Multiplex APLP System for High-Resolution Haplogrouping of Extremely Degraded East-Asian Mitochondrial DNAs

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Abstract

Mitochondrial DNA (mtDNA) serves as a powerful tool for exploring matrilineal phylogeographic ancestry, as well as for analyzing highly degraded samples, because of its polymorphic nature and high copy numbers per cell. The recent advent of complete mitochondrial genome sequencing has led to improved techniques for phylogenetic analyses based on mtDNA, and many multiplex genotyping methods have been developed for the hierarchical analysis of phylogenetically important mutations. However, few high-resolution multiplex genotyping systems for analyzing East-Asian mtDNA can be applied to extremely degraded samples. Here, we present a multiplex system for analyzing mitochondrial single nucleotide polymorphisms (mtSNPs), which relies on a novel amplified product-length polymorphisms (APLP) method that uses inosine-flapped primers and is specifically designed for the detailed haplogrouping of extremely degraded East-Asian mtDNAs. We used fourteen 6-plex polymerase chain reactions (PCRs) and subsequent electrophoresis to examine 81 haplogroup-defining SNPs and 3 insertion/deletion sites, and we were able to securely assign the studied mtDNAs to relevant haplogroups. Our system requires only 1×10⁻¹³ g (100 fg) of crude DNA to obtain a full profile. Owing to its small amplicon size (<110 bp), this new APLP system was successfully applied to extremely degraded samples for which direct sequencing of hypervariable segments using mini-primer sets was unsuccessful, and proved to be more robust than conventional APLP analysis. Thus, our new APLP system is effective for retrieving reliable data from extremely degraded East-Asian mtDNAs.
**Introduction**

Mitochondrial DNA (mtDNA) is a powerful tool for exploring matrilineal phylogeographic ancestry, as well as for analyzing highly degraded samples, because of its polymorphic nature and high copy numbers per cell. The recent advent of complete mitochondrial genome sequencing has led to improved techniques for phylogenetic analyses based on mtDNA, and many multiplex genotyping methods have been developed for the hierarchical analysis of phylogenetically important mutations [1–7].

However, few multiplex genotyping systems for analyzing East-Asian mtDNA lineage can be applied to extremely degraded samples [2, 5–7]. Even in these studies, haplogroup D, which exhibits the highest frequency and incidence of variations in many East-Asian populations, is not sufficiently classified. For example, Coutinho et al. [6] divided haplogroup D into 8 sub-haplogroups (the highest number among the above-mentioned studies). However, with the exception of sub-haplogroups D4b1 and D4e, these sub-haplogroups are exclusively observed in Native Americans; moreover, many sub-haplogroups of haplogroup D that are phylogenetically important in East-Asian populations are missing (e.g., haplogroup D4a). Therefore, there is a need to establish higher resolution multiplex systems for the hierarchical analysis of phylogenetically important mutations in East-Asian populations.

Among the methods for analysis of single nucleotide polymorphisms in mtDNA (mtSNPs), amplified product-length polymorphism (APLP) [8, 9] is considered one of the simplest and most robust. To detect mtSNPs, APLP employs two allele-specific primers, one of which has a few non-complementary bases in the 5’-terminus. The detection consists of assessing the difference in the length of the amplicons, which are obtained by polymerase chain reaction (PCR) and subsequent electrophoresis. We previously showed the effectiveness of APLP-based multiplex mtSNP analyses [9] for highly degraded samples when we successfully clarified the genealogy of individuals, and the relationship between populations excavated from different archaeological sites [10–16].

However, with respect to the successful analysis of extremely degraded samples, the conventional mitochondrial APLP (mtAPLP) system [9] has at least four drawbacks. First, conventional mtAPLP systems examine 35 haplogroup-diagnostic mtSNPs and a 9-bp repeat variation in the non-coding cytochrome oxidase II/tRNA^Lys^ intergenic region. This number of polymorphic sites is too small for classifying mtDNAs to sub-haplogroup level without using the sequence data of the hypervariable segments (HVS). Second, in each set of a conventional mtAPLP system, the mtSNPs are not selected in accordance with the phylogenetic order. For instance, the macro-haplogroup examined in set A is N despite the fact that seven out of nine haplogroups examined in this set stem from macro-haplogroup M: haplogroup D, its branches (D4, D4a, D4b, D4g, and D4e), and haplogroup M12. Third, the competitiveness of some primers is low. For example, haplogroup F mtDNA always shows an extra 66-bp band on gel. Fourth, the amplicon size is considered inappropriate. In practice, amplicons longer than 120 bp frequently disappear when analyzing extremely degraded samples. To overcome such limitations, a more accurate, detailed, and sensitive mtDNA haplogrouping system is required.

Here, we present a novel multiplex inosine-flapped APLP system that is specifically designed for haplogrouping extremely degraded East-Asian mtDNAs.

**Materials and Methods**

**DNA samples**

To obtain modern-day DNA samples, intraoral epithelial cells were collected from eight healthy Japanese adults. Before cells were collected, volunteers were informed, in writing that
their DNA would be anonymized and that it would be used only for haplogrouping of its mtDNA. Written consent was then obtained from each volunteer to use his or her DNA in the study. Both the consent procedure and, the written forms, were approved by the ethics committee of the Faculty of Medicine of the University of Yamanashi.

In order to establish the current APLP system, in addition to the samples from the volunteers, we also used ancient and modern-day DNA samples for which the mtDNA haplogroups had been securely determined in previous studies [9–17]. DNA samples provided by the University of Malaya, Tokai University School of Medicine, and the Yamagata University had all been anonymized before arriving at our research facility at the University of Yamanashi. We obtained permission to conduct this study using these DNA samples from each of the respective universities.

The intraoral epithelial cells were collected using a forensic swab (Sarstedt Inc., Nümbrecht, Germany). DNA extraction was performed using a MonoFas genome DNA extraction kit VIII (GL Science Inc., Tokyo, Japan), and the manufacturer’s protocol was followed. The quantity and purity of the DNA was evaluated by optical density (OD)260 and OD260/280 measurements, obtained using a spectrophotometer (Nano Drop 1000; Thermo Fisher Scientific Inc., Waltham, MA, USA).

To determine the mtDNA haplogroups from these DNA samples, segments of mtDNA that cover parts of the tRNAPro gene, the hypervariable segments (HVS) 1 (nucleotide position (np) 15999–16366, relative to the revised Cambridge reference sequence (rCRS) [18]), and HVS 2 (np 128–256) were analyzed as described previously [12]. Moreover, to confirm our ability to identify mtDNA haplogroups from modern-day samples, we also analyzed haplogroup-diagnostic mtSNPs and a 9-bp repeat variation in the non-coding cytochrome oxidase II/tRNALys intergenic region by using the conventional mtAPLP system [9]. Nucleotide changes observed in eight modern-day samples are shown in Fig 1. Thereafter, we assigned each modern-day mtDNA under study to the relevant haplogroup by using Phylotree, the updated comprehensive phylogenetic tree of global human mitochondrial DNA variation (www.phylotree.org; mtDNA tree Build 17) [19]. Basically, Phylotree is built based on the Reconstructed Sapience Reference Sequence (RSRS) [20] to avoid inconsistencies, misinterpretations, and errors in medical, forensic, and population genetic studies. However, the description of nucleotide changes in the conventional mtAPLP system [9] is based on rCRS. Therefore, we used an

| specimen Number | Mutations in the segments | SNPs in the cording region |
|-----------------|---------------------------|---------------------------|
| Haplogroup      | (16000–)                  |                           |
| Modern 1 D4j3   | 184 223 311              | A A T A                   |
| Modern 2 D4a    | 129 223 362              | A A T C                   |
| Modern 3 F2d    | CRS                       | 235 249d                  |
| Modern 4 N9b    | 129 182C 183C            | CRS                       |
| Modern 5 G3a2   | 223 274 362              | 143 152 204               |
| Modern 6 M7a1   | 209 223 324              | 204                       |
| Modern 7 C1a    | 093 223 298 325          | 249d                      |
| Modern 8 D4g1   | 223 278 362              | 150                       |

Fig 1. Nucleotide changes observed in modern-day DNA samples. aAll polymorphic sites are numbered according to the rCRS [18]. CRS denotes that the sequence of the segment is identical to the rCRS. The suffix A indicates a transversion, and d indicates a deletion. Deletions are recorded at the last possible site. Diagnostic polymorphisms are emphasized by bold italic type. bAll polymorphic sites are numbered according to the rCRS. Diagnostic polymorphisms are emphasized by bold italic type. The sites that did not show polymorphisms are omitted.

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rCRS-oriented version of mtDNA tree Build 17 [19] as a classification tree for the modern-day samples.

Furthermore, in order to evaluate the effectiveness of our system for the analysis of extremely degraded samples, we also tested ancient DNA samples extracted from one early Kofun (approximately 1,600 years old) and 11 Middle Jomon (approximately 4,000 years old) skeletons excavated from the Kusakari shell midden site, Chiba, Japan. DNA was extracted from the teeth of these skeletons according to the method described by Adachi et al. [14].

Hierarchical analysis of phylogenetically important East-Asian mtSNPs

To securely assign East-Asian mtDNAs to the relevant haplogroups, we analyzed 81 haplogroup-diagnostic SNPs, and three deletion/insertion polymorphisms (Fig 2): for haplogroup B, a 9-bp deletion in the non-coding cytochrome oxidase II/trNAlyg intergenic region; for haplogroup C5, a cytosine insertion just after nucleotide position 595 (595.1 C); for haplogroup

![Fig 2. Scheme of mitochondrial DNA haplogroup assignment based on the haplogroup-defining mutations.](image-url) The color coding of the frames is consistent with that employed to identify electrophoretograms in Figs 3 and 4. Superscript numbers correspond to the electrophoretogram lane numbers shown in Figs 3 and 4. The primer sets of multiplexes M-I to M-VIII and N-I to N-VI are shown in Tables 1 and 2, respectively.

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C1, a 2-bp deletion at positions 290–291. Each nucleotide position is numbered according to the RSRS [20]. These mutation sites were selected based on the RSRS-oriented version of mtDNA tree Build 17 [19], in consideration of the frequency and significance of the haplogroups observed in modern-day and ancient East Asian populations, as suggested by previous studies [9, 13–17, 21–27].

Phylogenetically important mutations were examined using fourteen 6-plex PCR sets (Table 1 and Fig 3; Table 2 and Fig 4). We recently developed novel APLP primers with a short inosine extension added to the 5′-terminus. This modification improves the competitiveness of allele-specific primers to the template DNA, resulting in enhanced reliability of the analysis of SNPs [28]. In the present study, we designed the primers based on this inosine-flapped APLP method. Moreover, to maximize the robustness of the PCR, we used amplicons with a length of <110 bp, which is shorter than the amplicon length used in the conventional system (<151 bp) [9]; we also examined fewer polymorphic sites in each set (6 sites, compared to 9 in the conventional system) [9].

First of all, reactions using primers of multiplexes M-I and N-I were performed for all samples, because most East-Asian mtDNAs stem from macro-haplogroups M and N, and these multiplexes can identify major branches of macro-haplogroups M and N that are widely observed in East-Asian populations.

Following SNP typing using multiplexes M-I and N-I, each mtDNA under study was classified on the basis of the criteria shown in Fig 2, by using the mtDNA haplogroup nomenclature from Phylotree [19]. Thereafter, each mtDNA underwent subsequent haplogrouping based on the result of multiplexes M-I and N-I. For example, if an mtDNA was designated to haplogroup B, further haplogrouping of this mtDNA was performed using multiplex N-V. If the SNPs observed in a sample did not represent a haplogroup motif, i.e., they were apparently incongruent with Phylotree, the data was discarded because, as we reported previously [29], such incongruence often stems from contamination of the sample.

PCR conditions and detection of PCR products
The formula of the amplification reaction and the PCR condition were the same for all multiplexes; only the primers differed. Each reaction was performed in a total volume of 10 μl, containing a 1 μl aliquot of the sample DNA solution, optimum concentrations of each primer (Tables 1 and 2), and reagents of the QIAGEN multiplex PCR kit (QIAGEN, Hilden, Germany).

The amplification reaction was conducted in a TaKaRa PCR Thermal Cycler FAST (TaKaRa, Shiga, Japan). The condition for PCR included: incubation at 95°C for 15 minutes; 5 cycles at 94°C for 30 seconds, and at 64°C for 5 minutes (ramp speed > 2.5°C/sec); 33 cycles at 94°C for 30 seconds, and at 64°C for 90 seconds (ramp speed > 2.5°C/sec); and a final extension at 72°C for 3 minutes.

A 2 μl aliquot of the PCR product was separated by electrophoresis in a precast native polyacrylamide gel (10% T, 5% C) containing 1 × TBE buffer with running buffer (1 × TBE) (TEFCO, Tokyo, Japan) using an electrophoretic apparatus STC-808 (TEFCO). The voltage at electrophoresis was 150 V (constant voltage), and the electrophoretic time was approximately 98 minutes. PCR bands were visualized fluorographically after staining with SYBR Green (Bio-Rad Laboratories, Hercules, CA, USA).

Testing the sensitivity of the new APLP system
To evaluate the sensitivity of our new APLP system, various amounts of crude DNA (1.0 × 10⁻⁹–0.1 × 10⁻¹⁵ g), which included genomic DNA and mtDNA with known haplogroups (D4j and F2), were examined using multiplexes M-I and M-III for D4j mtDNA, and
### Table 1. Primers of multiplexes M-I to M-VIII used for haplogrouping mtDNAs that stem from macro-haplogroup M.

#### Multiplex M-I

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| M          | M-15043A    | I III ATC CGT AAT ATA GGC CTC GT | 0.2                  | 104                  |
| non-M      | M-15043G    | ATC CGT AAT ATA GGC CTC GC       | 0.2                  | 100                  |
| M-15043F   |             | GTA AAT TAT GGC TGA ATC ATC CGC | 0.2                  |                      |
| G/M12      | G/M12-14569A| I I TAA CAC ACC CGA CCA CAC CA | 0.2                  | 94                   |
| non-G/M12  | G/M12-14569G| ACA CAC CGG ACC ACA CGG          | 0.2                  | 90                   |
| G/M12-14569R|            | TTT AGT AAT GGG GTT TGT GGG GT   | 0.2                  |                      |
| M8         | M8-7196A    | I I AAT TTT CTT CCC ACA ACA TCT C| 0.2                  | 84                   |
| non-M8     | M8-7196C    | CTC TCT CCC CAC AAC ACT TTC TC   | 0.2                  | 80                   |
| M8-7196F   |             | GTA AAT TAT GGC TGA ATC ATC CGC | 0.2                  |                      |
| G/M12      | G/M12-14569A| I I TAA CAC ACC CGA CCA CAC CA | 0.2                  | 94                   |
| non-G/M12  | G/M12-14569G| ACA CAC CGG ACC ACA CGG          | 0.2                  | 90                   |
| G/M12-14569R|            | TTT AGT AAT GGG GTT TGT GGG GT   | 0.2                  |                      |

#### Multiplex M-II

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| non-M29/Q  | M29/Q-13500T| I I I GGA ATA CCT TTC CTC ACA GGT | 0.2                  | 104                  |
| M29/Q      | M29/Q-13500C| GAA TAC CTT TCC TCA CAG GC       | 0.2                  | 100                  |
| M29/Q-13500R|            | GGC ATG AGA GTA ATA GAT AGG G     | 0.2                  |                      |
| non-M10    | M10-8793T   | I I I GTG GTT GGT GTA AAT GAG TGA | 0.2                  | 94                   |
| M10        | M10-8793C   | GTG GTT GGT GTA AAT GAG TGG      | 0.1                  | 90                   |
| M10-8793F  |             | AAG GGA GGG TGG ATG GAA TTA A    | 0.2                  |                      |
| non-M11    | M11-1095T   | I I I GGA TTA GAT ACC CCA CTA TGC T | 0.1                  | 84                   |
| M11        | M11-1095C   | GAT TAG ATA CCC CAC TAT GCT C    | 0.2                  | 80                   |
| M11-1095R  |             | GTG GCT GTG AGT GTT CCG G        | 0.2                  |                      |
| M9         | M9-4491A    | I I I GCA AAG ATG GTA GAG TAG ATG A | 0.2                  | 74                   |
| non-M9     | M9-4491G    | GCA AAG ATG GTA GAG TAG ATG AC   | 0.2                  | 70                   |
| M9-4491F   |             | ATG TTG GTT ATA CCC TTC CCG T    | 0.2                  |                      |
| non-M9a    | M9a-14308T  | I I I GTG GTG GTA AAT TTT AAT AGT GTA GGA | 0.2                  | 64                   |
| M9a        | M9a-14308C  | GTG GTG GTA AAC TTT AAT AGT GTA GGG | 0.2                  | 60                   |
| M9a-14308F |             | CCC TGA CCC CTC TCC TTC A        | 0.2                  |                      |
| non-E      | E-3705G     | I I I GAA TTA GAG TTA GGG AGG C  | 0.05                 | 54                   |
| E          | E-3705A     | GTG GCT GCT ACT CCT CCT C        | 0.2                  | 50                   |
| E-3705F    |             | TCA AAG TCA AAG TAC GCC CTC GA   | 0.2                  |                      |

#### Multiplex M-III

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| non-D6     | D6-3714A    | I I I CTT CAT ATG AGA TTA TTT GGG CT | 0.2                  | 104                  |
| D6         | D6-3714G    | TAC ATA TAG GTG TGT GGG C          | 0.2                  | 100                  |
| D6-3714F   |             | CCT AGC GTG TTA GTC AAT CCT        | 0.2                  |                      |
| non-D5     | D5-5301A    | I I I TAC ACA AAA AAC ACTG CTC ATC A | 0.2                  | 94                   |
| D5         | D5-5301G    | CAC AAA AAA CAA TAG CCT CAT GG     | 0.2                  | 90                   |
| D5-5301R   |             | GTG GAG TAG ATT AGG GGT AGG        | 0.2                  |                      |

(Continued)
Table 1. (Continued)

| Multiplex M-IV | Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|----------------|------------|-------------|----------|--------------------|---------------------|
| non-G          | G-4833A    | IIII CCA GAG GGT ACC CAA GGC A | 0.2      | 104                |
| G              | G-4833G    | CCA GAG GGT ACC CAA GGC G       | 0.2      | 100                |
| G              | G-4833R    | GTG AGG GAG AGA TTT GGT GTA TG  | 0.2      |                    |
| G2             | G2-5601T   | IIII TAA CAG CTA AGG ACT GCA AAA T | 0.2      | 94                 |
| non-G2         | G2-5601C   | TAA CAG CTA AGG ACT GCA AAA C   | 0.2      | 90                 |
| G              | G2-5601R   | CCC ATT GGT CTA GTA AGG GC       | 0.2      |                    |
| G1             | G1-15323A  | IIII AAT AGG AGG TGG ACT GT C   | 0.2      | 84                 |
| non-G1         | G1-15323G  | AAT GGA GGT GGA GTG CTG C       | 0.2      | 80                 |
| G1             | G1-15323F  | TCC CAC CCT CAC AAC TGT CT      | 0.2      |                    |
| G3             | G3-16274A  | IIII GGG TGG GTA GGT TTG TTG GTA TTA T | 0.1      | 74                 |
| G1a            | G1a-7867T  | IIII GGC CAA TTG ATT TGA TGG TAA GA | 0.2      | 64                 |
| non-G1a        | G1a-7867C  | GCC AAC TGA TTT GAT GTG AAG G   | 0.2      | 60                 |
| G1             | G1a-7867F  | GCC ATC CTT TAC ATA ACA GAC G   | 0.2      |                    |
| G1b            | G1b-12361A | IIII TAA CCA TGC ACA CTA CTA TAA CCA | 0.2      | 54                 |
| G1b            | G1b-12361G | ACC AGT CAC ACT ACT ATA ACC G   | 0.2      | 50                 |
| non-M7c1       | M7c1-5442T | IIII TGA ACA TAC AAA ACC CAC CCC AT | 0.1      | 84                 |
| M7c1           | M7c1-5442C | GAA CAT ACA AAA ACC CCC ACC CCA C | 0.2      | 80                 |
| M7a            | M7a-2772T  | IIII GCA AAC AGT ACC TAA CAA ACC T | 0.1      | 74                 |
| non-M7a        | M7a-2772C  | GCA AAC AGT ACC TAA CAA ACC C   | 0.2      | 70                 |
| M7a            | M7a-2772R  | TCG CCC CAA CGG AAA TTA TTA AT  | 0.2      |                    |
| M7a1           | M7a1-14364A| IIII CAT CAT ACT CTT TCA CCC ACA A | 0.2      | 64                 |

(Continued)
Table 1. (Continued)

| Multiplex M-VI | Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|----------------|------------|-------------|----------|--------------------|----------------------|
| non-M7a1       | M7a1-14364G| ATC ATA CTC TTT CAC CCA CA | 0.2      | 60                 |
| M7a2           | M7a2-15422G| I I I CCG AGG GCC TCT TGG A C | 0.1      | 54                 |
| non-M7a2       | M7a2-15422A| I CCG AGG GCC TCT TGG A T | 0.2      | 50                 |
| non-M7a1       | M7a1-15422F| CCG ATA AAA TCA CCT TCC ACC C | 0.1      |                    |

| Multiplex M-VII | Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|-----------------|------------|-------------|----------|--------------------|----------------------|
| non-D4a3        | D4a3-5466A | I I I ATA GGT AGG AGT AGC GTG G | 0.1      | 104                |

| Multiplex M-VII | Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|-----------------|------------|-------------|----------|--------------------|----------------------|
| non-D4a3        | D4a3-5466A | I I I ATA GGT AGG AGT AGC GTG G | 0.1      | 104                |

(Continued)
multiplexes N-I and N-VI for F2 mtDNA (Table 1 and Fig 3; Table 2 and Fig 4). The results of the experiments were confirmed by three independent assays. To detect the possibility of contamination, negative PCR controls were also analyzed.

**Application to highly degraded samples**

To validate the effectiveness of our new APLP system for highly degraded samples, we analyzed one early Kofun (approximately 1,600 years old) and 11 Middle Jomon (approximately 4,000 years old) skeletons. At first, the ancient DNAs were examined by using multiplexes M-I and N-I. Thereafter, the samples underwent subsequent haplogrouping using multiplexes M-V and N-III. The results of the experiments were confirmed by three independent assays.

Before performing the analysis using our new APLP system, we checked the quality of the ancient DNA samples by using a conventional APLP system [9]; we also checked the direct sequencing of the hypervariable segment I (15999–16366) using our mini-primer sets [29], for which the amplicon length is shorter than 139 bp. The results of the preliminary analyses revealed that 3 out of 12 samples could be assigned to relevant haplogroups using the conventional APLP system: sample B192 assigned to haplogroup N9b, sample B516C to haplogroup M7a, and sample B516D to haplogroup M7a; only one sample (B192), which is ascribed to the early Kofun period, could be analyzed by direct sequencing (mutations identified at the nucleotide positions 15999–16366: A16183C-T16189C-C16223T, relative to rCRS [18]). Along with the ancient samples, negative extraction and negative PCR controls were also analyzed.

**Results**

**Hierarchical analysis of mtSNPs**

As shown in Figs 3 and 4, our new APLP system correctly identified the genealogy of the mtDNAs for which the haplogroups had been determined in advance.
Fig 3. Electrophoretogram of PCR products from multiplexes M-I to M-VIII. The primer sets of multiplexes M-I to M-VIII are shown in Table 2. Yellow, light blue, light green, red, green, purple, orange, and blue frames indicate multiplexes M-I, II, III, IV, V, VI, VII, and VIII, respectively. This color coding corresponds to that given in Fig 2. LM indicates the 10-bp ladder marker.

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### Table 2. Primers of multiplexes N-I to N-VI used for haplogrouping mtDNAs that stem from macro-haplogroup N.

#### Multiplex N-I

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| B or non-B | 9 bp-F      | TGC CCA TGG TCC TAG AAT TAA TTC | 0.2 | 110 (non-B) 101 (B) |
| B or non-B | 9 bp-R      | GCT AAG TGT TTA CAG TGG GC | 0.2 | |
| N          | N-10873T    | I I I AGC CTA ATT ATT AGC ATC ATC CCT | 0.2 | 94 |
| non-N      | N-10873C    | GCC TAA TTA TTA GCA TCA TCC CC | 0.2 | 90 |
| non-R      | N-10873R    | GGG GGT CGG AGG AAA AGG T | 0.2 | |
| R          | R-12705T    | I I I I GGT AAC TAA GAT TAG TAT GGT AAT TAG GAA A | 0.2 | 84 |
| R          | R-12705C    | GGT AAC TAA GAT TAG TAT GGT AAT TAG GAA G | 0.2 | 80 |
| non-F      | F-6392T     | I I I I I CTC TAT CTT AGG GCC CAT CAA A T | 0.1 | 74 |
| F          | F-6392C     | TCT ATC TTA GGG GCC ATC AAC C | 0.2 | 70 |
| non-N9     | N9-5417G    | I I I I I I CAT ATC TAA CAA CGT AAA AAT AAA ATG ACA G | 0.2 | 64 |
| N9         | N9-5417A    | CCA TAT CTA ACA AGC TAA AAA TAA AAT GAC AA | 0.2 | 60 |
| N9         | N9-5417R    | GGA ATG GGG TGG GTC TTG TAT G | 0.2 | |
| non-A      | A-4248T     | I I I I I I ACC CAT CAT AAT CTC CAG CAT T | 0.2 | 54 |
| A          | A-4248C     | ACC CAT TAC AAT CTC CAG CAT C | 0.2 | 50 |
| A          | A-4248R     | TCA GAC ATA TTT CTT GGT TTT GAG G | 0.2 | |

#### Multiplex N-II

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| non-I      | I-10034T    | I I I TAC GGT TAA CTT CCA ATT AAC TAG T | 0.2 | 104 |
| I          | I-10034C    | CCG TTA ACT TCC AAT TAA CTA GC | 0.2 | 100 |
| I          | I-10034R    | ATT AGT AGT AAG GCT AGG AGG GT | 0.2 | |
| W          | W-15884C    | I I I I I I ATT AGT TTA TAC TAC AAG GAC AGG G | 0.2 | 94 |
| non-W      | W-15884G    | TTA GGT TAT ACT ACA AGG ACA GG C | 0.2 | 90 |
| W          | W-15884F    | TAC TCC ACA ACA ATC CTA ATC CTA ATA | 0.2 | |
| X          | X-6371T     | I I I TTA CAC CTA CCA GGT GTC TTG T | 0.18 | 84 |
| non-X      | X-6371C     | TAC ACC TAG CAG GTG TCT C C | 0.2 | 80 |
| X          | X-6371R     | TAT GGC AGG GGG TTT TAT ATT GAT | 0.2 | |
| non-L3     | L3-1018A    | I I ATA TGT TAA AGC CAC TTG CGT AGT T | 0.1 | 74 |
| L3         | L3-1018G    | AGT TTA AAG CCA CTT TCG TAG T C | 0.2 | 70 |
| L3         | L3-1018F    | AGC TAA AAT CTA CCT GAG TTG TAA | 0.2 | |
| Y2         | Y2-7859A    | I I I TAC ATA ACA GAC GAG GTC AAC A | 0.1 | 64 |
| non-Y2     | Y2-7859G    | CAT AAC AGA CTA GGT CAA CG | 0.2 | 60 |
| Y2         | Y2-7859R    | TAC CAT TGG TGC ATG TGT G | 0.2 | |
| N9a        | N9a-12358G  | I I I I I GAA GTT AGG GTG ATG GTG GC | 0.2 | 54 |
| non-N9a    | N9a-12358A  | GAA GTC AGG GTG ATG GTG GTT | 0.2 | 50 |
| N9a        | N9a-12358F  | ATA AAA GTA ATC ACC ATG CAC ACT AT | 0.2 | |

#### Multiplex N-III

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|----------------------|
| Y          | Y-14178C    | I I I I I CCG AGC AAT CTC AAT TAC AAC | 0.2 | 104 |
| non-Y      | Y-14178T    | CCC GAG CAA TCT CAA TAA CAA T | 0.2 | 100 |
| Y          | Y-14178R    | ATT GGT GCG GGG GCT TTG TAT | 0.2 | |
| Y1         | Y1-3834A    | I I I AGA ACA CCT CTG ATT ACT CCT A | 0.2 | 94 |
| non-Y1     | Y1-3834G    | GAA CAC CTC TGA TTA CTC CTC G | 0.2 | 90 |
| Y1         | Y1-3834R    | GTC GAA GGG GGT TTG GTT | 0.2 | |
| non-N9b    | N9b-13183A  | I I I ACA CTA TGC TTA GGC GCT A | 0.1 | 84 |

(Continued)
### Table 2. (Continued)

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|---------------------|
| N9b        | N9b-13183G  | CAC TAT GCT TAG GCG CTG | 0.2 | 80 |
|            | N9b-13183R  | GCC TAC GAT TTT TTT GAT GTC ATT TT | 0.2 | |
| N9b2       | N9b2-16294T | I I I A TA CCA ACA AAC CTA CCC A T | 0.2 | 74 |
| non-N9b2   | N9b2-16294C | ATA CCA ACA AAT CTA CCC A C | 0.2 | 70 |
|            | N9b2-16294R | GTA ATG TGC TAT GTA GCG TAA ATG | 0.2 | |
| N9b3       | N9b3-14996A | II TGA ATC ATC GCC TAC CTT CAC A | 0.1 | 64 |
| non-N9b3   | N9b3-14996G | AAT CAT CGG CTA CTT TCA CG | 0.2 | 60 |
|            | N9b3-14996R | TGT AGG AAG AGG CAG ATA AAG AAT A | 0.2 | |
| N9b1       | N9b1-12501A | II I CTC TCC CCC ACA ACA ATA TCC A T | 0.1 | 54 |
| non-N9b1   | N9b1-12501G | TGT TCC CCA CAA CAA AT T CTA TCA TG | 0.2 | 50 |
|            | N9b1-12501R | CGA GAT AAT AAC TTC TTG GTC TAG G | 0.2 | |

#### Multiplex N-IV

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|---------------------|
| non-R0     | R0-11719A   | I I ATT CTC ATA ATC GCC CAC GGA | 0.2 | 104 |
| R0         | R0-11719G   | TCT CAT AAT CGG CCA CGG G | 0.2 | 100 |
|            | R0-11719R   | TCC TTG AGA GAG GAT TAT GAT GC | 0.2 | |
| non-HV     | HV-14766T   | I I I CAA TGA CCC CAAC TAC GCA AAA T | 0.2 | 94 |
| HV         | HV-14766C   | CAA TGA CCC CAA TAC GCA AAA C | 0.2 | 90 |
|            | HV-14766R   | ATG CCG AGA AGT TGG TAG ATG GG | 0.2 | |
| U          | U-12372A    | I I I CTA CTA TAA CCA CCC TAA CCC CA A T | 0.1 | 84 |
| non-U      | U-12372G    | TAC TAT AAT ACG CAC TCT AAT CCT G | 0.2 | 80 |
|            | U-12372R    | ATG AGT TTT TTT TTT TAG GGT TAA CGA | 0.2 | |
| JT         | JT-15452A   | I I I CTT CGG CCT ACT TCT CCT CA | 0.2 | 74 |
| non-JT     | JT-15452C   | CTT CGG CCT ACT TCT CCT G C | 0.2 | 70 |
|            | JT-15452R   | GTG GCC TAG GAG GTC TGG | 0.2 | |
| R9         | R9-13928C   | I I I ACA TAC TCG GAT TCT ACC CTA C | 0.2 | 64 |
| non-R9     | R9-13928G   | ACA TAC TCG GAT TCT ACC CTA G | 0.2 | 60 |
|            | R9-13928R   | TAA GAA GCC GCT ATA GAT AGG GGA T | 0.2 | |
| non-P/T    | P/T-15607A  | I I I TCC GAT CGG TCC CTA ACA A A | 0.1 | 54 |
| P/T        | P/T-15607G  | CCG ATC CGG CCC TAA CAA G A | 0.2 | 50 |
|            | P/T-15607R  | TGG ATA GTA ATG CAA CAA G G | 0.2 | |

#### Multiplex N-V

| Haplogroup | Primer name | Sequence | Concentration (μM) | Amplicon length (bp) |
|------------|-------------|----------|--------------------|---------------------|
| B5         | B5-8584A    | I I I GGG AAA TAG AAT GAT CAG TAC TG T | 0.1 | 104 |
| non-B5     | B5-8584G    | GGA AAT AGA ATG ATC AGT ACT GC | 0.2 | 100 |
|            | B5-8584F    | AAC AAA CCC TGA GAA CCA AAA TGA | 0.2 | |
| non-B4c    | B4c-1119T   | I I I AGC CTT AAA CCT CAA CAG TTA AAT T | 0.1 | 94 |
| B4c        | B4c-1119C   | GCC CTA AAC CTC AAT AGT ACT AC | 0.2 | 90 |
|            | B4c-1119R   | TGA AGC ACC GCC AGG TCC | 0.2 | |
| B4a        | B4a-9123A   | I I I CGA CAG CGA TTT CTA GGA TAG TT | 0.2 | 84 |
| non-B4a    | B4a-9123G   | CAG AGC GAT TAG TAC GAT C | 0.2 | 80 |
|            | B4a-9123F   | CAA TAT CCA CCA TTA ACC TTC CCT | 0.2 | |
| B4b        | B4b-13590A  | I I I CGA GCG CTA GAT GCG CTT GTG T | 0.1 | 74 |
| non-B4b    | B4b-13590G  | GAG TGC TAT AGG CGC TTG T C | 0.2 | 70 |
|            | B4b-13590F  | GGA GTC CTA GCC ACA GCT | 0.2 | |
| B2         | B2-6473T    | I I T A GGA GAA GTA GGA CTG TAG TA | 0.2 | 64 |
| non-B2     | B2-6473C    | GGA GAA GTA GGA CTG TAG TG | 0.2 | 60 |

(Continued)
Sensitivity of the new APLP system

Although the copy number of mtDNA exhibits some variation among individuals, the detection limit was $1.0 \times 10^{-13}$ g of crude DNA for multiplexes M-I, M-III, and N-I, and $1.0 \times 10^{-14}$ g for multiplex N-VI. Consequently, our new APLP system correctly identified haplogroups D4j and F2 from $1.0 \times 10^{-13}$ g (100 fg) of crude DNA templates, which corresponds to less than 10 copy numbers of mtDNA (Fig 5). In the analysis of these samples, negative PCR controls were negative throughout the experiment.

Robustness of the new APLP system with respect to highly degraded mtDNA

By using our new APLP system, 10 out of 12 mtDNAs from the ancient skeletons were successfully assigned to relevant haplogroups (Fig 6). In the analysis of the ancient skeletons, negative extraction and negative PCR controls were negative throughout the experiment (data not shown).

Discussion

The results showed that, following a hierarchical analysis of 81 haplogroup-defining mtSNPs and 3 insertion/deletion sites performed using fourteen 6-plex multiplexes and subsequent
Fig 4. Electrophoretogram of PCR products from multiplexes N-I to N-VI. The primer sets of multiplexes N-I to N-VI are shown in Table 2. Yellow, light blue, light green, red, green, and purple frames indicate multiplexes N-I, II, III, IV, V, and VI, respectively. The color coding corresponds to that given in Fig 2. LM indicates the 10-bp ladder marker.

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electrophoresis, our new APLP system correctly identified the genealogy of the mtDNAs with known haplogroups. Previously, 15 to 36 mtSNPs and insertion/deletion polymorphisms were examined in East-Asian mtDNA by using conventional means such as SNaPshot minisequencing assays [2, 6, 7] or APLP [5, 9]. However, the number of mtSNPs examined in these previous studies is too small for detailed haplogrouping of East-Asian mtDNAs. In particular, haplogroup D, which is the predominant haplogroup in many East-Asian populations, was not sufficiently classified in the previously reported assays. By using our new APLP system, many major mtDNA lineages including haplogroup D can be securely classified to the sub-haplogroup level.

Moreover, using our new APLP system, hierarchical examination of many mtSNPs can help identify contamination or misinterpretation of the results on the basis of congruence with PhyloTree, the updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Therefore, as we reported previously [29], our new APLP system can improve the reliability of sequencing and SNP analysis of mtDNA.

The recent advent of high throughput sequencing (HTS) technology based on so-called Next Generation Sequencers has allowed analyses of the complete mitochondrial and chromosomal genome sequences even in very degraded samples like those from archaeological skeletons [30, 31]. However, HTS is very costly, and thus it is difficult for most laboratories to
perform such analyses routinely. Therefore, to maximize the success rate of HTS, it is important to evaluate the quality and quantity of DNA in the samples before subjecting them to HTS. Our new APLP system correctly identified the haplogroup of mtDNAs in only 100 fg \((1.0 \times 10^{-13} \text{ g})\) of crude DNA. This sensitivity is over 10 times higher than that reported in previous studies, where quantities of crude DNA in the order of at least pico \((1.0 \times 10^{-12})\) grams were required for accurate genotyping \([3, 5–7]\). This extremely high sensitivity may be attributable to the reduced number of SNP sites analyzed in each multiplex in our system compared to that analyzed in other systems. Our new APLP system is thus expected to serve as a time- and cost-efficient tool to evaluate the quality and quantity of DNA in samples before HTS analysis.

In the present study, we show how an inosine-flapped APLP system can be efficiently applied for the hierarchical multiplex analysis of mtSNPs. Adding a short inosine extension to the 5'-terminus of APLP primers improves the competitiveness of allele-specific primers to the template DNA, resulting in enhanced reliability of the SNP analysis \([28]\). Furthermore, the thermodynamics of the primers with inosine flaps have been proven to be less influenced by the sequence of PCR templates than the thermodynamics of the primers with 5' flaps containing ordinary bases \([28]\). These features of inosine-flapped primers are likely to have contributed to the high sensitivity observed for our new APLP system.

The robustness of our APLP system was verified by the analysis of 12 archaeological skeletons; only 3 such samples could be successfully assigned to relevant haplogroups using the conventional APLP system, whereas a total of 10 samples were successfully assigned using our new APLP system. Our inosine-flapped APLP primers generated shorter amplicons (\(<110 \text{ bp}\)) compared to those generated by conventional APLP primers (\(<151 \text{ bp}\)), and we believe it is the

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Fig 6. Electrophoretograms of the multiplex PCR products for mtDNA of ancient skeletons. Each lane gives results for a single sample: lane LM, 10-bp ladder marker; lane 1, B516C; lane 2, B516D; lane 3, B202C; lane 4, B228D; lane 5, B192; lane 6, B202A; lane 7, B202B; lane 8, B509A; lane 9, B516A; lane 10, B228C; lane 11, B511; lane 12, B585. Using the conventional APLP system, 3 out of 12 samples could be assigned to relevant haplogroups (B192 to N9b, B516C to M7a, and B516D to M7a). Arrows indicate subsequent haplogrouping flows based on the results obtained using multiplexes M-I and N-I. Yellow frames identify results obtained using multiplexes M-I and N-I, while results obtained using multiplexes M-V and N-III are framed in green and light green, respectively (color coding corresponds to that given in Fig 2).

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shorter amplicon length that is the source for the higher success rate observed for our new APLP system.

The haplogroups observed in the samples excavated from the Kusakari shell midden site were N9b and M7a. These haplogroups are observed in the Jomon people unearthed from Hokkaido, the northern island of Japan. Notably, haplogroup N9b is the most predominant haplogroup in these Hokkaido Jomon people (64.8%, 35 out of 54 individuals) [13]. The fact that these haplogroups are also observed in the Kusakari Jomon people, who were excavated from Honshu, the main island of Japan, indicates that these haplogroups are strong candidates for the so-called “Jomon genotype” as suggested by the previous studies [9, 12, 13]. Moreover, the fact that haplogroup N9b is observed in the Kofun sample (B192), excavated from the same site, may hint at genetic continuity in this site extending from the Jomon era to the Kofun era.

In addition, at the sub-haplogroup level, one Kusakari Jomon sample (B516C) was assigned to M7a1, which was not observed in the Hokkaido Jomon people [13]. Intriguingly, this sub-haplogroup is the most predominant one found in modern-day Japanese and Korean M7a mtDNAs [22, 32, 33]. It has its highest frequency (44 out of 156 individuals) in Okinawa islanders living in the southern-most islands of Japan [32]. However, haplogroup M7a is rare in Southeast Asian populations, whereas the frequencies of its sister haplogroups (e.g., M7b and M7c) are relatively high in these populations [24, 27, 34]. We have previously hypothesized that haplogroup M7a may have diversified from its ancestral M7 haplogroup in the southern part of the Japanese archipelago [13]. Given the findings here, the fact that haplogroup M7a1 is observed in Honshu Jomon people, but is absent in Hokkaido Jomon individuals, gives some support to this hypothesis.

Unfortunately, we could not compare the robustness of our new system with that of SNaPshot analysis or HTS, mainly because of the residual volume of the samples. However, our system generates short amplicons similar to those reported in the study of Coutinho et al. [6], which focuses on the ancient DNA analysis of the skeletons excavated from South America. Therefore, our system is expected to be as effective as that of Coutinho et al. [6] for the analysis of fragmented mtDNA.

As described earlier, in the case of extremely degraded samples like archaeological skeletons, it is often very difficult to obtain reliable mtDNA sequences. Despite such difficulties, it is worth trying to obtain as much mtDNA data as possible from those samples, because such data is important for phylogeographic analysis, and, in some cases, personal identification. Therefore, our new APLP system is expected to be very useful in analyzing extremely difficult forensic samples, as well as for molecular anthropological studies of ancient populations.

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Author Contributions

Conceived and designed the experiments: NA HS. Performed the experiments: NA HS MT TK. Analyzed the data: NA HS. Contributed reagents/materials/analysis tools: PN KM KU NA. Wrote the paper: NA HS TK KM.
References

1. Quintás B, Álvarez-Iglesias V, Salas A, Phillips C, Lareu MV, Carracedo A. Typing of mitochondrial DNA coding region SNPs of forensic and anthropological interest using SNaPshot minisequencing. Forensic Sci Int. 2004; 140: 251–257. PMID:15036446

2. Álvarez-Iglesias V, Jaime JC, Carracedo A, Salas A. Coding region mitochondrial DNA SNPs: Targeting East Asian and Native American haplogroups. Forensic Sci Int Genet. 2007; 1: 44–55. doi: 10.1016/j.fsigen.2006.09.001 PMID: 19083727

3. Van Oven M, Vermeulen M, Kayser M. Multiplex genotyping system for efficient inference of matrilineal genetic ancestry with continental resolution. Investig Genet. 2011; 2: 6. http://investigativegenetics.biomedcentral.com/articles/10.1186/2041-2223-2-6 doi:10.1186/2041-2223-2-6 PMID: 21429198

4. Ballantyne KN, Van Oven M, Ralf A, Stoneking M, Mitchell RJ, Van Oorshot RAH, Kayser M. MtDNA SNP multiplexes for efficient inference of matrilineal genetic ancestry within Oceania. Forensic Sci Int Genet. 2012; 6: 425–436. doi:10.1016/j.fsigen.2011.08.010 PMID: 21940232

5. Lee HY, Yoon JA, Yang WI, Shin KJ. A one step multiplex PCR assay for rapid screening of East Asian mtDNA haplogroups on forensic samples. Legal Med. 2013; 15: 50–54. doi:10.1016/j.legalmed.2012.08.002 PMID: 22981178

6. Coutinho A, Valverde G, Fehren-Schmitz L, Cooper A, Romero MIB, Espinoza IF, et al. AmericaPlex 26: A SNaPshot multiplex system for genotyping the main human mitochondrial founder lineages of the Americas. PLoS ONE. 2014; 9: e93292. doi: 10.1371/journal.pone.0093292 PMID: 24671218

7. Ren Z, Luo H, Song F, Wei W, Yang Y, Zhai X, et al. Developing mtSNP assay for forensic application in Han Chinese based on mtDNA phylogeny and hot spot. Electrophoresis. 2015; 36: 633–639. doi: 10.1002/elps.201400396 PMID: 25382174

8. Shinoda K, Adachi N, Guillen S, Shimada I. Mitochondrial DNA analysis of ancient Peruvian highlanders. Am J Phys Anthropol. 2006; 131: 98–107. PMID: 16485299

9. Adachi N, Shinoda K, Umetsu K, Hayakawa T, Takeyasu T, Hanazawa Y, et al. Multiplex amplified product-length polymorphism analysis for rapid detection of human mitochondrial DNA variations. Electrophoresis. 2001; 22: 3533–3538. PMID: 11669538

10. Adachi N, Umetsu K, Takigawa W, Sakaue K. Phylogenetic analysis of the human ancient mitochondrial DNA. J Archaeol Sci. 2004; 31: 1339–1348.

11. Shinoda K, Adachi N, Guillen S, Shimada I. Mitochondrial DNA analysis of ancient Peruvian highlanders. Am J Phys Anthropol. 2006; 131: 98–107. PMID: 16485299

12. Adachi N, Shinoda K, Umetsu K, Matsumura H. Mitochondrial DNA analysis of Jomon skeletons from the Funabodami site, Hokkaido, and its implication for the origin of Native American. Am J Phys Anthropol. 2009; 138: 255–265. doi: 10.1002/ajpa.20923 PMID: 18951391

13. Adachi N, Shinoda K, Umetsu K, Kitano T, Matsumura H, Fujiyama R, Sawada J, Tanaka M. Mitochondrial DNA analysis of Hokkaido Jomon skeletons: Remnants of archaic maternal lineages at the southwestern edge of former Beringia. Am J Phys Anthropol. 2011; 146: 346–360. doi: 10.1002/ajpa.21561 PMID: 21953438

14. Adachi N, Sawada J, Yoneda M, Kobayashi K, Itoh S. Mitochondrial DNA Analysis of the Human Skeleton of the Initial Jomon Phase Excavated at the Yugura Cave Site, Nagano, Japan. Anthropol Sci. 2013; 121: 137–143.

15. Adachi N, Shinoda K, Umetsu K. Ethno-history of the Hokkaido Ainu inferred from mitochondrial DNA data. DNA polymorphism. 2013; 21: 130–133 (in Japanese).

16. Shinoda K, Kakuda T, Kanzawa-Kiriyama H, Adachi N, Tsai PY, Tsai HK. Mitochondrial genetic diversity of Pithecanthropus from the Fanandama site, Hokkaido, and its implication for the origin of modern humans in Japan. Int J Legal Med. 2010; 124: 165–170. doi: 10.1007/s00414-009-0355-6 PMID: 19533161

17. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet. 1999; 23: 147. PMID: 10508508

18. Van Oven M, Kayser M. Updated comprehensive phylogenetic tree of global human mitochondrial DNA variation. Hum Mutat. 2009; 30: E386–E394. http://www.phylotree.org. doi: 10.1002/humu.20921 PMID: 18853457

19. Behar D, Van Oven M, Rosset S, Metspalu M, Hoogvli EL, Silva NM, et al. A “Copernican” reassessment of the human mitochondrial DNA tree from its root. Am J Hum Genet. 2012; 90: 675–684. doi: 10.1016/j.ajhg.2012.03.002 PMID: 22482806
21. Yao YG, Kong QP, Bandelt HJ, Kivisild T, Zhang YP. Phylogenetic differentiation of mitochondrial DNA in Han Chinese. Am J Hum Genet. 2002; 70: 635–651. PMID:11836649

22. Maruyama S, Minaguchi K, Saitou N. Sequence polymorphisms of the mitochondrial DNA control region and phylogenetic analysis of mtDNA lineages in the Japanese populations. Int J Legal Med. 2003; 117: 218–225. PMID:12845447

23. Starikovskaya EB, Sukernik RI, Derbeneva OA, Volodko NV, Ruiz-Pesini E, Torroni A, et al. Mitochondrial DNA diversity in indigenous populations of the southern extent of Siberia, and the origins of Native American haplogroups. Ann Hum Genet. 2005; 69: 67–89. PMID:15638829

24. Trejaut JA, Kivisild T, Loo JH, Lee CL, He CL, Hsu CJ, Li ZY, Lin M. Traces of archaic mitochondrial lineages persist in Austronesian-speaking Formosan populations. PLoS Biol. 2005; 3: e247. PMID:15984912

25. Sato T, Amano T, Ono H, Ishida H, Kodera H, Matsumura H, et al. Mitochondrial DNA haplogrouping of the Okhotsk people based on analysis of ancient DNA: an intermediate of gene flow from the continental Sakhalin people to the Ainu. Anthropol Sci. 2009; 117: 171–180.

26. Sukernik RI, Volodko NV, Mazunin IO, Eltsov NP, Dryomov SV, Starikovskaya EB. Mitochondrial genome diversity in the Tubalar, Even, and Ulchi: Contribution to prehistory of Native Siberians and their affinities to Native Americans. Am J Phys Anthropol. 2012; 148: 123–138. doi: 10.1002/ajpa.22050 PMID: 22487888

27. Delfin F, Ko AMS, Li MK, Gunnarsdóttir ED, Tabbada KA, Salvador JM, et al. Complete mtDNA genomes of Filipino ethnolinguistic groups: a melting pot of recent and ancient lineages in the Asia-Pacific region. Eur J Hum Genet. 2014; 22: 228–237. doi: 10.1038/ejhg.2013.122 PMID: 23756438

28. Shojo H, Tanaka M, Takahashi R, Kakuda T, Adachi N. A unique primer with an inosine chain at the 5'-terminus improves the reliability of SNP analysis using the PCR-Amplified product length polymorphism method. PLoS ONE. 2015; 10: e0136995. doi: 10.1371/journal.pone.0136995 PMID: 26381262

29. Adachi N, Uematsu K, Shojo H. Forensic strategy to ensure the quality of sequencing data of mitochondrial DNA in highly degraded samples. Legal Med. 2014; 16: 52–55. doi: 10.1016/j.legalmed.2013.10.001 PMID: 24262654

30. Prüfer K, Racimo F, Patterson N, Jay F, Sankararaman S, Sawyer S, et al. The complete genome sequence of a Neanderthal from the Altai Mountains. Nature. 2014; 505: 43–49. doi: 10.1038/ nature12886 PMID: 24352235

31. Rasmussen M, Sikora M, Albrechtsen A, Korneliussen TS, Moreno-Mayer JV, Poznik GD, et al. The ancestry and affiliations of Kennewick Man. Nature. 2015; 523: 455–458. doi: 10.1038/nature14625 PMID: 26087396

32. Sekiguchi K, Imaizumi K, Fuji K, Mizuno N, Ogawa Y, Akutsu T, et al. Mitochondrial DNA population data of HV 1 and HV 2 sequences from Japanese individuals. Legal Med. 2008; 10: 284–286. doi: 10.1016/j.legalmed.2008.02.002 PMID: 18442943

33. Lee HY, Yoo JE, Park MJ, Chung U, Kim CY, Shin KJ. East Asian mtDNA haplogroup determination in Koreans: haplogroup-level coding region SNP analysis and subhaplogroup-level control region sequence analysis. Electrophoresis. 2006; 27: 4408–4418. PMID: 17058303

34. Hill C, Soares P, Mornia M, Macaulay V, Clarke D, Blumbach PB, et al. A mitochondrial stratigraphy for island Southeast Asia. Am J Hum Genet. 2007; 80: 29–43. PMID: 17160892