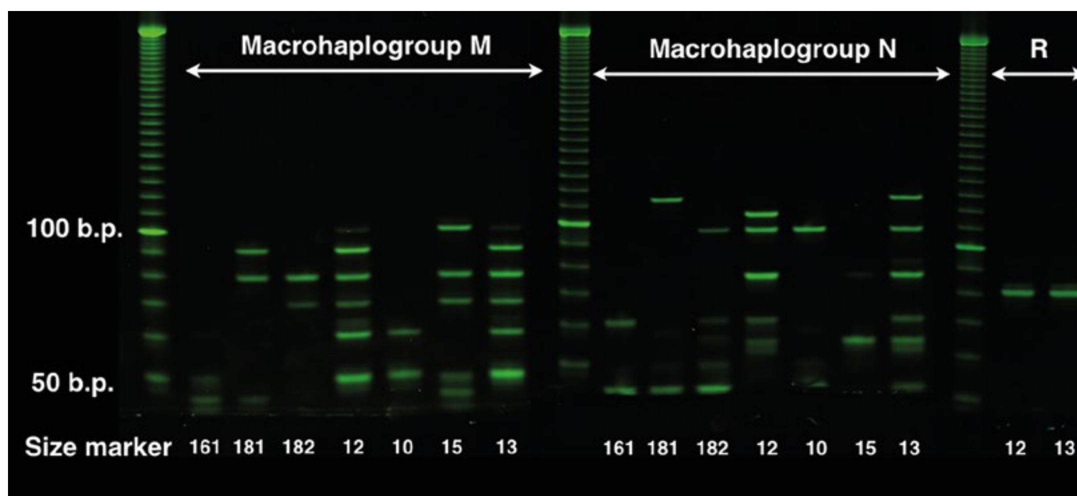

Figure 3.3 Results of amplified product length polymorphism (APLP) analysis. Number of each lane shows the sample number.

Source: Ken-ichi Shinoda.

Though small in number, the distribution of mtDNA haplogroups in this period provides insights into regional population history. Haplogroups B4 and R are the most prevalent in Southeast Asia, especially in the coastal region (Trejaut et al. 2005), indicating that this haplogroup may have been introduced to Japan from Southeast Asia. Interestingly, haplogroup B was also found in ancient Chinese samples (Fu et al. 2012). It seems that the ancestral population of coastal East Asia and Island Southeast Asia was enriched by the founder lineages of haplogroup B4, and the Ryukyu Islands may be one of the northernmost regions where this population arrived in the Palaeolithic period. This finding indicates that the southeast influx into the ancient Ryukyu population affected their genetic makeup and that the ancestors of the Aboriginal Taiwanese or Asian coastal region populations might be the main source of this haplogroup in the Ryukyu Islands.

The geographic specificity of haplogroup M7a is the most intriguing result of this study. Its ancestral haplogroup M7, although a characteristic of East Asian populations, was not found in the northeast region of the continent (Torroni et al. 1993; Derenko et al. 2007). Haplogroup M7a is absent or scarce in the East and Southeast Asian populations outside Japan. Moreover, M7a is one of the prevailing haplogroups not only among modern Japanese, including Honshu, Okinawa islanders, and Ainu populations (Tanaka et al. 2004), but also in the Jomon population (Adachi et al. 2011). The frequency of haplogroup M7a among modern Japanese is highest in the Okinawa islanders (23.3 per cent; Umetsu et al. 2005) – gradually decreasing towards the northern part of Honshu (Shinoda 2007). This finding indicates that this haplogroup may have a southern origin. Moreover, the age of haplogroup M7a was calculated to be *ca*. 23,000 BP, and the age likely falls within the onset of the Last Glacial Maximum (LGM; Adachi et al. 2011).

Our results confirm that the haplogroup M7a entered Japan, with the earliest settlers more than 20,000 years ago from Southeast Asia or the southern region of the Asian continent. The fact that positive proof of human occupation by haplogroup M7a on Ishigaki Island appears about 30,000–20,000 BP fits this scenario.

The peopling in Japan can be seen as a complex process, as the earliest settlements and recent migrations affected the resident populations differently. Although we can draw limited conclusions from this study, our results of ancient mtDNA analysis help to shed light on late Palaeolithic human migrations to, and within, the Japanese archipelagos from Southeast Asia.

Since hot and humid conditions are unfavourable for DNA preservation, there is a low possibility of finding well-preserved DNA in regions with a tropical climate, like the Ryukyu Islands. However, the present study shows that sufficient amounts of DNA were available in the human skeletal samples from the late Palaeolithic period obtained from the Ryukyu Islands because the remains were protected within caves. It seems that caves are favourable burial sites from the viewpoint of DNA preservation (Fehren-Schmitz et al. 2011). However, further studies are necessary to obtain more details on the human skeletal remains excavated from this region.

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