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Reconstruction of a historical genealogy by means of STR analysis and Y-haplotyping of ancient DNA

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Archaeological excavations in St Margaretha's church at Reichersdorf, Germany, in 1993 led to the discovery of eight skeletons, so far assumed to be of the Earls of Königsfeld, who used the church as a family sepulchre over a period of seven generations from 1546 to 1749. DNA-based sex testing and analysis of autosomal short tandem repeat systems (STR) was carried out to confirm the assumption of kinship. Since five of the individuals were determined as males, analysis of Y-specific STRs seemed feasible. A comparison of Y-haplotypes revealed that one individual could not be linked to the Königsfeld patrilineage, an observation supported by autosomal STR evidence. Two individuals typed as females posed an identification problem, since supposedly only male members of the family were buried in St Margaretha's. Nevertheless, these individuals could tentatively be identified as members of the House of Königsfeld through genetic fingerprinting.

Keywords: aDNA; Y-haplotyping; STR-typing; Königsfeld; paternity; kinship

Introduction

In 1993, during archaeological excavations in St Margaretha's Church at Reichersdorf, Lower Bavaria, Germany, skeletons of eight individuals were recovered. The church was used by the Earls of Königsfeld as their traditional family burial place from 1546 to 1749, documented by seven memorial stones in the chancel. According to the inscriptions, eight male members of the House of Königsfeld, spanning altogether seven

generations, were laid to rest in St Margaretha's. Based on this information and by evaluating the archaeological finds, seven of the discovered skeletons were identified as the individuals whose names are inscribed on the tombstones. Unfortunately, one skeleton had been destroyed by grave robbers.

Morphological traits (and the fact that remains of female garments were still discernible) gave reason to believe that the individual recovered from grave Ma 1 was a female approximately 30 years old. This observation posed an identification problem, since apparently no proof of a burial of a female in the church existed. It could only be speculated that this individual was affiliated to the House of Königsfeld.

The genealogical positions of the seven identified individuals were known from historical sources¹ so that the family tree in Figure 1 could be reconstructed,

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indicating the relationships between the individuals recovered in St Margaretha's.

Genetic typing by analysis of PCR-amplifiable short tandem repeat (STR) loci is the most promising approach for forensic DNA profiling and has become the method of choice for the identification of human remains²⁻⁵ and reconstruction of kinship.⁶⁻⁸ The comparatively short length of the STR loci (up to 250 bp) makes them especially suitable for typing ancient DNA (aDNA), characteristically highly degraded. Successful typing of DNA extracted from skeletal material well over a thousand years old is therefore possible.⁹⁻¹¹ According to archaeological findings, seven of the eight individuals were considered to be male descendants of the House of Königsfeld, connected patrilineally. Therefore we followed a dual strategy in the attempt to determine relationships between the individuals. In addition to analysing autosomal STRs to verify the presumed kinship structures, Y-specific STRs were examined to seek confirmation that all male individuals were representatives of the same patrilineage.

The special properties of the male Y-chromosome make it potentially useful in forensic studies¹² and paternity testing.¹³ Due to the haploidy of the Y-chromosome, most of it does not recombine during meiosis, so the chromosome is transferred from father to son unchanged, modified only by gradual accumula-

tion of mutations. Therefore, the analysis of polymorphisms located in the non-recombining region on the Y-chromosome allows retracing of paternal lineages^{14,15} comparable with the analysis of mitochondrial DNA to retrace maternal lineages.¹⁶ Y-chromosomal STRs are apparently as highly variable and short in length as their autosomal counterparts,¹⁷ which qualifies them as an investigative tool in the study of aDNA.¹⁸

Materials and Methods

Samples

The skeletal individuals from the graves were identified from archaeological data. The tombstones of the Earls of Königsfeld were on the walls and not on top of the graves, as might be expected. Thus the contents of the graves could not be directly identified. Archaeological identification relied on the evaluation of burial objects and on morphological age and sex determination.

The analysis was performed on DNA extracted from bone fragments and isolated teeth. For detailed information on sample material see Table 1.

Contamination Prevention Precautions commonly taken for aDNA work were followed during all stages of sample preparation.^{19,20} To prevent any possible risk of contamination, several precautions were adhered to: separation of pre- and post-PCR working areas to avoid PCR-product carry-over; dedication of equipment to the different laboratory rooms. No-template controls were regularly processed

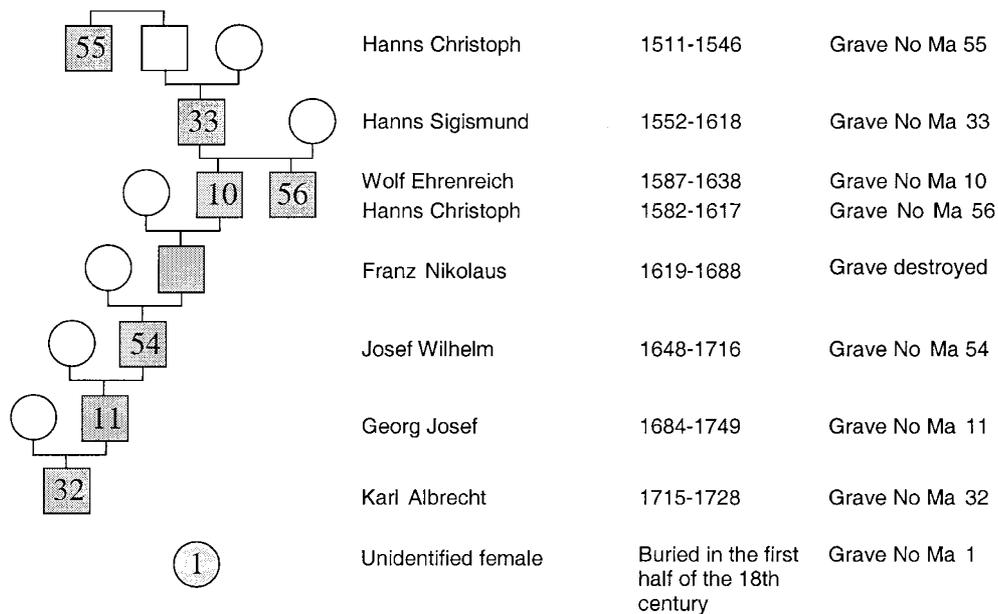


Figure 1 Lineage of the Earls of Königsfeld. Individuals in the family tree who were buried in St Margaretha's are depicted in grey. A grave number was assigned to the skeletal individuals for whom an identification could be achieved. Individual Ma 1 was identified by morphological traits as a 30-year-old female. An affiliation with the Königsfeld individuals was hitherto unknown. A relationship is suggested (see text)

throughout the DNA extraction to exclude possible contamination of the extraction buffers. Furthermore, no-template controls were used in every PCR run to monitor contamination of reagents.

DNA-Extraction

For sample preparation, the surfaces of the bone and tooth samples were removed. After exposure to UV irradiation (245 nm) for 20 min, the samples were ground to a fine powder. Aliquots of 0.3 g of bone and tooth powder were suspended in 1.5 ml 0.5 M EDTA (pH 8.0) and constantly shaken for 24 h at room temperature in order to decalcify. After centrifugation (6000 rpm/4 min), the supernatants were transferred to an automated nucleic acid extractor Gene Pure Type 341A (Applied Biosystems, Weiterstadt, Germany), which performs a proteinase K digestion followed by phenol/chloroform extraction. The DNA was concentrated by binding to silica powder Glassmilk™, Dianova (Hamburg, Germany) in the presence of isopropanol and sodium acetate (2.0 M, pH 4.5). The Glassmilk™-DNA was collected on filtrate membranes, then eluted into 50 µl of sterile water Ampuwa®, Fresenius (Bad Homburg, Germany).

Sex Determination

To determine the sex of the individuals, amplification of a sequence of the X-Y homologous gene amelogenin was carried out. 4 µl of aDNA extract were amplified in a 50 µl reaction mix comprising 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂, 175 µM dNTPs, 0.12 µM of each primer²¹ and 2 U AmpliTaqGold™ polymerase. PCR cycling conditions in a DNA Thermal Cycler (PE Applied Biosystems, Weiterstadt, Germany) were as follows: 60 cycles of 94°C 1 min, 50°C 1 min, 72°C 1 min with an initial denaturation step of 94°C 10 min.

Amplification of the Autosomal STR-loci

Loci HUMTH01,²² HUMCD4²³ and D1S1656²⁴ were amplified individually. For loci HUMVWA31/A,²⁵ HUMFES/FPS²⁶ and HUMF13A1²⁷ a triplex PCR approach was employed. One primer at each locus was labelled with a fluorescent dye marker. All PCRs were conducted in a DNA Thermal Cycler (PE Applied Biosystems). Each amplification was carried out in a 50 µl reaction volume. Due to the lower amplification efficiency of aDNA compared with modern DNA an increased number of PCR cycles is required. Several studies demonstrate that if only a few DNA targets are present a high number of PCR cycles are inevitable.^{28,29,30} If the DNA is

degraded, amplifications with less than 50 cycles usually show no detectable products.^{31,9-11} Therefore samples were amplified for at least 55 cycles. Every reaction was initiated by 10 min at 94°C. Reaction parameters were as follows.

D1S1656 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂, 175 µM dNTPs, 0.08 µM of each primer, 6 µl aDNA extract, 2 U AmpliTaqGold™ polymerase; 55 cycles: 94°C 1 min, 56°C 1 min, 72°C 1 min;

TH01: 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂, 175 µM dNTPs, 0.14 µM of each primer, 4 µl aDNA extract, 2.5 U AmpliTaqGold™ polymerase; 55 cycles: 94°C 1 min, 50°C 1 min, 72°C 1 min;

CD4: 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂, 175 µM dNTPs, 0.12 µM of each primer, 5 µl aDNA extract, 2.5 U AmpliTaqGold™ polymerase; 55 cycles: 94°C 1 min, 53°C 1 min, 72°C 1 min;

Triplex PCR VWA/FES/F13A1: 50 mM KCl, 10 mM Tris-HCl, 2.0 mM MgCl₂, 175 µM dNTPs, 0.1 µM for each primer of FES and F13A1 and 0.04 µM of each primer of VWA, 5 µl aDNA extract, 2 U AmpliTaqGold™ polymerase; 60 cycles: 94°C 1 min, 50°C 1 min, 72°C 1 min.

Amplification of the Y-chromosomal STR-loci

The Y-specific STR-loci DYS19, DYS390, DYS389I/II^{32,33} were amplified in a quadruplex-PCR. Usage of the primer pair DYS389 as described by Kayser *et al*³² and de Knijff *et al*³³ results in the amplification of two products of different sizes. Allele lengths of system DYS389I are in the range 239–263 bp, the length range of alleles of the larger system DYS389II is 353–385 bp. To accommodate the primer pair to the usage on aDNA, the reverse primer was modified so that the generated products were 94 bp shorter.¹⁸ The loci were coamplified in a 50 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, 2.0 mM MgCl₂, 175 µM dNTPs, 0.1 µM of each primer of DYS19, DYS390 and DYS389, 6 µl aDNA extract, 2.5 U AmpliTaqGold™ polymerase. PCR cycling conditions were as follows: 55 cycles of 94°C 1 min, 51°C 1 min, 72°C 1 min.

Fragment Length Determination

Analysis of the amplified fragments was carried out by using an automated fluorescence fragment length detection system GeneScan 672 (Applied Biosystems, Weiterstadt, Germany) on an ABI Prism 373A stretch DNA Sequencer (PE Applied Biosystems, Weiterstadt, Germany). Electrophoresis was carried out in denaturing 6% polyacrylamide gels. Length of the DNA fragments was determined from the internal lane standard GeneScan 350-ROX (Perkin Elmer, Weiterstadt, Germany).

Results

Chromosomal DNA Analysis

Sex Testing The sex of the individuals was determined by amplification of Y-specific loci and a segment of the amelogenin gene which exhibits a length dimorphism, resulting in X-specific fragments of 106 bp and Y-specific products 112 bp long.

Table 1 Sample materials

Sample no.	Skeletal element
Ma 1	Fibula, tooth 36
Ma 10	Femur ^a
Ma 11	Fibula ^a
Ma 32	Os ischium, tooth 47
Ma 33	Fibula, metatarsal III
Ma 54	Radius, metatarsal V, tooth 28
Ma 55	Ulna, metatarsal IV
Ma 56	Tooth 47, tooth 44

^aSeveral samples from different parts of the skeletal element were used for molecular analysis.

Reproducible results of the amplifications of Y-specific STR loci clearly identified five individuals as male. All samples, except for Ma 56, yielded products after amelogenin amplification (Table 2).

Repeated amplifications (five times) of the amelogenin sequence from samples of Ma 32 generated the X-specific product. Since all amplifications were successful, a general good quality of the DNA can be assumed, so that drop-out of the Y-specific fragment due to DNA degradation²⁰ is unlikely. Consequently, this individual was typed as female, a result which challenges the archaeological identification of Ma 32 as Karl Albrecht.

In the case of individual Ma 33, an inconsistency arises between the results of the amplification of the amelogenin sequence and the Y-specific loci. Although the male status of this individual was clearly manifested by determination of a Y-haplotype, amelogenin amplifications yielded only X-specific fragments. It can be assumed that drop-out of the Y-specific fragment has occurred here. Samples from the left fibula of Ma 33 used for amelogenin amplification showed products in only 50% of the amplifications, indicating a high degree of DNA degradation for this skeletal element. For Y-haplotyping, samples derived from a metatarsal of the same individual showed reproducible results. The discrepancy between the results of Y-haplotyping and amelogenin amplification can thus be explained by the usage of two DNA extracts, with differing suitability for molecular genetic analysis.³⁴

Short Tandem Repeats Amplifications of six STR loci showed amplification products for all skeletal samples, with the exception of Ma 56. Figure 2 shows an electrophoretogram displaying PCR products of a multiplex amplification of a sample.

DNA extracted from historical bone material exhibits several characteristic features, namely the limited number of targets and an increased degree of DNA degradation. Low concentrations of templates may lead to allelic or complete locus drop-out, due to stochastic differences in allele copy number.³⁵ All samples were therefore analysed at least five times to avoid false-homozygote typing.³⁶ Another characteristic of STR amplifications lies in the generation of stutter bands. These extra bands are caused by enzyme slippage during PCR.^{37,38} In general, the artefactual bands can be easily identified, since they exhibit a weaker product signal. With highly degraded aDNA, however, the amount of stutter products may exceed the quantity of the correct product^{39,34} impeding determination of the samples' allelic composition. However, since only the true alleles will be reproduced, the authentic genotype of the sample should be determinable after repeated amplifications. Nevertheless, for some of the individuals, a distinct identification of allele peaks was not possible for every locus. In cases where an unequivocal determination could not be made, more than two alleles are listed in Table 3, representing the STR genotypes of the Königsfeld individuals.

Y-chromosomal STR Systems – Y-haplotypes In a quadruplex-PCR, the tetrameric Y-STR systems were amplified from five of the skeletons. A four-locus Y-haplotype could be set up for all individuals (Table 4). Four individuals exhibit the same Y-haplotype, indicating that they belong to the same patrilineage, presumably the lineage of descendants of the House of Königsfeld. However, a different haplotype of individual Ma 11 renders its affiliation to the Königsfelder paternal lineage impossible.

Table 2 Sex determination of the eight skeletons

<i>Skeleton</i>	<i>Y-specific STR results</i>	<i>Amelogenin</i>	<i>Sex determined by molecular examination</i>	<i>Sex expected from archaeological findings</i>
Ma 1	–	X	Female	Female
Ma 10	Yes	XY	Male	Male
Ma 11	Yes	XY	Male	Male
Ma 32	–	X	Female	Male
Ma 33	Yes	X	Male	Male
Ma 54	Yes	XY	Male	Male
Ma 55	Yes	XY	Male	Male
Ma 56	–	–	–	Male

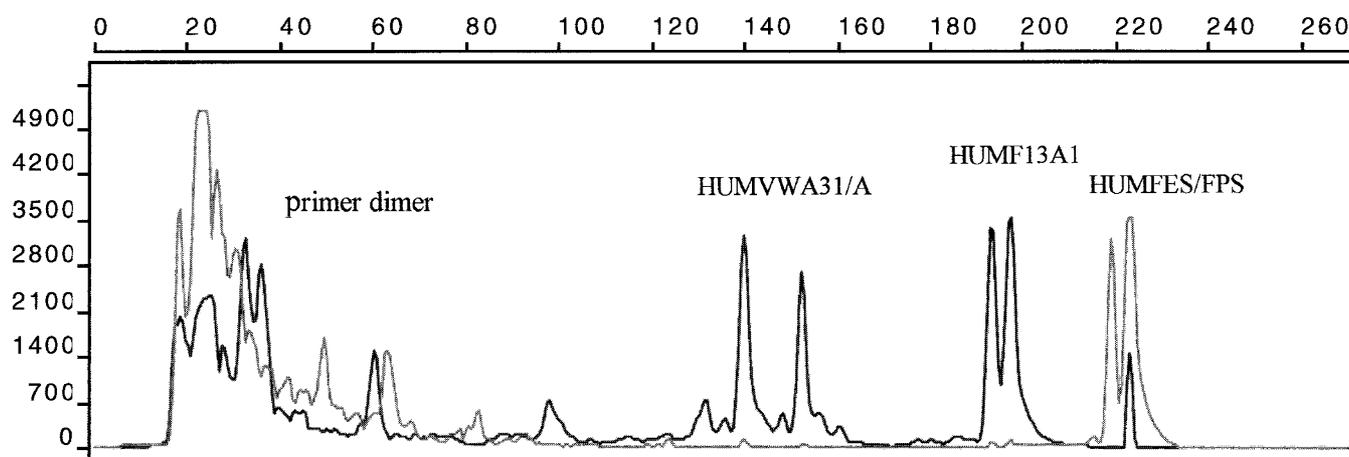


Figure 2 Electrophoretogram displaying PCR products of a triplex amplification of a tooth sample from individual Ma 32. Peaks represent fluorescent intensities of dye-labelled DNA products. The size of the products, in bases, is shown along the x axis. For locus HUMVWA31/A, alleles 14/17 were determined; for HUMF13A1, the alleles 6/7 were amplified. For HUMFES/FPS, the allelic designation was 10/11. Small stutter peaks one repeat unit shorter than the true allele products can be made out in system HUMVWA31/A. A pull-up signal can be distinguished in the second allele peak of system HUMFES/FPS. The peaks on the left up to 80 bp long, so called primer dimer, are the result of non-specific primer-primer interactions

Table 3 STR genotypes of the eight skeletons^a

Individual	HUMVWA31/A 135bp–167bp	HUMF13A1 181bp–235bp	HUMFES/FPS 213bp–237bp	HUMCD4 86bp–121bp	HUMTH01 154bp–178bp	DIS1656 122bp–160bp
Ma 1	14/17	6/7	10/11	10/11	6/8	15.3/19.3
Ma 10	16/18	5/7	(10/11/12)	5/11	6/(8)	12/(18.3/19.3)
Ma 11	14/17	6/7	10/(11)	5/12	–/–	11/(18.3/19.3)
Ma 32	14/17	6/7	10/(11)	5/11	(9)–	(17.3/18.3/19.3)
Ma 33	15/17	5/(5)	10/11	6/10	9/(9)	–/–
Ma 54	14/17	5/7	10/11	6/11	–/–	(18.3)/19.3
Ma 55	16/17	5/5	10/10	(5/6/8)	6/9.3	–/–
Ma 56	14/–	–/–	–/–	–/–	–/–	–/–

^aAllele designation for all loci is based on the number of repeat units.⁴⁵ Fragment lengths were determined after electrophoresis in PAA gels. To be typed unequivocally as homozygous for a given locus, at least five amplifications must show consistent reproduction of the same allele, otherwise the second allele is in parentheses. Allele products which could not be detected in at least three amplifications are also in parentheses. In some cases, determination was not possible: this is signified by a dash.

Table 4 Y-haplotypes of five Königsfeld individuals^a

Individual	DYS19 174bp–210bp	DYS390 191bp–227bp	DYS389I 145bp–169bp	DYS389II 259bp–291bp
Ma 10	14	23	12	28
Ma 11	14	(24)	13	33
Ma 33	14	23	12	28
Ma 54	14	23	12	(28)
Ma 55	14	23	12	(28)

^aWe were provided with only a very limited amount of skeletal material. Results for the Y-specific STR systems were repeated by quadruplex PCR until at least two verifying results per STR system were obtained. Results in parentheses indicate that only a single result could be produced, due to the scarcity of material.

Evaluation of the Genetic Evidence

In Figure 3, results of autosomal and Y-specific STR genotyping are presented in combination with the family tree. The following deductions can be made.

Individual 33 cannot be considered the biological father of individual 10. Three apparent exclusions of paternity were obtained.

The skeletal remains of the presumed father of Ma 54 were not available for typing, since the grave was destroyed prior to the archaeological excavations. In such a case, typing of a male relative in the paternal line will offer an indirect proof of paternity.¹⁵ Since individual Ma 54 and his alleged grandfather Ma 10 exhibit the same Y-haplotype, affiliation of Ma 54 with the Königsfeld patrilineage can be assumed.

Individual 54 is excluded from paternity of individual 11, based on an exclusion in system HUMCD4 and by the results of Y-STR typing.

Due to autosomal evidence, individual 11 can be included as putative father of individual 32. A formula for computation of the probability of paternity (W)⁴⁰ in cases lacking a mother is described elsewhere.⁴¹ Allele frequencies were derived from data gathered in recent

German populations.⁴² For the putative father-son pair, a probability of paternity of $W = 85\%$ was computed.

It is interesting to note that individual 33 and individual 10 exhibit an identical Y-haplotype; nevertheless a definite exclusion of paternity for Ma 33 can be obtained from the autosomal STR evidence. Yet it can be observed that Ma 55 exhibits the same Y-haplotype as Ma 10 and reveals an autosomal STR genotype that would not be in contradiction with paternity of Ma 55 to Ma 10. These results led to the suggestion that the skeletons of Ma 55 and Ma 33 had been mistaken for each other. This seems plausible considering that the tombstones were not directly affiliated with the graves, but were attached to the walls of the chancel. A probability of paternity of $W = 79\%$ can be derived for the putative father-son pair Ma 55 and Ma 10.

Identification of Individual Ma 1

Archaeological data could not identify individual Ma 1 although historical sources¹ do present evidence of the burial of a female in St Margaretha's.

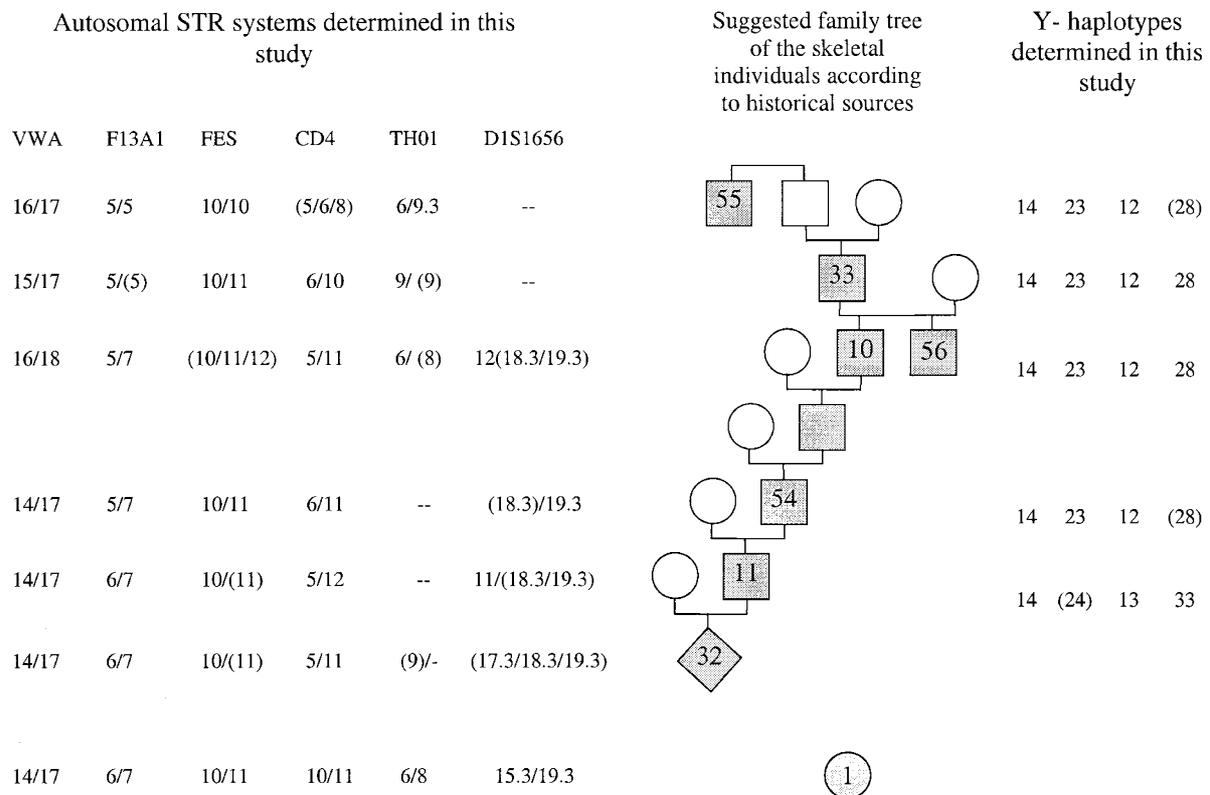


Figure 3 Presentation of the STR genotypes and Y-haplotypes in combination with the family tree, which was reconstructed according to archaeological and historical findings. For individual Ma 56, a determination of the genotype was not possible, due to the severe degree of DNA degradation in the skeletal material

Archaeological and morphological findings determined individual Ma 1 as an approximately 30-year-old female buried at the beginning of the 18th century. Therefore it is possible to fit Ma 1 into the family tree as putative mother of individual Ma 32. The observed genotypes of the three individuals (Ma 1, Ma 11 and Ma 32) exhibit a pattern which would be expected in a family group (Table 5). For this trio, a probability of $W = 85\%$ for Ma 11 and $W = 98\%$ for Ma 1 can be obtained.

We interpreted the results to the effect that Ma 1 is Maria Anna von Königsfeld, wife of Georg Josef von Königsfeld (Ma 11). According to historical sources,¹ Maria Anna was born in 1686. After marriage to Georg Josef for nine years, she died in 1722.

For Ma 32 female sex was clearly determined, thus excluding the identification of this individual as Karl Albrecht. Further studies of historical sources revealed that four of his sisters were buried in St Margaretha's. All of them died as infants and were possibly laid to rest in the same grave as their brother. So it is not unlikely that Ma 32 is indeed one of the sisters of Karl Albrecht.

Discussion and Conclusion

Up to now the reconstruction of kinship in (pre)historic family groups or societies has relied upon the comparison of phenotypic traits.⁴³ Thus the results have been of a more or less tentative nature. The case of the Königsfeld individuals presented an ideal opportunity of showing that kinship structures in skeletal remains can most feasibly be validated by examining the genetic variation between individuals. DNA typing led to the establishment of the paternal lineage of the descendants of the House of Königsfeld. It could be deduced from the results of Y-haplotyping that one individual buried in the Königsfeld sepulchre cannot be considered consanguine with the other male individuals. We can only speculate that this individual is the product

of adultery or that the identification of Ma 11 as a member of the House of Königsfeld is simply incorrect.

However, the following can be concluded from the STR analysis. It is very probable that Ma 11, Ma 1 and Ma 32 present a family trio. In accordance with archaeological experience and historical knowledge, we believe it is rather unlikely that a family not belonging to the Königsfeld genealogy was laid to rest in the sepulchre.

For Ma 32, a morphological sex-determination was not possible. Nevertheless, the age of the individual was determined as 15 years old at most (Schröter P, personal communication). Presumably due to this, archaeologists identified Ma 32 as Karl Albrecht von Königsfeld, who died at age 13. However, molecular typing of Ma 32 as female challenges this assumption. It might be speculated that the Y-encoded amelogenin gene is deleted in this individual. A frequency of 0.6% is reported for this deletion polymorphism in population samples from around the world.⁴⁴ Although the frequency in past populations is unknown, it seems rather unlikely that a deletion of this kind should have occurred in this individual. Since the DNA derived from samples of Ma 32 proved to be well preserved, it is also rather unlikely that a drop-out of the Y-specific fragment has occurred in the amelogenin amplifications. Therefore, it can be assumed that Ma 32 is indeed a sister of Karl Albrecht.

It would have been interesting to compare Y-haplotypes of living paternal relatives with the results obtained from the historical specimens. Alas, the last paternal descendant of the Königsfelder lineage died in 1815.¹

Corroboration of archaeological, morphological and molecular evidence led to the tentative identification of Ma 1 as Maria Anna of Königsfeld. In this and other cases where inclusive statements concerning relationships are made, it cannot be emphasised too strongly that the calculated probabilities of paternity and maternity represent hypothetical values because data

Table 5 STR genotypes of the putative family trio Ma 1 (mother), Ma 11 (father), Ma 32 (child)

<i>Skeleton</i>	<i>HUMVWA31/A</i>	<i>HUMF13A1</i>	<i>HUMFES/FPS</i>	<i>HUMCD4</i>	<i>HUMTH01</i>	<i>DIS1656</i>
Ma 1	14/17	6/7	10/11	10/11	6/8	15.3/19.3
Ma 11	14/17	6/7	10/(11)	5/12	–/–	11/(18.3/19.3)
Ma 32	14/17	6/7	10/(11)	5/11	(9)/–	(17.3/18.3/19.3)

on historical allelic frequencies is not available. Moreover, the individuals examined were members of a fairly small aristocratic class. Marriages among closely related individuals were presumably rather common. This might result in allelic frequencies deviating from frequencies in recent populations assumed to be in Hardy-Weinberg equilibrium.

However, despite these objections and characteristic features of aDNA mentioned above, it can be shown that the molecular approach is the most powerful tool for the identification and reconstruction of kinship of skeletal human remains.

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