Introduction

The Shomyoji shell midden site is located in Yokohama City, Kanagawa Prefecture, Japan (Figure 1). This site is famous for its distinctive Shomyoji-style pottery, which is recognized as an archaeological marker for the beginning of the late Jomon era.

In 2017, excavations at this site disinterred 25 grave pits (dokoubo) with human skeletons (Figure 2, Figure 3). These skeletons were not accompanied by grave goods that might have clearly indicated their chronological age, and radiocarbon dating was therefore used to date all of the specimens (Yokohama City Board of Education, in print). The radiocarbon age of most of the skeletons was calculated as approximately 4000 years calBP, corresponding to the early phase of the late Jomon era (Table 1).

The age of two skeletons (Dokoubo 1 and 2) unearthed from just above the Jomon-era skeletons (Figure 2) differed and corresponded to the Heian era and the beginning of the Kofun era (almost equal to the end of the Yayoi era), respectively.

Recent studies have revealed that Jomon people are considerably genetically different from any other population, including modern-day Japanese (Adachi et al., 2011; Adachi and Nara, 2018). This observation gives rise to an intriguing question: when after the Jomon era did this drastic change of genetic features occur? The Shomyoji shell midden site in Kanagawa, Japan can provide some clues to address this question. The skeletons buried at this site include some that are more recent than the Jomon-era skeletons with whom they are almost contiguously buried. We tested the genetic continuity of the Shomyoji shell midden people by analyzing mitochondrial DNA (mtDNA). Our results show that the mtDNA haplogroups of the Kofun and Heian skeletons vastly differ from those of the Jomon skeletons. This finding implies that the genetic conversion of the Japanese people may have occurred during or before the Kofun era, at least at the Shomyoji site. To confirm this hypothesis, nuclear genome analysis of the Shomyoji people is considered promising.

Materials and Methods

Skeletal materials

Well-preserved skeletons excavated in 2017 from the Shomyoji shell midden D point 3 site were used for the analyses. The locations and dates of the skeletons are shown in Figure 2 and Figure 3, and in Table 1. The skeletal locations from which the DNA samples were isolated are indicated in Table 2.
Tokyo. Gelatinization was used to extract collagen from bone (Longin, 1971; Yoneda et al., 2002). First, the bone surface was cleaned by sandblasting and ultrasonic washing in ultrapure water (Milli-Q water) for 10 min. Cleaned bone pieces were submerged in weak alkali (0.2 M NaOH) overnight to remove soil-derived humic and fluvic acids. After washing with Milli-Q water to a neutral pH, dried bone pieces were crushed to form a fine powder that was sealed in a cellulose tube and gently reacted with hydrochloric acid (1.2 M) for 15–18 h. After washing with Milli-Q water to a neutral pH, samples were recovered by centrifugation. The precipitate was heated to 90°C in acidified water (pH 4) for 12 h to extract gelatin. Dissolved gelatin was purified by passing through a Whatman GF/F filter and lyophilized.
DRASTIC GENETIC CONVERSION IN THE SHOMYOJI SITE

Extracted gelatin, containing 1 mg of carbon, was combusted to form CO₂ using a Vario ISOTOPE SELECT elemental analyzer (Omori et al., 2017). The CO₂ was introduced into a vacuum line and sealed in a reaction vessel with sufficient H₂ (2.2 times the amount of CO₂) and 2 mg of iron powder for catalysis. The graphite was reduced on the surface of the catalyst at 650°C for 6 h (Kitagawa et al., 1993). The carbon and nitrogen concentrations in the gelatin were measured by an elemental analyzer, and atomic C/N ratios were compared with the biologically expected range (2.9–3.6) for collagen quality assessment (DeNiro, 1985).

Reduced graphite was pressed in an aluminum holder for accelerator mass spectrometry (AMS) analysis (Compact AMS System, National Electrostatics Corp.). AMS was performed at The University Museum, The University of Tokyo. A series of international standards were simultaneously

Table 1. Calibrated date of skeletons analyzed

| Specimen | Atomic C/N | Lab ID    | Conventional carbon date | Marine (%) | Calibrated carbon date (1 SD) | Calibrated carbon date (2 SD) |
|----------|------------|-----------|--------------------------|------------|-------------------------------|-------------------------------|
| Dokoubo 1 | 3.3        | TKA-18927 | 1132 ± 22                | 32%        | 957–900 (68.2%)               | 965–823 (93.1%)               |
|           |            |           |                          |            | 813–801 (2.3%)                | 1694–1544 (95.4%)             |
| Dokoubo 2 | 3.3        | TKA-18928 | 1826 ± 22                | 32%        | 1683–1674 (5.1%)              | 1625–1557 (63.1%)             |
|           |            |           |                          |            | 3869–3763 (68.2%)             | 3897–3712 (95.4%)             |
| Dokoubo 7 | 3.4        | TKA-18932 | 3741 ± 24                | 55%        | 4222–4197 (15.3%)             | 4185–4136 (33.5%)             |
|           |            |           |                          |            | 4128–4097 (19.7%)             | 4256–4071 (95.4%)             |
| Dokoubo 9 | 3.3        | TKA-18934 | 3980 ± 24                | 52%        | 4222–4197 (15.3%)             | 4185–4136 (33.5%)             |
|           |            |           |                          |            | 4128–4097 (19.7%)             | 4256–4071 (95.4%)             |
| Dokoubo 10| 3.3        | TKA-18935 | 4098 ± 24                | 71%        | 4267–4155 (68.2%)             | 4351–4115 (95.4%)             |
| Dokoubo 11| 3.3        | TKA-18936 | 4104 ± 24                | 59%        | 4373–4370 (1.3%)              | 4412–4218 (94.4%)             |
| Dokoubo 13| 3.3        | TKA-18938 | 3887 ± 23                | 51%        | 4076–3978 (68.2%)             | 4140–4114 (3.8%)              |
| Dokoubo 14| 3.3        | TKA-18939 | 3760 ± 23                | 49%        | 3907–3834 (68.2%)             | 3971–3814 (93.7%)             |
| Dokoubo 15| 3.3        | TKA-18940 | 3852 ± 23                | 53%        | 4059–4041 (7.9%)              | 4078–3888 (95.4%)             |
| Dokoubo 17| 3.2        | TKA-18943 | 3923 ± 24                | 70%        | 4058–4033 (12.7%)             | 4082–3887 (95.4%)             |
| Dokoubo 19| 3.3        | TKA-18947 | 3941 ± 24                | 44%        | 4178–4169 (4.8%)              | 4243–4069 (94.0%)             |
| Dokoubo 21| 3.3        | TKA-18949 | 3949 ± 24                | 62%        | 4094–3990 (68.2%)             | 4150–3965 (95.4%)             |
| Dokoubo 23| 3.2        | TKA-18951 | 3877 ± 24                | 60%        | 4057–4044 (6.0%)              | 4079–3882 (95.4%)             |

* Adopted from Yokohama City Board of Education (in press)
measured, and δ13C measured by AMS was applied to correct the isotopic fractionations during preparation and measurement to calculate the conventional radiocarbon age (CRA) (Stuiver and Polach, 1977). The CRA was determined with 1 SD error. Calibration was conducted with a mixed calibration curve using IntCal13 and Marine13 datasets (Reimer et al., 2013). We used the ‘Mix Curve’ function of OxCal4.3 (Bronk Ramsey, 2009) with a local correction sets (Reimer et al., 2013). We used the ‘Mix_Curve’ function mixed calibration curve using IntCal13 and Marine13 data- sets (Reimer et al., 2013). We used the ‘Mix_Curve’ function mixed calibration curve using IntCal13 and Marine13 data-

## Table 2. DNA samples and amplified product-length polymorphism (APLP) analysis results

| Sample name | DNA sample | Result of APLP analysisa |
|-------------|------------|--------------------------|
| Dokoubo 1   | left temporal bone | B5                      |
| Dokoubo 2   | right temporal bone | B4*                     |
| Dokoubo 7   | right temporal bone | fail (full profile was not obtained) |
| Dokoubo 9   | right temporal bone | fail (full profile was not obtained) |
| Dokoubo 10  | right temporal bone | fail (full profile was not obtained) |
| Dokoubo 11  | right temporal bone | fail (full profile was not obtained) |
| Dokoubo 13  | lower left third molar | M7a1                   |
| Dokoubo 14  | left temporal bone | M7a2                     |
| Dokoubo 15  | lower right third molar | M7a2                   |
| Dokoubo 17  | left temporal bone | fail (full profile was not obtained) |
| Dokoubo 19  | right temporal bone | fail (full profile was not obtained) |
| Dokoubo 21  | left temporal bone | N9 (full profile was not obtained) |
| Dokoubo 23  | lower left second molar | N9b*                   |

Table 2. DNA samples and amplified product-length polymorphism (APLP) analysis results

Dokoubo 23 lower left second molar | M7a1

*The asterisk indicates that the haplogroup status could not be further identified.

## Extraction and purification of DNA

Strict precautionary methods were taken to avoid contamination with modern DNA as previously described (Adachi et al., 2018). In brief, skeletal samples were collected while wearing a face mask, laboratory coat, cap, and gloves. DNA-based studies were performed in a laboratory dedicated exclusively to ancient DNA analysis. We employed standard precautions to avoid contamination, such as the separation of pre- and post-polymerase chain reaction (PCR) experimen-
tal areas, the use of disposable laboratory-ware and filter-
plugged pipette tips, treatment with DNA contamination re-
move solution (DNA Away; Molecular Bio Products, San
Diego, CA), UV irradiation of equipment and benches, and
the use of negative extraction and PCR controls.

Moreover, to identify and exclude any possible contami-
nation with modern DNA, mtDNAs of eight individuals who

formed consent for the use of their DNA. This analysis was
approved by the Ethics Committee of the Faculty of Medi-
cine of the University of Yamanashi.

Teeth and temporal bones of the skeletons were used for
DNA extraction. For the teeth, DNA samples were obtained
as described in Adachi et al. (2018). In brief, each sample
from which DNA was to be extracted was dipped in a 13%
bleach solution (Nacalai Tesque Inc., Kyoto, Japan) for
15 min, rinsed several times with DNase/RNase-free dis-
tilled water (Thermo Fisher Scientific, Waltham, MA),
and allowed to air-dry. Thereafter, the outer surface of the
samples was removed to a depth of 1 mm using a dental drill.
Next, the samples were rinsed with DNase/RNase-free dis-
tilled water and allowed to air-dry under irradiation in a UV
crosslinker for 90 min. Samples were then encased in Exaf-
ine silicone rubber (GC, Tokyo) as described by Gilbert et al.
(2003). Once the rubber was consolidated, the tip of the root
of the tooth sample was removed with a horizontal cut and
powdered using a mill (Multi-beads shocker®; Yasui Kikai,
Osaka). Thereafter, the dentin around the dental cavity and
the dental pulp were powdered using a dental drill.

For the temporal bones, around 0.4 g of compact bone
surrounding the otic capsule was removed because it has
been reported to contain a high level of endogenous DNA
(Pinhasi et al., 2015). Before powdering, the bone samples
were processed as described for the teeth. However, instead
of being encased in silicon rubber, the bone samples were
directly powdered by the mill.

Powdered samples were decalcified with 0.5 M EDTA
(pH 8.0) (Thermo Fisher Scientific) at 56°C overnight.
EDTA buffer was then refreshed with fresh buffer, and sam-
ples were decalcified for a further 36 h at 56°C. Decalcified
samples were lysed in 1000 μl of Genomic Lysis buffer
(Genetic ID, Fairfield, IA) with 50 μl of 20 mg/ml protein-
ase K (QIAGEN, Hilden, Germany) overnight at 56°C.
nally, the lysate was extracted twice with 1200 μl of UltraPure™ phenol/chloroform/isoamyl alcohol (25:24:1) (Thermo Fisher Scientific) and 1200 μl of Infinity Pure Chloroform (FUJIFILM Wako Pure Chemical Corporation, Osaka). DNA was extracted from the lysate using a FAST ID DNA Extraction Kit (Genetic ID) in accordance with the manufacturer’s instructions.

**mtDNA analysis**

To securely assign mtDNAs of the Shomyoji skeletons to the relevant haplogroups, 84 haplogroup-diagnostic polymorphisms were analyzed for all samples using multiplex amplified product-length polymorphism (APLP) (Kakuda et al., 2016).

For the samples with successful APLP analysis, segments of mtDNA that covered parts of the tRNAPro gene, the hypervariable segments (HVS) 1 [nucleotide position (np) 15999–16366, relative to the revised Cambridge reference sequence (Andrews et al., 1999)] and HVS 2 (np 128–256) were amplified and sequenced using previously described primers (Adachi et al., 2013, 2014). Primer sequences are listed in Table 3.

Three primer sets were initially used for the PCR amplification of HVS 1: L15998 and H16142, L16120 and H16239, and L16208 and H16367. Bone samples other than Dokoubo 1 and 2 could not be amplified using these primer sets, suggesting that the residual DNA in these samples was highly fragmented. Based on this observation, five additional primer sets were used: L15998 and H16079, L16055 and H16139, L16120 and H16225, L16208 and H16291, and L16288 and H16367. Primers L127 and H257 were used for HVS 2 PCR amplification.

PCRs were performed in a total volume of 25 μl containing a 2.5 μl aliquot of DNA extract, 50 nM of each primer, and reagents of Platinum® Multiplex PCR Master Mix (Thermo Fisher Scientific). The cycling conditions comprised one cycle of DNA denaturation and activation of Platinum® Multiplex PCR Master Mix at 95°C for 2 min; 36 cycles of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. All PCRs were repeated twice independently.

PCR products were purified using the Monarch® PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, MA) and used as a sequencing template. Sequencing was performed by Fasmac Co., Ltd (Kanagawa), using Applied Biosystems 3130xl DNA analyzers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). All sequences were evaluated bidirectionally. Only sequences that did not contradict the results of APLP analysis were used as data.

We then assigned mtDNAs to the relevant haplogroup based on the APLP and sequencing data using the classification tree built by van Oven and Kayser (2009).

**Results**

**Radiocarbon dating**

The atomic C/N ratios and radiocarbon ages are shown in Table 3. Primers used for mtDNA amplification and sequencing

| Primer | Sequence (5’–3’) |
|--------|------------------|
| L127   | AGC ACC CTA TGT CGC AGT AT |
| H257   | TCT GTG TGG AAA GCG GCT GT |
| L15998 | CCA TTA GCA CCC AAA GCT A |
| L16055 | GAA GCA GAT TTG GGT ACC AC |
| L16120 | TTG CTG CCA GCC ACC ATG AA |
| L16128 | CCA GCC ACC ATG AAT ATT GTA C |
| L16208 | CCC CAT GCT TAC AAG CAA G |
| L16288 | CCA CTA GGA TAC CAA CAA ACC T |
| H16079 | TGT ACG AAA TAC ATA GCG GTT G |
| H16139 | TAC TAC AGG TGG TCA AGT AT |
| H16142 | ATG TAC TAC AGG TGG TCA AG |
| H16225 | GCA GGT GAT GTG TGA TAG TTG |
| H16239 | TGG CTT TGG AGT TGC AGT TG |
| H16291 | ATG TAC TAT GTA CTG TTA AGG GTG G |
| H16367 | ATC TGA GGG GGG TCA TCC AT |

Figure 4. Haplogrouping of the Shomyoji samples using the Multiplex APLP system (Kakuda et al., 2016). LM denotes a 10-bp ladder (Thermo Fisher Scientific). Lane numbers indicate the *dokoubo* skeleton number.
Table 1. Conventional radiocarbon ages, with 1 SD, and calibrated age, with 1 or 2 SDs, were calculated. All samples had biologically reasonable C/N ratios, suggesting that collagen was preserved in a good condition and that the determined radiocarbon dates are reliable. The calibrated ages (+2 SD) of the skeletons, with correction for partial marine reservoir effects, were assigned between 4420–3800 calBP except for Dokoubo 1 and Dokoubo 2, which correspond to the early part of the Late Jomon era (4420–3400 calBP: Kobayashi, 2008). The calibrated radiocarbon ages of Dokoubo 1 and Dokoubo 2 correspond to the Heian era (early 11th century AD) and early Kofun (late 3–4th century AD) era, respectively.

mtDNA analysis

Both negative extraction and PCR controls consistently showed negative results throughout the experiment. The results of APLP analyses are shown in Figure 4 and in Table 2 and Table 4. Nine of the 13 Shomyoji skeletons were successfully analyzed.

The mtDNAs of Dokoubo 1 and 2 were successfully sequenced, and we submitted these sequences to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.html) under accession numbers LC461209–LC461212. However, sequence analysis of the Shomyoji Jomon samples failed because of the ambiguity of the data and/or because some target DNA segments could not be sequenced. Based on these analyses, we assigned Shomyoji mtDNAs to the relevant haplogroups (Table 4).

In the detection of possible contamination by modern DNA, the mtDNA haplogroups observed in seven individuals [D4j3, N9b1, D4a1 (two individuals), G2a, D4e, and B5a2a1] were confirmed to be different from those of Shomyoji individuals (data not shown). However, the haplogroup of one individual (M7a1) was identical to that of the Shomyoji Jomon individuals (Dokoubo 13 and 23; Table 4). Because this individual was only engaged in sequencing analysis, and in conjunction with negative control results, the mtDNA data of Shomyoji skeletons are considered to be authentic.

Discussion

In the present study, we performed mtDNA analyses of 13 skeletons excavated from the Shomyoji site, and we obtained the data from nine individuals. mtDNA data of the skeletons excavated in the Kanto district during or before the Kofun era are still very limited (Adachi and Nara, 2015, 2018; Kakuda et al., 2016). In this context, our study serves as a meaningful addition to the genetic information of the ancient people who lived in the Kanto region.

In the Shomyoji Jomon individuals, we observed mtDNA haplogroups D4b2, M7a1, M7a2, and N9b* (Table 4). Because Dokoubos 13 and 23, 14 and 15, and 17 and 21 shared the same haplogroup, these individuals might have belonged to the same maternal line. However, we could not obtain reliable hypervariable region data for these individuals, and therefore it is not possible to refer to their detailed kinship at this stage. The haplogroups observed in the Jomon individuals of the Shomyoji site have been previously reported in

![Table 4. Nucleotide changes observed in the Shomyoji D site people](image)
mtDNA haplogroup studies of the Jomon people (Adachi et al., 2011, 2013; Kanzawa-Kriyama et al., 2013; Saeki et al., 2016; Kakuda et al., 2016).

However, haplogroups B5b3a and B4f observed in Dokoubo 1 and 2, respectively, have not been previously reported in mtDNA haplogroup studies of the Jomon people described earlier. Moreover, we did not find any sequences that could be assigned to haplogroups B5b3a and B4f in previously reported hypervariable region data of Jomon mtDNAs (Horai et al., 1989; Shinoda and Kanai, 1999; Shinoda, 2003).

Our results, in combination with those previously reported, indicate that Shomyoji individuals who lived in the Kofun and Heian eras differed genetically from the Jomon people at the same site. This implies that genetic conversion of the Japanese people occurred during or before the Kofun era, at least at the Shomyoji site. This view is consistent with that of Shinoda (2004), who pointed out the discontinuity between Jomon and Yayoi people.

However, haplogroups B5b3a and B4f are absent not only in the Jomon people but also in the Yayoi (Ota et al., 1995; Shinoda, 2004; Shinoda et al., 2017) and the Kofun people (Adachi and Nara, 2015; Kakuda et al., 2016). In fact, mtDNAs of Dokoubo 1 and 2 are the first, and hitherto the oldest and only, examples of haplogroups B5b3a and B4f in ancient mtDNA data of Japan. Additionally, haplogroups B5b3a and B4f are observed only at low frequencies (0.08% and 0.3%, respectively) in modern-day main-island Japanese (Tanaka et al., 2004).

These findings suggest that haplogroups B5b3a and B4f are not representatives of the Japanese mtDNA pool. Moreover, it is still likely that these haplogroups have existed in the Japanese population at low frequency since the Jomon era because the number of genetically analyzed Jomon skeletons is not sufficient to detect rare haplogroups. To clarify the timing of the appearance of haplogroups B5b3a and B4f in Japan, more ancient skeletons should be analyzed. Moreover, to confirm the possible abovementioned genetic conversion, nuclear genome analysis of the Shomyoji people should also be performed.

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