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ORIGINAL ARTICLE

Phylogenetic classification of Japanese mtDNA assisted by complete mitochondrial DNA sequences

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Abstract We investigated control and coding region polymorphisms in mitochondrial DNA (mtDNA) in 100 unrelated individuals from a Japanese population and determined the basal phylogenetic haplogroup lineages in all samples under updated information. Many of the basal phylogenetic haplogroup lineages assigned on East Asian mtDNA haplogroups corresponded to those previously established. However, new haplogroup lineages such as M7a2a, M7a2b, M7a2*, M7c1b, M11b2*, G2b*, D4c1b1a, D4g2b, A4*, A9, N9b*, B4d1, B4d2, and F1e were identified and established by complete sequencing. Although sequence comparison of the 1.15-kb control region identified 84 mitochondrial haplotypes, examination of coding region polymorphisms increased the total number of haplotypes to 91. Determination of the basal haplogroup lineages increased the discrimination power of mtDNA polymorphisms for personal identification and their usefulness in determining geographic origin in forensic casework in Japanese and other East Asian populations.

Keywords Mitochondrial DNA polymorphism · Control and coding region · Haplogroup · Complete sequence · Japanese

Introduction

Knowledge of the frequencies of specific mitochondrial DNA sequences and haplogroups in a given population is of crucial importance in the application of mitochondrial markers to forensic studies, as mitochondrial DNA (mtDNA) sequence types and haplogroups are strongly correlated with geographic origin. The classification of mtDNA lineages in East Asian populations based on control and coding region information and complete sequence information is currently being expanded [1–13]. More reliable phylogenetic trees for mtDNA differentiation based on complete sequencing of the mtDNA genome in East Asian populations have been presented [13]. These studies provide very useful information for forensic casework not only for personal identification but also for determining geographic origin from biological evidence based on mtDNA analysis.

Although the number of databases on mtDNA control region polymorphisms in Japanese, Korean, and Chinese populations is growing [3–5, 9, 10, 14–26], more detailed classification including coding region polymorphisms and more databases obtained from different regions within each country are necessary to increase the value of mtDNA polymorphic data in forensic casework.

Therefore, we have carried out a phylogenetic analysis of mtDNA polymorphisms, expanding previous data on Japanese populations [5, 10], by determining basal haplogroup lineages and establishing new lineages by complete sequencing.

Materials and methods

Samples

Genomic DNA was extracted from blood samples from 100 healthy unrelated Japanese individuals. Informed consent was obtained from blood donors. This study was approved by the ethics committee of Tokyo Dental College. Names of prefectures corresponding to birthplaces and numbers of

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individuals investigated are shown in Fig. S1. Leukocyte preparations from blood were digested with proteinase K (Sigma-Aldrich, Missouri) at 55°C overnight, followed by treatment with RNAse at 55°C for 2 h. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris–HCl, 1 mM EDTA at pH 7.6).

PCR amplification and sequencing of control region

We determined the nucleotide sequences in the 1.15-kb control region from nt 16024 to 579 in 100 samples. The PCR primer sequences used in this study are shown in Table S1. The complete control region was amplified essentially using primer pairs L15978 and H601. When amplification efficiency was low, two overlapping fragments (L15978-H68 and L8-H601) were amplified.

Each amplification was performed in a 30 μl mixture containing 10 ng genomic DNA, 10 mM Tris–HCl at pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.02% gelatin, 200 μM dNTP, 400 nM each primer, and 2.5 U AmpliTaq Gold (Applied Biosystems, NJ, USA). PCR amplification was performed by a two- or three-step method as shown in Table S1. Amplicons were purified with the QIAquick PCR purification kit (Qiagen, MA, USA) according to the manufacturer's instructions. PCR for sequencing was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Excessive dye was removed using the DyeEx TM Spin Kit (Qiagen). Sequence analysis was performed on an ABI 373A, 310, or 3130 DNA Sequencer (Applied Biosystems). Primers used for DNA sequencing were essentially the original PCR primer pairs, H68 and L8. When extension in the sequencing reaction was unsatisfactory, the additional primers given in Table S1 were used. In the phylogenetic analysis, the haplogroup motif and private mutations were confirmed by sequencing of additional PCR products and checking against our own previously established database [5] and databases reported by others [10], even when there was little doubt as regards to their accuracy [5, 10]. Control sequence data have been submitted to the EMPOP database (www.empop.org) [27, 28].

Typing of other polymorphisms

We investigated nucleotide variation in coding region polymorphisms to determine phylogenetic lineages by sequencing, single-strand conformation polymorphism analysis, allele-specific PCR amplification, PCR-RFLP, or size comparison in a non-denaturing gel. The nucleotide sequence from nt 10172–10659 was determined in all samples. The intergenic COII/tRNALys 9-bp deletion [29] and 3,010 and 5,178 mutations [30] were determined in many samples. More than 35% of the samples were estimated to belong to the D4 haplogroup, in which determination of the subgroup is difficult from only information on the control region. Therefore, the sequence from nt 7662–9900 was also determined in this haplogroup. Primers used for these PCR are shown in Table S1. To sequence the coding region, we selected primers from our primer sets depending on the region targeted for analyzing each haplogroup. Information for primers is available upon request. In 14 samples, in which a rare or new lineage was indicated, the full genome sequence was determined.

Data analysis

Gene diversity was calculated as follows: $h = n\left(1 - \sum x_i^2\right)/(n-1)$ (where n is sample size, and x_i is the frequency of the i-th mtDNA type) [31]. The probability of two randomly selected individuals from a population having identical mtDNA types was calculated using $P = \sum x_i^2$.

Results and discussion

In the comparison of nucleotide sequences within the 1.15-kb control region from 100 Japanese individuals (Table S2), a total of 84 different haplotypes were observed, not including variations in the length of C-stretch in the HV1 and HV2 regions. Of these, the most common type was observed in seven individuals, one type each was observed in four and three individuals, five types were shared by two individuals, and 76 types were observed in only one individual each. Gene diversity was estimated to be 0.9929, and the probability of two randomly selected individuals from the population having identical mtDNA types was 1.7%. When comparison of variation was limited to the HV1 (nt 16024–16400) and HV2 (nt 29–369) regions, which are the regions usually targeted in forensic work, the total number of haplotypes decreased to 80. The number of cytosines in the HV2 C-stretch is sometimes included in determining genotype in the population database. Because this variation frequently shows length–heteroplasmy in most haplogroups [5] and haplotypes with a different length of C-stretch have been obtained from biological materials from the same individuals, we did not include this variation in calculating number of haplotypes. However, we did include differences in the number of CA-repeats from nt 514–523, as this is comparatively restricted to certain haplogroups, suggesting that the mutation rate may be lower than that of the HV2 C-stretch. We further established the basal haplogroup lineages under the updated information in all samples by examining coding region polymorphisms. Finally, all haplotypes were classified into 69 haplogroup lineages defined by specific coding and

control region polymorphisms. The characteristic sites defining haplogroup lineages and diagnostic mutations defining the basal haplogroups are shown in Table S2. Examination of coding region polymorphisms revealed a total of 91 types, among which two types were shared by three individuals, five types were observed in two individuals, and 84 types were unique. Gene diversity increased to 0.9978, and the probability of two randomly selected individuals from the population having identical mtDNA types decreased to 1.22%.

Sub-grouping of the D4 haplogroup using coding region polymorphisms is particularly useful for Japanese and Korean populations, as the D4 haplogroup is found in high frequency in both populations, and many common haplotypes in the control region occur in this haplogroup. In the present study, haplotypes found in more than two individuals in the analysis of control region polymorphisms belonged to one type each in M7a1a and M7a1a+M7a1a5 and one type each in the D4a1, D4b2b+D4b2b1, D4c1b1+D4c1b1a, D4e1a2, D4e2, and D4e2+D4f+D4j haplogroups. In short, many frequent haplotypes were included in D4. Therefore, we determined the sequences from nt 7662–9900 in the samples in the D4 haplogroup. In 38 D4-type individuals, ten mutations found in this region were diagnostic mutations defining the basal haplogroups (Table S2). The frequent haplotype in D4, 16223–16362–16519–73–263–489, found in four individuals, was classified into D4e2, D4f, and D4j by analysis of coding region polymorphisms. Another similar haplotype, 16223–16362–73–263–489, was found in three individuals in D4e2. These two haplotypes could not be distinguished from each other without examination of the 16519 mutation. The region between HV1 and HV2 is not commonly examined in the forensic field. However, it is worth examining this mutation when 16223–16362 is found by examination of HV1 and HV2 alone. Another frequent haplotype, 16129–16223–16362–16519–73–152–263–489, found in seven individuals, was discriminated into D4a1, D4a1a, and D4a1b by coding region mutations at 5261 and 13651. These factors suggest that information on the coding region polymorphisms of these haplogroups would increase the usability of mtDNA polymorphisms in forensic casework.

We found and established 13 new haplogroup lineages, including one known but not yet established lineage in the Japanese population, by complete sequencing. In order to compare and designate new haplogroups, we referred to the nomenclature for new haplogroups compiled by Kong et al. [13]. We proposed new haplogroup names when more than two samples shared new coding region polymorphic sites in addition to haplogroup-specific mutations.

Three individuals, CB1082, GM1009, and SO1097, shared the 15422 mutation, which is characteristic for M7a2, in addition to the common mutations for M7a. One of them, CB1082, also shared the characteristic 16140–16209–146 motif to M7a2 shown in the updated phylogenetic tree by Kong et al. [13]. Determination of the complete sequence of this individual revealed the same specific mutations at 8176 and 12234 as the complete sequence of a Japanese individual (Hnsq0213) reported by Tanaka et al. [10]. Therefore, we designated this haplotype M7a2a. Another individual, GM1009, also shared 16140–16209, but lacked 146 and had a 249 mutation in the HV2 region. The complete sequencing of this sample (GM1009) revealed the same specific mutations at 961, 2218, and 8005 as those of two samples (NDsq0178 and NDsq0168) in the M7a2 haplogroup reported by Tanaka et al. [10]. Because one of them (NDsq0168) had artificial recombination from 12406 to 14002 derived from the F1a1b sequence, we excluded this information. We designated this haplotype M7a2b. The third individual, SO1097, shared the 15422 mutation with the M7a2 lineage, but did not share the mutations specific to the M7a2a and M7a2b haplogroups. This individual showed novel mutations at 12140 and 13596. Because this type of sequence had not yet been found in the M7a2 haplogroup, we designated it M7a2*, although it may constitute a different lineage in this haplogroup. The M7a2 haplogroup lineages have been found in Japanese [5, 10, 18, 19, 25, 26], but not in Koreans and Chinese. [9, 14–17, 20–24].

One individual, FS1014, had a 16223–16294–16295–146–199–489 motif in the control region, characteristic to M7c1b, as proposed by Kivisild et al. [4]. Because characteristic coding region mutation sites to M7c1b have not yet been reported, we determined the complete sequence of this individual. The same haplogroup lineage found in a sample (TCsq0077) in haplogroup M7c by Tanaka et al. shared mutations at 6053, 7961, 12804, and 14755 with our M7c1b. M7c1b is rare in Japan and China, but has been found in 1–2% of Korean populations [5, 9, 10, 14–26].

One individual, HO1019, shared all specific mutations for the M11 haplogroup shown in the phylogenetic tree by Kong et al. [13]. Complete sequencing of this individual revealed the same 13890 mutation as that in the M11b sequences by Tanaka et al. [10] (HNsq0152) and Kong et al. [8] (accession no.: AY255156), but absence of the 14790 and 10685 mutations is found in the M11b sequence. The sequence had novel 5192 and 12285 mutations, which had not yet been reported in this haplogroup. Therefore, we designated it M11b*, although it may constitute a different lineage of this haplogroup. M11 is rare in Japan [5, 10, 18, 19, 25, 26] and is found in 0.36% of Korean [14, 15, 23, 24] and 0.9% of Chinese populations [9, 16, 17, 20–22].

One individual, CB1022, had a 16189–16223–16362–16390 motif in the HV1 region, which is characteristic of the D5a1 haplogroup, but did not share 150 in HV2. After complete sequencing, it was found to have mutations

specific to the G2 haplogroup and, further, possessed an 8877 mutation, which is a characteristic mutational site for the G2b haplogroup [13]. However, other mutations were completely different from those in the G2b sequence by Kong et al. [8]. The present individual had 6932, 12678, 12892, 13590, 15034, 15525, 15852, and 15930 mutations. In contrast, G2b by Kong et al. [8] had 263A, 3593, 4853, 11151, and 16172 mutations. Therefore, we designated it G2b*, although it may constitute a different lineage of this haplogroup. A sequence sharing similar mutations has yet to be found among Japanese, Korean, and Chinese populations [5, 9, 10, 14–26].

Two individuals, HG1045 and AC1044, showed the same haplotype in the control region, 16224–16245–16292–146, and possessed the 14692 mutation characteristic to the D4c1b1 haplogroup. One of these samples, AC1044, further possessed a 15924 mutation, and the same complete sequence was found in a Japanese sample (NDsq0163) reported by Tanaka et al. [10]. Therefore, we designated this haplogroup branch D4c1b1a, although this haplogroup cannot be discriminated by only the control region with D4c1b1. The D4c1b1 control region motif is found in 0.95% of Japanese [5, 10, 18, 19, 25, 26], 0.29% of Korean [14, 15, 23, 24], and 0.15% of Chinese populations [9, 16, 17, 20–22].

One individual, OY1056, had the 195, 298, and 5231 mutations, which are characteristic for the D4g2 haplogroup lineage. It also had 504, 4131, 5460, 6719, 9386, and 13359 mutations, and the identical haplotype was found in a complete sequence of a Japanese sample (PDsq0098) by Tanaka et al. [10]. Therefore, we designated this haplotype D4g2b. The D4g2b-like control region haplotype has been found in 0.2% of Japanese [10, 25], but not in Korean [14, 15, 23, 24] or Chinese populations [9, 16, 17, 20–22].

One individual, EH1061, possessed characteristic mutations for the A haplogroup (152, 235, 16290, 16319, and 8794) and also possessed the 16362 mutation. The 16362 mutation in the A haplogroup was first shown to be specific for the A4 haplogroup [4]. Derenko et al. [12] recently revised the classification of haplogroup A in northern Asian populations using full genome information, designating A4a, A4a1, A4b, A4c, and A4d subclades, in which A4a was characterized by mutations 1442–9713–16249. A complete sequencing of EH1061 revealed an identical control region motif, 16234–16249–16290–16319–16362–152–235, to that of Japanese A4a (ONsq0125) reported by Tanaka et al. [10], which shared 1442–9713–16249 mutations. Although EH1061 did not share the 1442 and 9713 mutations specific to A4a, it did have novel 3408, 8348, 8409, 8459, 14062, and 14067 mutations, which suggested that it diverged after obtaining the 16249 mutation, constituting a new subclade. We provisionally designated this haplogroup A4*, although we had already found a similar type of mutation in a Japanese population (not shown). The control region haplotype of this linage, which includes the A4a1 and A4* haplogroups (16223–16234/16249–16290–16319–16362), was found in 0.34% of Japanese [5, 10, 18, 19, 26], 0.14% of Korean [23, 24], and 0.18% of Chinese populations [21].

Another individual, TK1057, also possessed characteristic mutations for the A haplogroup (152–235–16223–16290–16319 and 8794), but did not share 11536 specific to A5, 16362 specific to A4, or 16242 and 64 specific to the A8 haplogroup lineages. It did possess 2857, 8692, and 9711 mutations, and identical mutations were found in a complete sequence of a Japanese sample (NDsq0028) in the A haplogroup reported by Tanaka et al. [10], suggesting that it constitutes a new lineage. Therefore, we designated it the A9 haplogroup. Because the A9 haplogroup does not have characteristic mutations in addition to A-specific mutations in the control region and the haplotype with the 152–235–16223–16290–16319 motif is common in the Japanese, Korean, and Chinese populations [5, 9, 10,14–26], it is impossible to estimate the frequency of this haplogroup without coding region information.

One individual, KN221, possessed the 12501 mutation, which is characteristic for N9b1. Complete sequencing of the sample revealed five new mutations, 5457, 5492, 9548, 13154, and 15061, which had not yet been found in the N9b1 haplogroup. Although it may constitute a new lineage in this haplogroup, we designated it N9b1* since no other samples sharing similar mutations in the control region have been found in Japanese, Korean, or Chinese populations [5, 9, 10, 14–26].

We also completely sequenced two individuals, CB1068 and IR1089, in the B haplogroup. Both samples shared the 11914, 13942, and 15930 mutations characteristic to the B4d haplogroup. One individual, CB1068, further shared the 316 and 15038 mutations with the sequence LY7589 (accession no. AY255140) reported by Kong et al. [8]. Therefore, we designated this haplogroup branch B4d1, although it may further branch, as CB1068 possessed additional novel mutations at 12372 and 15262. A very similar control region haplotype was also found in two samples in the Korean population [23] and one more sample in the ancient Chinese sequence [20]. The other individual, IR1089, possessed 546, 5084, 5418, 9120, 12135, 16185, and, 16234 mutations in addition to the B4d coding motif. The same haplotype was found in the complete sequence of a Japanese sample (HNsq0238) reported by Tanaka et al. [10]. Therefore, we designated this haplotype B4d2. A very similar control region haplotype to B4d2 was found in 0.2% of Japanese [10, 18] and 0.22% of Korean populations [14, 23] and in one sample of the Ancient Chinese sequence [20].

One individual, TK1079, shared 10609 and 16189 mutations, suggesting that this haplotype belongs to F1bd. However, the HV2 sequence did not have any of the characteristics of the F1b or F1d haplogroups. Complete sequencing revealed mutations at positions 3027, 5587, 10007, 12618, 13260, 13749, 15022, 15024, 15496, and 16355, which was identical to those in the complete sequence of a Japanese (KAeq0070) reported by Tanaka et al. [10]. Therefore, we designated it the F1e haplogroup. This haplogroup branch has only been found in these two samples, and a similar control region haplotype has not yet been found in other Japanese [5, 18, 19, 25, 26], Korean [14, 15, 23, 24], or Chinese populations [9, 16, 17, 20–22].

Finally, by examining coding region polymorphisms to determine the basal haplogroup branches, in addition to complete control region information, the total number of haplotypes increased from 84 to 91 and gene diversity increased from 0.9929 to 0.9978.

In this study, we examined coding region polymorphisms in the genome of mtDNA in a Japanese population and extended subdivision of the haplotypes obtained, thereby increasing the usefulness of mtDNA polymorphisms for personal identification. The present data also provide information on reliable mtDNA phylogeny in several haplogroups by complete sequencing. The recent trend toward global travel has accentuated the necessity of finding the means to determine geographic origin in addition to personal identification in forensic work involving individual cases or mass disasters. The present database will help extend the applicability of mtDNA polymorphisms for such purposes in forensic casework in Japanese and other East Asian populations.

Access to complete sequence data

The data of the 14 complete nucleotide sequences are available under the DDBJ accession numbers AP010824-AP010837.

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After contribution of the revised manuscript, we noticed that an article by QP Kong et al. was published in PLoS ONE 3(8);e3016, 2008. Errors in the sequences in sample names NDsq0178 and NDsq0168 pointed out in the above description have been corrected in the article.