doi: 10.3325/cmj.2009.50.286

# Kinship and Y-Chromosome Analysis of 7th Century Human Remains: Novel DNA Extraction and Typing Procedure for Ancient Material

Daniel Vanek[1], Lenka Saskova[1], Hubert Koch[2]

[1]Forensic DNA Service, Prague, Czech Republic

[2]Bavarian State Department of Monuments and Sights, Regensburg, Germany

**Aim** To develop novel DNA extraction and typing procedure for DNA identification of the 7th century human remains, determine the familiar relationship between the individuals, estimate the Y-chromosome haplogroup, and compare the Y-chromosome haplotype with the contemporary populations.

**Methods** DNA from preserved femur samples was extracted using the modified silica-based extraction technique. Polymerase chain reaction amplification was performed using human identification kits MiniFiler, Identifiler, and Y-filer and also laboratory-developed and validated Y-chromosome short tandem repeat (STR) pentaplexes with short amplicons.

**Results** For 244A, 244B, 244C samples, full autosomal DNA profiles (15 STR markers and Amelogenin) and for 244D, 244E, 244F samples, MiniFiler profiles were produced. Y-chromosome haplotypes consisting of up to 24 STR markers were determined and used to predict the Y-chromosome haplogroups and compare the resulting haplotypes with the current population. Samples 244A, 244B, 244C, and 244D belong to Y-chromosome haplogroup R1b and the samples 244E and 244F to haplogroup G2a. Comparison of ancient haplotypes with the current population yielded numerous close matches with genetic distance bellow 2.

**Conclusion** Application of forensic genetics in archaeology enables retrieving new types of information and helps in data interpretation. The number of successfully typed autosomal and Y-STR loci from ancient specimens in this study is one of the largest published so far for aged samples.

Received: January 21, 2009

Accepted: April 27, 2009

**Correspondence to:**
Daniel Vanek
Forensic DNA Service
Janovskeho 18
170 00 Prague 7, Czech Republic
daniel.vanek@DNA.com.cz

Anthropological determination of sex and age in ancient human remains entails an unacceptable level of uncertainty, especially in cases of infant burials (1). The analysis of DNA recovered from archaeological remains is not only used to determine sex (2) but also to assemble the commingled remains (3), establish the mating or burial patterns (4), or make a kinship analysis for the reconstructing genealogy of historical burial sites (5,6). Comparison of older archaeological and historical hypotheses with the exact anthropological and genetic findings is a current trend that can bring more objectivity to historical anthropology. Currently available archaeogenetic techniques (7) can be combined with modern molecular-biological techniques to improve the quality of information retrieved from the ancient artifacts (8).

DNA analysis of bone samples is considered to be very difficult due to the numerous factors influencing the success or failure of testing, such as composition and humidity of soil, age of the specimen, presence of microorganisms, pH and temperature, and the treatment after exhumation. Therefore, there is an understandable need for a multidisciplinary approach which would solve problems related to DNA analysis of ancient material (7).

In this study, we describe the DNA extraction and typing procedure developed for successful DNA identification of ancient bone samples. The whole process comprises the laboratory set-up, counter cross-contamination procedures, improved extraction of nucleic acids, sensitive DNA quantitation, polymerase chain reaction (PCR) using MiniFiler, Identifiler, and Y-filer kits (Applied Biosystems, Foster City, CA, USA) and the short Y-STR amplicon miniplexes, and Y-chromosome haplogroup prediction.

Analyzed bone samples from the second half of the 7th century were found in a burial-place in Ergolding, Germany. Previous archaeological research revealed that the artifacts found with the individual 244A (9) are of very high historic value, suggesting that this individual was a very important person. Therefore, it was decided to perform DNA analysis of all 6 skeletons from the central grave 244 in order to find out more about the familiar relationship and the geographic origin of the individuals.

## MATERIALS AND METHODS

### Burial place

Early-medieval burial place in Ergolding (Bavaria, Germany) was archaeologically examined in the period 1997-2002 by the Bavarian State Department of Monuments and Sights, Germany. The archaeological research revealed more than 440 graves (9). DNA analysis was performed on the remains of 6 men of early adult age found in the central grave number 244. Men from the grave 244 (marked 244A to 244F) were buried together into a single wooden burial chamber. Individuals found in the western part of the chamber (244A, 244B, and 244C) lied straight on the back, body-by-body, and all 3 men were buried with swords, spears, shields, and spurs, like heavily armored mounted warriors (9). Historic value of the artifacts found in the grave 244 makes this place one of the richest Bavarian burial sites from the late-Merowig period (9). The grave 244 dates to the period around 670 AD. The eastern part of the burial chamber with the individuals 244D, 244E, and 244F was robbed and therefore no valuable artifacts were found.

### Laboratory set-up and counter cross-contamination procedures

Cleaning of the human remains and the DNA extraction was performed in a specialized laboratory dedicated specifically for the analysis of the ancient samples. Only staff with known DNA profile was allowed to enter this laboratory.

The monitoring of background contamination by the modern DNA was performed using the 4N6 XC Test (Biologicals, Ricany, Czech Republic). Critical areas and items (floor, tables, bone cleaning tent, surface of pipettes, interior of the laminar box, and centrifuge and blender cups) were regularly monitored for the presence of contamination. Wet swabs from the selected areas and items were taken using the 4N6 DNA Swabs (Copan, Brescia, Italy) and the DNA was extracted using the standard ChargeSwitch® procedure (Invitrogen, Carlsbad, CA, USA). The design of the 4N6 XC test assay is based on the simultaneous (2-in-1) real-time PCR quantification and short tandem repeat (STR) typing in one tube, and allows identifying the source of contamination and therefore accepting appropriate decontamination procedures.

All consumables entering the laboratory were purchased as DNA-clean and were further UV irradiated before use for at least 10 minutes in a UV crosslinker CL-1000 (UVP, Upland, CA, USA). DNA extraction was performed in laminar flow box. All staff wore laboratory coats, facemasks, hairnets, and gloves during the whole stay in the laboratory.

### Bone sample preparation

Bags with samples from the grave 244 were examined upon delivery to the laboratory and only the suitable (at least 10-cm long) fragments of the femur or the whole femur were used for the sampling. The sampling was performed in the plastic tent Captair Pyramid® (Erlab, Köln, Germany), that was wiped by the 10% solution of the sodium hypochlorite prior to use. Using the micro-tool Dremel® (Dremel, Racine, WI, USA), we cut off approximately 8 cm-long half-round piece of the femur. In order to remove the contaminants from the bone sample surface, we ground away 2-3 mm of the bone. Cleaned semicular bone sample was further cut to smaller pieces approximately 5 mm thick. The sample was then transferred to 50-mL tube and further chemically cleaned by inversion for 30 seconds in 5% commercial bleach, followed by 2-3 washes in distilled water (molecular biology grade) and 1 thorough wash in 96% ethanol. Cleaned and wet samples were covered by 30 mL of HaR buffer (Biologicals) and stored for a minimum of 4-6 weeks at ambient temperature. Samples removed from the HaR buffer were rinsed by 96% ethanol and dried in Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 56°C for 12 hours. Completely dried samples were transferred to bleach-cleaned and UV-irradiated 75-mL blender cup (Warring, Torrington, CT, USA) and ground to a fine powder. The resulting bone powder was transferred to a clean 50-mL tube and its mass was recorded. The bone powder was stored at +4-8°C prior to DNA extraction.

### Silica-based DNA extraction from bone powder

The extraction procedure used was based on the protocol reported by Davoren (10), with significant modification at the beginning of the method. The bone powder (approximately 1.5 g per extraction) was incubated at 56°C for 18-24 hours in Thermomixer Comfort (Eppendorf) in 14 mL of DeX buffer (Biologicals) and 20 mg of Proteinase K (Roche, Basel, Switzerland). Starting from the second digestion with AL buffer (Qiagen, Hilden, Germany), we followed the original protocol (10) but in the last step 6 mL of eluted DNA was concentrated to approximately 50 µL using Amicon® 50kDa ultracel column (Millipore, Billerica, MA, USA) and a vacuum concentrator 5301 (Eppendorf). A minimum of 2 independent extractions per sample were performed.

### DNA quantitation and quality assessment

Extracted DNA was quantified by real-time PCR, using the DeG 4N6 Quant kit (Biologicals) on MasterCycler® ep realplex S instrument (Eppendorf). This quantitation chemistry employs 2 sets of primers for different targets of human ALU sequence in the combination with the SYBR green intercalation dye. The concept of the DeG 4N6 Quant quantitation system allows to detect as little as 1 pg of human DNA and also to estimate the relative level of DNA degradation and to determine the presence of PCR inhibitors.

### PCR amplification

Samples from extractions A-F were PCR-amplified using human identification kits MiniFiler, Identifiler, and Y-filer and the laboratory-developed Y-miniplex I and II. PCR conditions for MiniFiler, Identifiler, and Y-filer kits were in accordance with manufacturer's recommendations, with the exception that in order to increase the expected yield of the PCR product, the recommended cycle number was increased by one. All amplifications were performed on MasterCycler® ep gradient S termocycler.

The newly designed Y-chromosome miniSTR pentaplexes included 8 "non-core" Y-STR loci: DYS388, DYS426, DYS444, DYS446, DYS447, DYS449, DYS459, DYS481 plus additional 2 Y-STR loci – DYS392 and DYS438, that overlap with the of Y-filer™ kit (Table 1). Primers for all the selected loci were obtained from a published source (11) or re-designed using Primer Design and Primer Select programs (DNASTAR, Madison, WI, USA) to produce as small as possible amplicon sizes. Primer sequences are listed in Table 2. PCR mix contained 2.5 µL of Gold Star 10× buffer (Promega, Madison, WI, USA), 2.5 µL of either 10× MiniPrimer mix I or 10× MiniPrimer mix II, 2.5 U of AmpliTaq Gold DNA Polymerase

TABLE 1. Definition of Y chromosome short tandem repeat loci used in the Y-miniplex I and Y-miniplex II

| Miniplex | Repeat motif | Allele range | Product size (bp) |
|---|---|---|---|
| **Y-miniplex I** | | | |
| DYS446 | (TCTCT)n | 8-23 | 85-160 |
| DYS444 | (ATAG)n | 9-16 | 287-315 |
| DYS438 | (TTTTC)n | 7-16 | 95-140 |
| DYS388 | (ATT)n | 10-15 | 151-166 |
| DYS481 | (CTT)n | 18-31 | 115-158 |
| **Y-miniplex II** | | | |
| DYS426 | (GTT)n | 9-13 | 85-110 |
| DYS459 | (ATTT)n | 6-10 | 136-156 |
| DYS392 | (TAT)n | 6-18 | 94-130 |
| DYS449 | (TTTC)n (N)n (TTTC)n | 26-37 | 222-262 |
| DYS447 | (TAATA)n (TAAAA)n | 22-29 | 200-245 |

TABLE 2. Primers used in the Y-miniplex I and Y-miniplex II

| Miniplex/ Locus | Orientation | Primer sequences (5'to 3') | Dy label |
|---|---|---|---|
| Y-miniplex I | | | |
| DYS446 | F | TATTTTCAGTCTTGTCCTGTC | FL |
| | R | GAGACTCTGTCTGAAGAGAG | |
| DYS444 | F | TCTAAGGGATCCAAAGGCAGAA | FL |
| | R | GTGTGAACCATTTGGCATGTTTA | |
| DYS438 | F | TGGGGAATAGTTGAACGGTAA | JOE |
| | R | GGCAACAAGAGTGAAACTCCA | |
| DYS388 | F | GAATTCATGTGAGTTAGCCGTT-TAGC | JOE |
| | R | GAGGCGGAGCTTTTAGTGAG | |
| DYS481 | F | AGGAATGTGGCTAACGCTGT | TAMRA |
| | R | ACAGCTCACCAGAAGGTTGC | |
| Y-miniplex II | | | |
| DYS426 | F | GGTGACAAGACGAGACTTT-GTGT | FL |
| | R | CTCAAAGTATGAAAGCATGACCA | |
| DYS459 | F | CAGGTGAACTGGGGTAAATAAT | FL |
| | R | TTGAGCAACAGAGCAAGACTTA | |
| DYS392 | F | AAAAGCCAAGAAGGAAAA-CAAA | JOE |
| | R | GAAACCTACCAATCCCATTCCTT | |
| DYS449 | F | CTTGCTCTTTTTCTTTTCTCTCTT | JOE |
| | R | GCACTCTAGGTTGGACAACAA | |
| DYS447 | F | GGTCACAGCATGGCTTGGTT | TAMRA |
| | R | GGGCTTGCTTTGCGTTATCTCT | |

(Applied Biosystems), genomic DNA, and water in a final volume of 25 µL. The minimum quantity of male DNA required to acquire reliable results was 50 pg per PCR reaction. PCR amplification was performed using the following cycling protocol: 11 minutes of initial denaturation at 95°C, followed by a 3-step profile consisting of 30 seconds of denaturation at 94°C, 1 minute and 30 seconds of annealing at 59°C, and 1 minute and 30 seconds of extension at 72°C for 31 cycles. A final extension at 60°C took 90 minutes. All amplifications were performed on MasterCycler® ep gradient S termocycler.

### Capillary electrophoresis

Amplified STR fragments were separated on ABI PRISM® *310* Genetic Analyzer (Applied Biosystems) under standard conditions. Samples were injected (5 kV injections) for 5-10 seconds. The resulting data were analyzed using GeneMapper ID software, version 3.2 (Applied Biosystems) with a 50 relative fluorescent units (RFU) analysis threshold.

### Interpretation of results

A minimum of 2 independent extractions per sample were performed and the resulting extract was used for DNA quantitation and a set of PCRs. In order to perform a reliable interpretation of results, we strictly followed the rule that an allele can only be reported if observed at least twice in replicated samples. This method of replicated analyses is widely used in the interpretation of LCN and mixed samples results (12,13).

### Kinship analysis

In order to establish the kinship relationships among all individuals, statistical analyses using the Patcan version 1.2. software (14) were performed. All calculations were based on allelic frequencies estimated for German population by Huckenbeck (15).

### Y-haplogroup prediction and database searches

For all haplotypes, Y-chromosome haplogroups were predicted using the method described by Athey (16). The resulting Y-chromosome haplotypes were searched against Y-chromosome haplotypes in publicly accessible databases (www.ysearch.org, www.genebaze.cz, and www.yhrd.org).

## RESULTS

The initial examination of femur samples from the Ergolding 7th century burial place revealed that the outer surface of samples 244A, B, C, D, and F was more porous, most probably as a result of demineralization (Figure 1). The core of the bone was, on the other hand, very hard and the cut to the bone had unusual opalescent, mother-of-pearl appearance (Figure 2). The femur from the individual 244E had no signs of fragmentation of the surface and the cut to the body of the femur had a standard ivory white appearance.

The initial extractions performed with the standard protocol (10) were not successful. We either obtained no measurable amount of extracted DNA or the extracted DNA contained a massive concentration of inhibitors that completely blocked both the RT-PCR quantitation and PCR amplification. In order to remove soil-born inhibitors, we covered previously cleaned samples by 30 mL of HaR buffer and stored for a minimum of 4-6 weeks at ambient temperature. The employment of HaR completely solved the problem of inhibitors.

Figure 1.

The appearance of the surface of bone samples selected for DNA analysis. Figure 1 shows the sample 244B.

Figure 2.

The nacreous appearance of the cut to the bone samples selected for DNA analysis. Figure 2 shows the sample 244B."

In order to increase the yield of DNA per gram of bone powder, we exchanged the original ATL buffer used in the first digestion step with the DeX buffer (Biologicals). The modified extraction protocol with HaR and DeX buffers yielded approximately 0.5-3.0 ng of DNA from 1.5 g of bone powder used for extraction.

By combining the data from MiniFiler and Identifiler typing, for samples 244A, 244B, 244C we obtained full autosomal DNA profiles (15 STR markers and Amelogenin) and for samples 244D, 244E, and 244F we obtained MiniFiler profiles based on 8 STR and Amelogenin markers (Table 3). The resulting electopherograms (EPG) were free of contamination signs and artificial peaks, and the peak heights were sufficient for reliable analysis of data. Figure 3 shows EPGs obtained for sample 244B. The results of autosomal STR typing were further used for statistical evaluation of kinship analysis.

TABLE 3. DNA profiles obtained using the combination of forensic kits MiniFiler and Identifiler

| | Sample | | | | | |
|---|---|---|---|---|---|---|
| Locus* | 244A | 244B | 244C | 244D | 244E | 244F |
| D13S317 | 12/13 | 12 | 9/12 | 12 | 10/12 | 8/(13) |
| D7S820 | 8/11 | 8/11 | 10/11 | 9/11 | 9/12 | 11 |
| AMELOGENIN | X/Y | X/Y | X/Y | X/Y | X/Y | X/Y |
| D2S1338 | 17/24 | 17/24 | 17/20 | 20/24 | 17 | 19/23 |
| D21S11 | 28/29 | 28/29 | 28/29 | 29 | 29/31 | 28.2 |
| D16S539 | 9/12 | 11/12 | 11/14 | 10 | 8/11 | 13 |
| D18S51 | 12/17 | 12/17 | 16/17 | 12/15 | 13/17 | 12/15 |
| CSF1PO | 10/12 | 10/12 | 12/14 | 10/12 | 11/11 | 10 |
| FGA | 22/24 | 21/22 | 20/23 | 21/22 | 21/25 | 23/24 |
| D8S1179 | 10/14 | 10/14 | (11/13) | – | – | – |
| D3S1358 | 17/18 | 16/18 | 15/18 | – | – | – |
| THO1 | 6/8 | 6/8 | 9 | – | – | – |
| D5S818 | 11/13 | 11/13 | (11/12) | – | – | – |
| TPOX | (8/9/11) | 8/11 | 8/11 | – | – | – |
| vWA | 17/19 | 17/19 | (17/18/19) | – | – | – |
| D19S433 | 13/15 | 13/15 | 14/16 | – | – | – |

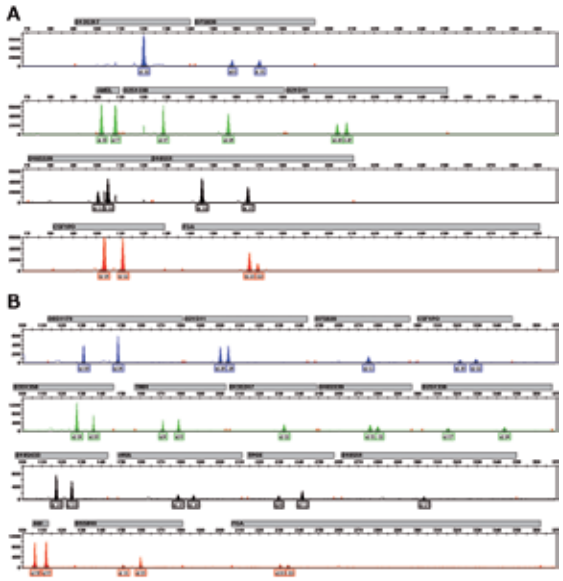
*Alleles in table were observed at least twice in replicate samples. Alleles in brackets were observed just once.

By combining the data from Y-filer and Y-miniplex I and II, we obtained Y-chromosome haplotypes consisting of 24 (samples 244B, 244C), 21 (sample 244A), 19 (sample 244D), and 17 (samples 244E, 244F) STR markers (Table 4). Figures 4 and 5 show EPGs obtained for sample 244B. None of the tested samples provided results in all 26 loci. All samples provided results in loci DYS389 I, DYS458, DYS393, DYS392, DYS438, DYS426, and DYS459; on the contrary, none of the samples yielded results in DYS444 (included in Y-miniplex I). We also observed that sample 244E had a very rarely observed duplication in the locus DYS19 (alleles 14 and 15). Samples 244A and 244B were identical in the respect of their Y-chromosome haplotypes. Sample 244C was closely related to the 244A/B haplotype (genetic distance 1 is caused by 1 repeat difference in DYS458). No other close relationship according to paternal lineage was found among the investigated samples.

Y-chromosome haplogroups were successfully predicted for all samples. Samples 244A, 244B, 244C, and 244D belonged to the Y-chromosome haplogroup R1b and samples 244E and 244F belonged to haplogroup G2a.

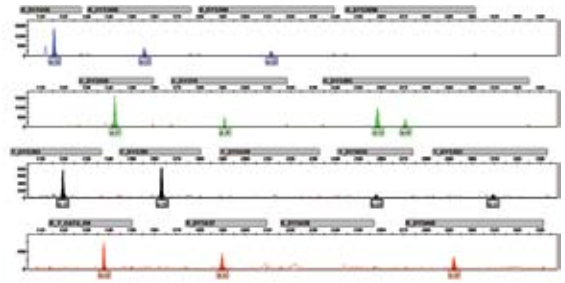
Resulting Y-chromosome haplotypes were stored in publicly accessible database www.ysearch.org (for sample identifiers

Figure 3.




Electropherograms of DNA typing results of the sample 244B using Minifiler (A) and Identifiler (B) amplification kits. Peak labels represent alleles obtained; intensity of the peaks is expressed in relative fluorescent units.

Figure 4.



Electropherograms of DNA typing result of the sample 244B using Y-filer amplification kit. Peak labels represent alleles obtained; intensity of the peaks is expressed in relative fluorescent units.

see the Table 5). The comparison of Y-chromosome haplotypes for the samples from the grave 244 with the database www.ysearch.org resulted in numerous close matches with living individuals. Table 6 summarizes the results for sample 244A/B and matches with genetic distance 0 and 1.

The statistical analysis of the kinship relationship between the individuals from the grave 244 revealed the following:

TABLE 4. Y-chromosome haplotypes obtained using the Y-filer forensic kit and the Y-miniplex I and Y-miniplex II. Alleles in the table were observed at least twice in replicate samples

| | Sample | | | | | |
|---|---|---|---|---|---|---|
| Locus* | 244A | 244B | 244C | 244D | 244E | 244F |
| DYS456 | 16 | 16 | 16 | 15 | – | – |
| DYS389 I | 13 | 13 | 13 | 13 | 12 | 12 |
| DYS390 | 23 | 23 | 23 | 24 | – | - |
| DYS389 II | – | 29 | – | 29 | 29 | 29 |
| DYS458 | 17 | 17 | 18 | 18 | 16 | 19 |
| DYS19 | – | 14 | 14 | – | 14, 15 | – |
| DYS385 | 11, 14 | 11, 14 | 11, 14 | 11, 14 | - | 15 |
| DYS393 | 13 | 13 | 13 | 13 | 14 | 13 |
| DYS391 | 10 | 10 | 10 | 11 | – | 10 |
| DYS439 | – | – | 12 | 12 | 11 | – |
| DYS635 | – | 23 | 23 | 23 | – | – |
| DYS392 | 13 | 13 | 13 | 13 | 11 | 11 |
| Y-GATA-H4 | 12 | 12 | 12 | 11 | – | 12 |
| DYS437 | 15 | 15 | 15 | 15 | 16 | – |
| DYS438 | 12 | 12 | 12 | 12 | 10 | 11 |
| DYS448 | 19 | 19 | 19 | – | – | 22 |
| DYS446 | 13 | 13 | 13 | – | 21 | 16 |
| DYS444 | – | – | – | – | – | – |
| DYS388 | 12 | 12 | 12 | – | 13 | 12 |
| DYS481 | 22 | 22 | 22 | – | 21 | 21 |
| DYS426 | 12 | 12 | 12 | 12 | 11 | 11 |
| DYS459 | 9, 10 | 9, 10 | 9, 10 | 8, (10)* | 9, 9 | 9, 9 |
| DYS449 | 29 | 29 | 29 | 29 | – | 27 |
| DYS447 | 24 | 24 | 24 | – | 23 | – |
| Estimated haplogroup | R1b | R1b | R1b | R1b | G2a | G2a |

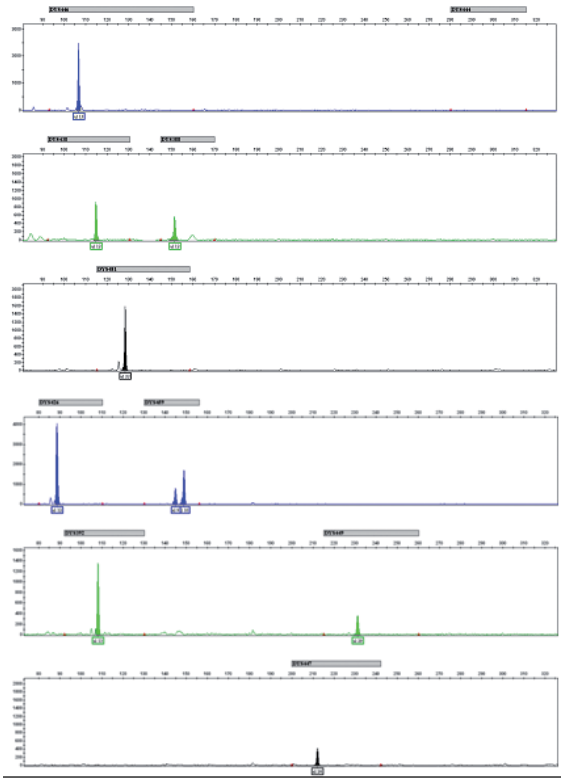
*Alleles in brackets were observed just once. Please note the duplication event observed in the locus DYS19 of sample 244E.

most likely, samples 244A and 244B were from full siblings (brothers). Calculated probability was 99.99979%. Based on calculations using the autosomal markers, all other individuals from the same grave were unrelated. However, even if there was no significant kinship relationship between samples 244A or 244B and 244C based on the analysis of the autosomal STR markers, their Y-chromosome haplotypes were closely related. Therefore, we concluded that the sample 244C was coming from a closer unspecified paternal relative of the individuals 244A and 244B (eg, distant cousin).

## DISCUSSION

The main goals of this study were to develop novel DNA extraction and typing procedure that would enable to perform DNA identification of 7th century human

Figure 5.



Electropherograms of DNA typing results of the sample 244B using Yminiplex I and Y-miniplex II quintuple systems. Peak labels represent alleles obtained; intensity of the peaks is expressed in relative fluorescent units.

remains using currently available techniques, to set the familiar relationship between the individuals, to estimate the Y-chromosome haplogroup and to compare the Y-chromosome haplotype with the contemporary populations.

Appropriate DNA extraction technique is condition sine qua non for the majority of ancient samples. It is obvious that the chemical composition of soil, site humidity, pH, temperature, presence of microorganisms, as well as the age of the specimen will influence the success rate of the DNA extraction from bone samples. According to our previous experience with historical (8) and samples from the mass graves found in former Yugoslavia (10), only middle part of the femurs were used for the extraction of DNA. The initial extraction attempts were not successfully performed by according to the standard protocol (10). We either obtained non measurable amounts of DNA or extracted DNA showed the presence of PCR inhibitors.

TABLE 5. Identifiers assigned to Y-chromosome haplotypes in YSEARCH database

| Sample | YSEARCH identifier |
|---|---|
| 244A/244B | 6QUDR |
| 244C | H78RA |
| 244D | ZYRUU |
| 244E* | H6KZ2, QCX3T |
| 244F | 2SRMA |

*244E has duplication at the locus DYS19 (alleles 14 and 15). Ysearch settings do not allow entering two values for this locus, therefore the data were submitted to the database twice: once with allele 14 (User ID=QCX3T) and the second time with allele 15 (User ID=H6KZ2). The database was searched for both IDs and the resulting table is the compilation of these two searches. The original genetic distances were increased by 1, which accounts for the duplicated DYS19 in 244E.

The use of the newly developed and validated HaR buffer, which removed the inhibitors prior to the extraction, helped to surmount the inhibition problems. At the same time, substitution of ATL tissue lysis buffer (Qiagen) with specially designed DeX buffer improved the extraction efficiency and we were able to get approximately 0.5-3.0 ng of DNA from 1.5 g of bone powder. Therefore, the effectiveness of DeX buffer in decalcification and protein digestion was entirely proven. The amount of extracted DNA was sufficient for DNA quantitation and quality assessment using the DeG 4N6 Quant kit, as well as for both autosomal and Y-chromosome STR typing. The silica-based extraction procedure used in this study provided sufficient amount of nuclear DNA free of protein inhibitors (17) and we had no need to use further cleaning procedures to be able to remove the extracts (18). Employment of any other measures to minimize PCR inhibitory effect (19) was unnecessary. Despite the fact that we achieved remarkable success in analyzing bones from the grave 244, it is logical to anticipate that some excavation sites with different influencing conditions might require further modifications of the proposed protocol, especially concerning the removal of different inhibitors and sample digestion.

MiniFiler PCR Amplification Kit proved its suitability for highly degraded and inhibited samples. We achieved 100% success rate (full profile) for all samples typed. Kinship analysis performed on the MiniFiler results revealed the full sibling relationship between individuals 244A and 244B, and Y-chromosome results showed the relatedness of the 244A/244B and 244C according to the paternal line. The extended DNA typing with Identifiler performed on samples 244A, 244B, and 244C helped us to get the full 15-loci autosomal profile and more accurate results in kinship analysis. We have no knowledge about the popula-

TABLE 6. Results of the Y-chromosome haplotype of the samples 244A/244B search against the records in the publicly accessible database www.ysearch.org

| User ID | Last name | Origin | Haplogroup | Markers compared | Genetic distance* |
|---|---|---|---|---|---|
| 6QUDR | Unknown skeleton 244AB | Germany | R1b | 25 | 0 |
| 5KGUJ | Null425-S21 | Unknown | R1b1b2g | 24 | 1 |
| BKS6Y | Pilkinton | England | R1b | 24 | 1 |
| H78RA | Unknown skeleton 244C | Germany | R1b | 24 | 1 |
| R2NPS | Drake | Ireland | R1b1 (tested) | 22 | 0 |
| 3DPK6 | Lener | Pennsylvania, USA | R1b1 (tested) | 22 | 0 |
| 55NY4 | Knowles | England | R1b (tested) | 22 | 1 |
| ABP3E | Fullwood | North Carolina, USA | R1b1b2 (tested) | 22 | 1 |
| BCDEJ | Bland | North Carolina, USA | R1b1b2 (tested) | 22 | 1 |
| 7M32W | Coale | England | R1b (tested) | 22 | 1 |
| BQFW4 | Moore | Virginia, USA | R1b1b2 (tested) | 22 | 1 |
| Y9DB5 | Morris | North Carolina, USA | Unknown | 22 | 1 |
| ZBG6C | Bland | South Carolina, USA | R1b1b2 (tested) | 22 | 1 |
| CXCCK | Duff | South Carolina, USA | R1b1b2 (tested) | 22 | 1 |
| H9V9F | Bland | Kentucky, USA | Unknown | 22 | 1 |
| K5F2C | Lowery | Unknown | Unknown | 22 | 1 |
| KMMMD | Ratcliff | England | R1b | 22 | 1 |
| MNRQ2 | Lowery | Unknown | Unknown | 22 | 1 |
| NST5Q | Austin | Unknown | Unknown | 22 | 1 |
| SB7ZF | Ogden | Unknown | Unknown | 22 | 1 |
| TRSR8 | Middlebrooks | England | R1b1b2 (tested) | 22 | 1 |
| U9ZSP | Bland | England | Unknown | 22 | 1 |
| 9SAY7 | Meeks | USA | R1b1 (tested) | 22 | 1 |
| KKREK | Speed | Barbados | R1b (tested) | 22 | 1 |
| Y5TTM | Peyton | USA | R1b1 (tested) | 22 | 1 |
| HHH5E | Ridley | Ireland | Unknown | 22 | 1 |
| J5S97 | Radcliffe | England | Unknown | 22 | 1 |
| MR9PE | Middlebrook | England | R1b1b2g (tested) | 22 | 1 |
| SRU6A | Radcliff | Ireland | R1b1b2 (tested) | 22 | 1 |
| RS3ND | Cheetham | England | Unknown | 22 | 1 |
| T25ST | Martin | Canada | R1b | 21 | 1 |

*Only matches with genetic distance 0 and 1 are shown.

tion structure around Ergolding in 7th century and therefore cannot exclude possible inbreeding and selection trends. The statistical calculations were performed using the contemporary population data for Germany (15) and therefore we can expect some level of uncertainty caused by the non-existence of reliable 7th century population data.

Currently available forensic kits for degraded DNA (miniSTRs) do not cover the Y-chromosome STR loci and therefore we had to develop and validate a set of Y-chromosome mini-STR systems that would allow performing DNA typing on highly degraded DNA samples (11,20).

None of the samples tested using Y-filer and laboratory-developed Y-miniplex I and II were successfully typed in all 26 loci. However, we were able to obtain Y-chromosome haplotypes consisting of 24 (samples 244B and 244C), 21 (sample 244A), 19 (sample 244D), and 17 (samples 244E and 244F) STR markers. All samples were successfully typed just in loci DYS389 I, DYS458, DYS393, DYS392, DYS438, DYS426, and DYS459. On the contrary, none of the samples processed yielded results in DYS444 (included in Y-miniplex I). The most probable explanation for the complete amplification failure of the system DYS444 might be the relatively long amplicon. The number of successfully typed autosomal and Y-STR loci from ancient specimen re-

ported herein is one of the largest published so far. Previous studies used only mtDNA typing (21,22) or found Y-chromosome haplotypes consisting of fewer loci (4,23,24).

We also observed that sample 244E had previously very rarely observed duplication in the locus DYS19 (alleles 14 and 15). As the same results of independent extractions and PCRs were obtained, we concluded that this finding might be related to the duplicated region of the Y chromosome that is highly homologous with the DYS19 flanking region (25). Balaresque (26) reported that DYS19 duplications were mainly found in Y-haplogroups G and C3c and this finding is in a perfect accordance with the haplogroup predicted for sample 244E (G2a).

Y-chromosome haplotypes obtained for ancient bone samples allowed us to perform a database search for the close matches and thus find possible distant relatives of the bodies found in the grave 244. Even if this finding has no direct scientific relevance, the distribution of closely related Y-haplotypes can support the migration patterns suggested for different Y-chromosome haplogroups (27).

One of the key factors influencing the testing quality and reliability of the DNA obtained from the ancient material is the set-up of the DNA extraction laboratory (28). It is mandatory not only to use DNA-free chemicals and consumables, but also to exercise strict contamination controls (29) and evaluate all safety measures taken. In the presented study, the utilization of 4N6 XC test for the monitoring of contamination control and firm laboratory rules resulted in problem-free evaluation of analyzed samples.

Developed DNA extraction and typing procedure enabled us to successfully perform the kinship and Y-chromosome analysis of the 7th century human remains from the late-Merowig period. The described procedure should be applicable in other archaeological, anthropological, or forensic studies.

## Acknowledgments

We acknowledge the assistance of Biologicals, namely Dr Martin Pospisek, in developing the HaR and DeX buffers used in the extraction protocol and quantitation chemistry. We also acknowledge the Prague Castle Administration, namely Dr Bravermanova, for providing the laboratory space for DNA extraction from the ancient material. This work was supported by the Bavarian State Department of Monuments and Sights and in part by Research Grant No. FI-IM3/115 from the Ministry of Industry and Trade, Czech Republic.

## References

1 Lassen C, Hummel S, Herrmann B. Molecular sex identification of stillborn and neonate individuals ("Traufkinder") from the burial site Aegerten. Anthropol Anz. 2000;58:1-8. Medline:10816779

2 Hummel S, Bramanti B, Finke T, Herrmann B. Evaluation of morphological sex determinations by molecular analyses. Anthropol Anz. 2000;58:9-13. Medline:10816780

3 Schultes T, Hummel S, Herrmann B. Classification of isolated skeletal elements using aDNA typing [in German]. Anthropol Anz. 1997;55:207-16. Medline:9341088

4 Schultes T, Hummel S, Herrmann B. Amplification of Y-chromosomal STRs from ancient skeletal material. Hum Genet. 1999;104:164-6. Medline:10190328 doi:10.1007/s004390050930

5 Alt K, Vach W. Kinship studies in skeletal remains – concepts and examples. In: Alt KW, Rösing FW, Teschler-Nicola M, editors. Dental anthropology. Fundamentals, limits and prospects. Wien: Springer; 1998.

6 Melchior L, Gilbert MT, Kivisild T, Lynnerup N, Dissing J. Rare mtDNA haplogroups and genetic differences in rich and poor Danish Iron-Age villages. Am J Phys Anthropol. 2008;135:206-15. Medline:18046774 doi:10.1002/ajpa.20721

7 Cipollaro M, Galderisi U, Di Bernardo G. Ancient DNA as a multidisciplinary experience. J Cell Physiol. 2005;202:315-22. Medline:15389521 doi:10.1002/jcp.20116

8 Kubalek P, Stolz D, Saskova L, Vanek D. A Near Eastern haplotype in the burial of a young man with a fissure in the skull from the early medieval cemetery at Tetin [in Czech]. Archaelogy in Central Bohemia. 2008;1:645-50.

9 Koch H. Chronology of Bavarian graves from Ergolding, Hagnerleiten, Landshut. Proceedings 24 [in German]. Niederbayerischen Archäologentages. 2006;6:191-9.

10 Davoren J, Vanek D, Konjhodzic R, Crews J, Huffine E, Parsons TJ. Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. Croat Med J. 2007;48:478-85. Medline:17696302

11 Hanson EK, Ballantyne J. An ultra-high discrimination y chromosome short tandem repeat multiplex DNA typing system. PLoS One. 2007;2:e688. Medline:17668066 doi:10.1371/journal.pone.0000688

12 Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, et al. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res. 1996;24:3189-94. Medline:8774899 doi:10.1093/nar/24.16.3189

13 Gill P. Application of low copy number DNA profiling. Croat Med J. 2001;42:229-32. Medline:11387628

14 Riancho JA, Zarrabeitia MTA. Windows-based software for common

paternity and sibling analyses. Forensic Sci Int. 2003;135:232-4. Medline:12927402 doi:10.1016/S0379-0738(03)00217-2

15 Huckenbeck W, Scheil HG. The distribution of the human DNA-PCR polymorphisms. Available from: http://www.uni-duesseldorf.de/WWW/MedFak/Serology/database.html. Accessed: April 28, 2009.

16 Athey TW. Haplogroup prediction from Y-STR values using an allele-frequency approach. Journal of Genetic Genealogy. 2005;1:1-7.

17 Scholz M, Giddings I, Pusch CM. A polymerase chain reaction inhibitor of ancient hard and soft tissue DNA extracts is determined as human collagen type I. Anal Biochem. 1998;259:283-6. Medline:9618211 doi:10.1006/abio.1998.2676

18 Kemp BM, Monroe C, Smith DG. Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. J Archaeol Sci. 2006;33:1680-9. doi:10.1016/j.jas.2006.02.015

19 Shutler GG, Gagnon P, Verret G, Kalyn H, Korkosh S, Johnston E, et al. Removal of a PCR inhibitor and resolution of DNA STR types in mixed human-canine stains from a five year old case. J Forensic Sci. 1999;44:623-6. Medline:10408119

20 Butler JM, Shen Y, McCord BR. The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci. 2003;48:1054-64. Medline:14535668

21 Haak W, Forster P, Bramanti B, Matsumura S, Brandt G, Tanzer M, et al. Ancient DNA from the first European farmers in 7500-year-old Neolithic sites. Science. 2005;310:1016-8. Medline:16284177

22 Bouwman AS, Brown KA, John A, Prag NW, Brown TA. Kinship between burials from Grave Circle B at Mycenae revealed by ancient DNA typing. J Archaeol Sci. 2008;35:2580-4. doi:10.1016/j.jas.2008.04.010

23 Garcia-Bour J, Perez-Perez A, Alvarez S, Fernandez E, Lopez-Parra AM, Arroyo-Pardo E, et al. Early population differentiation in extinct aborigines from Tierra del Fuego-Patagonia: ancient mtDNA sequences and Y-chromosome STR characterization. Am J Phys Anthropol. 2004;123:361-70. Medline:15022364 doi:10.1002/ajpa.10337

24 Schultes T, Hummel S, Herrmann B. Ancient DNA-typing approaches for the determination of kinship in a disturbed collective burial site. Anthropol Anz. 2000;58:37-44. Medline:10816784

25 Butler JM, Schoske R. Duplication of DYS19 flanking regions in other parts of the Y chromosome. Int J Legal Med. 2004;118:178-83. Medline:15069570 doi:10.1007/s00414-004-0436-5

26 Balaresque P, Parkin EJ, Roewer L, Carvalho-Silva DR, Mitchell RJ, van Oorschot RA, et al. Genomic complexity of the Y-STR DYS19: inversions, deletions and founder lineages carrying duplications. Int J Legal Med. 2009;123:15-23. Medline:18553096 doi:10.1007/s00414-008-0253-3

27 Wiik K. Where did European men come from? Journal of Genetic Genealogy. 2008;4:35-85.

28 Handt O, Hoss M, Krings M, Paabo S. Ancient DNA: methodological challenges. Experientia. 1994;50:524-9. Medline:8020612 doi:10.1007/BF01921720

29 Yang DY, Watt K. Contamination controls when preparing archaeological remains for ancient DNA analysis. J Archaeol Sci. 2005;32:331-6. doi:10.1016/j.jas.2004.09.008