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Mitochondrial DNA Sequencing of Shed Hairs and Saliva on Robbery Caps: Sensitivity and Matching Probabilities

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ABSTRACT: Sequencing of mitochondrial DNA (mtDNA) has been used for human identification based on teeth and skeletal remains. Here, we describe an amplification system for the mtDNA control region (D-loop) suited for the analysis of shed hair, which constitutes the most common biological evidence material in forensic investigations. The success rate was over 90% when applied to evidence materials such as shed hair, saliva stains and saliva on stamps. The analysis of evidence materials collected from three similar robberies revealed the presence of mtDNA sequences identical to those of the suspects in the three crimes. The use of mtDNA control region sequences for individual identification was evaluated. The probability of identity by chance for the mtDNA types of the suspects in the robberies was found to vary between $Pr = 0.017 - <0.0017$, depending on the reference population used, emphasizing the need for large population databases to obtain the appropriate estimate.

KEYWORDS: forensic science, DNA typing, mitochondrial DNA, DNA sequencing, shed hair, individual identification

Analysis of mtDNA sequences has become an important tool both in the study of human evolution (1,2) and in forensic medicine (3,4). Following the demonstration that a single hair suffices to determine the mtDNA sequence of an individual (3), a number of studies have explored this potential for non-invasive sampling both in the fields of anthropology and animal behavior (5,6). Several characteristics of the mitochondrial genome makes it particularly well suited for genetic analyses from limited materials. The high mtDNA copy number per cell (1000 to 10,000 copies/cell) (7) allows for the analysis of materials with limited amounts of, or partly degraded, DNA. Indeed, mtDNA sequence information has been obtained from ancient human remains (8). The higher nucleotide substitution rate of mtDNA relative to most nuclear genes also increases the potential for individual identification (9). Finally, mtDNA is inherited uniparentally, through the maternal parent (10). This pattern of inheritance allows for individual identification, even when the reference material used is separated from the evidence material by several generations. Since the mtDNA of siblings and maternal relatives is expected to be identical, with the exception of new mutations, individual identification is limited to the maternal lineage. Due to the haploid, or clonal nature, of mtDNA, jumping PCR (11), which commonly occurs in degraded DNA, does not cause erroneous results. By contrast, in analysis of nuclear loci jumping PCR may result in the presence of chimeric sequences that complicate the analysis of the allelic phase of polymorphisms.

mtDNA sequences have been used for human identification from remains such as teeth, skeletal fragments, and dried tissue (12–17). Several studies of mtDNA from plucked human hair have also been performed (5,18). In forensic investigations shed hair is among the most frequent types of biological evidence. Shed hairs contain less than 10 ng of DNA while the amount of DNA in hairs several months old and without a root are below the detection limit (<1 ng DNA) (19). Reliable analysis of such materials requires a very sensitive and robust amplification system. A method was recently described for the analysis of DNA from human hair shafts (20). Forensic investigators should be wary of analyses of mtDNA from specimens with low yields which involve several labor-intensive steps due to the possibility for introducing contamination (20).

Here, we describe a different PCR amplification system for the mtDNA control region than that previously described. The method is particularly sensitive and thus well suited to analyze shed hair. We applied the method to four forensic cases with evidence materials consisting of shed hairs, saliva stains, nail scrapes and small bloodstains. In particular, evidence materials were analyzed from a series of robberies conducted in Sweden in 1990 and 1991; these resulted in a total booty of close to 1 billion SEK (120 million US dollars). The mtDNA sequence analysis was successful and informative in all four forensic cases. We also evaluate some of the statistical aspects of using mtDNA sequences for individual identification and for the estimation of geographic origin of a suspect based on mtDNA analysis.

Methods

DNA Extraction

Extraction of DNA from shed hair, hair samples with roots, and nail scrapes was performed by digestion of a 1 to 2 cm portion of

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the hair, or the nail scrapes, in 200 μL PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.45% Nonidet P40 and 0.45% Tween 20). Five μL proteinase K (10 mg/mL) was added to the sample and the solution was made 35 mM with respect to DTT and then incubated for 3 h at 56°C. The proteinase K was inactivated at 95°C for 10 min and 10 μL from the solution was used in the PCR without further purification (19). Saliva stains were cut out from the robbers hoods and extracted in 1 mL of the buffer above with the addition of 10 μL proteinase K. After inactivation of the proteinase K, 20 μL of the solution was taken directly into the PCR. Blood stains on cloth were excised and incubated in 1 mL H_2O, followed by pelleting of the cloth and washing of nuclear cells. The samples were then mixed with 200 μL of 5% Chelex solution (21), incubated for 30 min at 56°C and then boiled for 10 min. From this solution, 10 μL was taken directly into a 50 μL PCR. Blood samples were extracted in Chelex as described above.

The method for extraction of DNA from the stamps and letters have been described previously (22). Briefly, materials from stamps and letters (0.5 to 1.0 cm^2) were excised and incubated in 1 mL of 5% Chelex solution. The samples were boiled for 5 min followed by incubation for 30 min at 56°C and thereafter boiled again for 10 min followed by centrifugation. The volume of the samples was then reduced from 1 mL to 100 μL by centrifuge driven dialysis using a Millipore Ultrafree -MC. From this solution, 20 μL was taken directly into a 50 μL PCR.

Nested mtDNA Amplification

The 50 μL PCR contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 200 μM of each dNTP, 0.5% Tween-20, 0.5% NP40, 10% glycerol, 2.5 units Taq polymerase (AmpliTaq, Perkin-Elmer, Norwalk, CT, USA) and 0.5 μM of each primer. Initially, segments I and II of the mtDNA control region were amplified in one fragment using the primers L15926 and H580 (23) (Table 1). An aliquot of 2 μL from this amplification was subsequently used as template for two nested PCRs. Segment I was amplified with the biotinylated primer L15997 and M13(-21)-H16401, while segment II was amplified with the biotinylated primer L29 and M13(-21)-H408. The primers H16401 and H408 contain a 5′-tail with the M13(-21) universal sequencing primer sequence (Table 1) (18,24,25).

For shed hair the above strategy proved unsuccessful, presumably because the 1.3 kb product generated by the primer pair L15926 and H580 was not amplified with sufficient efficiency. The system was therefore designed to amplify the two smaller fragments in two separate first PCRs. The primers L15965 and H16491 were used to amplify a 567 bp fragment from segment I and the primers L16527 and H448 used to amplify a 520 bp fragment from segment II, to be used as templates for the nested reaction (Table 1). The amplification was performed in a Perkin-Elmer 9600 PCR instrument for 25 and 32 cycles, for the first and nested reaction, respectively. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and an extension at 72°C for 30 s. The regions sequences were between nt 15998–16400 and nt 30–270 or nt 30–407. Segment II contains a stretch of 12 cytosines that for some samples ($n = 4$) proved difficult to determine. The region prior to this stretch was, however, analyzed using an internal primer, M13-21-H271, that together with the L29 primer amplifies a 299 bp fragment.

mtDNA Control Region Sequencing

Biotinylated PCR product was immobilized on 40 μL streptavidine-coated Dynabeads™ (Dynal A.S., Oslo, Norway) and incubated together with 40 μL BBextra (6 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) for 15 min. The beads were washed once with 50 μL Samag (10 mM Tris-HCl pH 7.5, 1M NaCl, 0.1% Triton X-100) followed by removal of the non-biotinylated strand through denaturation in 50 μL alkali solution (0.1 M NaOH, 1 M NaCl, 0.1% Triton X-100) for 15 min. Beads were then washed once in alkali solution and twice in Samag. The immobilized single-stranded DNA (ssDNA) was resuspended in 24 μL dH_2O and used in a sequencing reaction based on the Sequenase Dye Primer Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing of double-stranded PCR products was also performed. For this, purification of PCR products for sequencing was performed using Millipore Ultrafree MC-30.000 filters (Millipore Corp., Bedford, MA, USA) and sequencing was performed using the Dye Primer Cycle Sequencing FS kit (Applied Biosystems).

TABLE 1—*Summary of primers used for amplification of mtDNA control region.*

Primer/Region	Sequence
Segments I and II	
L15926*	5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′
H580*	5′-TTGAGGAGGTAAGCTACATA-3′
Segment I	
External	
L15965	5′-CCAAGGACAAATCAGAGAAAA-3′
H16491	5′-GTAGGAACCAGATGTCGGATA-3′
Internal	
L15997*	5′-Biotin-CACCATTAGCACCCAAAGCT-5′
M13-21H16401*	5′-ACATGTAAAACGACGGCCAGTTGATTTCACGGAGGATGGTG-5′
Segment II	
External	
L16527	5′-GGTCATAAAGCCTAAATAGCC-3′
H448	5′-GAGTGGGAGGGGAAAATAAT-3′
Internal	
L29*	5′-Biotin-GGTCTATCACCCTATTAACCAC-3′
M13-21-H408*	5′-ACATGTAAAACGACGGCCAGTCTGTTAAAAGTGCATACCGCCA-3′
M13-21-H271	5′-ACATGTAAAACGACGGCCAGTTTTTGTTATGATGTCTGT-3′

*Primer previously described (5,14,24,25).

Electrophoresis and sequence analysis were performed on an Applied Biosystems 373 DNA sequencer. The DNA sequences were aligned and compared using the SeqEd™ program (Applied Biosystems).

Nested DRB1 Amplification

PCR was performed in a 50 μL reaction containing the same reagents as for the mtDNA, with the exception of the primers. In the first PCR, a fragment containing the second exon of the DRB genes was amplified using the primer pair GH 46 (26,27) and UG 203 (22). The amplification was performed for 25 cycles, each cycle consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Two μL of the product from this PCR was transferred to each of two new reactions: In the first reaction, a shorter fragment of the second exon of the DRB1, 3, 4, 5, 6 genes was amplified using the primer pair GH 46 and GH 50. In the second amplification the second exon of the DRB1 gene was amplified, using the primer pair GH 46 and CRX 37 (26). The semi-nested amplification reactions were performed for 30 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). All PCR products were analyzed electrophoretically in a gel containing 3% Nusieve and 1% regular agarose. The gel was stained with ethidium bromide and the fragments visualized and photographed under ultraviolet (UV) light.

DRB1 Typing

The PCR product from the amplification with DRB general primers (GH 46 and GH 50) was applied to a nylon membrane and hybridized to each of 19 probes, while the DRB1 product (GH 46 and CRX 37) was hybridized to a second set of eight probes (26).

Statistical Analysis

The mtDNA segment I sequences of forensic materials were compared with a database comprising over 2500 sequences from European (EU), African (AF), Asian (AS), Native American (NA), and Australian-Oceanic (AU) populations, compiled from the literature. The number of sequences included in a particular comparison varied depending on the length of the available sequence, but was in no case less than 2173 and with the following distribution: EU, 844; AF, 577; AS, 494; NA, 201; AU, 57. The number of complete matches between forensic sequences and the database was calculated, as was the number of sequences differing by one and two nucleotides from the forensic sequence.

The probability for a DNA sequence coming from a geographic region *i* was calculated as follows. Let E be a sequence matching that from a forensic evidence and E_i a particular sequence from the *i*th region. The conditional probability of a sequence E coming from region *i* can then be expressed as

$$Pr(\mathrm{I}|\mathrm{E}) = \frac{(Pr(\mathrm{I}) \times Pr(\mathrm{E}|\mathrm{I}))}{\sum_{i=1}^{n} (Pr(\mathrm{I}) \times Pr(\mathrm{E}|\mathrm{I}))}$$

where *Pr* (I) is the probability of randomly selecting a sequence from region *i*, *Pr* (E|I) the probability of selecting sequence E from all the sequences from region *i* and, finally, *n* the total number of regions. To compute the probability that an individual with a certain sequence, E, belongs to a certain part of the reference population (in the present setting, one of the regions), two probability distributions are necessary:

1. The a priori distribution *Pr*(I).
2. The conditional probability that, given an individual stems from region *i*, has the sequence E, that is, *Pr*(E|I).

Pr(E|I) is estimated from the available database as the number of sequences in the database matching E over the number of sequences from region *i*. This estimate is affected by factors such as sample size and sampling strategy. If the reference population is all people of the world, the prior *Pr*(I) can be estimated by the relative frequency of the number of people from region *i* divided by the total world population (for the regions included). As a first approximation of the population numbers for the regions defined in the paper, we used the 1996 population numbers from the United Nations web site (http://www.undp.org/popin/wdtrends/pop/fpop.htm). For example, if eight of a total of ten matches with a forensic sequence were from the AF region and the remaining two from the EU region, the probability for an AF origin of the sequence was calculated as $Pr = ((0.216) \times (8/577)) / ((0.216) \times (8/577)) + ((0.213) \times (2/844)) = 0.86$, where 0.216 and 0.213 represent the proportion of the two populations. Similarly, the probability for an EU origin was $Pr = 0.14$. These probabilities, when they displayed a consistent pattern for identical matches and those with one and two nucleotide differences, were used to indicate geographic origin.

To calculate the probability of identity by chance (*Pr*IBC) for a match between a forensic sequence and that of a suspect, the estimated frequency of the corresponding sequence x_i in the database was calculated as $Pr = x_i/n$, where *n* is the number of sequences sampled from a region. When no match was found the probability was estimated as $Pr = < (1/n)$. In cases where a number of independent evidence materials were analyzed from each crime scene, a corrected value (Pr_c) was calculated by adding up the *Pr*IBC values for all genetically distinct materials investigated from a particular crime scene as

$$Pr_c\mathrm{IBC}(\mathrm{E}) = \sum_{i=1}^{n} Pr\mathrm{IBC}(\mathrm{E}_i)$$

where *Pr*IBC(E_i) represents the *Pr*IBC for the forensic sequence E_i (when compared with the total database) and *n* the number of genetically distinct materials investigated from the crime scene.

The 95% confidence interval for the probability of identity by chance was calculated as $Pr \pm 1.96 \times ((Pr \times (1 - Pr))/n)^{0.5}$, where *Pr* is the estimated probability of identity by chance and *n* is the sample size (number of sequences).

Results

Sensitivity of the Amplification and Sequencing Method

To develop a suitable method for the analysis of shed hairs, a number of primer pairs for the D-loop were compared for their sensitivity. Amplification of the D-loop in one fragment, using the primers L15926 and H580, followed by amplifications of segments I and II in two independent nested PCRs produced sequence only from blood and hairs containing root (Table 1). However, by limiting the amplification to either segment I or II in the initial PCR, followed by a nested PCR, the sequence analysis was successful from the majority of materials (Table 1). This amplification system was found to produce sequence from samples with between 30 ng and 30 fg of total genomic DNA (Table 2). The threshold for obtaining a readable sequence of segment I was found to be as

TABLE 2—*PCR efficiency and quality of DNA sequence obtained with different primer pairs and amplification systems, with and without nesting.*

Primer pair(s)		Amount of Genomic DNA in PCR							
		33 ng	3.3 ng	0.33 ng	33 pg	3.3 pg	0.33 pg	33 fg	3.3 fg
16965/16491*–15997/1640†									
	PCR product§	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	–
	Sequencing‖	3/420	3/420	3/420	3/420	3/420	3/420	4/420	–
15527/448*–29/408†									
	PCR product§	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	–
	Sequencing‖	3/400	3/400	3/400	4/400	3/400	6/400	–	–
15527/448*–29/271†									
	PCR product§	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+	–
	Sequencing‖	–/260	–/260	2/260	1/260	1/260	2/260	–	–
15997/16401‡									
	PCR product§	+ + +	+ + +	+ +	+ +	+	–	–	–
	Sequencing‖	2/380	2/380	4/340	1/340	5/310	–	–	–
29/408‡									
	PCR product§	+ + +	+ + +	+ +	+ +	+	+	–	–
	Sequencing‖	0/400	0/400	0/400	0/400	4/400	–	–	–

*Outer primer pair in nested reactions.
†Inner primer pair in nested reactions.
‡Reactions without nesting.
§Amount of PCR product: + + + high yield, + + moderate yield, + low yield, – no product.
‖Sequencing: number of ambiguous positions/total number of readable nucleotides.

low as 33 fg of genomic DNA, corresponding to about 10 copies of mtDNA (using an estimate of 1×10^3 copies of mtDNA per cell) (Table 2).

Application to Forensic Cases

The evidence materials from four forensic cases were analyzed. In the first and second case, typing of nuclear genes had failed presumably due to insufficient amounts of DNA. In the third and fourth case, HLA-DRB1 typing had previously been performed and the suspects could not be excluded.

Case #1: Shed Hair and Robber Hoods—This case concerned a series of three armed bank robberies that were carried out in Stockholm in 1990 and 1991. Four individuals were, based on other evidence, suspected of having performed the robberies. A total of 19 different evidence materials were investigated in connection with Robbery No. 1. Close to the bank where Robbery 1 was conducted, two robber hoods, containing one saliva stain each (evidence materials No. 2832/93 and 2833/93), were found and 17 shed hairs in other clothes. Sequences of segment II were obtained from 17 of the 19 evidence materials. Of the 17 materials that yielded an amplification product, two materials had a segment II sequence identical to that of suspect S1 (Table 3, Fig. 1). Two other materials had a segment II sequence identical to that of suspect S2. In ten materials the segment II sequence was identical to that of S4. In addition, three other sequences, not matching any of the suspects, were found in three evidence materials. Segment I sequences were obtained from 12 of the materials. In one material no amplification product was obtained, and in six other materials there was not enough sample available to perform the PCR. Among the 12 materials that yielded a PCR product, two had a segment I sequence identical to that of suspect S1. An additional two evidence materials had a segment I sequence identical to that of S2. Four materials had a segment I sequence identical to that of S4. Finally, four materials had sequences that did not match each other or the sequence of any of the suspects. Combining the segment I and II sequence data, two evidence materials had a sequence identi-

TABLE 3—*Summary of results of mitochondrial sequencing of Case #1.*

Material	Segment I	Segment II	HLA-DRB1
Suspect #1 (S1)	seq. #1	seq. #1	DR2/0401
Suspect #2 (S2)	seq. #2	seq. #2	0101/0301
Suspect #3 (S3)	seq. #3	seq. #3	1001/0403
Suspect #4 (S4)	seq. #4	seq. #4	0405/1201
Robbery 1			
2343/93 hair	seq. #13	seq. #13*	NR
2345/93 hair	seq. #14	seq. #14*	NR
2342/93 hair	seq. #12	NR	NR
2337/93 hair	identical to S1	identical to S1	NR
2339/93 hair	seq. #11	seq. #11*	0701
2335/93 hair	identical to S4*	identical to S4	NR
OXT 11—1 hair	NA	identical to S4	NR
OXT 11—2 hair	identical to S4*	identical to S4	NR
OXT 11—3 hair	NA	identical to S4	NR
OXT 11—4 hair	NA	identical to S4	NR
OXT 11—5 hair	NA	identical to S4	NR
OXT 11—6 hair	NA	identical to S4	NR
OXT 11—7 hair	NA	identical to S4	NR
OXT 11—8 hair	identical to S1	identical to S1*	NR
OXT 11—9 hair	identical to S4*	identical to S4	NR
1234/93 hair	NR	NR	NR
2333/93 hair	identical to S2	identical to S2*	NR
2832/93 saliva	identical to S4*	identical to S4*	NR
2833/93 saliva	identical to S2	identical to S2*	NR
Robbery 2			
2319/93 hair	seq. #8	seq. #8	NR
2321/93 hair	seq. #9	seq. #9	NR
2323/93 hair	seq. #10	NR	NR
2325/93 hair	identical to S2	identical to S2	0301
1754/93 saliva	seq. #6	seq. #6	0801
1755/93 saliva	seq. #7	NR	0701
1756/93 saliva	identical to S1	identical to S1	DR2/0401
Robbery 3			
2327/93 hair	identical to S2	identical to S2	NR
2329/93 hair	identical to S1	identical to S1	NR
2331/93 hair	identical to S1	identical to S1	NR
1749/93 saliva	seq. #5	seq. #5	DR2

NOTE—For sequence # see Fig. 1, * Indicates segment I sequences analyzed from position 16172 to 15998 and segment II sequences analyzed from position 30 to 270. NA, not analyzed. NR, no results obtained.

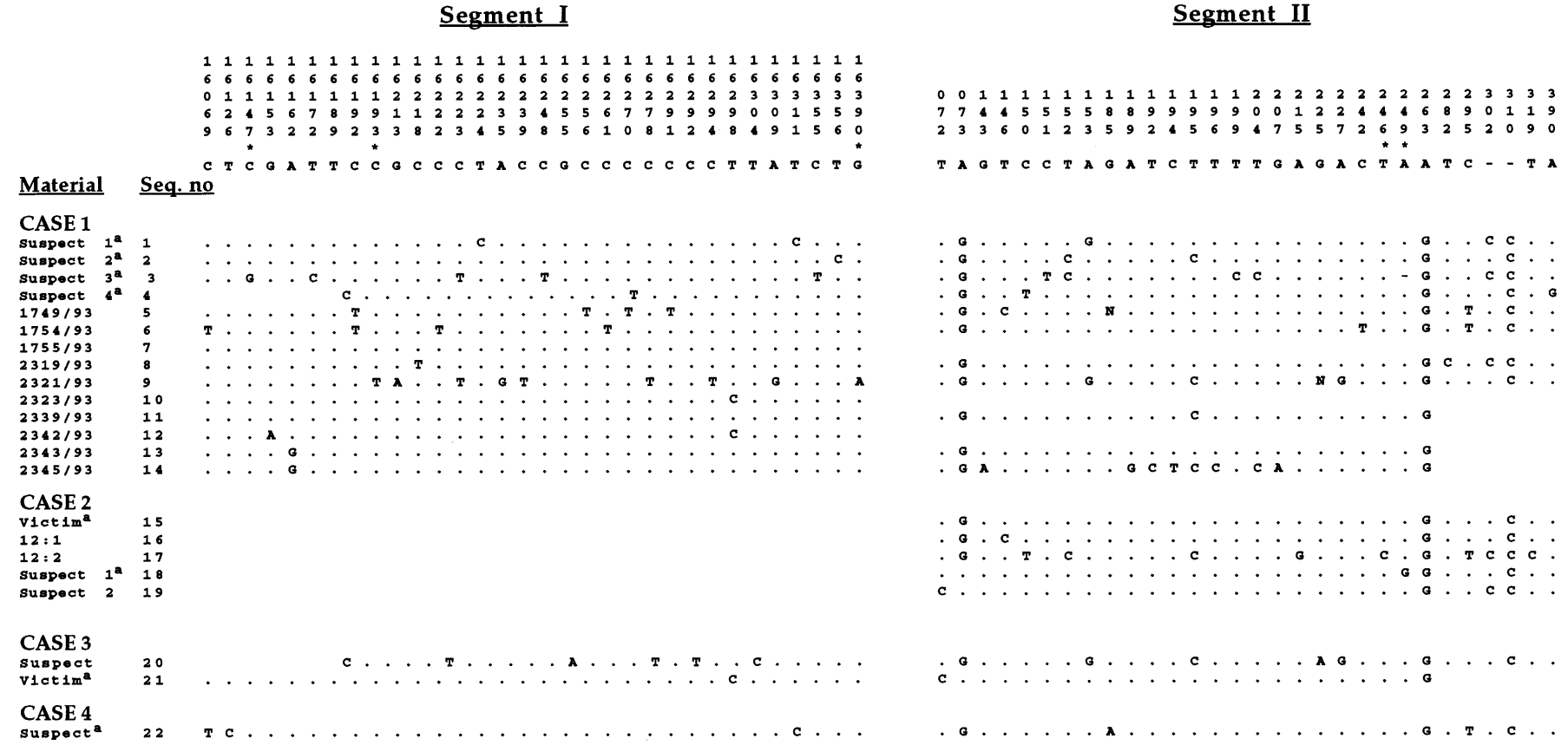

FIG. 1—*Mitochondrial DNA sequences of segments I and II of control region from the four forensic cases analyzed. The numbering of polymorphic positions is according to Andersson et al. (1981) (23). ''*'', indicate polymorphic sites not found among 152 reference sequences (14,47).* [a] *denote sequences identical to those of some of the evidence materials.*

cal to that of S1, two materials a sequence identical to that of S2, and four materials a sequence identical to that of S4. In every case where both segment I and II sequences were available from the same material, they matched the same suspect.

From Robbery 2, seven evidence materials were available. These consisted of three saliva stains and four shed hairs on a robber hood found in the vicinity of the bank. Segment II sequences were obtained from five of the evidence materials. The segment II sequence of the saliva stain No. 1756/93 was identical to that of suspect S1. The segment II sequence of the shed hair No. 2325/93 was identical to that of S2 (Table 3, Fig. 1). Three other segment II sequences were obtained from three of the other evidence materials. The segment I sequence of evidence No. 1756/93 was identical to that of S1. The sequence of evidence No. 2325/93 was identical to that of S2. Thus, the segment I and II sequences of evidence No. 1756/93 both matched S1 and the sequences of 2325/93 both matched that of S2. Several other sequences were also obtained in the remaining evidence materials.

In Robbery 3, four evidence materials were available, consisting of one saliva stain on a robber hood and three shed hairs found in clothes connected with the crime. Amplification products were obtained from all four materials. The segment I and II sequences of evidence materials No. 2329/93 and 2331/93 were identical to those of S1 (Table 3, Fig. 1). Also, the segment I and II sequences of evidence No. 2327/93 were identical to those of S2. A single evidence material had a sequence that did not match any of the suspects.

In all three robberies evidence materials containing sequences that did not match any of the four suspects were found. At present the origin of sequences that did not match any of the suspects is unknown. None of these sequences is identical to those of the people handling the laboratory analysis.

DRB1 amplification products were obtained in six of the evidence samples (Table 3). Evidence No. 1756/93 showed an identical DRB1 type to that of suspect S1 (DRB1*DR2/0401). For the other evidence materials, only a single DRB1 allele was found in each material. This indicates that allelic exclusion has occurred in the PCR from these samples. Precautions were taken to ascertain that the $MgCl_2$ in the PCR buffer was not reduced by improper storage (28). Allelic exclusion has previously been attributed to the stochastic nature of the PCR, when initiating the reaction from a very low copy number (29).

Case #2: Shed Hair—This case involved the investigation of the murder of an elderly man in his home. Based on other evidence, two men were suspected of having committed the crime. At the scene of the crime, one shed hair was secured in the hand of the victim (evidence material No. 10), three shed hairs on the sweater of the victim (12:1, 12:2 and 12:3) and two shed hairs were found on the victim's undershirt (M2a1, M2b1). Due to the lack of sufficient material, it was possible to analyze only the segment II sequences. The segment II sequence of evidence M2a1 was found to be identical to that of suspect S1. The sequence of the two evidence materials 10 and M2b1 was identical to that of the victim. The sequences of evidence materials 12:2 and 12:3 were identical. The sequences of evidence materials 12:1, 12:2 and 12:3 were not identical to either those of the victim or suspects (Fig. 1).

Case #3: Nail Scrapes and Bloodstains—The investigation concerned the murder of a woman, with her husband being the prime suspect. The evidence material consisted of nail scrapes from the victim and two small bloodstains (2 mm) on the underwear of the suspect. Typing of the evidence materials for HLA-DRB1 showed that both the nail scrapes and one of the bloodstains (K297) contained the DRB1*0401 allele, identical to that of the victim (DRB1*0401/DRB1*0401), while no trace of the alleles of the suspect (DRB1*0701, DRB1*1301) was found. The other bloodstain (K296) contained DRB1 alleles identical to those of both the suspect and the victim, with the strongest signal for the DRB1*0401 allele, identical to that of the victim. The segment I and II sequences of the nail scrapes and the K297 bloodstain were identical to that of the victim (Fig. 1). The bloodstain (K296) showed a mixture of nucleotides at two positions (positions 225, 227; Fig. 1). At both these positions, the sequence obtained showed an indication of a mixture (50%) of the two nucleotides present in the suspect and the victim. At the other four nucleotide positions in segment II differing between the suspect and the victim, the proportion of nucleotides other than those of the victim was considerably lower than 50%, and therefore these positions were interpreted by the analysis software as a single nucleotide. The difference between the positions in the ability to detect a mixture of nucleotides indicate that both the particular pair of nucleotides involved and the sequence context may affect the signal level. These results are consistent with the presence of a mixture of genetic material from the victim and the suspect in evidence K296. However, final proof of such a mixture must rely on the isolation by cloning and sequencing of individual DNA sequences matching the suspect and victim from this evidence material.

Case #4: Threatening Letters—The investigation concerned two threatening letters, suspected to have been sent by a former girlfriend of the receiver. The stamps as well as the sealing of the envelopes served as evidence material. As reference material, letters known to have been sent by the suspect were used. The HLA-DRB1 typing of evidence as well reference materials had previously revealed an inclusion of the suspect (22). The mtDNA segment I and II sequences of the three evidence materials were identical to that of the reference materials (Fig. 1).

Probability of Sequence Identity by Chance

The number of matches between the segment I sequences of forensic evidence materials and those in the population database varied between 0 and 128; for six of the forensic sequences no matches were found (Table 4). The probability of identity by chance between a suspect and a forensic sequence was found to vary between $Pr = 0.017$ and <0.0017, depending on the reference population (Table 5). If an estimate is calculated only for those regions with which a sequence match was found, the range of values becomes much more narrow ($Pr = 0.0069$ to 0.0083). Thus, the upper limit estimate of the probability of identity by chance is determined by the smallest sample size, in our case from the AU region. For the cases including a number of genetically distinct evidence materials, the estimate of the probability of identity by chance, corrected for the number of distinct materials at the crime scene, was calculated (Pr_c). For example, the Pr_c for S1 in Robbery 1, where seven genetically distinct genetic materials were found, was calculated as $Pr_c = 0.0051 + 0.0055 + 0.0032 + 0.0580 + 0.0032 + 0.0039 + 0.0039 = 0.083$. In general,

TABLE 5—*Probability of identity by chance for some of the segment I sequences from forensic materials. 95% confidence interval in parentheses.*

	Probability of Identity by Chance per Geographic Region*						Corrected Prob.†
	EU	AF	AS	NA	AU	Total	Total
Case #1							
Robbery 1							
suspect S1	0.0083 (0.0022–0.0144)	0.0069 (0.0002–0.0136)	<0.0020 (0.0010–<0.0059)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	0.0051 (0.0002–0.00809)	0.083
suspect S2	0.0095 (0.003–0.016)	<0.0017 (0.0000–<0.0050)	0.0080 (0.0002–0.0158)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	0.0055 (0.0024–0.0086)	0.083
suspect S4	0.0083 (0.0022–0.0144)	<0.0017 (0.0000–<0.0050)	<0.0020 (0.0010–<0.0059)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	0.0032 (0.0008–0.0056)	0.083
Robbery 2							
suspect S1	0.0083 (0.0022–0.0144)	0.0069 (0.0002–0.0136)	<0.0020 (0.0010–<0.0059)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	0.0051 (0.0002–0.00809)	<0.097
suspect S2	0.0095 (0.003–0.016)	<0.0017 (0.0000–<0.0050)	0.008 (0.0002–0.0158)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	0.0055 (0.0024–0.0086)	<0.097
Robbery 3							
suspect S1	0.0083 (0.0022–0.0144)	0.0069 (0.0002–0.0136)	<0.0020 (0.0010–0.0059)	<0.0049 (0.0000–0.0145)	<0.017 (0.0000–0.0502)	0.0051 (0.0002–0.00809)	<0.011
suspect S2	0.0095 (0.003–0.016)	<0.0017 (0.0000–0.0050)	0.008 (0.0002–0.0158)	<0.0049 (0.0000–0.0145)	<0.017 (0.0000–0.0502)	0.0055 (0.0024–0.0086)	<0.011
Case #3							
suspect	<0.0012 (0.0000–<0.0035)	<0.0017 (0.0000–<0.0050)	<0.002 (0.0010–<0.0059)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	<0.00046 (0.0000–<0.0014)	<0.00046
Case #4							
suspect	<0.0012 (0.0000–<0.0035)	<0.0017 (0.0000–<0.0050)	<0.002 (0.0010–<0.0059)	<0.0049 (0.0000–<0.0145)	<0.017 (0.0000–<0.0502)	<0.00046 (0.0000–<0.0014)	<0.00046

NOTE—*Number of matches over number of sequence from individual regions (EU, European populations; AF, African populations; AS, Asian populations; NA, Native American populations; AU, Australian/Oceanic populations). †Sum of the matching probabilities for all genetically distinct evidence materials from crime scene.

TABLE 6—*Number of matches between evidence materials and segments I and II sequences of different populations.*

		Case #1				Case #2			Case #3		Case #4
	Σx_i^2	S1	S2	S3	S4	S1	S2	V	S	V	S
Population/D-loop region											
Caucasian (n = 19)*											
segment I	0.058	1	1	0	0	—	—	—	0	0	0
segment II	0.064	0	1	0	0	0	0	0	0	0	0
both	0.053	0	1	0	0	—	—	—	0	0	0
British (n = 100)†											
segment I	0.034	2	1	0	0	—	—	—	0	2	0
segment II	0.047	0	0	0	0	0	2	3	1	2	1
both	0.014	0	0	0	0	—	—	—	0	2	0
Asian (n = 23)*											
segment I	0.038	1	0	0	0	—	—	—	0	0	0
segment II	0.038	0	0	0	0	0	0	1	0	0	0
both	0.038	0	0	0	0	—	—	—	0	0	0
African (n = 10)*											
segment I	0.1	0	0	0	0	—	—	—	0	0	0
segment II	0.1	0	0	0	0	0	0	0	0	0	0
both	0.1	0	0	0	0	—	—	—	0	0	0

NOTE—S, suspect; V, victim. *, from Ref (2) †, from Ref 47.

number of matches was very small. The comparison of the origin of sequences differing by zero, one and two base-pair differences yielded consistent indications of geographic origin. When applying this approach to the eleven forensic materials of unknown origin in Table 4, two showed no matches even when two bases were allowed to differ, seven appeared to be of EU origin, and two either of EU or AS origin.

Informativeness of Control Region Segments

For some of the evidence materials the number of matches with segment I sequences was very high (such as Case #1, evidence No. 1755/93, 126 matches). To increase the discrimination power, both segments of the control region were analyzed for most of our evidence materials. Consistent with this the number of matches between the sequences of suspects in our cases and the published segment I and II sequences decreased when both segment were examined (Table 6). A measure of the added information obtained by analyzing both segments relative to only one can be obtained by calculating the average probability of identity by chance, as $Pr = \Sigma x_i^2$, where x_i is the frequency of the ith mtDNA type (14). Using published sequences to compute this measure, we note that the average probability of identity by chance can be reduced by a factor of 2 to 3 when examining both segments (Table 6). It will be possible to obtain a more accurate measure of the informativeness of analyzing both relative to only one region once additional control region sequence databases for both segments become available.

Discussion

Sensitivity

The amplification system described permits the analysis of mtDNA sequences from as little as 33 to 330 fg of genomic DNA, equivalent to 10 to 100 copies of mtDNA. The amplification system thus requires only about 1/400 of the amount of genomic DNA needed for the analysis of the nuclear gene DRB1 (22). The difference in the required amount of DNA is roughly consistent with the copy number difference per cell between the nuclear and the mtDNA genomes.

When applied to shed hairs, bloodstains, nails scrapes, and saliva on stamps and sealed letters, the amplification system showed a very high success rate. Only one of 33 evidence materials examined for segment I, and four of 39 examined for segment II, did not yield a sequence, corresponding to a success rate of 97% and 91% for segments I and II, respectively. Difficulties in assigning bases were observed in only four samples and confined to a stretch of cytosines in segment II. Using additional primers, the sequence up to this stretch could be determined in these samples.

Contamination

The use of a nested amplification system increases the potential for contamination and thereby false positive amplifications. To manage this problem the experiments were performed in a laboratory with dedicated areas for the pre-PCR, nested, and post-PCR steps. Multiple negative controls were included at each step, from DNA extraction to the PCR. Three negative controls were run on each side of each sample, and taken through the whole procedure, from the extraction of DNA to the sequencing. All materials were analyzed at least twice, and on different occasions. The evidence materials were analyzed first, and the analysis repeated, before the reference material was analyzed. We consider the most likely step for introducing contaminating molecules to be the transfer from the first to the second PCR, due to the very large copy number available in tubes with successful amplifications. For routine analysis of forensic samples, a nested method without the need to open the tubes between the two reactions would be preferable. Methods for one-tube nested PCR, with a sensitivity similar to that of regular nested PCRs, have been developed for other targets (30–32).

Probability of Sequence Identity by Chance

The strategy for selecting the appropriate reference population for estimating the probability of identity by chance for a match has been a matter of intense debate (33,34). In order not to underestimate the probability of a match, even the most conservative

approach of using the highest allele frequency estimated for any population (the ceiling principle) has been suggested (NRC 1992) (35). More recently, though, other approaches have been recommended (NRC 1996) (36). An alternative approach is to calculate a range of estimates based on a worldwide set of human populations, to be used to assess the variability due to the choice of reference population. In applying this strategy to the forensic cases we found the probability of identity by chance for the segment I sequences to vary tenfold when data from all populations were included, but less than twofold when including only populations for which a match was found. This emphasizes the need to establish large databases that are balanced with respect to the sample size from different populations.

Under the assumption of independence between mtDNA and HLA-DRB1, a combined estimate of the probability of identity by chance was calculated for evidence materials where both markers could be typed. The combined probability of matching by chance for the mtDNA segment I sequence and the DRB1 genotype was estimated to $Pr = 2.5 \times 10^{-3}$ for evidence No. 1756/93 in Case #1, $Pr = 9 \times 10^{-4}$ for evidence No. K297 in Case #3 and to $Pr = 4.6 \times 10^{-6}$ for the evidence in Case #4, using published information of the DRB1 frequencies in the Swedish population (27). An alternative means of reducing the probability of matching by chance is to examine a larger portion of the mtDNA. Extending the analysis to both segments I and II of the control region will be particularly useful for populations with limited mtDNA diversity. However, to achieve more than a twofold to threefold reduction of the probability, a substantially larger portion of the molecule will have to be examined.

The estimates of the probability of a match by chance in the preceding sections were contingent on the recovery of a single type of DNA sequence at each crime scene. In forensic cases involving multiple evidence materials, it is appropriate to provide the probability of a particular match, corrected for the number of genetically distinct materials analyzed from this particular crime scene. When applied to Case #1, where a number of evidence materials were found, the corrected probability estimate is substantially higher than if only a single material is considered (Table 5). For cases where a large number ($N > 10$) of genetically distinct evidence materials have been found at a crime scene, the most informative result may actually be that of an exclusion.

Linking Suspects to Multiple Robberies

Due to similarities between the three robberies in Case #1, it was suspected that they were carried out by the same individuals. mtDNA sequences identical to those of suspects S1 and S2 were found at all three crime scenes, while sequences identical to that of S4 were found only in evidence connected with the third robbery. The probability that the evidence at the three crime scenes came from three independent individuals can be estimated from the combined probability of identity by chance for each of the three evidence materials. Thus, the probability of three independent individuals having deposited DNA evidence matching that of S1 is $Pr = (0.0050)^3 = 1.3 \times 10^{-7}$ and that of S2 is, similarly, $Pr = (0.0052)^3 = 1.4 \times 10^{-7}$. Thus, it is very unlikely that the evidence in the three cases came from three independent individuals.

Court Decision in Case #1

The mtDNA analyses of the materials in Case #1 were admitted as evidence in the Swedish district court and two of the suspects (S1 and S2) were, on the basis of genetic and other evidence, convicted in February 1996 (Court No. B12-9767-94). The court decision was appealed. The court of appeal in June 1996 decided in accordance with the decision of the district court (Court No. B13-9767-94).

Origin of Maternal Lineages

mtDNA diversity has been estimated to be fivefold to tenfold higher between Caucasian, African-American, and Asian populations as compared to within populations, using hybridization with sequence-specific probes (14). By applying a logistic regression model to the data of Stoneking et al. (1991), the ethnic group was correctly predicted for 65.3% of the individuals, although the success rate varied substantially among ethnic groups (37). This indicates a potential for using mtDNA sequences to infer geographical origin, at least with respect to the major human population groups. Similarly, a mtDNA distance analysis was used to assess the origin of the ''Iceman,'' relative to European and other populations (16). We used the origin of matching database sequences to indicate the origin of the forensic sequence and assessed for the similarity by allowing for 0 to 2 nucleotide differences between the forensic sequence and those in the database. The appropriate geographic region was indicated for four out of six individuals with known origin. The power of this approach, when applied to a comprehensive data set, remains to be determined. However, the limitations to this type of analysis have to be recognized. mtDNA is likely to provide only information of the maternal origin relative to major population groups only.

A particular problem arises for populations of mixed origin. For instance, the sequences in the database from region AU, for example, are not representative for the Oceanic population, since there are no samples from people that stem from Europe, which are a large part of the 29 million people living there. Within some of these groups the small genetic differences among populations make any positioning of individuals based on mtDNA sequences unreliable. In particular, the small genetic differences among European populations (38), with some exceptions (39), make it difficult to assign a mtDNA sequence to any particular geographic subarea within Europe. Further, mtDNA sequence comparisons will be useful only for populations with limited admixture, and are unsuited for populations that have been recently mixed. Finally, even in populations where admixture is not extensive, it is far from clear that the ''phenotype'' of an individual needs to go with the geographic origin of its mtDNA sequence.

Thus, a very cautionary use of the estimation of geographic origin of mtDNA sequences in, for example, tactical law enforcement work is of uttermost importance.

Future Considerations for the Use of mtDNA

In general, the use of mtDNA sequences in forensic medicine is currently at its infancy and there is a need for developments in several areas. The comparison of segments I and II indicated an increased information content by analyzing both regions. However, a much larger portion of the mtDNA needs to be examined to substantially reduce the probability of identity by chance. Analysis of the complete human mtDNA genomes available suggests that the number of nucleotide differences between individuals can be increased by at least threefold to fourfold by examining the entire mtDNA (Gyllensten et al., unpublished). Thus, even though parts of the coding sequences are less polymorphic than the control

region, the total number of sites differing between individuals can be increased substantially. The probability of identity by chance based on a whole genome comparison is not known at present, but is likely to be very low. The ability to examine the entire mtDNA, or large portions thereof, relies on the use of simple and automated means for sequence analysis. The rapid development of new techniques in the field of DNA sequencing will make such an endeavor more feasible (40).

Our understanding of essential features of mitochondrial genetics is still scanty. For instance, a more detailed examination of the level of, and mechanism for generation of, heteroplasmy, which may be more common than hitherto anticipated, is warranted (41,42). In particular, further studies of the mtDNA sequence mosaicism between tissues are necessary in order to gage the effect of this on the interpretation of forensic samples based on different tissue types. Also, a number of studies have reported high mutation rates in pedigree analyses (43,44). The consequences of such emerging evidence of a higher mutation rate, as well as the presence of mutational hotspots in mtDNA, also merit attention, since this may, at least theoretically, result in false exclusions (45). Related to this is the need to quantitate the number of molecules from which an amplification reaction is initiated, since both the risk of amplifying a molecule introduced by contamination (46), and a possible rare sequence variant in a heteroplasmic individual, are directly related to the number of original template molecules sampled by PCR. Efforts to resolve these issues will be needed in order to realize the full potential of mtDNA in forensic investigations.

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References

1. Cann RL, Stoneking M, Wilson AC. Mitochondrial DNA and human evolution. Nature 1987;325:31–6.
2. Vigilant L, Stoneking M, Harpending H, Hawkes K, Wilson AC. African populations and the evolution of human mitochondrial DNA. Science 1991;253:1503–7.
3. Higuchi R, von Berolding CH, Sensabaugh GF, Erlich HA. DNA typing from single hairs. Nature 1988;332:543–6.
4. Wilson MR, Holland MM, Stoneking M, DiZinno, JA, Budowle B. Guidelines for the use of mitochondrial DNA sequencing in forensic medicine. Crime Lab Dig 1993;20:68–77.
5. Vigilant L, Pennington R, Harpending H, Kocher TD, Wilson AC. Mitochondrial DNA sequences in single hairs from a southern African population. Proc Nat Acad Sci USA 1989;86:9350–4.
6. Morin PA, Moore JJ, Chakraborty R, Jin L, Goodall J, Woodruff DS. Kin selection, social structure, gene flow, and evolution of chimpanzees. Science 1994;256:1193–1201.
7. Bodenhagen D, Clayton DA. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. J Biol Chem 1974;249:7991–5.
8. Paabo S, Gifford JA, Wilson AC. Mitochondrial DNA sequences from a 7000-year old brain. Nucl Acid Res 1988;16:9775–87.
9. Brown WM, Prager EM, Wang A, Wilson AC. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 1982;18:225–39.
10. Hutchison III CA, Newbold JE, Potter SS, Edgell M. Maternal inheritance of mammalian mitochondrial DNA. Nature 1974;251: 536–8.
11. Paabo S, Higuchi RG, Wilson AC. Ancient DNA and the polymerase chain reaction. J Biol Chem 1989;264:9709–12.
12. Ginther C, Issel-Tarver L, King MC. Identifying individuals by sequencing mitochondrial DNA from teeth. Nat Genet 1992;2: 135–8.
13. Hagelberg E, Sykes B. Ancient bone DNA amplified. Nature 1989; 342:485.
14. Stoneking M, Hedgecook D, Higuchi RG, Vigilant L, Erlich HA. Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. Am J Hum Genet 1991;48:370–82.
15. Holland MM, Fisher DL, Mitchell LG, Rodriguez WC, Canik JJ, Merril CR, et al. Mitochondrial DNA sequence analysis of human skeletal remains: Identification of remains from the Vietnam War. J Forensic Sci 1993;38:542–53.
16. Handt O, Richards M, Trommsdorff M, Kilger C, Simanainen J, Georgiev O, et al. Molecular genetic analyses of the Tyrolean ice man. Science 1994;264:17758.
17. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, et al. Identification of the remains of the Romanov family by DNA analysis. Nat Genet 1994;6:130–5.
18. Hopgood R, Sullivan KM, Gill P. Strategies for automated sequencing of human mitochondrial DNA directly from PCR products. Biotech 1992;13:82–92.
19. Higuchi R. Rapid, efficient, DNA extraction for PCR from cells or blood. Ampl, Perkin-Elmer Cetus, 1989;2:1–3.
20. Wilson MR, Polanskey D, Butler J, DiZinno JA, Replogle J, Budowle B. Extraction, PCR amplification and sequencing of mitochondrial DNA from human hair shafts. BioTechn 1995; 18:662–9.
21. Walsh P, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotech 1991;10:506–13.
22. Allen M, Saldeen T, Gyllensten U. PCR-based DNA typing of saliva on stamps and envelopes. Biotech 1994;17:546–52.
23. Anderson S, Bankier AT, Barrell BG, de Bruijin MHL, Coulson AR, Drouin J., et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457–65.
24. Sullivan KM, Hopgood R, Lang B, Gill, P. Automated amplification and sequencing of human mitochondrial DNA. Electrophor 1991; 12:17–21.
25. Sullivan KM, Hopgood R, Gill P. Identification of human remains by amplification and automated sequencing of mitochondrial DNA. Int J Leg Med 1992;105:83–6.
26. Scharf SJ, Griffith RL, Erlich HA. Analysis of DNA sequence polymorphism at the HLA-DRB1 locus using the polymerase chain reaction and non-radioactive oligonucleotide probes. Hum Immun 1991; 30:190–201.
27. Allen M, Saldeen T, Pettersson U, Gyllensten U. Genetic typing of HLA class II genes in Swedish populations: application to forensic analysis. J Forensic Sci 1993;38:554–70.
28. Hu CY, Allen M, Gyllensten U. Effect of freezing of the PCR buffer on the amplification specificity: Allelic exclusion and preferential amplification of contaminating molecules. PCR Meth Appl 1992; 2:182–3.
29. Walsh PS, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: Mechanism and solution. PCR Meth and Appl 1992;1: 241–50.
30. Erlich HA, Gelfand D, Sninsky J. Recent advances in the polymerase chain reaction. Science 1991;252:1643–51.
31. Yourno J. A method for nested PCR with single closed reaction tubes. PCR Meth and Appl 1992;2:60–5.
32. Ylitalo N, Bergstrom T, Gyllensten U. Detection of genital HPV using single tube nested-PCR and type-specific oligonucleotide hybridization. J Clin Micro 1995;33:1822–8.
33. Levontin RC, Hartl DL. Population genetics in forensic DNA typing. Science 1991;254:1745–50.
34. Risch NJ, Devlin B. On the probability of matching DNA fingerprints. Science 1992;255:717–20.
35. National Research Council. DNA technology in forensic science. National Academic Press, Washington, DC, 1992.
36. National Research Council. The evaluation of forensic DNA evidence. National Academic Press, Washington, DC, 1996.
37. Connor A, Stoneking M. Assessing ethnicity from human mitochondrial DNA types determined by hybridization with sequence-specific oligonucleotides. J Forensic Sci 1994;39:1360–71.

38. Pult I, Sajantila A, Simanainen J, Georgiev O, Schaffner W., Pääbo, S. Mitochondrial DNA sequences from Switzerland reveal striking homogeneity of European populations. Biol Chem Hoppe-Seyler 1994;375:837–40.
39. Sajantila A, Lahermo P, Anttinen T, Lukka M, Sistonen P, Savontaus M-L., et al. Genes and languages in Europe: an analysis of mitochondrial lineages. Genom Res 1995;5:42–52.
40. Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, et al. Accessing genetic information with high-density DNA arrays. Science 1996;274:610–4.
41. Ivanov PL, Wadhams MJ, Roby RK, Holland MM, Weedn VW, Parson TJ. Mitochondrial DNA sequence heteroplasmy in the Grand Duke of Russia Georgij Romanov establishes the authenticity of the remains of the Tsar Nicholas II. Nat Genet 1996;12:417–20.
42. Jazin EE, Cavelier L, Eriksson I, Oreland L, Gyllensten U. Human brain contains high levels of heteroplasmy in the noncoding regions of mitochondrial DNA. Proc Natl Acad Sci USA 1996;93: 12382–7.
43. Howell N, Kubacka I, Mackey DA. How rapidly does the mitochondrial genome evolve? Am J Hum Genet 1996;59:501–9.
44. Parsons T, Muniec D, Sullivan K, Woodyatt N, Alliston-Greiner R, Wilson M, et al. A high observed substitution rate in the human mitochondrial DNA control region. Nat Genet 1997;15:363–8.
45. Pääbo S. Mutations in the mitochondrial microcosm. Am J Hum Genet 1996;59:493–6.
46. Handt O, Krings M, Ward RH, Pääbo S. The retrieval of ancient human DNA sequences. Am J Hum Genet 1996;59:368–76.
47. Piercy R, Sullivan KM, Benson N, Gill P. The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. Int J Leg Med 1993;106:85–90.

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