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DNA analysis in the case of Kaspar Hauser

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Abstract In 1828 a mysterious young man appeared in Nürnberg, Germany, who was barely able to speak or walk but could write down his name, Kaspar Hauser. He quickly became the centre of social interest but also the victim of intrigue. His appearance, his origin and assassination in 1833 were, and still are, the source of much debate. The most widely accepted theory postulates that Kaspar Hauser was the son of Grand Duke Carl von Baden and his wife Stephanie de Beauharnais, an adopted daughter of Napoleon Bonaparte. To check this theory, DNA analysis was performed on the clothes most likely worn by Kaspar Hauser when he was stabbed on December 14th, 1833. A suitable bloodstain from the underpants was divided and analysed independently by the Institute of Legal Medicine, University of Munich (ILM) and the Forensic Science Service Laboratory, Birmingham (FSS). Mitochondrial DNA (mtDNA) was sequenced from the bloodstain and from blood samples obtained from two living maternal relatives of Stephanie de Beauharnais. The sequence from the bloodstained clothing differed from the sequence found in both reference blood samples at seven confirmed positions. This proves that the bloodstain does not originate from a son of Stephanie de Beauharnais. Thus, it is becoming clear that Kaspar Hauser was not the Prince of Baden.

Key words Kaspar Hauser · Ancient DNA · Mitochondrial DNA · Sex determination

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Introduction

In 1828, Kaspar Hauser appeared in Nürnberg, Germany. According to his later descriptions, he was kept alone in a dungeon as long as he could remember. Speculation quickly surrounded the origin of this man and the possibility that he was the son of Grand Duke Carl von Baden and his wife, Stephanie de Beauharnais. Their first-born son had died soon after birth. The suggestion arose that the newborn Prince had been exchanged with a sick child in order to change the succession to favour another branch of the family. If this theory was right and the Prince was secretly raised, he would have been of the same age as Kaspar Hauser and therefore Kaspar Hauser could be the prince of Baden. This theory gives a motive for the fact that Kaspar Hauser was stabbed only five years after his public appearance. DNA analysis has been performed to check if he really was a Prince of Baden.

In the last few years, identification of human remains by DNA analysis has proven to be a powerful tool in forensic investigations. Analysis of mtDNA is especially useful, because it is present at a high copy number in cells [1] and is more likely to survive for prolonged periods compared to chromosomal DNA. It has been successfully applied to human remains such as a 7000-year-old human brain [2] or an approximately 5000-year-old man who was found mummified in an alpine glacier [3]. MtDNA is inherited only from the mother to the child and thus all members of a family who are maternally related would have the same mtDNA type. Because of this unique pattern of inheritance, mtDNA is very useful in identification cases [4-6]. MtDNA analysis and analysis of Y-chromosomes are methods in determining family relationships when a gap of several generations exists between an ancestor and a living descendant but mtDNA analysis is the only option in determining maternal family relationships such as in the identification of the remains of the Romanov family [7]. Therefore, we applied mtDNA analysis to a suspected bloodstain from Kaspar Hauser and maternally related descendants of Stephanie de Beauharnais to check if a relationship can be demonstrated.

In order to perform DNA analysis, authentic material from Kaspar Hauser was required. Suitable material for analysis would be the remains of Kaspar Hauser from the grave situated in Ansbach in a public cemetery. An exhumation would have been possible but was not performed because it was doubtful if the gravestone was still above the correct grave due to disturbances during the war. Therefore, it was not clear if bones found in the grave would originate from Kaspar Hauser or from other persons buried nearby.

The museum of Ansbach owns clothes which were worn by Kaspar Hauser when he was stabbed on December 14th, 1833. These clothes were seized by the prosecutor after his murder and were kept at the court until 1888. The clothes were then given to the Historical Society of Ansbach. They were photographed and exhibited for the first time shortly before 1930. In Spring 1996 the clothes were inspected by members of the Institute of Legal Medicine, University of Munich (ILM) and the Forensic Science Service Laboratory (FSS), Birmingham. The clothes seemed fairly clean and in a good state of preservation. The upper clothing was seen to bear a single stab cut, penetrating the several layers of cloth in the region of the left chest. The bloodstains on the clothing had the same outline as those in the pictures from the 1920s, but appeared faded. Bloodstaining was associated with the cut marks and also appeared to have run down and collected along the top edge of the underpants, where there was a substantial stain. The fabric of the underpants had two layers in the upper region where the bloodstain was situated. The bloodstain was cut out and divided for independent analysis in each laboratory. The inner part of the fabric was chosen because it was least likely that contamination from outside or environmental exposure such as sunlight would have occurred.

Materials and methods

Laboratory organisation

To exclude the possibility of contamination, mtDNA analysis was performed independently by the ILM and the FSS and each sample was analysed in duplicate. All results were compared with mtDNA databases containing all analysed sequences from each laboratory, including mtDNA sequences of persons working in the laboratories. To minimise the possibility of contamination in the laboratories, all extractions were performed using sterile aliquots of buffers once only, the remainder being rejected after use. Extraction and amplification were carried out in different rooms to ensure that amplified products could not contaminate the extraction laboratory. Negative controls without DNA added to the extraction mixture were used in all experiments. All experiments were rejected on the rare occasion that a signal was obtained in the negative control.

Sampling of bloodstained clothing

The fabric of the underpants had two layers in the upper region, where the substantial bloodstain was situated. In the region of the bloodstain, the two layers were stuck together. To separate the two layers of fabric, the underpants were put in a chamber with a controlled humidity of 80%. Under these conditions, the two sticky layers of fabric could be separated. Two pieces of approximately 10 cm^2 were removed from the inner layer of the bloodstain.

DNA extraction

Both laboratories performed independent extractions from two pieces of fabric each of which was 4 cm^2. At the ILM the pieces of fabric were first incubated overnight at 56 °C in 1 ml of mild lysis buffer I (0.02 M Tris-HCI, 0.01 M EDTA, 0.05 M KCI, 0.5% Tween 20 and 0.5 mg proteinase K) and then with 1 ml of lysis buffer II (0.02 M Tris-HCI, 0.01 M EDTA, 0.05 M KCI, 1% Tween 20, 1% NP-40, 0.04 M DTT, 1 mg proteinase K). After lysis the samples were extracted with phenol, phenol/chloroform and chloroform. DNA was precipitated with 2.5 vols ethanol and washed 3 times with 0.5 ml distilled water in a Microcon 30 concentrator (Amicon) for 30 min. Pieces of fabric without bloodstaining were extracted in the same way.

At the FSS the pieces of fabric were incubated overnight at 37 °C in 500 µl extraction buffer (0.01 M Tris HCl pH8.0, 0.01 M EDTA, 0.1 M NaCl, 2% SDS), 50 µl proteinase K (10 mg/ml) and 20 µl 1 M DTT. The buffer was recovered and the cloth re-extracted with 500 µl extraction buffer, 50 µl proteinase K and 20 µl DTT at 56 °C for 3 h. The buffer was again recovered. DNA was purified from both first and second recovered buffers with two washes each of phenol and phenol/chloroform and one of chloroform, precipitated with 2.5 vols of ethanol with incubation at –20 °C overnight. The DNA was collected by centrifugation at 13 000 rpm for 30 min, the pellet was washed with 70% ethanol and resuspended in 50 µl sterile deionised water. Results were obtained from the first and second extractions of the blood staining.

At the ILM blood samples from the maternal relatives of Stephanie de Beauharnais were extracted by a standard phenol/chloroform protocol [8]. At the FSS reference samples were extracted with Chelex 100 resin (Bio-Rad) [9].

Sex determination

The sex of the bloodstained clothing was tested by amplification of a segment of the X-Y homologous gene, amelogenin [10]. At the ILM the size of PCR products was then determined with a 373A DNA Sequencer (Applied Biosystems) as previously described [11]. At the FSS, the sex of the blood was determined after agarose electrophoresis.

Amplification and sequencing of mtDNA

At the ILM amplification of PCR products for sequencing was carried out in a single step reaction with 200–500 pg DNA of the blood samples or 1/10 of the main extractions from the bloodstained clothing as described [12] with the exception that AmpliTaqGold (Perkin Elmer) was used. The amplification conditions were 12 min at 95 °C in a first step, 30 s at 95 °C, 40 s at 57 °C (primer set II, III, IV) or 60 °C (primer set I) and 20 s at 72 °C for 32, 35 or 38 cycles depending on the amount of DNA. After controlling on ethidium bromide gels and purification, sequencing analysis of PCR products was performed using the AmpliTaqFS polymerase cycle sequencing with dye-labelled dideoxyterminators (Applied Biosystems). All results were confirmed by sequencing in both directions. The PCR amplification and sequencing primers for the mtDNA control region were [12]:

Primer set I:	F15990 5′ TTA ACT CCA CCA TTA GCA CC 3′,
	R16239 5′ TGG CTT TGG AGT TGC AGT TG 3′,
Primer set II:	F16163 5′ TGA CCA CCT GTA GTA CAT AA 3′,
	R16391 5′ GAG GAT GGT GGT CAA GGG AC 3′,
Primer set III:	F00034 5′ CAC CCT ATT AAC CAC TCA CG 3′,
	R00266 5′ GTT ATG ATG TCT GTG TGG AA 3′,
Primer set IV:	F00174 5′ TAT TTA TCG CAC CTA CGT TC 3′,
	R00370 5′ CTG GTT AGG CTG GTG TTA GG 3′.

To check for human specificity, 50 ng DNA from cow, pig, sheep and chicken were amplified under the same conditions with 38 cycles. No visible PCR products were obtained in an ethidium bromide gel.

Nested PCR is normally used by the FSS [13] however insufficient intact mtDNA was recovered for this procedure and sequence was determined after a single round amplification of 35 cycles with primer-pairs to give overlapping products of approximately 250 bases [6]. Sequencing was performed after solid phase capture of biotinylated primer product with dye labelled primers and Sequenase polymerase (Applied Biosystems). Electrophoresis was performed on a 377 DNA Sequencer (Applied Biosystems).

Results and discussion

Bloodstain on the clothing

The bloodstain was strongly fixed on the cloth and therefore practically insoluble in water. In each laboratory extraction was performed twice using similar methods. Only a minute amount of highly degraded DNA was obtained. PCR products of mtDNA from the degraded samples were obtained using sets of primer pairs which gave lengths up to approximately 300 basepairs. For sequencing mtDNA, the FSS used a modification of a solid phase sequencing protocol [14]. ILM performed mtDNA analysis using dye-terminator cycle sequencing [12]. DNA sequences of 568 basepairs (FSS) and 769 basepairs (ILM) were obtained (Table 1). The sequences of both laboratories were identical in the regions where comparison was possible. The quality of sequence using either dye-terminator cycle sequencing or dye-primer was comparable to that of fresh blood samples. One example for dye-terminator cycle sequencing is shown in Fig. 1. Additionally, pieces of fabric from the underpants without bloodstaining were extracted but no PCR products sufficient for mtDNA sequencing were obtained.

Verification

Analysis of the highly degraded DNA from the bloodstain was close to the detection limit of the methods used. Thus there was a high risk of contamination during sampling and analysis. No PCR products sufficient for mtDNA sequencing could be obtained when fabric from the underpants without bloodstaining was analysed. Using fabric with bloodstaining, sufficient DNA for mtDNA sequencing could be obtained. DNA samples from the first (mild) lysis and the following (complete) lysis gave identical results, therefore surface contamination seems very unlikely. There was also no indication of contaminating sequences in the bloodstain. From the independent analysis of the minute amount of highly degraded DNA in both laboratories, sequences were obtained which were identical in the regions where comparison was possible. Therefore, and due to the fact that contamination could not be detected, it is confirmed that the obtained sequence derives from the bloodstain on the cloth.

A sex test of the bloodstain was performed by amplifying portions of the X-Y homologous gene, amelogenin [10, 15] with a single primer pair. This provides a robust method for typing samples of a very degraded nature. The 106 and 112 bp products found by this test show that the bloodstain originated from a male.

Reference samples

In order to reconstruct the mtDNA sequence of Stephanie de Beauharnais, blood samples of two living maternal relatives were analysed by each laboratory. The samples were donated under notarial supervision.

Analysis of mtDNA sequences was carried out in duplicate at both laboratories and resulted in identical mtDNA sequences. This result is to be expected from two descen-

Table 1 Mitochondrial DNA sequences compared to the Anderson [27] reference sequence

Origin of sample	DNA source	Length sequenced (bp)	Positions within the hypervariable regions (HRV) of mitochondrial DNA												
			HVR1			HVR2									
			16220	16223	16292	73	189	195	204	207	228	263	309.1	309.2	315.1
Reference sequence			A	C	C	A	A	T	T	G	G	A	*	*	*
Kaspar Hauser	Bloodstained clothing, FSS	568	.	T	T	G	G	C	–	A					
Kaspar Hauser	Bloodstained clothing, ILM	769	.	T	T	G	G	C	C	A	.	G	C	.	C
Gt. Gt. Gt. granddaughter 1 of Stephanie de Beauharnais	Blood sample	781	C	.	.	.	.	.	.	.	.	G	C	C	C
Gt. Gt. Gt. granddaughter 2 of Stephanie de Beauharnais	Blood sample	781	C	.	.	.	.	.	.	.	.	G	C	C	C

·, Sequence unchanged from reference seuquence; –, No nucleotide assignment; *, Nucleotide absent from reference sequence; FSS, Forensic Science Service, Birmingham; ILM, Institute of Legal Medicine, Munich

Bloodstained clothing: the FSS determined sequences between 62–226 and 15998–16400 bp, the ILM between 35–400 and 15998–16400 bp

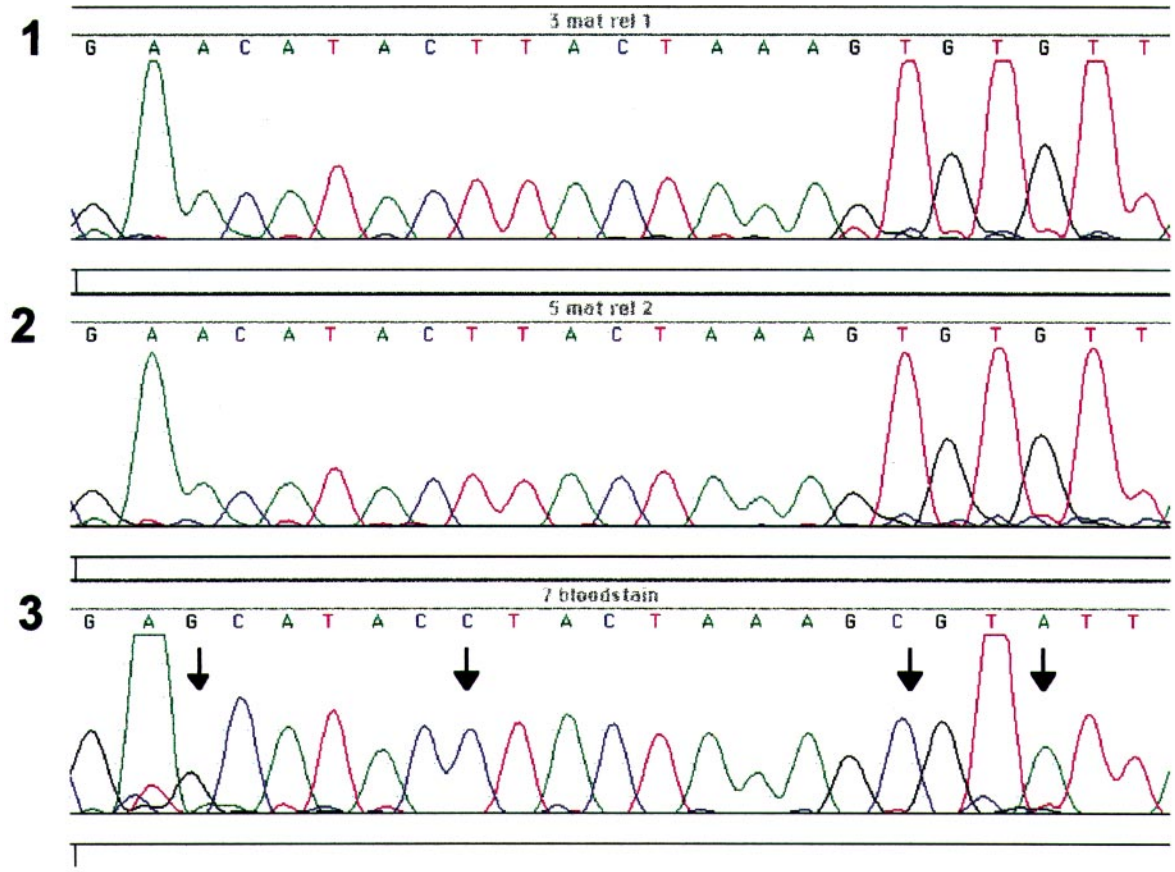

Fig. 1 Comparison of mtDNA sequences (nt 186–210, obtained using dye-terminator cycle sequencing) of the bloodstain extract from clothes worn by Kaspar Hauser (3) with two maternal relatives of Stephanie de Beauharnais (1 and 2). The sequence from the bloodstained clothing of Kaspar Hauser shows four differences in this region at position 189, 195, 204 and 207 (arrows) to the identical sequences of the two living relatives of Stephanie de Beauharnais

dants of the same maternal ancestor. From the family tree (Fig. 2) it can be seen that this sequence would have originated from their great, great, great grandmother Stephanie de Beauharnais. To assess the strength of evidence we compared the DNA profile from the living relatives with over 500 unrelated Caucasian sequences in published and unpublished databases [13, 16–18; unpublished databases are from the Forensic Science Service, Birmingham and the Institute of Legal Medicine, Munich]. We did not find a match, which suggests that the DNA sequence may be rare (possibly less than one in 500). Between generations it is theoretically possible that sequences might differ due to a mutation [19, 20]. The identical mtDNA sequences from both living maternal relatives demonstrate that in the 10 generations separating them from Stephanie de Beauharnais no mutation has occurred. Therefore, the identical mtDNA sequences from the maternal relatives represent the sequence of Stephanie de Beauharnais. The same sequence would be expected to be present in all of her children including her eldest son, who, as legend has it, re-appeared in 1828 named Kaspar Hauser.

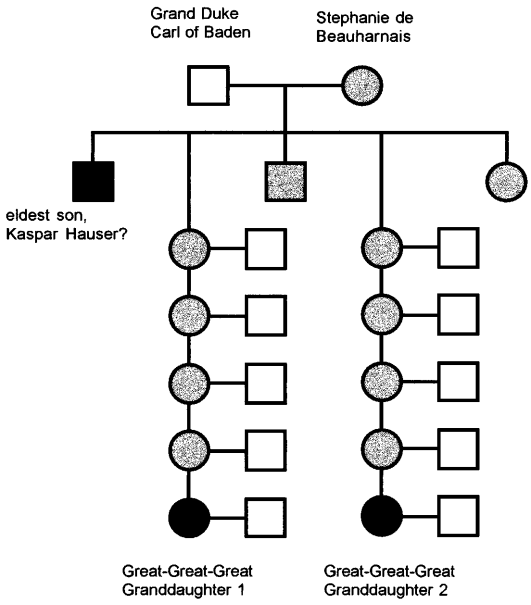

Fig. 2 Reduced lineage of Stephanie de Beauharnais showing the relationship to the two maternal relatives tested. According to the legend, Kaspar Hauser should be identical with her eldest son

The mtDNA sequence from the bloodstained clothing differed from the reconstructed sequence of Stephanie de Beauharnais (Table 1) at 7 and 9 confirmed positions depending on the length of sequence obtained by the two

laboratories. These differences between mtDNA sequences cannot be explained by mutation within only one generation. Different mutation or substitution rates for the two segments and even single nucleotide positions for the hypervariable mtDNA control region have been reported [21]. They range from about 1/800 per generation [22–25] down to 1/33 [26], or 1/25 per generation [20], assuming a generation time of 15 years. Therefore, the person from whom the bloodstain on the underpants arose cannot be a son of Stephanie de Beauharnais. Together with the knowledge we have about the clothing and its history, Kaspar Hauser is therefore not the son of Stephanie de Beauharnais. Thus, it is becoming clear that Kaspar Hauser is not the Prince of Baden and the legend can be disproved.

With this analysis, the sequence of mtDNA from bloodstained clothing, which had been subjected to various environmental exposures for more than 160 years was determined. Moreover, the results of the sex determination demonstrate that not only mtDNA but also chromosomal DNA can be successfully analysed after such a long time.

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