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# Genetic comparison of the head of Henri IV and the presumptive blood from Louis XVI (both Kings of France)

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ABSTRACT

A mummified head was identified in 2010 as belonging to Henri IV, King of France. A putative blood sample from the King Louis XVI preserved into a pyrographically decorated gourd was analyzed in 2011. Both kings are in a direct male-line descent, separated by seven generations. We have retrieved the hypervariable region 1 of the mitochondrial DNA as well as a partial Y-chromosome profile from Henri IV. Five STR loci match the alleles found in Louis XVI, while another locus shows an allele that is just one mutation step apart. Taking into consideration that the partial Y-chromosome profile is extremely rare in modern human databases, we concluded that both males could be paternally related. The likelihood ratio of the two samples belonging to males separated by seven generations (as opposed to unrelated males) was estimated as 246.3, with a 95% confidence interval between 44.2 and 9729. Historically speaking, this forensic DNA data would confirm the identity of the previous Louis XVI sample, and give another positive argument for the authenticity of the head of Henri IV.



## 1. Introduction

In 2010, a mummified head was identified as belonging to the French King Henri IV, according to 22 scientific and historical arguments (Fig. 1) [1]. Since this date, as frequently seen for such identification of famous remains causing media excitement and professional criticism, few counter-arguments were given by some researchers [2]: especially, the absence of skull vault opening (presented as frequently carried out during the French royal embalming process, but not systematical, and attested on a text by Alexandre Lenoir describing the exhumation of the Henri IV body in 1793), and the absence of DNA comparison with further body samples (hairs, for example). Such elements were refuted in subsequent publications [3–5]: the text by Alexandre Lenoir (describing a skull cut with a saw in the case of the Henri IV body) is in fact largely posterior to the exhumation (published in 1801, for an event of 1793); besides, an analysis of the original texts written by direct and objective witnesses of the graves' profanations (Dom Druon, Dom Laforcade, Latreilhe and Kohler) do not mention an opened skull for Henri IV (this detail being judged as a latter adjunction to the original text by Claude Tinthouin, associated with some obvious errors and aproximations). Moreover, as stated by the responsible of the Medici project, there is no evidence of craniotomy before the Grand Ducal Branch (1537) and many exceptions later [6], testifying of a frequent practice of non-skull opening for Florentine, but also French kings' embalming (an explanation for the intact skull of Henri IV, married to Marie de Medici).

Since the publication of a genetic analysis of a putative sample of the blood of the French King Louis XVI in 2011 [7], we hypothesize that the DNA comparison of this sample with that of the Henri IV head would be of double interest: adding an additional argument for the identification of the head and also confirming the identity of the blood.

## 2. Materials and methods

### 2.1. DNA Extraction and amplification

A 200 mg tissue sample was taken from the inner part of the putative Henri IV head during a fiberscopy through the trachea undertaken in 2010 (Fig. 2). The sample was digested overnight at 50 °C in a lysis solution composed of 0.5% SDS, 50 mM TRIS, and 1 mg/mL of proteinase K in $H_2O$. Afterwards, the DNA was extracted three times with phenol, phenol–chloroform and chloroform–isoamilic and concentrated using an Amicon Ultra centrifugal filter (Millipore). Finally, the

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**Fig. 1.** General aspect of the mummified head of the French King Henri IV.

extract was purified with a silica-extraction kit (Fermentas) and eluted to 30 μl volume. Standard precautions designed for controlling contamination with modern DNA, including a physically isolated DNA extraction room with positive-air pressure and overnight UV lights, sterile lab gear (coveralls, face mask, face shield and gloves), inclusion of two blank controls for each amplification and genotyping of researchers involved in the laboratory analyses were adopted during the experimental procedures [8].

The mitochondrial DNA (mtDNA) hypervariable region 1 (HVR1) was amplified by polymerase chain reaction (PCR) in two overlapping fragments with the L16,055-H16,218 and L16,185-H16,378 primers (numbered according to the Cambridge Reference Sequence), along with extraction and negative controls to monitor against possible contamination. The amplification was based in a two-step amplification protocol, as described in Lalueza-Fox et al. [9]. The reaction was performed in a total 20 μl volume containing: 5 μl of DNA extract, 1X PCR buffer, 2.5 mM $MgCl_2$, 500 mM of each dNTP, 2 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), 150 nM of each primer in the first multiplex step and 1.5 μM of each primer in the second step. The annealing temperature used was 50 °C. The PCR products were visualized in a 1% low-melting point agarose gel and purified after being excised from the gel with a silica-binding method. Subsequently, the purified PCR products were cloned into bacteria using TOPO-TA cloning kit (Invitrogen); inserts of the expected size were sequenced in a ABI3730 capillary sequencer (Applied Biosystems) following manufacturer's instructions. Thirty-two clones were generated for the mtDNA HVR1.

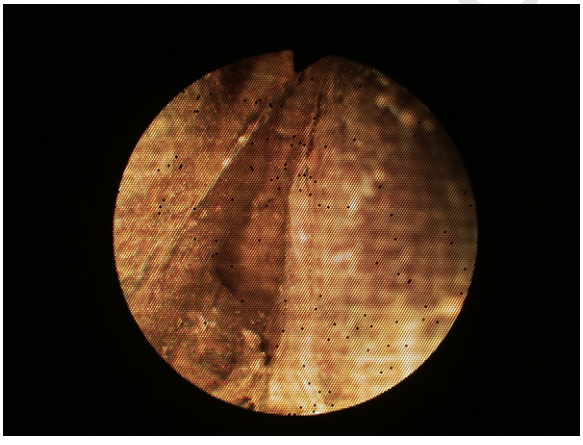

**Fig. 2.** Exact site of the sampling for these genetic analyses (fiberscopy view into the trachea).

### 2.2. Y-STR loci

A Y-STR genotype was performed on 5 μl from the extract by generating a 17 loci Y-STR profile (DYS19, DYS189I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385I/II, DYS438, DYS439, DYS437, DYS448, DYS456, DYS458, DYS635 and Y GATA H4) with the AmpFlSTR Yfiler™ PCR amplification kit (Applied Biosystems, Foster City, CA), following manufacture's instructions. All Y-STR amplification products were analyzed on an ABI PRISM 3100 Genetic Analyzer machine (Applied Biosystems). The size of each fragment was calculated automatically with the GeneMapper software version 3.2 and the alleles assigned by comparison to an internal size ladder included in the Y-filer kit. Haplotype matches were sought for in the YHRD database rel. 40 (www.yhrd.org). The likelihood ratio of a match between Henri IV and Louis XVI, which are separated by seven generations (Fig. 3), and carrying haplotypes that are different by one repeat unit at one locus, was estimated as $LR = (7m(1-m)^6)/(1/p)$, where $m$ is the mutation rate of any mismatched locus (as reported in YHRD), and $p$ is the frequency of the partial haplotype, again in YHRD. We considered that to generate a one-step difference in seven meioses, mutation may have happened in any one of the seven meioses, but that the allele must have been transmitted faithfully in the remaining six. Other possible pathways involving a higher number of forward and backward mutations have been not taken into account as their probability would be proportional to $m^3$, and, thus, much less likely. A partial confidence interval on the LR was estimated by using the 95% confidence of the haplotype frequency provided by YHRD.

## 3. Results

### 3.1. mtDNA

The majority of the clones generated show an U5b* mtDNA haplotype defined by three nucleotide changes at positions 16239T 16270T 16311C (see Supplementary material). The three HVR1 diagnostic positions were confirmed in two different amplifications of the L16185-H16378 HVR1 fragment, proving that the results are reproducible. This mtDNA haplotype is present so far in one single individual from France (originally published in [10]) in an in-house database of 22,807 published European sequences, and it is absent in all people involved in the laboratory analysis. Additional clones showed no consistent nucleotide substitutions (e.g. clones with singletons attributable to postmortem damage), suggesting that there is some diffuse background contamination in the sample. This certain heterogeneity is consistent with previous, failed attempts to retrieve mtDNA sequences from the head of Henri IV [1], although in our case the obtained mtDNA haplotype is phylogenetically consistent and reproducible.

In addition, neither the mtDNA haplogroups (one CRS and two T2) nor the specific nucleotides substitutions of the three people involved in the laboratory analyses are coincident with the sequence obtained from the mummified tissue.

### 3.2. Y-chromosome

A partial Y-STR profile with 6 loci was obtained for the Henri IV sample (Table 1), which can be attributed to the degradation of the original DNA. A second genotyping attempt yielded only three loci amplifications that nevertheless were coincident with the previous ones. The lack of multiple peaks indicates that there was only one majoritary profile in the extract, as opposed to what would be expected if the sample would be heavily contaminated with exogenous DNA. None of the researchers involved in the DNA extraction had an identical profile; the person who did the Y-STR analysis is a female. Allelic dropouts are a major concern while applying the Yfiler™ ~~filerc drop~~PCR amplification kits to highly degraded genetic material; while we do not have enough evidence to ascertain how important this problem can be, the fact that the tissue's Y-STR profile is partially reproducible and that this includes the rare (18) long DYS385 allele is suggestive of a non-artifactual profile.



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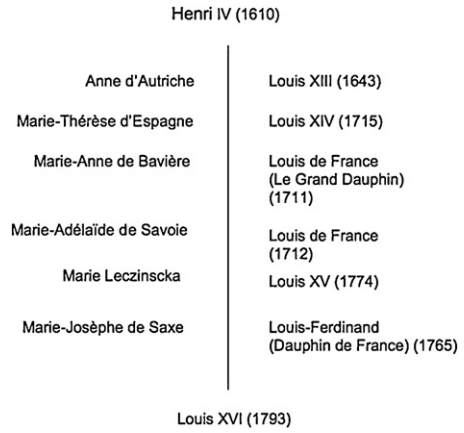


**Fig. 3.** Simplified genealogic tree from Henri IV to Louis XVI.

**Table 1**
Y-STR profile for the putative Louis XVI sample [7] and the partial Y-STR profile of Henri IV mummified head in two different Y-filer tests.

| Marker | Louis XVI | Henri IV (1st test) | Henri IV (2nd test) |
|---|---|---|---|
| DYS389I | 12 | 13 | 13 |
| DYS389II | 30 | – | – |
| DYS390 | 22 | – | – |
| DYS456 | 15 | – | – |
| DYS19 | 15 | – | – |
| DYS385 | 13, 18 | –, 18 | –, 18 |
| DYS458 | 21 | – | – |
| DYS437 | 15 | 15 | –15 |
| DYS438 | 10 | – | – |
| DYS448 | 21 | 21 | – |
| YGATAH4 | 12 | – | – |
| DYS391 | 10 | 10 | – |
| DYS392 | 11 | – | – |
| DYS393 | 14 | 14 | – |
| DYS439 | 12 | – | – |
| DYS635 | 21 | – | – |

The loci that gave positive results were: DYS389I, DYS385 (only the long allele), DYS437, DYS448, DYS391 and DYS393. Interestingly, all show a complete identity with the previously published putative Louis XVI profile, except for the first marker that shows allele 13 instead of 12 (obtained in two independent Y-filer tests) (Table 1). This discrepant allele is nevertheless coherent with a mutation event within a family line that usually involves gaining or loosing one single STR copy. The matching portion of the haplotype was found in only one of 14,158 Europeans ($p = 7.06 \times 10^{-5}$). Given that the mutation rate of DYS389I per generation is $m = 2.523 \times 10^{-3}$ the likelihood ratio of the two samples belonging to males separated by seven generations (as opposed to unrelated males) was estimated as 246.3 (see Section 2), with a 95% confidence interval of (44.2–9729).

## 4. Discussion

Although a previous analysis failed to retrieve DNA from the mummified head of Henri IV [1], the reproducible mtDNA sequences and the partial Y-chromosome profile obtained in the current study suggest that the specimen contains endogenous DNA. At present we cannot explain the discrepancy in the success between both studies, although it could be attributed to endogenous differences in the two samples. The fact that the sample from this study was taken from the inside of the head instead of the neck [1], could help explain this discrepancy.

Some physical traits of the king such as auburn hair and blue eyes (the latter trait is depicted differently in several portraits) had to be associated to already described alleles in particular genes such as MC1R and HERC2 respectively. However, attempts to retrieve diagnostic SNPS in these two nuclear genes by PCR have so far yielded no amplification, probably due to the degradation of the endogenous DNA. Therefore, we cannot rely on any phenotypical identification from the genetic analysis.

Even if partial, the Y-chromosome profile is of interest because of the previously published Louis XVI haplotype [7]. Thanks to the fact that some of the alleles in the retrieved loci (such as the DYS385 long allele) have very low frequencies in the general European populations, and also the extremely rare presence of the alleles found in the partial Y-STR profile, it is likely that Henri IV's sample and the putative Louis XVI blood sample are paternally related. In the current Y chromosome haplotype reference database (YHRD) [11], the partial Henri IV profile has no matches among 40,988 individuals worldwide or among 16,734 Eurasians. If instead of the 13 allele at locus DYS389I, we search the partial profile with the Louis XVI 12 allele at this locus, we only obtain one single match in the whole YHRD database (i.e. 1 in 40,988 individuals worldwide), corresponding to an Italian male from Marche [11].

Thus, this genetic analysis provides further support to the authenticity of the blood kept into a decorated gourd and attributed to the king Louis XVI (and yet another argument for the authenticity of the mummified head of Henri IV [1]).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2012.11.018.

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