Int J Legal Med (2002) 116:68–73

ORIGINAL ARTICLE

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A new database of mitochondrial DNA hypervariable regions I and II sequences from 162 Japanese individuals

Received: 10 August 2000 / Accepted: 7 January 2001

Abstract A database of mitochondrial DNA (mtDNA) hypervariable region 1 (HV1) and region 2 (HV2) sequences of the mtDNA control region was established from 162 unrelated Japanese individuals. The random match probability and the genetic diversity for this database were 0.96% and 0.997, respectively. Length heteroplasmy in the C-stretch regions located around position 16189 in HV1 and 310 in HV2 was observed in 37% and 38% of the samples, respectively. A strategy using internal sequencing primers was devised to obtain confirmed sequences in these length heteroplasmic individuals. This database, combined with other mtDNA sequence databases from the Japanese population, will permit the significance of mtDNA match results to be properly reported in mtDNA typing casework in Japan.

Keywords Mitochondrial DNA · Sequence database · Hypervariable region 1 (HV1) · Hypervariable region 2 (HV2) · Heteroplasmy

Introduction

PCR-based mtDNA typing has become widely established as a powerful tool in forensic science (reviewed in Holland and Parsons 1999). Mainly because of a higher success rate for amplification compared to nuclear DNA typing, this technique is applied to highly degraded specimens such as bone, teeth, and hair samples. In addition to systematic validation studies including inter-laboratory studies (Fisher et al. 1993; Wilson et al. 1995; Carracedo et al. 1998), many successful identification cases have been reported (e.g. Sullivan et al. 1992; Holland et al. 1993; Gill et al. 1994; Stoneking et al. 1995; Ivanov et al. 1996; Lutz et al. 1996). Because the mtDNA molecule is a single linked unit, the significance of mtDNA matching in forensic cases requires comparison to large mtDNA sequence databases, to determine the relative rarity of the mtDNA type in question. Furthermore, there is strong geographic and ethnic structure to mtDNA variation globally and within Asian populations (Melton and Stoneking 1996; Melton et al. 1997a, 1997b). In recent years, many databases of HV1 and HV2 sequences from the mtDNA control region have been published to permit mtDNA forensic casework in particular countries or geographic regions (e.g. Piercy et al. 1993; Lee et al. 1997; Sekiguchi et al. 1997; Parson et al. 1998; Lutz et al. 1998; Baasner et al. 1998; Pfeiffer et al. 1998, Rousselet and Mangin 1998; Seo et al. 1998; Budowle et al. 1999). The strength of mtDNA evidence that can be reported is very often limited by the size of the database available for comparison, as most mtDNA types are very rare, seen only a single time in even the largest databases yet compiled (Holland and Parsons 1999). For this reason, it is important that mtDNA sequence databases continue to be generated and published, to extend mtDNA typing capability to additional populations and to increase the size of existing databases.

We report here a new database of mtDNA HV1 and HV2 sequences from 162 randomly selected, unrelated individuals from Japan and describe the general patterns of variation observed. Additionally, we describe an efficient strategy for obtaining confirmed sequence information from individuals that are length heteroplasmic in polycytosine stretches (C-stretches) in HV1 and/or HV2. Individuals with C-stretch length heteroplasmy are difficult to

Electronic supplementary material to this paper can be obtained by using the Springer Link server located at http://dx.doi.org/10.1007/s004140100211

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sequence by standard chain termination methods because the sequences of different templates become out of register once the length heteroplasmy is encountered by the sequencing polymerase (Bendall and Sykes 1995; Marchington et al. 1997; Parson et al. 1998). It is important that mtDNA typing laboratories be able to overcome this difficulty on a routine basis. The database presented here will, in combination with other databases of the Japanese population, contribute to the utility of forensic mtDNA testing in Japan.

Materials and methods

Samples and DNA extraction

A total of 162 blood samples from unrelated Japanese individuals were obtained from random anonymous donors residing in Tokyo at an urban Tokyo blood bank and 10 µl of each was dropped onto cotton cloth. DNA was extracted from a half of each bloodstain using the Chelex extraction method (Walsh et al. 1991).

Polymerase chain reaction

PCR was performed on 1 µl of each DNA extract. The two hypervariable regions of mtDNA control region (HV1 and HV2) were each amplified in a total of 50 µl of PCR mix consisting of 0.4 µM each primer, 1 × PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 200 µM each dNTP, 5 U AmpliTaq DNA polymerase (Perkin Elmer) and 8 µg BSA. The primers used are listed in Table 1. For HV1, F15971 and R16410, and for HV2, F15 and R389 were used for PCR. Amplification was carried out in a 9600 GeneAmp thermal cycler (Perkin Elmer) under the following conditions for both regions: 94 °C for 30 s followed by 32 cycles of 94 °C for 20 s, 56 °C for 10 s and 72 °C for 30 s. After PCR, 5 µl of products was separated by electrophoresis on a 2% agarose gel for 30 min and PCR products were visualised by ethidium bromide staining followed by UV transillumination.

Sequencing

Prior to sequencing, PCR products were purified using Centricon-100 filtration units (Amicon). Depending on the band intensity of the PCR product on agarose test gels, 0.5–1.0 µl of purified products were used for sequencing. Cycle sequencing was performed in a 9600 GeneAmp thermal cycler in a total reaction volume of 20 µl consisting of template amplicon, ABD Prism Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase, FS (Perkin Elmer) and 0.5 µM primers under the following condition: 25 cycles of 96 °C for 15 s, 50 °C for 5 s and 60 °C for 2 min. After the sequencing reaction, residual dye terminators were removed using AGCT columns (Edge BioSystems) and the products were dried in vacuum concentrator. The samples were run on an ABD 373 Stretch automated DNA sequencer, separated by electrophoresis on 6% (19:1 acrylamide:bisacrylamide) gels at a constant power of 28 W. DNA sequences were analysed by the ABI Prism Sequencing Analysis software (version 2.1) and consensus sequences were obtained semi-automatically using ABI Sequence Navigator software (Ver.1.0.1). Pairwise comparison of all sequences was performed using the MitoSearch program (C. Stauffer, FBI Laboratory).

Confirmation of point heteroplasmy

Samples that showed evidence of heteroplasmy via direct sequencing were amplified a second time and sequenced using dye-labeled primers. Dye primer sequencing produces more uniform peak heights allowing better detection and characterisation of heteroplasmic mixtures. Depending on the heteroplasmic position, appropriate tagged primers were selected for PCR (Table 1). PCR was performed in a total of 25 µl of PCR mix consisting of 0.5 µM each primer, 1 × PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$), 200 µM each dNTP and 1.25 U AmpliTaq Gold DNA polymerase (Perkin Elmer). Amplification was carried out under the following conditions: 95 °C for 9 min, followed by 33 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 2 min. Dye primer cycle sequencing was carried out using BigDye Primer Cycle Sequencing FS Ready Reaction kits (-21M13 and M13Rev., Perkin Elmer) according to the manufacturers instructions. Sequencing products were separated on an ABD PRISM 310 Genetic analyser.

Criteria of DNA sequence determination

DNA sequences were determined from position 16024 to 16365 in HV1 and from 73 to 340 in HV2 (Fig. 1). Sequences were confirmed from both forward and reverse sequence data (Wilson et al. 1993). For samples with length heteroplasmy in C-stretch regions, additional sequencing was performed using internal primers (R16175 and F16190 for HV1, R270 for HV2) to obtain sequence data following the C-stretch in each direction. Even after performing this sequencing, short regions immediately adjacent to the C-stretch cannot be confirmed from both strands (Fig. 1). For these regions, a separate set of PCR and sequencing reactions was performed for confirmation using sequence information in the same direction, but from independent amplifications.

Results and discussions

A sequence database of the mtDNA HV1 and HV2 regions was established from 162 randomly selected Japan-

Table 1 Sequences of primers used for amplification and sequencing. The lower four are primers used for dye primer sequencing. The sequence indicated with lower-case letters is tag for –21 M13 forward or M13 reverse primers

Primer	Nucleotide sequence	
F15971	5′ TTAACTCCACCATTAGCACC	3′
F16190	5′ CCCCATGCTTACAAGCAAGT	3′
R16175	5′ TGGATTGGGTTTTTATGTA	3′
R16410	5′ GAGGATGGTGGTCAAGGGAC	3′
F15	5′ CACCCTATTAACCACTCACG	3′
R270	5′ TGGAAAGTGGCTGTGCAGAC	3′
R389	5′ CTGGTTAGGCTGGTGTTAGG	3′
F15978 (–21M13)	5′ tgtaaaacgacggccagtCACCATTAGCACCCAAAGCT	3′
F16190 (–21M13)	5′ tgtaaaacgacggccagtCCCCATGCTTACAAGCAAG	3′
R16188 (M13 Rev.)	5′ caggaaacagctatgaccGGGGGTTTTGATGTG	3′
R16420 (M13 Rev.)	5′ caggaaacagctatgaccTGATTTCACGGAGGATGGTG	3′

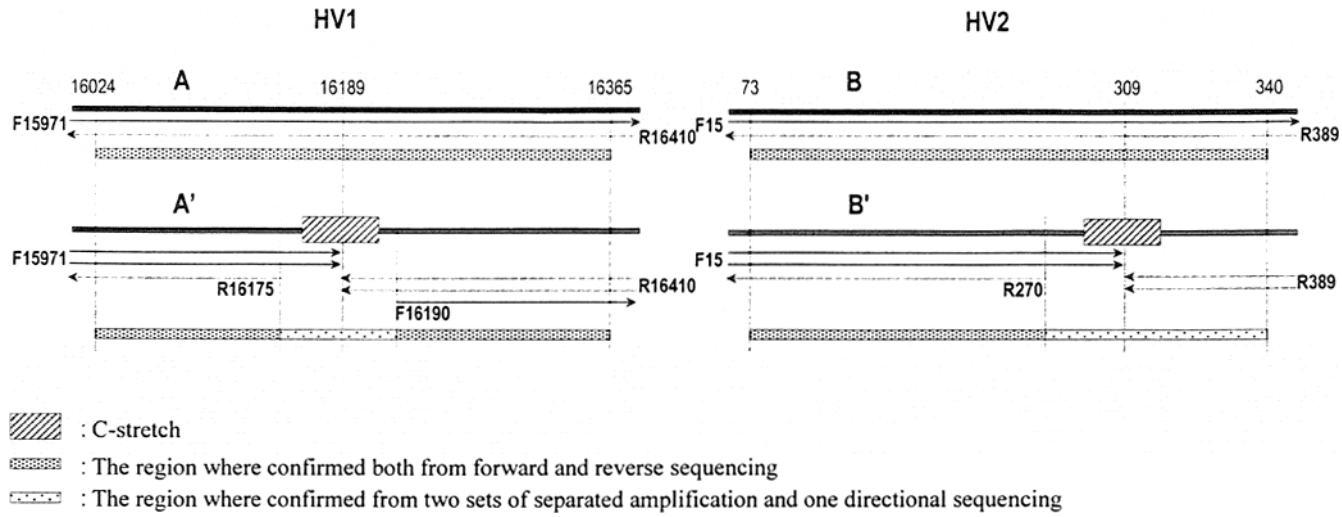

Fig. 1 A, B Schematic representations of HV1 and HV2 region. Positions and primer names are shown. Directions of sequencing are indicated by arrows. **A** and **B** sequencing strategy for non-heteroplasmic samples, **A'** and **B'** sequencing strategy for the samples containing length heteroplasmy at C-stretch region

ese individuals residing in metropolitan Tokyo. Complete sequence information of the mtDNA types observed is shown in Table S1; each sequence was compared with the reference sequence of Anderson et al. (1981) and substituted bases are shown (these sequences have been incorporated into a shared forensic mtDNA database, see Budowle et al. 1999 and are currently in submission to GenBank). A total of 140 different mtDNA types was observed of which 129 types were seen only once. Of the 11 types seen in multiple individuals, 6 were shared by 2 individuals, 2 were shared by 3 individuals, 2 were shared by 4 individuals, and 1 was shared by 7 individuals (Table 2). Such a distribution with a small number of common mtDNA types and a large number of rare mtDNA types is completely typical of mtDNA population structure. In this database, genetic diversity according to Tajima (1989) and random match probability (RMP) according to Stoneking et al. (1991) were estimated as 0.996 and 0.96%, respectively. The average number of polymorphic differences between individuals in the database is 8.8. Comparison of all possible sequence pairs gave 45 matches out of 13,041 comparisons, for an empirical random match frequency of one in 290.

We can combine our data with two previously published Japanese mtDNA sequence databases (100 individuals: Seo et al. 1998, 55 individuals Sekiguchi et al. 1997) for a total of 317 Japanese sequences. For the regions common to all three databases, from position 16051 to 16365 in HV1 and from 73 to 340 in HV2, there were 262 different mtDNA sequence types and 233 types that occurred only once in the combined database. Of the 29 types present in multiple individuals, 17 were shared by 2 individuals, 8 were shared by 3 individuals, 2 were shared by 5 individuals, 1 was shared by 6 individuals, and 1 was shared by 10 individuals (Table 2). Genetic diversity and RMP were estimated as 0.998 and 0.56%, respectively. This low RMP value is similar to that of the US Caucasian population estimated from a large sample size (N = 604) (Budowle et al. 1999). Comparison of all possible sequence pairs (50,086 pairs) gave 121 matches, for an empirical random match frequency of one in 414.

Table 2 Frequency distribution of mtDNA types in the Japanese database

Number of times observed	This study		Combined database[a]	
	Number of mtDNA types	Total	Number of mtDNA types	Total
1	129	129	233	233
2	6	12	17	34
3	2	6	8	24
4	2	8	0	0
5	0	0	2	10
6	0	0	1	6
7	1	7	0	0
10	0	0	1	10
Total	140	162	262	317

[a] Results from combined Japanese database with Sekiguchi et al. (1997) and Seo et.al. (1998)

Length heteroplasmy in C-stretch regions was observed both in HV1 and HV2. In HV1, 37% (60/162) of samples showed length heteroplasmy in the C-stretch region. Length heteroplasmy was easily identified because of the dramatic decrease in sequence quality that occurs beyond the heteroplasmic region, due to template molecules that are out of register (Parson et al. 1998). Moreover, all of these samples contained a T to C transition at position 16189. This creates an unbroken series of 10 or more Cs that is apparently replicated with poor fidelity within mitochondria. This effect of HV1 length heteroplasmy is pronounced and we agree with other authors that it is not possible to identify a predominant length variant within the mixture from only the direct sequencing electropherograms (Parson et al. 1998).

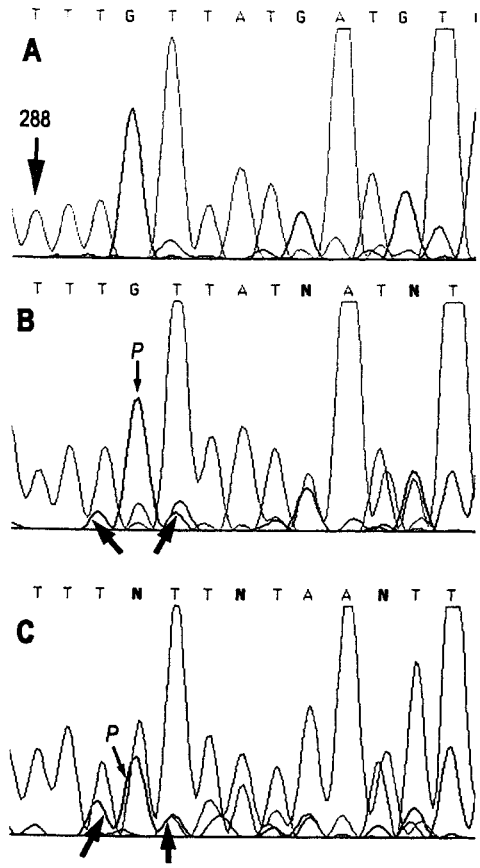

Fig. 2 A–C Electropherograms showing different degrees of mixed peaks in HV2 (reverse sequencing) due to variable degrees of C-stretch length heteroplasmy. A no detectable heteroplasmy (one of the type JP135), B discernable heteroplasmy (type JP029), C pronounced heteroplasmy (type JP058). Signal coming from the predominant length variant is labeled "P" and corresponding signal coming from alternative longer and shorter variants is indicated by arrows

Fig. 3 Electrophenograms obtained by dye primer cycle sequencing (type JP049). The mixed peaks were clearly observed both in forward (Fwd.) and reverse (Rev.) sequencing

In contrast to HV1, the degree of length heteroplasmy varies among individuals in HV2 (Marchington et al. 1997) such that a predominant variant can often be called even when some length heteroplasmy is apparent. In other cases, the length-variant mixture involves two or more prevalent components, and greatly disrupts further sequence resolution beyond the region (Fig. 2). We observed that individuals with only seven Cs in this region as a rule did not manifest length heteroplasmy, while those C-stretches of eight or more Cs tended to have heteroplasmic mixtures. Because the heteroplasmic ratios vary greatly from sample to sample (ranging from no detectable heteroplasmy, to barely discernible, to pronounced) it is difficult to state the total number of samples heteroplasmic for HV2. However, we estimated that HV2 length heteroplasmy was noticeably observed in 61/162 individuals (38%). Virtually all of these were within the subset of individuals who had eight or more Cs prevalent in the HV2 C-stretch (90/162 individuals or 56%). As with HV1, additional amplification and sequencing with an internal primer (R270) was required for double-strand confirmation in cases where length heteroplasmy significantly reduced base-calling accuracy.

We also observed four instances of heteroplasmic point mutations within HV1, adding to a growing number of reports of control region point mutation heteroplasmy detected by direct sequencing (Gill et al. 1994; Comas et al. 1995; Bendall et al. 1996; Marchington et al. 1996; Ivanov et al. 1996; Wilson et al. 1997; Bendall et al. 1997; Hühne et al. 1998; Parson et al. 1998). Once the point heteroplasmy was identified, samples were subjected to dye primer cycle sequencing. The presence of heteroplasmy was confirmed by careful examination in both the forward and reverse directions, with a consistent mixed signal well above background levels (Fig. 3). Heteroplasmic positions were designated according to the IUB code. The positions of heteroplasmy observed are as follows: 16093 Y; 16172 Y; 16197 S; 16287 Y. Position 16093 is known to be an extreme hotspot for C/T heteroplasmy, but often at levels that are undetectable by direct sequencing (Tully et al. 2000). Position 16172 is known to have a high rate of evolutionary substitution whereas 16197 and 16287 have relatively low evolutionary rates (Wakeley 1993; Meyer et al. 1999; Excoffier and Yang 1999).

In this study, we established a Japanese mtDNA sequence database of HV1 and HV2 regions for 162 individuals. This data is now available for use in assessing the significance of matches in mtDNA forensic casework in Japan, as well as for population and evolutionary analyses. The data has been generated under strict criteria for confirmation, requiring unambiguous base calls from both strands. For the specific instances where that was not possible – for example, immediately adjacent to length-heteroplasmic C-stretches – confirmation was made by multiple unambiguous calls made from the same strand, but from different PCR amplifications of the same sample. We feel it is important to maintain high standards of sequence determination not only in forensic casework, but

in maintenance of databases as well. Continued establishment and publication of high-quality mtDNA sequence databases from diverse populations will abet the expanding role of mtDNA typing in forensic casework.

Acknowledgements This work was supported by a grant from the Japan Science and Technology Agency. We thank all the staff members at US Armed Forces DNA Identification Laboratory for their kind assistance, Ikuko Sakai at NRIPS for sample collection and two anonymous reviewers for their helpful comments. The opinions and assertions expressed herein are solely those of the authors and are not to be construed as official or as the views of the United States Department of Defense or the United States Department of the Army.

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