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Pitfalls in the analysis of mitochondrial DNA from ancient specimens and the consequences for forensic DNA analysis: the historical case of the putative heart of Louis XVII

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Abstract Amplification of mtDNA D-loop fragments with a length of 200 bp or more from ancient and even from fairly recent biological samples, can lead to erroneous results. This was clearly illustrated in our investigation of the putative heart of Louis XVII. By selecting different sets of primers which amplified shorter fragments of mtDNA (length 109 bp–201 bp), authentic polymorphisms could be visualised which remained undetected with the more classical primers for fragment sizes > 210 bp. Here we have extended those findings to other biological materials. A competitive PCR assay for quantitation of the amount of mtDNA for different fragment lengths, using a 10 bp deletion construct, was applied to ancient material and on a set of hairs of various ages of sampling (1966 up to the present). The results showed that DNA degradation started a few years after sampling. In the DNA extracts of the older hair shafts (1983–1995), the proportion of the number of short fragments to the number of long fragments is on average 4 in contrast to the most recent hair shafts. The numbers of amplifiable mtDNA copies for the hairs from 1975 and older were too small to show a clear difference. Use of long PCR fragments in such cases can yield misleading results. Use of short PCR fragments for the analysis of mtDNA from shed hair, in combination with a competitive PCR assay to determine the state of degradation, should improve the reliability of forensic mtDNA analysis considerably.

Keywords Ancient DNA · DNA degradation · Mitochondrial DNA · Genetic identification · Louis XVII

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Introduction

Identification of human remains by DNA analysis has proven to be a powerful tool in forensic and historical investigations (Hagelberg et al. 1991). mtDNA is especially useful, because it is present at a high copy number in cells (Bogenhagen and Clayton 1974) and is more likely to survive for prolonged periods compared to nuclear DNA. It has been shown that mtDNA analysis can be performed successfully on human remains up to 12,000 years old (Hagelberg et al. 1989) and even on Neanderthal skeletons (Krings et al. 1997; Ovchinnikov et al. 2000). Because of its unique maternal inheritance (Orrego and King 1990), mtDNA is also very useful in forensic identification cases (Orrego and King 1990; Sullivan et al. 1992) and for determining maternal family relationships when a gap of several generations exists between an ancestor and a descendant (Ivanov et al. 1996).

Recently, we were involved in one of the most intriguing historical mysteries concerning the Royal House of France with mtDNA analysis on the putative heart of Louis XVII, son of Louis XVI and Marie-Antoinette (Jehaes et al. 2000). The results showed that the mtDNA D-loop sequence of the heart was identical to those of maternal relatives of Louis XVII. This supported the official version that Louis XVII and not a substitute, died in the Temple in Paris on June 8, 1795. During our investigations, we obtained reproducible results when small PCR fragments (< 200 bp) were analysed in contrast to large PCR fragments (> 210 bp). By using only long PCR fragments, we would have missed some of the authentic polymorphisms. Although "jumping" PCR might overcome fragmented DNA (Pääbo et al. 1990), a minor contamination may be picked up preferentially when long PCR fragments are amplified. In this report, we discuss this observation in more detail and provide evidence that this pitfall also has some consequences when dealing with old cases in forensic hair DNA analysis.

Materials and methods

DNA extraction

From the outset of this study extreme efforts were made to recover ancient DNA samples free of contamination by contemporary DNA. Precautions and controls were as described in Jehaes et al. (1998 a) and mtDNA analysis of the heart was independently performed in two laboratories. The DNA was extracted at the Center for Human Genetics (Leuven) from one aorta segment and four segments of the heart muscle (together ~ 500 mg) using the Geneclean for Ancient DNA kit (BIO 101, Vista, Calif.) described by Jehaes et al. (2000). In the Institut für Rechtsmedizin (Münster) also ~ 500 mg heart tissue was extracted: two segments of the heart tissue (one aorta and one heart muscle segment) were extracted using the phenol-chloroform extraction method and another three segments (two heart muscle and one aorta segment) were extracted using NucleoSpin Funnel Columns (Machery-Nagel, Düren, Germany) as described by Jehaes et al. (2000).

Hair shafts (2 cm from the end) were extracted as described in Jehaes et al. (1998 b).

Analysis of the mtDNA D-loop

At the Center for Human Genetics (Leuven) amplification of two overlapping fragments (between 214 and 293 bp) for each of the two hypervariable regions of the non-coding D-loop (Decorte et al. 1996) was done by a single hot start PCR of 45 cycles using AmpliTaq Gold™ polymerase (Perkin Elmer, Emeryville, Calif.). Hot start PCR was carried out using 5 U of AmpliTaq Gold™ polymerase, 0.4 μM primers, 2.5 mM $MgCl_2$, 1 mg/ml BSA, 0.2 mM of dTTP, dATP and dCTP, 200 μM dITP, 40 μM dGTP, 1 × PCR buffer (Perkin Elmer, Emeryville, Calif.) and 10% of DNA extract in a 100 μl reaction volume. PCR amplification was performed on a Perkin-Elmer 480 Thermal Cycler for 45 cycles after a pre-heat step at 94 °C for 10 min in order to activate the enzyme. The temperature profile consisted of denaturation at 94 °C for 45 s, annealing for 1 min at 56 °C and elongation at 72 °C for 1 min. In addition to the amplification of long fragments, six shorter fragments (between 109 and 201 bp) were amplified according to the protocol described above. The PCR products were directly sequenced according to a solid phase protocol with the Sanger dideoxy chain termination method (Sanger et al. 1977) on the automated ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden).

At the Institut für Rechtsmedizin (Münster) amplification and sequencing were performed for the complete hypervariable regions HV1 and HV2 as described before (Holland et al. 1995; Pfeiffer et al. 1999 a). In addition, five short overlapping fragments were in-

Table 1 Oligonucleotides used for the amplification of the mtDNA D-loop (*Ls* short PCR fragments analysed in Leuven, *Ms* short fragments analysed in Münster, *HV* long PCR fragments)

PCR fragment	Nucleotide position[a]	Primers used	Sequence
Ls1	16082–16220 (16105–16205)	L16099-M13 H16201-BIO	5′-GTCCTTTGTCGATACTGCCGCTATGTATTTCGTAC-3′ 5′-biotin-TTGATTGCTGTACTTGCTTG-3′
Ls2	16139–16247 (16173–16237)	L16157-M13 H16228-BIO	5′-GTCCTTTGTCGATACTGATACTTGACCACCTGTAGT-3′ 5′-TTGCAGTTGATGTGTGATAG-3′
Ls3	16266–16384 (16290–16372)	L16285-M13 H16366-BIO	5′-GTCCTTTGTCGATACTGCCCACTAGGATACCAACAAA-3′ 5′-biotin-TGAGGGGGGTCATCCATG-3′
2HV1	16091–16384 (16130–16378)	L16110-M13 H16366-BIO	5′-GTCCTTTGTCGATACTGATTTCGTACATTACTGCCAG-3′ see Ls3
Ls4 and Ms4	47–174 (65–167)	L66-M13 H115-BIO	5′-GTCCTTTGTCGATACTGGCATTTGGTATTTTCGTCTG-3′ 5′-biotin-TGAACGAGGTGCGATAAATA-3′
Ls5 and Ms5	70–271 (135–260)	L88-M13 H252-BIO	5′-GTCCTTTGTCGATACTGGGTATGCACGCGATAGCAT-3′ 5′-biotin-GTGGAAAGTGGCTGTGCAGA-3′
Ls6	154–271 (170–260)	L174-M13 H252-BIO	5′-GTCCTTTGTCGATACTGTATTTATCGCACCTACGTTC-3′ see Ls5
1HV2	2–271 (55–260)	L21-M13 H252-BIO	5′-GTCCTTTGTCGATACTGATCACAGGTCTATCACCCTA-3′ see Ls5
2HV2	154–368 (205–360)	L174-M13 H348-BIO	see Ls6 5′-biotin-TTCTTTGTTTTTGGGGTTTGG-3′
Ms1	15989–16175 (16009–16159)	Ms1-F Ms1-R	5′-CCCAAAGCTAAGATTCTAAT-3′ 5′-TGGATTGGGTTTTATGTA-3′
Ms2	16144–16251 (16164–16234)	Ms2-F Ms2-R	5′-TGACCACCTGTAGTACATAA-3′ 5′-GGAGTTGCAGTTGATGT-3′
Ms3	16190–16410 (16210–16390)	Ms3-F Ms3-R	5′-CCCCATGCTTACAAGCAAGT-3′ 5′-GAGGATGGTGGTCAAGGGAC-3′
HV1	15989–16410 (16009–16390)	Ms1-F Ms3-R	see Ms1 see Ms3
HV2	15–484 (35–464)	HV2-F HV2-R	5′-CACCCTATTAACCACTCACG-3′ 5′-TGAGATTAGTAGTATGGGAG-3′

[a] Positions between brackets indicate the sequence that could be read

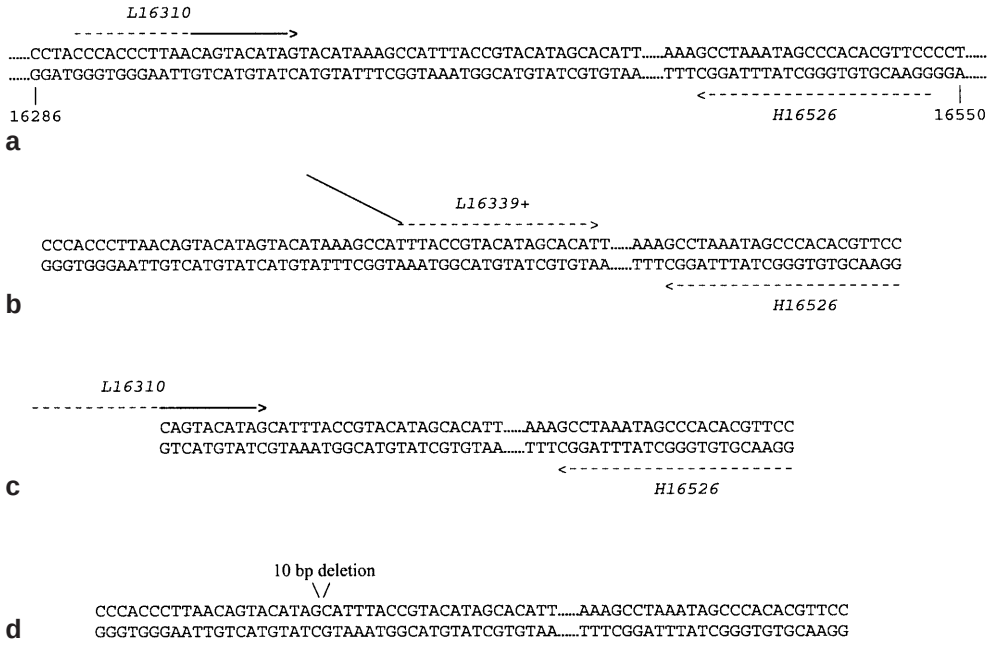

Fig. 1 a–d Schematic view describing the generation of the internal standard by PCR. The reverse primer H16526 is used in all amplification steps. **a** Amplification of a 258-bp fragment, position of original 5′ and 3′ primer (L16310); **b** first reamplification step: 3′ linker primer (L16339+) replaces 3′ primer; the 258 bp fragment is used as a template; **c** second reamplification step: original forward primer replaces 3′ linker primer; the 237 bp fragment is used as a template; **d** 248 bp standard generated with 10 bp deletion

vestigated (Holland et al. 1995; Pfeiffer et al. 2000). The phenol-chloroform extracts were amplified in 30 cycles and when no product was obtained, reamplification was done in 25 cycles (1 µl PCR product in 25 µl reaction volume). The Funnel Column extracts were amplified using 35 cycles in a total reaction volume of 25 µl. DNA sequencing was carried out on an ABI Prism 310 automated sequencer using BigDye Terminator sequencing reagents (ABI-Perkin Elmer, Weiterstadt, Germany). All fragments were sequenced in both directions.

The primer sequences used in this study are listed in Table 1.

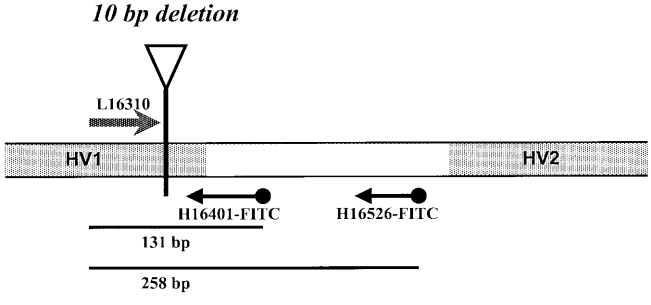

Fig. 2 Position of the amplification primers for the competitive quantitation PCR relative to the two hypervariable regions, HV1 and HV2, of the human mtDNA D-loop region

Quantitation of the number of amplifiable mtDNA molecules for PCR

In order to determine the number of amplifiable mtDNA molecules for PCR in DNA extracts, a competitive PCR method was developed (Decorte et al. 1998). Therefore, an internal control, a molecule of 248 bp, was constructed encompassing positions 16290–16547 in the human mitochondrial control region with bases 16311–16320 (10 bp) deleted. This construct was made by three amplification steps (Fig. 1 a–d). In a first PCR reaction primer pair L16310 and H16526 (Table 2) was used to amplify a fragment of 258 bp. Amplification was done in a reaction volume of 25 µl for 25 cycles as described in the previous section, except for the annealing temperature which was decreased to 55 °C. In a second PCR, the forward primer was replaced with a hybrid primer containing 10 nucleotides from the 3′ end of the forward primer in the first PCR and 19 nucleotides downstream starting 10 bases from the 5′ end of the previous forward primer. The reverse primer and the conditions were identical to the first PCR. In a third PCR reaction, the two primers from the first PCR reaction were used together with the diluted PCR fragments from the second PCR. The annealing temperature was decreased to 37 °C in order to allow the short primer sequence overlap (10 nucleotides) to anneal. The resulting molecule with a 10 bp deletion was cloned into pUC18 vector with the SureClone Ligation Kit (Amersham-Pharmacia-Biotech, Uppsala, Sweden), as described by the manufacturer. The concentration of the purified construct (not linearised) was determined with the PicoGreen® dsDNA quantitation kit (Molecular Probes, Leiden, The Netherlands) on the Fluor-Imager™SI (Amersham-Pharmacia-Biotech, Uppsala, Sweden) and λDNA, provided with the kit, was used as a calibration standard. A dilution series of 500 copies/3 µl–25 copies/3 µl (5 dilutions) of the internal control was used for the quantitation of the ancient samples and a series of 1,000 copies/3 µl–500 copies/3 µl (5 dilutions) for

Table 2 Oligonucleotides used for quantitation of the number of amplifiable mtDNA molecules

Primer	Sequence
L16310	5′-CCCACCCTTAACAGTACATAG-3′
H16401-FITC	5′-fluorescein-TGATTTCACGGAGGATGGTG-3′
H16526-FITC	5′-fluorescein-GGAACGTGTGGGCTATTTAGGC-3′
L16339-linker	5′-CAGTACATAGCATTTACCGTACATAGCAC-3′

Table 3 MtDNA D-loop sequence obtained by amplification of long and short fragments of the different DNA extracts of the putative heart of the Louis XVII (*Y* = C/T, *R* = A/G, ? = sequence could not be determined, / = positions outside the amplified PCR fragments, * identical to the Anderson sequence, ** this study, *ra* = reamplified, *p/c* = phenol/chloroform extraction, *MN* = NucleoSpin Funnel Columns, °*blank* PCR control positive, when two nucleotides are written the nucleotide in lower case is present in minor amounts)

Origin of sample		PCR fragments	A16051	A16141	A16162	T16189	C16223	C16256	T16311	PCR fragments	A73	T152	A189	C194	A263	–315.1
Results of Leuven																
Aorta		Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4)	*	Y	/	/	/	/
		Long (2HV1)	/	*	*	*	T	*	Y	Long (1HV2)	R	*	*	*	/	/
										Long (2HV2)	/	/	/	?	G	C
Muscle	Extract 1	Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls5)	*	C	*	Tc	/	/
		Long (2HV1)	/	*	*	C	?	*	*	Long (1HV2)	*	*	*	*	/	/
										Long (2HV2)	/	/	/	?	G	C
	Extract 2	Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls5)	*	C	*	T	/	/
		Long (2HV1)	/	G	*	Ct	?	*	*	Long (1HV2)	*	Ct	Ga	*	/	/
										Long (2HV2)	/	/	/	Y	G	C
	Extract 3	Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls5)	*	C	*	T	/	/
		Long (2HV1)	/	*	*	*	*	?	*	Long (1HV2)	Ag	Ct	*	Y	/	/
										Long (2HV2)	/	/	/	T	G	C
	Extract 4	Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls5)	*	C	*	T	/	/
		Long (2HV1)	/	*	*	*	*	*	*	Long (1HV2)	Ag	Ct	*	Tc	/	/
										Long (2HV2)	/	/	/	T	G	C
Johanna-Gabriela (hair)**		Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls6)	*	C	*	T	/	/
Marie-Antoinette (hair,Nijmegen)**		Short (Ls1, Ls2, Ls3)	/	*	*	*	*	/	*	Short (Ls4, Ls6)	*	C	*	*	/	/
Results of Münster																
Aorta	Extract 1(p/c)	Short [Ms3 (ra)]	/	/	/	/	*	*	*	Short (Ms4, Ms5)	Not determined					
		Long [HV1(ra)]	G	*	Ga	*	*	*	*	Long (HV2)	No PCR product					
	Extract 2 (MN)	Short (Ms1, Ms2, Ms3)	No result[0]							Short (Ms4, Ms5)	?	C	*	T	/	/
		Long (HV1)	No PCR product							Long (HV2)	No PCR product					
Muscle	Extract 3 (p/c)	Short [Ms2, Ms3 (ra)]	/	/	/	*	*	*	*	Short (Ms4, Ms5)	Not determined					
		Long [HV1(ra)]	G	*	Ga	*	*	*	*	Long (HV2)	Ag	*	*	*	G	C
	Extract 4 (MN)	Short (Ms1, Ms2, Ms3)	*	*	/	*	*	*	*	Short (Ms4, Ms5)	*	C	*	T	/	/
		Long (HV1)	No PCR product							Long (HV2)	No PCR product					
	Extract 5 (MN)	Short (Ms1, Ms2, Ms3)	*	*	/	*	*	*	*	Short (Ms4, Ms5)	*	C	*	T	/	/
		Long (HV1)	No PCR product							Long (HV2)	No PCR product					

The sequencing results of the long PCR fragments are listed in the Naundorff study (Jehaes et al. 1998a)

the modern samples. In order to determine the approximate number of template molecules in a DNA extract, a hot start PCR was performed with 3 µl of the dilution series to which a constant amount of the DNA extract (2 µl) was supplied. Amplification was done with AmpliTaq Gold™ polymerase as described in the previous section (hot start PCR, Leuven). The reverse primer was labelled with fluorescein, which allowed us to quantify the resulting PCR products on the ALF DNA-sequencer. Two primer sets (L16310/H16401-FITC and L16310/H16526-FITC; Fig. 2) were selected to determine the amount of amplifiable fragments with different lengths (131 bp and 258 bp).

Results and discussion

Table 3 gives an overview of the mtDNA D-loop sequences obtained in Leuven for the five heart segments by amplifying long and short PCR fragments. Reproducible results were obtained for the short PCR fragments, in contrast to those of the long PCR fragments. Ambiguous sequences were seen in the long PCR fragments at several positions throughout the D-loop. The two long PCR fragments, 1HV2 (270 bp) and 2HV2 (214 bp), have nucleotide position 194 in their overlap. The polymorphism at position 194 was most often unambiguously detectable in the shorter 2HV2 and not in 1HV2, which indicated that we had to amplify short PCR fragments in order to obtain a reproducible authentic sequence. If our results had only been based on the sequences obtained from the long PCR fragments, we would have missed the authentic polymorphisms.

Table 3 gives an overview of the mtDNA D-loop sequences obtained for the five heart segments in Münster. The extractions with phenol-chloroform gave no results in the PCRs in HV1 performed with 30 cycles. Reamplification with the same primers of 1 µl PCR product in 25 µl PCR reaction mix gave positive but not reproducible results (contamination with the sequence of the performing technician). The amplification of the HV2 region (long fragment) did not give a reproducible product. With a more sensitive method (Toprak and Brinkmann unpublished results), using the NucleoSpin Funnel Columns (Machery-Nagel, Düren, Germany), DNA was successfully extracted from all tissues. The amplification of HV1 and HV2 with the long primers gave no result but with the short fragment primers, reproducible PCR products were obtained from all extractions. The NucleoSpin Funnel Columns allow the lysis of the tissue in a high amount of buffer and concentration of the DNA in a small volume of extract with a low risk of contamination. A smaller number of PCR cycles are needed for amplification and the risk of unwanted amplification of low copy contaminants is lower.

Quantitation results (Jehaes et al. 2000) indicated that in general much more DNA was obtained for the short mitochondrial fragments (131 bp) compared to long mito-

Table 4 Quantitation of mtDNA fragments in the DNA extracts of modern hairs (freshly plucked or not older than 6 months) and aged hairs of several individuals

Individual	Year	Number of extractions	Number of copies/µl DNA extract (± SD)	
			131 bp	258 bp
Forensic case 1	2000	1	598 (± 138)	1566 (± 662)
Forensic case 2	2000	1	240 (± 56)	400 (± 127)
Forensic case 3	2000	1	387 (± 17)	377 (± 70)
Individual 1	2000	1	246 (± 78)	250 (± 54)
Individual 2	2000	1	43 (± 6)	149 (± 20)
Individual 3	2000	1	218 (± 4)	591 (± 45)
Individual 4	2000	1	48 (± 19)	106 (± 39)
Individual 5	2000	1	32 (± 8)	131 (± 48)
Forensic case 4	1995	6	146 (± 39)	23 (± 19)
			180 (± 51)	75 (± 38)
			62 (± 22)	21 (± 6)
			167 (± 23)	93 (± 39)
			96 (± 32)	30 (± 13)
			27 (± 12)	15 (± 4)
Individual 2	1994	2	146 (± 36)	192 (± 82)
			55 (± 10)	53 (± 16)
Individual 5	1994	1	10 (± 3)	59 (± 25)
Forensic case 5	1983	6	38 (± 22)	2 (± 1)
			677 (± 135)	266 (± 85)
			131 (± 45)	30 (± 11)
			127 (± 34)	57 (± 16)
			271 (± 82)	46 (± 7)
			417 (± 69)	87 (± 29)
Individual 3	1975	2	3.5 (± 1)	10 (± 3)
			11 (± 6)	7 (± 4)
Individual 4	1966	2	0	0
			1 (± 1)	1 (± 1)

chondrial fragments (258 bp). Since the heart DNA was degraded into small fragments it is not surprising that amplification of long fragments was problematic. Jumping PCR can help to amplify longer fragments than are actually present in the extract (Pääbo et al. 1990). However, as seen in our results, if contamination occurs, jumping PCR will complicate the interpretation of the results. In two of the five heart DNA extracts, ambiguous sequences were obtained for the short PCR fragments, which indicated the presence of a minor contamination. In the quantitation results, however, no indication was present for a major modern DNA contamination. In contrast, the quantitation results indicated that authentic DNA was present because the amounts of long mitochondrial molecules was dramatically lower than the amounts of short molecules. Therefore, probably very small amounts of contamination are preferentially amplified when long PCR fragments are used. The construction of a mosaic sequence via jumping PCR will probably be at random each time since no reproducible results were obtained with the long PCR fragments.

In the Naundorff study (Jehaes et al. 1998a), where we analysed the maternal relatives of Louis XVII, two nucleotide differences in HV2 where seen between the living maternal relatives and the three daughters of Maria-Theresia. Subsequent analyses of short PCR fragments (Jehaes et al. 2000; Table 3) in new hair DNA extracts revealed that we most probably missed these two nucleotide differences. We even had to shorten the fragment encompassing position 194 (from 201 to 117 nucleotides) in order to reveal the underlying polymorphism.

While the problem of DNA degradation could have been predicted for the more than 200-year-old heart of Louis XVII, it could also complicate the analysis of old forensic samples. Shed hairs are often found at the scene of the crime and it might help in identifying the person who committed the crime. The number of mtDNA copies with a length of 131 bp and 258 bp were quantified in DNA extracts of 2 cm hair shafts (end of head hair) of different ages (Table 4). In the DNA extracts of hairs not more than 1 year old, either more long than short fragments were found or approximately the same proportion. Control blood samples showed similar results (data not shown). These results might indicate that there was a selection for longer DNA fragments during DNA isolation. However, the methods used for extracting DNA from blood (chelex) or hair samples (concentration of the DNA on Microcon 100) exclude this possibility. Inspection of the mtDNA sequence in the neighbourhood of the primer binding site for L16401-FITC revealed downstream the presence of a guanine tract (16388–16391) which could form a hairpin structure with a cytosine tract (16375–16380). The formation of this hairpin could decrease the binding efficiency of the primer L16401-FITC 9 bases upstream of the guanine tract and/or the binding of the Taq DNA polymerase. As a result, the efficiency of amplification for the short PCR fragment is reduced in contrast to the long PCR fragment where the Taq DNA polymerase could overcome the hairpin structure. Therefore, the amount of amplifiable short fragments is most probably an underestimation of the real amount, which will not affect our final conclusions.

In the DNA extracts of the older hair shafts (from 1983 to 1995), the proportion of the number of copies of 131 bp to the number of copies of 258 bp is on average 4 in contrast to the most recent hair shafts. The number of amplifiable mtDNA copies for 131 bp and 285 bp for the hairs from 1975 and older (including the quantitation results of the new DNA extracts of Johanna Gabriela and Marie-Antoinette, data not shown) were too small to show a clear difference. This additional experiment indicates that DNA degradation can start quite early in shed hair shafts. The presence of DNA degradation in hair shafts might also explain the lower success rate for mtDNA analysis reported for hair shafts (Wilson et al. 1995; Pfeiffer et al. 1999b; Dimo-Simonin et al. 2000). Amplification of the two hypervariable segments (400 bp; HV1 and HV2) or even the complete control region (1300 bp) might fail when mtDNA is too degraded. However, the presence of even a minor contamination with modern DNA, might lead to a result not representative for the hair shaft. Therefore, special attention needs to be given to the genetic identification of older forensic samples performed with long PCR fragments (> 210 nucleotides). A competitive PCR assay as described here should be helpful to determine the state of degradation of the mtDNA and provide evidence for the reliability of the applied method for PCR and sequencing. Use of short PCR fragments (< 200 bp) in all forensic cases should improve mtDNA analysis of forensic hair samples considerably and increase the success rate of analysis.

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