ARTICLE

Genetic genealogy reveals true Y haplogroup of House of Bourbon contradicting recent identification of the presumed remains of two French Kings

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Genetic analysis strongly increases the opportunity to identify skeletal remains or other biological samples from historical figures. However, validation of this identification is essential and should be done by DNA typing of living relatives. Based on the similarity of a limited set of Y-STRs, a blood sample and a head were recently identified as those belonging respectively to King Louis XVI and his paternal ancestor King Henry IV. Here, we collected DNA samples from three living males of the House of Bourbon to validate the since then controversial identification of these remains. The three living relatives revealed the Bourbon's Y-chromosomal variant on a high phylogenetic resolution for several members of the lineage between Henry IV and Louis XVI. This 'true' Bourbon's variant is different from the published Y-STR profiles of the blood as well as of the head. The earlier identifications of these samples can therefore not be validated. Moreover, matrilineal genealogical data revealed that the published mtDNA sequence of the head was also different from the one of a series of relatives. This therefore leads to the conclusion that the analyzed samples were not from the French kings. Our study once again demonstrated that in order to realize an accurate genetic identification of historical remains DNA typing of living persons, who are paternally or maternally related with the presumed donor of the samples, is required.

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INTRODUCTION

As all individuals are genetically different, modern DNA technology has increased the ability to perform human identity testing. Individual genetic identification is important in the determination of perpetrators of violent crimes, resolving uncertain paternity or maternity and identifying remains of missing persons or victims of mass disasters.¹ Next to forensic cases, genetic identification also strongly increases the opportunity to identify skeletal remains or other biological samples associated with historical figure.² In this way, the genetic analysis can give insight in several historical and archaeological research questions. The genetic approach was successful, for example, to check the verity of the death of the legendary outlaw Jesse James³ or Louis XVII, the son of Louis XVI and Marie-Antoinette, who died during the French Revolution.^{4,5} It also gave certainty about remains found at the burial place of the Romanov family members,^{6,7} about the biological relationship within the family of Austria's patron Saint Leopold III⁸ and about the reliability and commercial value of religious relics during the Middle Ages.^{9,10}

The main drawback of the genetic identification of presumptive remains from historical figures is that the DNA within these samples is often degraded and that DNA contamination can mask the original DNA of the person.^{11,12} When the person and his or her close relatives

died a long time ago, good quality DNA as reference material is usually not available, whereas this is rarely the case in forensic cases. Nevertheless, validation of the data remains essential to confirm the results of the genetic identification. For many of these studies, due to a lack of samples of relatives, only 'sub-optimal' validations can be performed, which can be very valuable but which are insufficient to confirm the identity. Sub-optimal validations can also be used to exclude identification and are feasible by several methods. One method is genetic sexing of the remains as done for the wrongly assumed remains of the Polish renaissance poet Jan Kochanowski.¹³ Another method is comparing genetic markers for several parts of the remains, because sometimes relics of several persons are mixed; this was found in the remains assumed to belong to the Italian poet Francesco Petrarca.¹⁴ Comparing the familial relationship between skeletons based on historical records is a third method that was successfully applied for Prince Branciforte Barresi and his family.¹⁵ The last method consists of determining the geographic region of origin of an individual based on the mtDNA and/or Y-chromosome (Y-chr) haplogroup as performed for the relics of Evangelist Luke.¹⁶

Optimal validations that guarantee the identification of historical remains are possible using different approaches. One such approach consists of performing genetic tests on different authenticated objects or samples from the same individual or from a direct relative but with

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a completely independent origin of the samples. This was done successfully for the identification of the Polish astronomer Nicolaus Copernicus.¹⁷ The most convincing validation to identify the donor of particular remains is the analysis of the DNA of living relatives of the presumptive donor. This will also detect DNA contamination. Because these relatives are often several generations away from the individual in question, only markers on the mtDNA or Y-chr may be studied, thanks to their haploid mode of inheritance.² This haploid mode means that any living maternal or paternal relative of the individual in question should have an identical mtDNA or Y-chromosomal type with the sample to be identified.¹⁸ This has been successfully realized in the identification of the grave of the Nazi official, Martin Bormann.¹⁹

A recent study reported the genetic identification of the remains of two kings of France by comparing their Y-STR profiles. One of these samples is a presumptive blood sample of King Louis XVI (1754–1793), who died on the guillotine during the French Revolution. After the execution, many spectators apparently soaked their handkerchief in the blood of the king. One of the many handkerchiefs so collected was apparently kept in a pyrographically decorated gourd, which is now in the possession of an Italian family. Careful examination confirmed that the gourd indeed contained a handkerchief with traces of human blood powder.²⁰ DNA analyses revealed

a 17 Y-STR profile and the confirmation of the Y-chromosomal haplogroup G(xG1,G2).²⁰ The other sample is a mummified head attributed to King Henri IV (1553–1610), the first king of France from the House of Bourbon and a direct paternal ancestor of Louis XVI (Figure 1). During the French Revolution, the mummified body of Henri IV was excavated from its burial place in the Basilique St Denis in Paris. Unconfirmed accounts mention that it was decapitated. This ‘mummified head’ of Henri IV then would have followed a complex route over the years and came finally in the hands of collectors of royal relics and antiquaries. Based on 22 scientific and historical arguments, the head was recently identified as belonging to the French King Henri IV.²¹ Nevertheless, this identification remains controversial as several historical counter-arguments have been formulated.^{22,23} Recently, after an initial failure to find DNA, a second effort to genotype the ancient DNA of the head was more successful. An mtDNA and a Y-chromosomal profile were obtained.²⁴ The similarity between the six Y-STRs of a sample of the head, whereby only three alleles could be reproduced in a second PCR analysis, with the Y-STR profile of the blood sample presumably of Louis XVI was an indication for the attribution of both samples to the two Bourbon kings of France.²⁴

The genetic identifications of both putative samples of two kings of France were only validated by the similarity of a limited number of

Figure 1 Overview of the patrilineal relationships between the three selected living members of the House of Bourbon and the French Kings Henri IV and Louis XVI. These five individuals are given in grey. The English names (if they are used in English literature) and titles of the members of the House of Bourbon are given in the genealogical tree together with their year of birth and death.

Y-STRs based on clearly degraded DNA. The aim of our study was to attempt to reconstruct the true Y-chromosomal variant of the genealogical lineage of the House of Bourbon from which these French kings originated. DNA samples of three living male relatives of the kings were analyzed. In addition, historical evidence was obtained that the mother of Henri IV was related in an uninterrupted female line to the Habsburgs.^{25,26} Their mtDNA was available from our previous investigations regarding Louis XVII.^{4,5} The results of the Y-chr and mtDNA analysis will show that the published genetic identification of the presumptive blood sample of Louis XVI and the presumed head of Henry IV cannot be confirmed.

MATERIALS AND METHODS

Three living male relatives of Henri IV and Louis XVI were selected for this study. Their official genealogical relationships with the two kings are given in Figure 1. They were Axel Prince of Bourbon-Parma (sample A), Sixte Henri Prince of Bourbon-Parma (sample SH) and João Henrique Prince of Orléans-Braganza (Sample JH). Buccal swab samples from the three DNA donors were collected with informed consent. DNA extraction was done by using the Maxwell 16 System (Promega, Madison, WI, USA) followed by real-time PCR quantification (Quantifiler Human DNA kit, Applied Biosystems, Foster City, CA, USA). In total, 38 Y-STR loci were genotyped for all samples as described in a previous study.²⁷ Alleles were assigned according to the nomenclature rules as used in the YHRD databank.²⁸ All haplotypes were submitted to the Whit Athey's Haplogroup Predictor²⁹ to obtain probabilities for the inferred haplogroups. Based on these results, the samples were assigned to a specific Y-SNP assay to confirm the haplogroup and to assign the sub-haplogroup to the most accurate level of the latest Y-chromosomal tree, according to the updated tree version 1.2 of AMY-tree.^{30,31} The Y-SNPs were genotyped using SNaPshot mini-sequencing assays (Applied Biosystems) according to previously published protocols.^{32,33} All primer sequences and concentrations for the analysis of the Y-SNPs are available as Supplementary Materials (Supplementary Table S1) or in van Oven *et al*.³⁴

RESULTS

The three Bourbon males were correctly assigned to the main Y-chromosomal haplogroup R1b (R-M343) using the Whit Athey's Haplogroup Predictor as it was confirmed by Y-SNP typing. The individuals were further assigned to sub-haplogroup R1b1b2a1a1b* (R-Z381*) based on the latest update of the Y-chromosomal phylogenetic tree of AMY 1.2.³¹ The 38 Y-STR haplotypes of the living donors were compared to each other (Table 1). A maximum of four mutational differences out of 38 Y-STRs was found between the living donors, namely between samples A and JH (Table 2). Next, these haplotypes were also compared with the haplotypes from the blood sample and the head (Table 1). There were 25–26 mutational differences between the 17 common genotyped Y-STRs of the living donors and the blood sample, assuming that each mutation leads to a gain or loss of one repeat unit on a Y-STR. There were eight mutational differences between the six common Y-STRs of the living donors and the head sample and even five out of the three confirmed STRs (Table 2). Based on the calculated mean mutation rate for each set of Y-STRs using the individual mutation rates measured in Ballantyne *et al*³⁵ and based on the formulas of Walsh,³⁶ the 95% confidence interval for the number of meioses between both individuals of each sample pair was calculated (Table 2). Finally, the mitochondrial DNA analysis of the head also did not support the attribution of the sample to Henri IV. According to Charlier *et al*,²⁴ the donor of the head belongs to mtDNA haplogroup U5b*. Our previous study of a series of living and deceased maternally related relatives of Louis XVII showed that the mtDNA haplogroup of the Habsburgs belonged to haplogroup H.^{4,5}

DISCUSSION

Detailed Y-chr haplotypes and haplogroups were obtained for three living relatives of the House of Bourbon from which the French kings originated from 1589 (Henri IV became King of Navarre in 1572 by his mother's rights, and King of France in 1589 at the death of Henri III, as the eldest descendant of Louis IX (1214–1270)) till the end of the monarchy. Based on these results, it is clear that their genealogical common ancestor (GCA) based on the official genealogy, namely King Louis XIII, was also their biological ancestor. First, the sub-haplogroups at the finest level of the Y-chromosomal phylogenetic tree were identical for all three relatives, namely R-Z381*. As there are no known recurrent mutations observed for SNP Z381 and as it is not lying in a Y-SNP conversion hotspot on the Y-chr,³¹ male individuals who share this Y-SNP must also share a common male lineal ancestor at the point of the SNP's first appearance.³⁷ Y-chromosomal sub-haplogroup R-Z381* is a subgroup of R-U106, which has been found in Western Europe with the highest frequency of around 35% in the north of the Netherlands and in Denmark but with a steep frequency fall to the south as the frequency of R-U106 is only 7% in France.^{38–40} The frequency of sub-haplogroup R-Z381* within Europe itself is not yet well known, except in Flanders where the frequency is around 9% within the autochthonous population.⁴¹ Second, also the 38 Y-STR haplotypes of the three individuals were highly similar. Differences were found only on four Y-STRs. These Y-STRs have a high mutation rate in comparison with all the other genotyped Y-STRs (Supplementary Table S2, Supplementary Materials). Moreover, the 17 Y-STR haplotype of the three individuals according to the AmpFISTR Yfiler kit (Applied Biosystems), which is a part of the genotyped 38 Y-STR haplotype, is compared with the other haplotypes in the YHRD database release 43 (www.yhrd.org). The 17 Y-STR haplotype of the three living donors was found in only 3 out of 53 576 individuals in the total database, with 1 individual from Poland, 1 from Italy and 1 European American. Based on earlier studies, it is common that individuals with a matching 17 Y-STR haplotype (identical by state) are not genealogically related with each other (identical by descent) within the radiation of sub-haplogroup R-M269*, in contrast to individuals with an (almost) matching 38 Y-STR haplotype.^{39,42} Based on the calculated mean mutation rate for all 38 genotyped Y-STRs and the formulas of Walsh,³⁶ the GCA is indeed most likely their true biological ancestor as well (Table 2). This means that no non-paternity event happened along the three studied in-depth paternal lineages although some rumors that the branch of Bourbon Orleans would be illegitimate (more details in Supplementary Materials). Therefore, the genetic analysis of the three DNA donors in this study revealed the Y-chromosomal variant of the Bourbon lineage, including King Louis XIII, King Louis XIV and Louis, le Grand Dauphin (Figure 1).

No paternal relationship was found between the living DNA donors and the donors of the blood sample of Louis XVI or the head of Henri IV. First, the Y-chr of the donor of the blood sample belongs to haplogroup G(xG1,G2) while the living Bourbon members belong to R-Z381*. Based on the time calibration of the Y-chromosomal phylogeny, the time of the most recent common ancestor (tMRCA) between individuals belonging to haplogroup G and R will be some 10 000 years ago.^{43,44} Second, the strong differentiation on the 17 Y-STR haplotypes between the living donors with the blood donor also suggests at least 260 meioses between them (Table 2). Although a low number of Y-STR results were obtained for the presumptive head of Henry IV, even the six Y-STRs and the three confirmed Y-STR haplotypes showed high differences with the living Bourbon donors, suggesting at least > 110 meioses between them (Table 2). Moreover,

Table 1 Y-chromosomal haplogroups and Y-STR profiles of the three analyzed members of the Bourbon family, the presumed blood sample of Louis XVI²⁰ and the presumed head of Henri IV²⁴

Sample	Axel, Prince of <i>Bourbon-Parma (sample A)</i>	Sixte-Henri, Prince of <i>Bourbon-Parma (sample SH)</i>	João Henrique, Prince of Orléans- <i>Braganza (sample JH)</i>	Presumed blood sample of Louis XVI	Presumed head of Henri IV
Y-chromosomal haplogroup	<i>R1b1b2a1a1* /R-Z381*</i>	<i>R1b1b2a1a1* /R-Z381*</i>	<i>R1b1b2a1a1* /R-Z381*</i>	<i>G (xG1,G2) /G (xM285,P287)</i>	—
DYS393	13	13	13	14	14
DYS390	23	23	23	22	—
DYS19	14	14	14	15	—
DYS391	10	10	10	10	10
DYS385	11,14	11,14	11,14	13,18	—,18
DYS426	12	12	12	ND	ND
DYS388	12	12	12	ND	ND
DYS439	12	12	12	12	—
DYS389I	13	13	14	12	13
DYS392	13	13	13	11	—
DYS389b	16	16	16	18	—
DYS458	18	18	18	21	—
DYS459	9,10	9,10	9,10	ND	ND
DYS455	11	11	11	ND	ND
DYS454	11	11	11	ND	ND
DYS447	25	25	25	ND	ND
DYS437	15	15	15	15	15
DYS448	19	19	19	21	21
DYS449	28	28	29	ND	ND
DYS464	15,15,16,16	15,15,16,16	15,15,16,16	ND	ND
DYS460	12	12	12	ND	ND
GATA H4	12	12	12	12	—
YCAII	19,23	19,23	19,23	ND	ND
DYS456	17	17	17	15	—
DYS607	19	19	19	ND	ND
DYS576	16	16	16	ND	ND
DYS570	16	17	17	ND	ND
DYS724	35,38	35,39	35,40	ND	ND
DYS442	18	18	18	ND	ND
DYS438	12	12	12	10	—
DYS635	23	23	23	21	—

Abbreviation: ND, not determined.

The alleles given in italics are those that were not confirmed in a second PCR reaction in the study of Lalueza-Fox *et al.*²⁰ DYS389b shows the number of repeats present in the second part of DYS389II (DYS389II minus DYS389I).

the genetic identifications of the presumptive head of Henri IV and the presumptive blood sample of Louis XVI were only based on a similar partial Y-STR profile of both samples.²⁴ Nevertheless, no evidence for a genealogical relationship between the DNA donors of the head and the blood sample could be found based on the calculations of the tMRCA due to the low number of analyzed Y-STRs and the mutational difference observed on one Y-STR (Table 2).³⁶ Therefore, the similarity between the partial Y-STR profiles of both samples as indicated by Charlier *et al.*²⁴ could have been just the result of coincidence. Finally, if the samples were indeed of both kings of France this would mean that there were at least two non-paternity events within the royal lineage of the House of Bourbon, namely that Henri IV was not the biological father of Louis XIII, next to another non-paternity between Louis, le Grand Dauphin and King Louis XVI (Figure 1). Non-paternity events—whether a man is not the biological father of his legal children—mainly occurs when the mother had sexual intercourse with a man other than the legal father or when an unreported adoption occurred. Nevertheless, the frequency of paternal discrepancy in the Western

European populations is at most 3% and is probably <1% of human births.^{45,46} Moreover, while there has been speculation about non-paternity cases in the family, the written records do not contain evidence for such events within this lineage of the House of Bourbon.^{47–49}

Next to the Y-chr, the mitochondrial DNA analysis of the head of Henri IV also does not support the presumed identification of the head sample. According to Charlier *et al.*²⁴ the donor of the head belongs to mtDNA haplogroup U5b* defined by three nucleotide changes at positions m.16239C>T m.16270C>T m.16311T>C (nucleotides are numbered according to the revision of the Cambridge Reference Sequence⁵⁰) of the mitochondrial hypervariable region 1 (HVR1). These three diagnostic positions were confirmed in two different amplifications of the L16185-H16378 (numbered also according to the revision of the Cambridge Reference Sequence⁵⁰) HVR1 fragment, proving that the results were reproducible. Henry IV was maternally related with Louis XVII—through his mother Jeanne III d' Albret, over Anna of Habsburg to Marie-Antoinette^{25,26} (Figure 2)—but this mtDNA

Table 2 Comparison between the Y-STR profiles of the three analyzed members of the Bourbon family and the partial Y-STR profiles of the presumed blood sample of Louis XVI and the presumed head of Henri IV

Sample pairs	N		Mutation rate (mutations/generation)	N_{exp} meioses	N_{calc} meioses (95% CI)
	N_{Y-STR}	mutations			
A vs SH	38	2	5.91E-03	5	3–33
A vs JH	38	4	5.91E-03	23	5–41
SH vs JH	38	3	5.91E-03	22	8–48
A vs Blood	17	25	3.23E-03	14	260–830
SH vs Blood	17	25	3.23E-03	13	260–830
JH vs Blood	17	26	3.23E-03	17	330–1050
A vs Head	6	7	2.82E-03	13	90–755
SH vs Head	6	7	2.82E-03	12	90–755
JH vs Head	6	8	2.82E-03	12	160–1110
Blood vs Head	6	1	2.82E-03	7	16–362
A vs Head	3	4	3.73E-03	13	30–630
SH vs Head	3	4	3.73E-03	12	30–630
JH vs Head	3	5	3.73E-03	12	100–1280
Blood vs Head	3	1	3.73E-03	7	30–630

A difference has been made between the six Y-STR profiles and the three verified Y-STR profiles for the presumed head of Henri IV as only three out of six Y-STR alleles were reproduced in a second PCR. Sample A: Axel, Prince of Bourbon-Parma; Sample SH: Sixte-Henri, Prince of Bourbon-Parma; Sample JH: João Henrique, Prince of Orléans-Braganza. N_{Y-STR} : number of commonly genotyped Y-STRs for each pair of samples. $N_{mutations}$: number of mutations assuming that each mutation leads to a gain or loss of one repeat unit on the commonly genotyped Y-STRs for each pair of samples. Mutation rate: estimated averaged mutation rate for the set of commonly genotyped Y-STRs based on the individual mutation rates measured in Ballantyne *et al.*³⁵ N_{exp} meioses: number of expected meioses between the two samples based on the official genealogy; N_{calc} meioses: number of meioses based on the Walsh formulae³⁶ with a 95% confidence interval (CI).

haplogroup belongs to haplogroup H and did not show the three nucleotide changes as seen for the head sample. According to the latest mtDNA phylogenetic tree⁵¹ and its most recent update on www.phylotree.com, the MRCA of the head donor and Louis XVII lived more than several tens of thousands years ago. If the head sample was indeed attributed to Henri IV, this would mean that a non-maternity event happened somewhere between Henri IV and Marie-Antoinette, an event which is known to occur very exceptionally but without any indication in written records.⁵²

Finally, there is also the other (genetic) data that conflict with the attribution of the presumptive blood sample of Louis XVI. The chance that the donor of the blood sample was a non-blue-eyed person is 84.2% because of the heterozygote SNP rs12913832 on the *HERC2* gene. As King Louis XVI was blue-eyed, Lalueza-Fox *et al.*²⁰ already mentioned that the *HERC2* gene result provided controversial evidence for the physical appearance of the donor of the blood sample. Moreover, the blood sample was part of a relic of the execution of the king, an object which was very popular at that time. As one of the purposes of this relic was economic (for financial gain), it is very likely that the donor of the blood sample was not really the king himself.²⁰

CONCLUSION

By using in-depth genealogical trees of living members of the House of Bourbon, the 'true' Y-chromosomal variant of Bourbon males of the French dynasty lineage was reconstructed. This Y-chromosomal variant was different from the ones of the presumptive head of Henry IV and of the presumptive blood sample of Louis XVI. As the genetic identifications of these samples were only based on the similarity between the partial Y-STR profiles of both samples, these identifications can no longer be accepted. Moreover, matrilineal genealogical

Figure 2 Overview of the matrilineal relationship between the French King Henri IV and Louis XVII, both given in grey. The English names and titles of the maternal ancestors are given in the genealogical tree together with their year of birth and death.

data revealed that the observed mtDNA variant for the head sample was not the one that could be expected. These discrepancies might indicate that the samples analyzed were actually contaminated with non-authentic DNA. As contamination is indeed always possible when dealing with samples with high DNA degradation,^{11,12,53} contamination cannot be excluded here, especially for the head as the first attempt to collect DNA from the sample was not successful.²¹ Alternatively, they would support the hypothesis that the French kings were not the donors of these biological samples. Y-chromosomal analysis on the already identified heart of Louis XVII, the son of Louis XVI,⁴ might further remove all doubts about the identification of the blood sample considered to be of Louis XVI and might also solve any controversy about the paternity of Louis XVI of this child. As was already stressed in earlier studies,⁵ DNA typing of living relatives who are paternally or maternally related should be a requisite to solve historical cases with ancient DNA. This is an essential extra quality control for DNA contamination as well as a guarantee for a more objective identification, as illustrated here by this study of the Bourbon family.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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