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Mitochondrial DNA Sequences of the Famous Karl Wilhelm Naundorff (1785 ?- 1845)

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Abstract: We have obtained mitochondrial DNA (mtDNA) sequences from Karl Wilhelm Naundorff's hair, who pretended to be the son of King Louis XVI (1754-1793) and Queen Marie-Antoinette (1755-1793). Authenticity of the hair is established by optic and electronic microscopy. Sequences of hypervariable regions of the mitochondrial DNA (extracted from two different hairs) show five mutations: 16298*C*; 72*C*, 152*C*, 195*C* and 263*G*; the corresponding mtDNA haplogroup is the sub-haplogroup **HVO**. Consequently, Naundorff cannot be excluded to be considered as being Louis XVII on the basis of mtDNA sequence of his humerus (as affirmed in Jehaes *et al*., 1998). Comparisons of mtDNA sequences between mtDNA extracted from Naundorff's hair reported here and those (Jehaes et *al*., 2001) of the living Anna of Roumania (of Habsburg descent) shows that both correspond to mtDNA haplogroups of the general **HV** cluster.

Keywords: Mitochondrial DNA haplogroups ; Karl Wilhelm Naundorff ; mutations in the hypervariable regions

INTRODUCTION

In 1793, during the French Revolution, the King of France Louis XVI (1754-1793), and the Queen Marie–Antoinette (1755-1793) were beheaded. Their children, Marie-Thérèse Charlotte (1778-1851) and Louis-Charles (1785-1795 ?), remained imprisoned in the Temple (in Paris) where they survived to the death of their parents. According to the official records Louis-Charles, who was proclaimed as the King of France Louis XVII immediately after the death of his father, died of tuberculosis in the Temple on 8 June 1795. But since then, the official version of his death has been repeatedly questioned ; one of the most persistent theories claims that it was a substitute who died on 8 June.

Just after the official death of Louis XVII, some individuals claimed to be Louis XVII. The most famous of them, Karl Wilhelm Naundorff (1785 ?-1845), came in Paris in 1833. He could apparently provide sufficient circumstantial evidence to convince ex-members of the courts of Versailles and Tuileries of his descent. Naundorff was deported to England by the French authorities in 1836 ; he died in 1945 in Delft (the Netherlands), where he was buried under the name of "Louis XVII, Roi de France et de Navarre, né à Versailles le 27 mars 1785, décédé à Delft le 10 août 1845".

About fifteen years ago, Jehaes et *al* (**1**) excluded Naundorff as the son of Marie-Antoinette on the basis of mitochondrial DNA (mtDNA) sequences of his remains (an assumed bone from Naundorff, 1950's exhumation, preserved in a jar that was never sealed because it was used by Pr Froeutjes as a "curiosity" and so far as "educational materials"), compared to those (all of the Habsburg type) obtained from the hair of two sisters of Marie-Antoinette, Johanna-Gabriela (1750-1762) and Maria-Josepha (1751-1767), from Marie-Antoinette herself, and to the sequences obtained from DNA samples of two living maternal relatives (Anna of Roumania and André de Bourbon Parme). In the present study we compare mtDNA sequences we obtain from authentic Naundorff's hair to those already published (**1,2**).

MATERIAL AND METHODS

The hairs

One of us (C.C.) furnished the material : a large envelope, containing a mean-sized envelope (with inside two locks of Naundorff's wig) and a little envelope containing his own hair. Inscriptions (in french) on the little envelope show (Figure 1 above) that the last owner (Anna Thomas) indicates the origin of these hairs : « Les cheveux, contenus dans cette enveloppe, sont ceux que j'ai coupé (ôtés) de ma propre main, de la tête de feu le prince Charles Louis de Bourbon, duc de Normandie. –Delft le 13 août 1845. Jan Soutendam, Med. Doctor. » Soutendam was effectively one of the three (with Snabilié and Kloppert) Medical Doctors who has

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examined two days before (the 10th of August) Naundorff's corpse, who passed away at this date.

On the front of the little envelope (not shown) an other inscription indicates that the antepenult owner was Otto Friedrichs, one of the best specialist in the past of Louis XVII and Naundorff.

The lock (Figure 1 below) comprises about one hundred hairs, varying in colour from white to darker. Five of them (numbers 1 to 5) were chosen for further analysis.

Microscopy

These five hairs were examined in confocal stereoscopic micrography, and by SEM-EDX (Philips XL30 model environmental version) ; probe Bruker AXS energy dispersive X-ray, PGT system analysis (Spirit Model, Princeton gamma technology).

DNA extraction

All of the molecular analyses were realized according to the methodology recommended in our previous study **(3)**concerning ancient DNA (a-DNA). Genomic DNA was extracted from the bulb of hair number 2 using a standard method (0.5 M EDTA, sarcosyl 20% and proteinase K 10mg/ml), and purified using a commercial kit (NucleosSpin $^{+}$ Kit ;
Macherey-Nagel, Duren, Germany), in accordance with the manufacturer's instructions (with some modifications).

Amplification of the hypervariable regions of the mtDNA

The mtDNA genomic sequence intervals for *HVR1* and *HVR2*(Hypervariable Regions 1 and 2) were amplified by PCR with primers F15971 and R16410, and with primers L15 and H484, respectively. For each PCR, the DNA extract for hair specimen was amplified in a 12.5 µl reaction mixture : 2mM MgCl2, 50 mM KCl, 10mM Tris / HCl pH 9, 0.1%Triton X-100, 0.2 mM each dNTP, 0.1 µM each primer, and 2.5 U of DNA polymerase (Ampli Taq Gold ; Applied Biosystems, Foster City, CA, USA). The amplification was carriedout with an initial denaturation step at 95°C for 6 min, followed by 35 cycles 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

HV1 and HV2 DNA sequences

PCR products were purified from agarose gel (QIA-Quick PCR Purification KIT ; Qiagen, Valencia, CA, USA). Both stands and all the amplified mtDNA fragments eluted from agarose gel slides were directly sequenced (Big Dye Terminator Cycle Sequencing Kit ; Applied Biosystems) and separated (ABI PRISM 3130 Genetic Analyzer ; Applied Biosystems).

The sequences obtained were aligned against the Revised Cambridge Reference Sequence (**4**) to identify the presence of polymorphic sites. Seqscape software (Applied Biosystems) and Clustal analysis (http://www.clustal.org) were used for pairwise alignment.

RESULTS

Table 1 summarizes the main microscopy characteristics of Naundorff's five hairs studied. Their colour are blond, brown-red or browner. Figure 2 shows an example of a SEM-EDX analysis of one of these hairs (hair number 1) : scales are well visible on the SEM photography. Very few impurity particles are seen on their surfaces, so the corresponding hairs were washed and cleaned.
The mean thickness of these five hairs = 46.6 microns (26-57). Hair number 2 is particularly fine.

Figure 3 shows a main characteristic of Naundorff's hair, signaled more than fifty years ago (**5**) : the excentricity of the medular canal, a relatively rare abnormality present in some sort of hair (when the trichogram is studied in high-resolution microscopy). All the five hairs (but hair number 2, too fine) show this abnormality.

All the five hairs studied, even the most cleaned, present some dandruff at their surfaces (Figure 4) ; the dandruff coverage is the most intense for hair number 2. Contrary to ordinary desquamed skin cells, dandruffs are aggregates of hundred or thousands (depending on size) corneocytes, with a high percentage of residual nuclei within the cells (**6**).

Figure 5 shows the bulb of hair number 2. The surface of this bulb is peculiarly rich in organic matter ; this part was chosen first for DNA extraction experiments.

Results on mtDNA HVR1 and HVR2 sequences

An approximate quantity of 20 ng of genomic DNA was obtained fromhair number 2 bulb. Preliminary experiments concerning the amelogenin gene (**7**) showed that the corresponding individual is a XY male.

We obtained DNA sequence (from 16025 to 16355 and from 67 to 299, respectively) of the *HVR1* (16032-16352) and *HVR2* (72-294) segments of the mtDNA extracted from the bulb of hair number 2. One mutation only (16298*C*) is present in the *HVR1* sequence ; but there are four mutations (72*C*, 152*C*, 195*C* and 263*G*) in the *HVR2* sequence. The same results were obtained in a replication study concerning mtDNA extracted from hair number 3.

In the modern european system of nomenclature (**8**), the 16298*C*, 72*C*, 152*C*, 195*C*, 263*G* combination corresponds to the mtDNA sub-haplogroup **HVO**.

DISCUSSION

It is on the basis of comparisons of consensus *HVR1* and *HVR2* mtDNA sequences (Table 2) between Naundorff's humerus and those of other Habsburg samples known at this time that Jehaes *et al.* (**1**) excluded the possibility that Naundorff could be considered as being Louis XVII. The Habsburg samples studied (and particularly Anna of Roumania) do not have any mutations in the *HVR1* sequence, sothey can belong to the vast mtDNA paragroup **H***, and Naundorff's osseous sample having at least the 16250*T* mutation in it, was so clearly excluded from any Habsburg's ancestry.

But the authors (**1**) discussed at length about difficulties they had to obtain reproducible mtDNA sequences from Naundorff's humerus (a right humerus ? removed from the coffin during the restoration of his burial place in 1950 in Delft). In our own experiments, based on reproducible results obtained on mtDNA extracted from two authentic Naundorff's hairs, we found a 16298*C* mutation in the *HVR1* sequences but not the 16260*T* variant.

If analogous for the 263*G* mutation of *HVR1* (and no mutation at the 194 site), Naundorff's mtDNA sequence extracted from the humerus is different to that from the hairs for the 72*C*, 152*C* and 195*C* mutations. It results that the mtDNA sequences are clearly different between the samples of the bone and of the hair.

The 16298*C* mutation of *HVR1* for the hair samples, by itself, justify its assignment to the mtDNA **HVO** sub-haplogroup. Compared to the sequences obtained previously for all the Habsburg individuals, *HVR2*sequences from Naundorff's hair are identical for the 263*G* and the 152*C* mutations, but the 72*C* mutation is not found in Habsburgs. The 194 site, not mutated in the samples from Naundorff's hair, is variable among the Habsburg samples : The 194*T* mutation is present in the sample extracted from the putative heart of Louis XVII and those from Johanna-Gabriella and from Anna of Roumania, but Marie-Antoinette has the 194*C* mutation. The 195*C* mutation, present in Naundorff's hair, is absent in Habsburgs.

The differential *HVR2* pattern of variation observed here in mtDNA sequences between Naundorff's hair and Habsburg samples seems in accordance to the diversity reported in modern populations of southern Germany (**9**), where 40% of individuals belong to the general **HV** cluster. Among them two individuals were assigned to the sub-haplogroup **HVO**. In this database of **HV** (mtDNA *HVR1* and *HVR2*) profiles, the 263*G* mutation is ubiquist and the 152*C* mutation is found in two **H***, in three **H5** and in one **H6** individuals (but the 194*C* mutation was not found in the database).

The published (**1**) profiles of both the two living Anna of Roumania (on blood sample) and André de Bourbon Parme (on hair sample) are sure, because confirmed on two different samples by two independent laboratories ; but there are seven generations of female mtDNA transmission between Marie-Antoinette and Anna of Roumania and André de Bourbon Parme, with non-negligibility that rare events of mutations happened between them.

Jehaes *et al* (**1**) relates at length in their publication the difficulties they had to obtain reproducible results concerning Marie-Antoinette (Louis XVII's mother) *HVR2* sequences at the 73, 143, 146, 195 and 199 sites, and for the 152*C*, 194*T* and 263*G* mutations. It is the reason why we are now working on Angoulême Countess' authentic hair (Louis XVII's sister), in order to clarify the important point of the reproducibility of results obtained from hair contemporary to those of Naundorff and Louis XVII.

CONCLUSION

Results reported in the present study concerning mtDNA *HVR1* and *HVR2* sequencesobtained from Naundorff's authentic hair establish that : 1/ Five mutations were obtained (16298*C* in *HVR1*; 72*C*, 152*C*, 195*C*, and 263*G* in *HVR2*) ; the corresponding mtDNA sub-haplogroup is **HVO**. 2/ This haplogroup is clearly different to that previously published (**1**) for a Naundorff's osseous sample ; so the affirmation of the exclusion ordered in (**1**) that Naundorff cannot be LouisXVII, based on this crireria, cannot be maintained. 3/ The comparison between Naundorff's hair mtDNA sequences and those (**2**) of Anna of Roumania (a living relative belonging to the maternal Habsburg lineage) shows that both correspond to related mtDNA sub-haplogroups inside to the general **HV** cluster.

List of abbreviations

a-DNA : ancient DNA ; mtDNA : mitochondrial DNA ; *HVR1* : HyperVariable Region1 of the mtDNA ; *HVR2* : HyperVariable Region 2 of the mtDNA ; **H** : the most common mtDNA haplogroup ; **H*** : the **H** paragroup ; **HVO** : an **H** sub-haplogroup ; **HV** : the general **H** cluster ; PCR : Polymerase Chain Reaction ; SEM-EDX : Scanning Electronic Microscopy – Energy Dispersive X-rays.

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historian B. Roy-Henry was one of those that are at the origin of the project. This study was included in our program of genetic investigations developed at the Institute of Molecular Anthropology concerning royal families in France.

Additional Information : The *HVR1* and *HVR2* mtDNA sequences of Naundorff's hairs are available on demand at : lucotte@hotmail.com

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Table 1. Microscopic characterizations of the five hairs

Numbers	Colour	Thickness (η) at the basis	Excentricity of the medular canal	Dandruff	Bulb
1	brown	56	visible in transversal sections	+	-
2	brown	26	not visible	numerous	+
3	blond	57	+	++	?
4	brown-red	48	+	+++	_
5	blond	46	+	+	-

Table 2. Consensus mtDNA HVR1 and HVR2 sequences obtained from different samples (stars indicate sites in conformity to Anderson sequence).

Names	**Samples**	**HVR1**		**HVR2**					**References**
		16260	16298	72	152	194	195	263	
Naundorff	humerus[1]	*T*	*	*	*	*	*	*G*	**1**
Naundorff	hair 2	*	*C*	*C*	*C*	*	*C*	*G*	Present study
Naundorff	hair 3	*	*C*	*C*	*C*	*	*C*	*G*	Present study
Louis XVII?	heart	*	*	*	*C*	*T*	*	*G*	**2**
Johanna	hairs[2]	*	*	*	*C*	*T*	*	*G*	**2**
Marie-Antoinette	hairs[3]	*	*	*	*C*	*C*	*	*G*	**2**
Anna	blood	*	*	*	*C*	*T*	*	*G*	**1**

[1] The sample sequenced at Nantes (**1**), the most reliable.
[2] New DNA extracts reanalysed in (**2**).
[3] The Nijmegen sample, new DNA extracts reanalysed in (**2**).