GENETIC ANALYSIS OF SINGLE HAIR SHAFTS BY AUTOMATED SEQUENCE ANALYSIS OF THE MITOCHONDRIAL D-LOOP REGION

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INTRODUCTION

Hair samples found at a crime scene can be important as evidence material in the identification of the offender. Sometimes, it represents the only biological evidence sample which connects a suspect to a crime. However, most hairs submitted for DNA analysis are shed hair which do not contain a hair root. Analysis with VNTR or STR markers is therefore problematic and in most cases no DNA profile is obtained. In contrast to nuclear DNA, sufficient amounts of mitochondrial DNA can be extracted from hair shafts to allow the analysis of the highly polymorphic mt d-loop region.

Here, we present a strategy for the analysis of the two hypervariable regions in the mt d-loop by PCR and automated sequence analysis on the A.L.F. DNA sequencer. A population database was constructed with 51 unrelated Caucasians of Belgian descent.

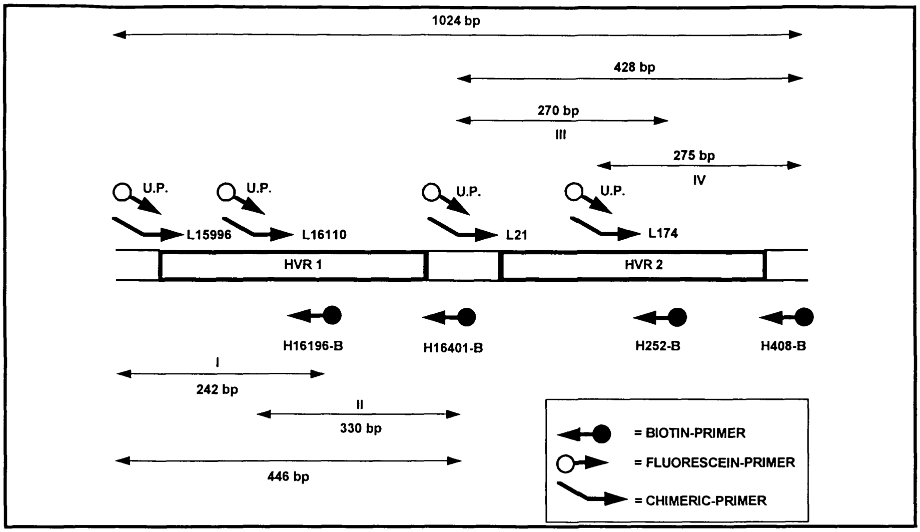
MATERIALS AND METHOD

DNA was extracted from hair shafts according to the procedure described by Gill (1985) except for concentration of the DNA extract. which was done by filtration on Microcon 30 or 100 devices (Amicon).

Five microliter of the DNA extract was added to a PCR reaction containing 2.5 units of Taq DNA-polymerase (Perkin-Elmer), 2.5 mM $MgCl_2$, 0.2 µM of oligodeoxyribonucleotides (dTTP, dATP and dCTP), 200 µM of dITP, 40µM dGTP, 0.2 mM of each primer (Fig. 1) and PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.4), 200 mg/ml gelatin and 170 µg/ml of BSA) in a volume of 100 µl. The reactions were processed for 35 cycles to denaturation at 94°C for 45 sec., annealing at 60°C for 30 sec. and extension at 72°C for 30 sec in a GeneAmp 2400 or 9600 Cycler (Perkin-Elmer).

Semi-nested PCR was performed as follows: a first PCR was done with primer pair L15996 and H16401-B or primer pair L20 and H408-B under the conditions described above except for the number of cycles which was reduced to 30. In a second PCR reaction 5 µl of the first PCR was used as a template with one of the two primers replaced by an internal primer. Quality control of the PCR products was done on 6% polyacrylamide gels in Tris-borate-EDTA buffer at 200 volt.

Single stranded templates were generated by binding of 70 µl of the biotinylated PCR products to streptavidin-coated beads (Dynabeads™, Dynal), as described by the manufacturer, followed by denaturation with 1N NaOH. Enzymatic sequencing with T7 DNA polymerase was done with the AutoRead Sequencing kit (Pharmacia-Biotech). In all the sequencing reactions, a single fluorescein-labelled primer (U.P.) was used which was complementary to the 3'end of the 3'PCR primer (Fig. 1).



PCR primers:	L15996	5'- GTCCTTTGTCGATACTGCTCCACCATTAGCACCCAAAGC
	L16110	5'- GTCCTTTGTCGATACTGATTTCGTACATTACTGCCAG
	H16196-B	5'- BIO-TTGCTGTACTTGCTTGTAAGC
	H16401-B	5'- BIO-TGATTTCACGGAGGATGGTG
	L21	5'- GTCCTTTGTCGATACTGATCACAGGTCTATCACCTA
	L174	5'- GTCCTTTGTCGATACTGTATTTATCGCACCTACGTTC
	H252-B	5'- BIO-GTGGAAAGTGGCTGTGCAGA
	H408-B	5'- BIO-CTGTTAAAAGTGCATACCGCCA
Sequencing primer:	U.P.	5'- FITC-GTCCTTTGTCGATACTG

Figure 1: Strategy of the amplification and sequencing procedure for the analysis of mtDNA variation in the d-loop region.
Primers are identified by a letter designating the strand of mtDNA (L is the non-coding and H is the coding strand) and a number corresponding to the reference sequence (Anderson 1981) of the base at the 3' end of the primer. U.P. is a universal primer not complementary to mtDNA.

Seven microliter of the sequencing reactions were loaded on a 7% Urea-Hydrolink Long Ranger gel (J.T. Baker) after denaturation for 3 min. at 85°C. Electrophoresis was done on the automated A.L.F. DNA sequencer (Pharmacia-Biotech).

RESULTS AND DISCUSSION

The developed strategy for the analysis of the d-loop region is outlined in Fig. 1. Depending on the source and the constitution of the biological sample either the two hypervariable egions (HVR1 or HVR2) or four overlapping segments (I to IV) are amplified. This approach allows also for a semi-nested PCR where one of the two hypervariable regions (HVR1 or HVR2) is amplified in a first PCR followed by a second amplification reaction of the two overlapping segments. The primers complementary to the H-strand are biotinylated which after capture with Dynabeads and alkali denaturation results in single stranded templates for enzymatic sequence analysis (solid-phase-approach). The non-biotinylated strand can be collected for further sequencing which will confirm the obtained sequence data from the biotinylated DNA strand. The unlabelled PCR primers are chimeric with a universal sequence (5') and a specific sequence complementary to the L-strand (3') (Fig. 1). This approach has the advantage that the sequence can be read

Table 1: Distribution of the mtDNA types observed in this study

	Number of mtDNA types		
Frequency (%)	**HVR1**	**HVR2**	**d-loop**
19.6	1	-	-
9.80	-	1	-
7.84	-	1	-
5.88	3	-	-
3.92	2	4	2
1.96	28	34	47

starting from the beginning of the amplified fragment. Sequence analysis of the PCR products revealed a number of 'stops' in the obtained sequence which was probably due to 'pausing' of the enzyme at regions with secondary structure. These 'stops' were resolved mainly by the inclusion of dITP, a nucleotide analogue for dGTP, in the PCR reaction (Dierick 1993).

Amplification of mtDNA from fresh hair shafts showed that it was possible to amplify a fragment of 1024 bp containing the complete d-loop with 40 cycles (data not shown). Evaluation on forensic hair samples resulted, however, in a low success rate of amplification even for the two hypervariable regions of 430 bp when a single amplification step was used. By using the semi-nested approach a success rate of 95-100% was obtained. This was a two to three fold increase of the success rate compared to the analysis with VNTR or STR markers.

In order to provide a statistical value in case of a match, we started with a survey for mtDNA variation in a small population of 51 unrelated Caucasians of Belgian descent. On average between 700 and 800 nucleotides were obtained for each individual. Differences with the reference sequence (Anderson 1981) were observed at 50 positions in the first hypervariable region (HVR1) and at 28 positions in the second (HVR2). In total, 49 different mtDNA types were identified: 47 sequences were observed only once in the population of 51 individuals while 2 mtDNA types were observed each 2 times (Table 1).

The mean pairwise sequence difference within the database was 3.52 for HVR1, 3.86 for HVR2 and 7.38 for the complete d-loop region. Therefore, a possible strategy for routine use in forensic analysis could be based on the analysis of HVR2 which is the most variable region (Table 1). In case of a match between the evidence sample and the reference sample from suspect or victim, further analysis of HVR1 will confirm or exclude a match. This approach has until now been used with success in several forensic cases. However, in those cases where close maternal relatives (brothers) may be suspected of a crime it is not possible to identify the offender between the relatives with mtDNA analysis alone. Only exclusion analysis with mtDNA is possible with 100% reliability.

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