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ORIGINAL ARTICLE

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Population data for 101 Austrian Caucasian mitochondrial DNA d-loop sequences: Application of mtDNA sequence analysis to a forensic case

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Abstract The sequence of the two hypervariable segments of the mitochondrial DNA (mtDNA) control region was generated for 101 random Austrian Caucasians. A total of 86 different mtDNA sequences was observed, where 11 sequences were shared by more than 1 individual, 7 sequences were shared by 2 individuals and 4 sequences were shared by 3 individuals. One of the four most common mtDNA sequences in Austrians is also the most common sequence in both U.S. and British Caucasians, found in approximately 3.0% of Austrians, 4.0% of British, and 3.9% of U.S. Caucasians. Of the remaining three common Austrian sequences, one was not observed in either U.S. or British Caucasians. However, three British Caucasians exhibited a similar sequence type. Therefore, this particular cluster of sequence polymorphisms may represent a common "European" mtDNA sequence type. In general, Austrian Caucasians show little deviation from other Caucasian databases of European descent. Finally, mtDNA sequence analysis was applied to a forensic case, where hairs found at a crime scene matched the control hairs from the suspect.

Key words Mitochondrial DNA · Heteroplasmy · mtDNA · Population frequency estimates · Forensic casework

Introduction

The identification of human biological evidence has undergone immense change since the development of PCR typing systems for forensic casework (Comey et al. 1993; Fregeau and Fourney 1993; Herrin et al. 1994; Sparkes et al. 1996 a, b). In addition, technical revolutions in the past decade have provided automated procedures for routine sequence analysis of PCR-generated templates (Brumbaugh et al. 1988; Hunkapillar et al. 1991). Consequently, sequence analysis of mitochondrial DNA (mtDNA) amplicons has become a powerful forensic tool for the following reasons:

1. Analysis of mtDNA has been performed by PCR amplification and automated DNA sequencing (Sullivan et al. 1992; Holland et al. 1995).
2. mtDNA is highly polymorphic due to a rapid rate of evolution (Brown et al. 1979; Cann et al. 1987) and can provide substantial support for the identification of biological evidence.
3. mtDNA is present in high copy number compared to chromosomal DNA (Robin and Wong 1988), and may provide results when genomic DNA does not, allowing for the analysis of hair shafts and old skeletal material (Holland et al. 1993; Wilson et al. 1995 a).
4. The maternal inheritance of mtDNA (Giles et al. 1980) allows for comparison of family members who share a common matrilineal ancestry, providing the basis for identification of biological evidence in human remains cases, or the maternal relationship of living individuals (Ginther et al. 1992; Gill et al. 1994; Holland et al. 1995; Stoneking et al. 1995; Boles et al. 1995; Ivanov et al. 1996).

Mitochondrial DNA sequencing has been validated for forensic casework analysis by several laboratories (Sullivan et al. 1992; Holland et al. 1993; Fisher et al. 1993; Wilson et al. 1993, 1995 b). Given that the frequency of mtDNA types in a given population is currently quantified by counting the number of occurrences in a sequence database and that mtDNA sequence types can be population or race dependent, the aim of this study was to establish an mtDNA database for both hypervariable segments within the control region for unrelated Caucasian individuals living in Western Austria. This database will facilitate interpretation of results from mtDNA sequence analy-

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sis in forensic casework performed in Austria. The mtDNA sequence distribution was evaluated and compared to the sequence distribution within racial groups (i.e. U.S. and British Caucasian databases), and between racial groups (i.e. Caucasian, African, Hispanic, and Asian databases). Finally, the application of this technique to the analysis of hair shafts in a forensic case is presented.

Materials and methods

Innsbruck Laboratory

Extraction

Blood was obtained from 101 unrelated Caucasian individuals born in Western Austria who were native German speakers. DNA was isolated from blood samples by digestion with Proteinase K (Boehringer Mannheim, Germany) and subsequent extraction with phenol/chloroform (Sambrook et al. 1989). The amount of DNA was determined by agarose gel electrophoresis in comparison to a lambda DNA standard (Boehringer Mannheim, Germany): 0.8% agarose in 10 mM Tris-borate, pH 8.3, 1 mM EDTA, with 5 µl of 10 mg/ml ethidium bromide and run at 150 V for 12 min. DNA was visualized under UV light.

Amplification

A total of 20 ng of DNA was used as template for the PCR amplification reactions. The two hypervariable segments (HV I and HV II) within the control region were amplified using the following primers HV I, L15 997 : 5′-CACCATTAGCACCCAAAGCT-3′ and H16 401 : 5′-TGATTTCACGGAGGATGGTG-3′, HV II, L00 029 : 5′-GGTCTATCACCCTATTAACCAC-3′ and H00 408 : 5′-CTGTTAAAAGTGCATACCGCCA-3′. (Piercy et al. 1993), where “L” stands for light strand, and “H” stands for heavy strand.

PCR was performed in a total volume of 25 µl consisting of 1 × PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM $MgCl_2$), 250 µM each dNTP, 0.25 µM each primer and 2 U Taq Polymerase (Perkin Elmer). Amplification was carried out on a 9600 GeneAmp Thermal Cycler (Perkin Elmer) at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s for 30 cycles. Amplicons were purified with a QIAquick spin PCR purification kit (Qiagen, Germany) to remove remaining amplification primers dNTP and buffer, and eluted from the column matrix with 30 µl bidistilled sterile water. Purified DNA was quantified on a Hitachi F 2000 spectro-fluorometer using the Picogreen dsDNA quantitation kit (Molecular Probes Europe).

Cycle sequencing and electrophoresis

Sequencing reactions were performed in a 9600 GeneAmp Thermal Cycler (Perkin Elmer) using the ABD (Applied Biosystems) Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer). Approximately 10 ng of amplified DNA was used as template in a total assay of 10 µl (one half the normal reaction volume and reaction kit reagents) at 96 °C for 15 s, 50 °C for 15 s and 60 °C for 2 min for 25 cycles. Each template was sequenced from both directions, in the forward direction with primer L15 997 and in the reverse direction with primer H16 401 for HV I, and with L00 029 and H00 408 for HV II. The primer concentration in the sequencing reactions was 10 µM. The sequencing products were extracted in 1/10 volume 3 M sodium acetate (pH 4.6) and 2.5 vols. absolute ethanol and purified from residual terminators by washing in 70% ethanol. DNA was resolved in 5 µl loading solution consisting of 4 µl deionized formamide and 1 µl 25 mM EDTA (pH 8.0), denatured at 90 °C for 2 min, snap-cooled on ice and loaded on a denaturing polyacrylamide sequencing gel (5% 29 : 1 acrylamide:bisacrylamide, 8 M urea, 1 × TBE, pH 8.3, 34 cm well-to-read). Electrophoresis was carried out on an ABD 373 Stretch Sequencer at constant power (27 W) for 10 h. Analysis was performed using ABD Prism Sequencing Analysis Software, Version 2.1 and sequences were aligned and analyzed with Sequence Navigator Software, Version 1.0.1 (Perkin Elmer).

Armed Foreces DNA Identification (AFDIL) Laboratory

Lab-to-lab comparison

DNA extracts from 30 individuals were amplified and directly sequenced to confirm the results obtained in the Innsbruck Laboratory and to illustrate the reproducibility of mtDNA sequence data in different laboratories using different methods.

Amplification

A total of 20 ng of DNA was amplified using equal concentrations (0.4 µM) of the primers F15 971/R16 410 and F15/R389 for HV I and HV II, respectively (Holland et al. 1995); HV I, F15 971 : 5′-TTAACTCCACCATTAGCACC-3′ and R16 410 : 5′-GAGGATGGTGGTCAAGGGAC-3′, HV II, F15 : 5′-CACCCTATTAACCACTCACG-3′ and R389 : 5′-CTGGTTAGGCTGGTGTTAGG-3′; where “F” stands for the forward direct primer on the light strand and “R” stands for the reverse direction primer on the heavy strand.

Amplification was carried out in a 9600 GeneAmp Thermal Cycler (Perkin Elmer) by a hold step at 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. Amplicons were purified using the Centricon-100 spin columns (Amicon, Beverly, MA) and sequence analysis performed directly on the purified products using the same primers (F15 971, R16 410, F15, and R389) and the ABD Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase FS (Perkin Elmer). Conditions were 96 °C for 15 s, 50 °C for 5 s and 60 °C for 2 min for 25 cycles. Sequencing reaction products were purified from residual dye terminators using AGCT columns and vacuum dried in a Hetovac mirocentrifuge (Alcatel, ATR, Laurel, MD). DNA was resuspended in 5 µl loading solution and loaded on a denaturing polyacrylamide sequencing gel as described with the following exceptions: a 6% 19 : 1 acrylamide:bisacrylamide gel was used and the gel was run at constant power of 28 W.

Results and discussion

Interpretation of sequencing data

The two hypervariable segments of the mtDNA control region were amplified and directly sequenced from 101 random Caucasians living in Western Austria (Table 1). mtDNA sequences were automatically analyzed by the ABI Prism Sequencing Analysis Software (Version 2.1) and manually inspected using the Sequence Navigator Software (Version 1.0.1). Each mtDNA sequence was a consensus sequence of the forward and the reverse sequencing reactions using the same primers as for PCR (Fig. 1). Sequence polymorphisms are reported as differences from a standard human mtDNA reference sequence (Anderson et al. 1981).

In general, a base call was confirmed when forward and reverse sequence showed clearly defined complementary peaks and only minor traces of other peaks were ob-

Table 1 Polymorphic nucleotide positions of the mitochondrial d-loop region (HV I/HV II) of 101 Austrian Caucasian samples compared to the reference sequence of Anderson et al. (1981). Dots (.) indicate no difference to the Anderson sequence, a base call (according to IUPAC recommendations) shows the substitution at a distinct position, a D indicates a deletion, an insertion compared to Anderson's sequence is designated as the appropriate position and numbers followed by a dot. The polycytosine region in HV II commonly has insertions compared to the reference sequence, an N indicates that the predominant sequence at this position could not be confirmed

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Table 1 (continued)

	16324	16325	16342	16343	16344	16353	16356	16360	16362	16366	16390	16391	16399	57.1	61.1	64	72	73	93	106	107	108	109	110	111	119	143	146	150	151	152	153	183	185	188	189	195	199	200	204	207	215	217	225	228	247	250	252	259	263	282	285	295	309.1	309.2	309.3	310	315.1	315.2	315.3	319	324	340	343
And.	T	T	T	A	C	C	T	C	T	C	G	G	A	I	I	C	T	A	A	G	G	A	G	C	A	T	G	T	C	C	T	A	A	G	A	A	T	T	A	T	G	A	T	G	G	G	T	T	A	A	T	C	C	I	I	I	T	I	I	I	T	C	C	C
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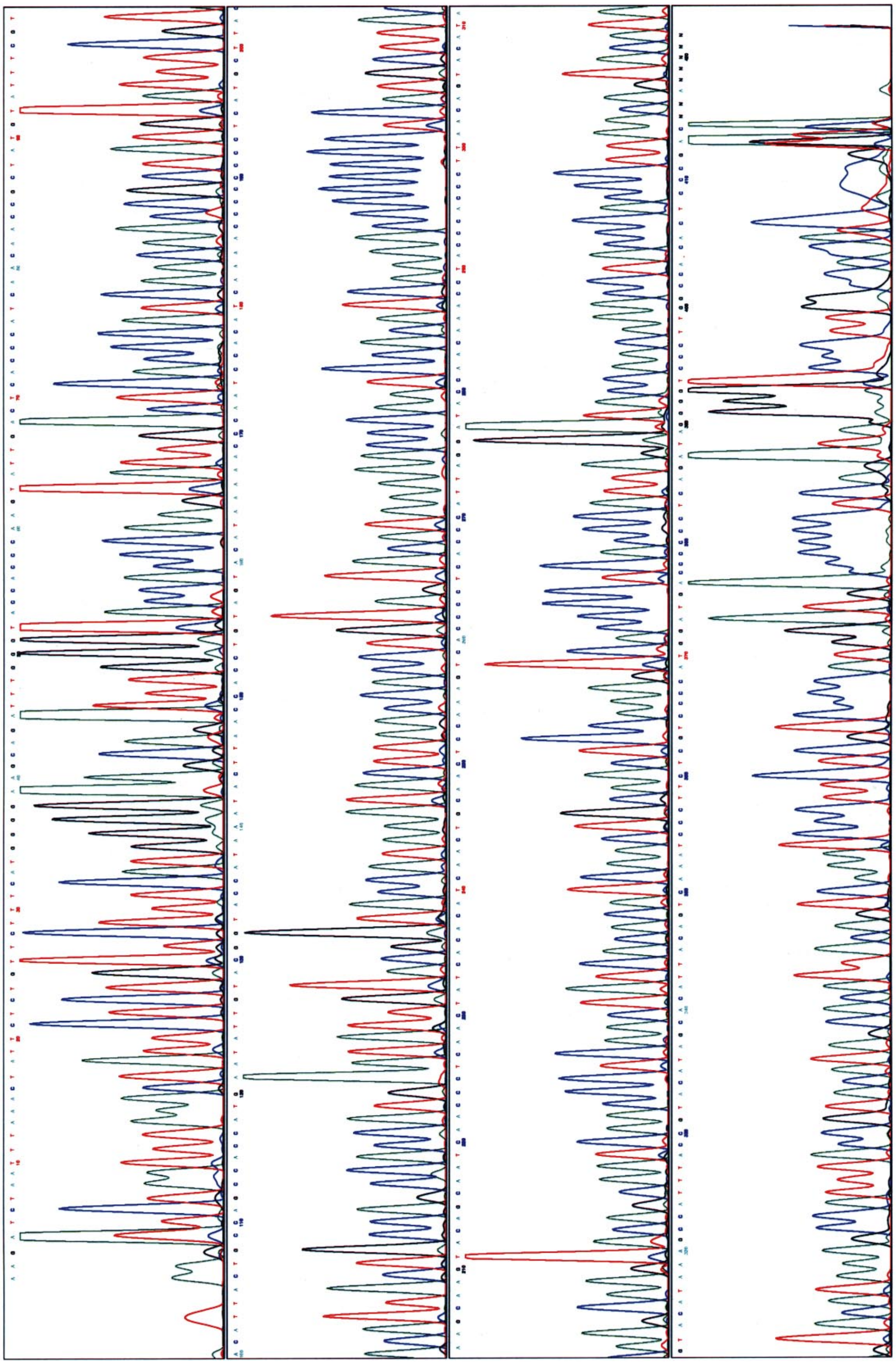

Fig. 1 Full-length sequence of the first hypervariable segment (HV I) of AUT32 sequenced using primer L15997

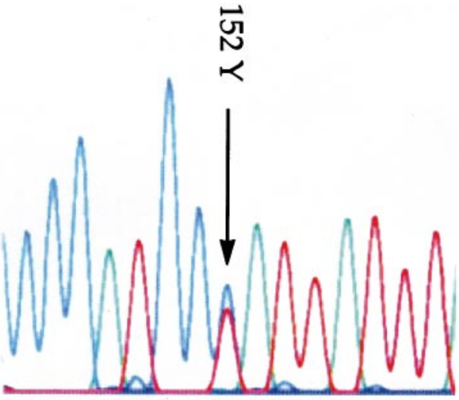

Fig. 2 The electropherogram of the mtDNA d-loop sequence of AUT68 shows two clearly defined peaks (C and T) at position 152 in HV II and was therefore called Y. Both nucleotide signals are well above background noise and reproducible through independent forward and reverse sequencing reactions. This shows a heteroplasmic situation, a mixture of two mtDNA populations. In all, we found three heteroplasmic events in our database

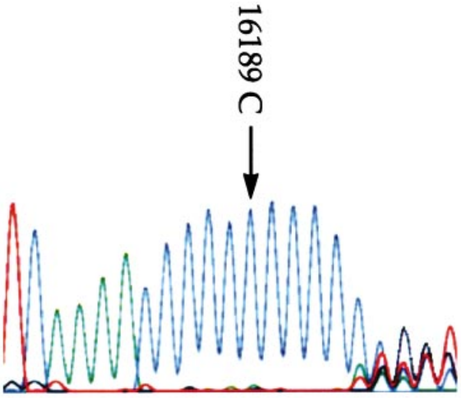

Fig. 3 The transition of thymine (T) to cytosine (C) at position 16 189 leads to the formation of a polycytosine region usually containing more than 10 cytosines. This causes a heteroplasmic situation in HV I. The mixture of the different length variants is often problematic to interpret, therefore all data following the C-stretch after position 16 193 were discarded, the sequence 5′ of the polycytosine region was confirmed by independent PCR and sequencing reactions

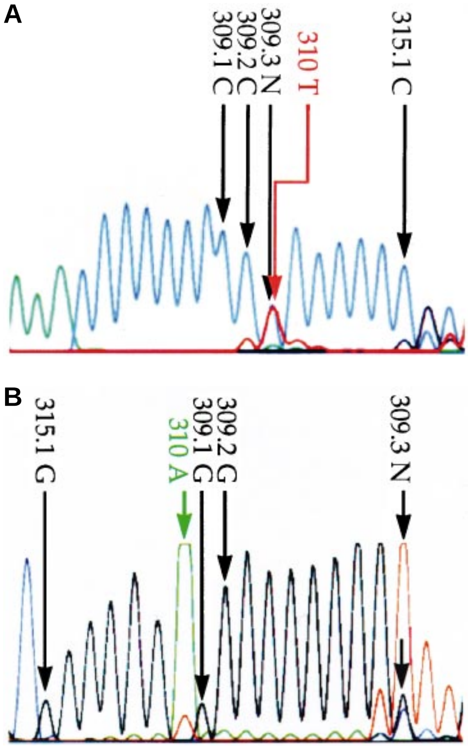

Fig. 4 A, B The insertion of cytosines (C) between positions 303 and 310 relative to the reference sequence (Anderson et al. 1981) leads to a heteroplasmic situation in HV II . Fig. 4**A** shows the forward sequencing reaction, the predominant mtDNA type has a 309.1 C and a 309.2 C insertion, which can clearly be identified by the reverse sequencing reaction (Fig. 4**B**, 309.1 G, 309.2 G). Another length variant including 3 cytosine insertions is apparent. However, it is not prominent, therefore this insertion was designated 309.3 N

served underneath the primary sequence (attributed to background "noise"). Forward sequencing reactions (matching the mtDNA light strand) usually produced better results than reverse sequencing reactions. This phenomenon was also observed in other mtDNA regions (e.g. cytochrome b and 16S-rRNA, data not shown) and is known to be a common characteristic of mtDNA sequencing (presumably related to the different ratios of purines and pyrimidines on the heavy and light strands). Thus, some positions consistently show the same ambiguous results in one direction, although in the other sequencing direction the base call can clearly be made (e.g. 317, 324, 330). We consider these to be problematic positions due to artifacts caused by the sequencing chemistry and empirical knowledge of sequence characteristics at these positions permits appropriate base calls to be made. In some cases problematic positions are "characteristic" of an individual sequence and are reproducible both within the same sample extract, between sample extracts, and even between maternal relatives. In such cases, unambiguous sequence information from one of the strands can be used to determine the correct base call. The basis for these conclusions has also been validated or confirmed through cloning experiments (data not shown).

It is important to note that in some samples, mixtures of nucleotide signals may be observed in sequence data from both strands, with both peak components well above typical background noise. Such results were clearly observed three times within our samples. Because they occurred at positions known not to be generally problematic and were reproducible within otherwise clean sequence profiles, these positions are considered to represent heteroplasmy, a genuine mixture of bases within an individual at a particular position. Heteroplasmic positions were designated with an IUB code [(e.g. sample AUT 68 at position 152 is a confirmed mixture of C and T, thus the IUB code of Y is given to this position (Fig. 2)]. While heteroplasmy was detected clearly in three instances in the present study, detection of unbalanced heteroplasmic mixtures by direct sequencing is problematic due to sequence back-

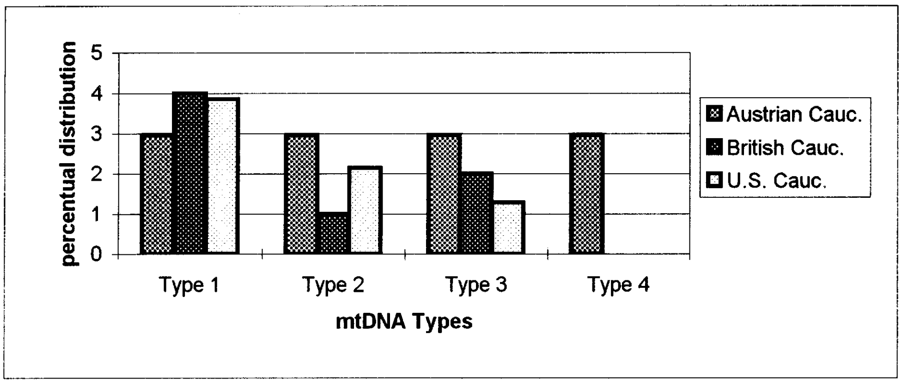

Fig. 5 Most frequent mtDNA types present in the Austrian Caucasian population sample (approximately 3%) compared to the British Caucasian and the U.S. Caucasian databases, inspecting ranges from 16025 to 16365 and 73 to 340. Type 1: 263 G, 315.1 C; Type 2: 263 G, 309.1 C, 309.2 C, 315.1 C; Type 3: 16069 T, 16126 C, 73 G, 185 A, 228 A, 263 G, 295 T, 315.1 C; Type 4: 16 069 T, 16126 C, 16145 A, 16231 C, 16261 T, 73 G, 150 T, 152 C, 195 C, 215 G, 263 G, 295 T, 309.1 T, 315.1 C, 319 C

ground. Once considered rare within the control region, heteroplasmy has recently been detected with increasing frequency due to expanded high-resolution sequencing efforts (Comas et al. 1995; Bendall et al. 1996). The relatively frequent detection of heteroplasmy is consistent with the rapid substitution rate of mtDNA between immediate maternal relatives (Howell et al. 1992; Comas et al. 1995; Bendall et al. 1996; Howell et al. 1996; Ivanov et al. 1996; Parsons et al. 1997). The tight biological bottleneck encountered during mtDNA transmission allows for rapid segregation of one apparent sequence type to another and from one ratio of heteroplasmic variants to another in a single generation (Hauswirth and Laipis 1982; Bendall et al. 1996; Howell et al. 1996; Ivanov et al. 1996; Parsons et al. 1997).

Polycytosine stretches occur in both hypervariable segments and are sometimes problematic to interpret. These regions are often heteroplasmic, having populations of mtDNA molecules differing in the number of cytosines within the cytosine stretch (C-stretch). In HV I, the transition of the T at position 16189 to a C leads to the formation of a C-stretch usually containing more than 10 cytosines and this substitution is almost always associated with pronounced heteroplasmy in HV I C-stretch length variants (Bendall and Sykes 1995). This situation was observed in 15% of the individuals sampled for the Austrian database. The sequence following the C-stretch usually cannot be analyzed properly due to the sequence being “out of register,” as the polymerase attempts to sequence through the different length variants (Fig. 3). In these cases, base calls were confirmed by second independent amplification and cycle sequencing with the same primers. Due to the difficulty in confirming the number of length variants, only the ten C’s starting at position 16184 are normally reported.

In HV II, a heteroplasmic situation often occurs with an insertion (relative to the reference sequence) of at least one cytosine between position 303 and 310. In most cases, the insertion can clearly be defined as a cytosine, however, sometimes there is an apparent mixture of cytosines and thymines at or around position 310 (Fig. 4 A). Again, this phenomenon can be explained by the presence of mtDNA populations differing in the number of cytosines. The confusion of whether there is sequence heteroplasmy at position 310 can normally be resolved by sequence analysis in the opposite direction (Fig. 4 B). Once again, cloning experiments have been conducted to confirm heteroplasmic mixtures in the HV II C-stretch (data not shown). Unlike the C-stretch region of HV I, heteroplasmic mixtures in the HV II C-stretch often have an identifiable prominent type and this information is included in our database [sometimes with an N indicating a position where nearly equal proportions of length variants is apparent (Fig. 4 b, Table 1)].

An inter-laboratory comparison study was conducted to evaluate sequencing results derived from different laboratory procedures. DNA extracts from 30 individuals were amplified and directly sequenced following the operating procedures of AFDIL. There were no differences in the base calls made for any of the common sequence data generated by the Innsbruck or AFDIL laboratories.

Population data

Within the sample of HV I and HV II sequences from 101 random Austrian Caucasians, a total of 86 different mtDNA sequences was observed. Of these, 11 sequences were shared by more than 1 individual, 7 sequences were shared by 2 individuals and 4 sequences were shared by 3 individuals (Fig. 5). One of the four most common mtDNA sequences, observed in approximately 3.0% of Austrians (Type 1, Fig. 5), is also the most common sequence in both U.S. (unpublished) and 4.0% of British Caucasians (Piercy et al. 1993). Type 1 is found in approximately 3% of Austrian, 4% of British, and 3.9% of U.S. Caucasians. Of the remaining three common Austrian sequences, two were also common sequences in US and British Caucasians (Type 2 and Type 3, Fig. 5). However, one sequence (Type 4) was not observed in either U.S. or British Caucasians, but was observed in approximately 3% of Austrian Caucasians. Of interest, two British Caucasians exhibited this sequence type with the addition of 309.1 C and one British Caucasian has the addition of 309.1 C and the omission of 215 G. The distinctive constellation of polymorphisms in this sequence was not observed in any non-Caucasian population in our forensic database (including 319 African and 90 Hispanic sequences). Therefore, this particular cluster of sequence polymorphisms may represent a common, uniquely "European" mtDNA sequence type.

Not surprisingly, when compared to other racial groups, Austrian Caucasians have a significantly different distribution of sequence polymorphisms in the control region than Africans, Hispanics and Asians. On the basis of shared common types, the Austrian Caucasian population has a similar sequence distribution to other Caucasian populations. Thus, our sequencing results are consistent with the conclusion of Melton et al. (1997), reached on the basis of a single-strand oligonucleotide assay of common mtDNA polymorphic sites, that there is minimal population substructure within European Caucasian populations detectable with non-phylogenetic methods. In addition, this is consistent with the results obtained for nuclear or chromosomal DNA markers (e.g. Alper et al. 1995).

Finally, 12 polymorphisms were observed between 16 366 and 16 399 in 11 Austrian Caucasian individuals, as well as 9 polymorphisms in 9 individuals in British Caucasians (Piercy et al. 1993). Many forensic laboratories are concentrating on the region between 16 024 and 16 365 and do not consider the potential polymorphic content between 16 366 and 16 399. In addition, positions 73 to 340 are routinely analyzed in HV II. However, there were three additional Austrians with polymorphisms outside this region. Consequently, the laboratories restricting their analysis to these defined regions should take this information into consideration.

Case example

The application of mtDNA sequence analysis to forensic casework was investigated. In October 1996 a burglary was committed in an apartment in Tyrol, Austria. Head hairs, which were substantially different in color and shape from the hairs of the owner of the apartment, were found at the crime scene. The hairs had no roots, and thus must have been shed or cut. The morphological description of the hairs matched the head hairs of an individual suspected of the crime. mtDNA sequence analysis of both control region hypervariable segments was performed on two of these hair samples. Two separate extractions using different reagents gave the same results for both samples and matched the sequence determined from two reference hairs of the suspect (16 298 C, 72 C, 263 G, 309.1 C, 315.1 C). Testing of the reference samples was performed only after analysis had been completed on the evidentiary samples. For frequency estimation, this nucleotide sequence was compared to mtDNA databases (i.e. the AFDIL database; 233 U.S. Caucasians, 100 British Caucasians, 90 African Americans, 115 Afro-Caribbeans, 114 Africans, 90 Hispanics) and the Austrian Caucasian database presented here. Although the U.S. Caucasian database does not include position 72, one U.S. Caucasian and two British Caucasians were included as matches to the "case sequence." However, the case sequence was not found in the Austrian Caucasian database. Thus, the frequency of this type could be reported as 3 in 434 reported Caucasian sequences (233 U.S. Caucasians, 100 British Caucasians and 101 Austrian Caucasians).

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