

# Molecular genetic identification of skeletal remains from the Second World War Konfin I mass grave in Slovenia

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**Abstract** This paper describes molecular genetic identification of one third of the skeletal remains of 88 victims of postwar (June 1945) killings found in the Konfin I mass grave in Slovenia. Living relatives were traced for 36 victims. We analyzed 84 right femurs and compared their genetic profiles to the genetic material of living relatives. We cleaned the bones, removed surface contamination, and ground the bones into powder. Prior to DNA isolation using Biorobot EZ1 (Qiagen), the powder was decalcified. The nuclear DNA of the samples was quantified using the real-time polymerase chain reaction method. We extracted 0.8 to 100 ng DNA/g of bone powder from 82 bones. Autosomal genetic profiles and Y-chromosome haplotypes were obtained from 98% of the bones, and mitochondrial DNA (mtDNA) haplotypes from 95% of the bones for the HVI region and from 98% of the bones for the HVII region. Genetic profiles of the nuclear and mtDNA were determined for reference persons. For traceability in the event of contamination, we created an elimination database including genetic profiles of the nuclear and mtDNA of all persons that had been in contact with the skeletal remains. When comparing genetic profiles, we matched 28 of the 84 bones analyzed with living relatives (brothers, sisters, sons, daughters, nephews, or cousins). The statistical analyses showed a high confidence of correct identification for all 28 victims in the Konfin I mass grave (posterior probability ranged from 99.9% to more than 99.999999%).

**Keywords** Forensic identification · Mass grave victims · STR · mtDNA · Second World War bones · Slovenia

## Introduction

The ability to recover DNA from old bones has become a valuable tool for identifying victims in mass graves and individual graves from the Second World War. When working with DNA from old bones, the main problem stems from the low amount of starting molecules, degradation of DNA, and the presence of polymerase chain reaction (PCR) inhibitors [1]. MtDNA testing is regularly employed in forensic identification of aged skeletal remains [2–4], but mtDNA typing alone is often insufficient for identification, and the analysis of nuclear short tandem repeat (STR) loci is required [5]. The identification of the 50-year-old skeletal remains of pilot James B. McGovern [6] serves as a good example, because the presence of a common mtDNA control region type limited the discriminatory power of the mtDNA data, and it was only after analyses of autosomal STR loci and Y chromosomal STR loci (Y-STRs) were performed that the likelihood ratio (LR) reached a value that supported the hypothesis that the bone was from an individual related to the family references, rather than from an unrelated individual. However, due to the rather long time span since the Second World War massacres, it is difficult to find living relatives to identify the victims in mass graves. If there are no close relatives, more distant relatives can also be very helpful because a combination of genetic markers may provide satisfactory probabilities of identity.

Molecular genetic methods have been used to identify victims of massacres that took place during and after the Second World War (1945) in Slovenia [7–10] and Croatia [11], and also for Finnish soldiers that lost their lives in the

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former Soviet Union [4]. Because of differences in DNA preservation in skeletal remains due to regional climatic conditions and chemical properties of the soil, the studies vary in the number of genetic markers that researchers analyzed. Palo et al. [4] obtained mtDNA haplotypes from bones for the HVI/HVII regions and compared them to mtDNA haplotypes of living maternal relatives. Definis Gojanović et al. [11] obtained Y-STR haplotypes and compared them to Y-STR haplotypes of living paternal relatives. Marjanović et al. obtained autosomal STR profiles in one study [7] and Y-STR haplotypes in another [10]; they did not analyze the polymorphisms in the control region of mtDNA. In identifying Second World War victims from two mass graves, we obtained mtDNA haplotypes from the HVI/HVII regions, Y-STR haplotypes, and autosomal STR profiles from the bones, which made it possible to compare both close and distant relatives in both the maternal and paternal lines. Among the skeletal remains from a small mass grave at Mount Storžič, we identified three victims with posterior probability (PP) from 99.999% to 99.99999% [8, 9]. This report discusses identification of skeletal remains from a large mass grave at the cave site Konfin I, where 88 victims were killed.

The Commission on Concealed Mass Graves in Slovenia has registered almost 600 hidden mass graves from the period during and after the Second World War [12]. For most mass graves, there are no documents to base victim identification on. The Konfin I mass grave is a rare exception because a list of the victims (88 Slovenian men taken from the Central Prison on the night of 24 June 1945 and brought to the execution site at Konfin I Cave) can be made based on archives (i.e., the prisoners' logbook of the Yugoslav secret police (OZNA) Central Prison and the registry of detainees with the list of wounded and patients). Among the victims were 40 wounded men and patients that had been transferred from the general hospital in Ljubljana to the OZNA Central Prison 14 days prior to execution and 48 men selected from among the prisoners. These men were not tried in a court and had not been convicted of any crime [13]. Their bodies were thrown into a 45-m-deep karst cave, and the entrance was dynamited. The bodies were not covered with earth that would have kept the skeletons in their original position. Water runoff ran unhindered into the cave, and its 20-m<sup>2</sup> bottom was completely covered with a 2-m-thick layer of mixed bones filled with mud.

The skeletal remains were excavated under the leadership of a local archeologist and anthropologist. Under a decree by the government of the Republic of Slovenia, the Commission on Concealed Mass Graves in Slovenia entrusted identification of the victims in the Konfin I mass grave to our institute. Thus, after excavation and anthropological study, the bones were sent to us to perform the molecular genetic identification. DNA typing involved all

excavated right femurs (67 complete and 17 proximal fragments). We collected buccal swabs from 41 family references (sisters, brothers, daughters, sons, wives, cousins, and nephews) that were close or distant relatives of 36 Konfin I massacre victims. The bone samples for DNA analysis were collected, labeled, and photo documented. The bones were numbered; we used the same numbers as the anthropologist.

Typing of nuclear DNA and mtDNA was carried out for the bones, reference persons, and persons to be included in the elimination database. For reference persons, mtDNA typing was carried out for maternal relatives and Y-chromosome typing for paternal relatives.

## Materials and methods

To ensure quality standards and prevent contamination in the molecular genetic laboratory, we followed recommendations by Alonso et al. [14], Tully et al. [15], Bär et al. [16], Carracedo et al. [17], Kemp and Smith [18], Wilson et al. [19], Kalmar et al. [20], Davoren et al. [21], Tamariz et al. [22], Shaw et al. [23], and Vanek et al. [24]. We created an elimination database containing all persons that had been in contact with the skeletal remains at any phase of excavation, storage, anthropological analysis, or molecular genetic analysis. The elimination database allows traceability in the case of contamination. The extraction-negative controls were included in every batch (usually 23 samples) of extraction to verify the purity of the extraction reagents. We performed at least two extractions for each bone, and together with the bones, we thus analyzed at least eight extraction-negative controls. The PCR-negative controls were included in every amplification reaction to verify the purity of the amplification reagents. The PCR-positive controls were also included in every amplification reaction. All negative and positive controls were carried through the sequencing process.

## DNA extraction

We collected buccal swabs on sterile cotton swabs from living relatives and persons included in the elimination database. For genetic investigations, an 8- to 10-cm fragment was taken from each bone. The bone samples were cleaned mechanically and chemically. The surface was decontaminated by physical removal of the surface using a rotary sanding tool (Dremel) and rinsing in 5% Alconox detergent, water, and 80% ethanol. Grinding in a TissueLyser (Retsch) homogenizer using liquid nitrogen followed. Genomic DNA was obtained from 0.5 g of bone powder incubated in 1.5 ml of 0.5 M EDTA pH 8.3 (Promega) for 24 h at 37°C in a Thermomixer comfort (Eppendorf), shaken at 950 rpm. After centrifugation at 13,400 rpm for 1.5 min in a MiniSpin

(Eppendorf) centrifuge, the supernatant was discarded. The incubation of bone material in EDTA was repeated two more times. After 72 h of decalcification, the precipitate was washed with 1 ml of ultrapure distilled water (Gibco) and centrifuged at 13,400 rpm for 1.5 min, and the supernatant was discarded. Then, 250  $\mu$ l of G2 buffer and 60  $\mu$ l of proteinase K (both EZ1 DNA Investigator Kit, Qiagen) was added to the precipitate, and it was incubated overnight at 56°C in a Thermomixer comfort (Eppendorf), shaken at 950 rpm. This was followed by centrifugation at 6,000 rpm for 4 min in a MiniSpin (Eppendorf) centrifuge. Up to 500  $\mu$ l of supernatant was transferred to the sample tube. The DNA was purified in a Biorobot EZ1 (Qiagen) device using the EZ1 DNA Investigator Card and EZ1 DNA Investigator Kit (Qiagen). Following the manufacturer's instructions [25], the Biorobot EZ1 was used to obtain genomic DNA from decalcified bone precipitate using the large-volume protocol, and from relatives' buccal swabs and elimination database samples using the "tip dance" protocol. The extraction-negative controls were included in the extraction process to verify the purity of extraction reagents. Genomic DNA extraction was carried out at least twice from each bone.

#### DNA quantification

DNA extracts from all samples were quantified, and levels of PCR inhibitors monitored, using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems). Reactions were carried out in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using SDS software, version 1.0 (Applied Biosystems) according to the manufacturer's instructions [26]. From at least two isolates obtained from a single bone, the one with the highest nuclear DNA content was used for nuclear STR typing, and others were used for mtDNA sequencing. When less than 16 pg DNA/ $\mu$ l was detected, autosomal and Y-STR loci were amplified in parallel, and only duplicated alleles were reported.

#### STR typing of nuclear DNA

STR typing of autosomal DNA was performed for bones using various amplification kits: the AmpF/STR Identifiler™ PCR Amplification Kit (Applied Biosystems), PowerPlex 16 System (Promega), and additionally, for degraded DNA samples, the AmpF/STR MiniFiler™ PCR Amplification Kit (Applied Biosystems). The AmpF/STR Identifiler™ PCR Amplification Kit and PowerPlex 16 System contain the same 13 core STR loci and amelogenin, whereas the AmpF/STR Identifiler™ PCR Amplification Kit also contains the loci D2S1338 and D19S433, and the PowerPlex 16 System, the loci Penta E and Penta D. Overall, 17 STR loci and amelogenin were amplified. The AmpF/STR MiniFiler™

PCR Amplification Kit (Applied Biosystems) contains eight STR loci shared with the AmpF/STR Identifiler™ PCR Amplification Kit, but uses shorter amplicons, which makes them more likely to be successful on fragmented DNA. When statistical analysis did not reveal a PP of 99.9%, the PowerPlex ESX 17 System (Promega) amplification kit was used to obtain the profiles on six additional STR loci (D10S1248, D1S1656, D22S1045, D2S441, D12S391, and SE33). Typing of the Y-STRs was performed using the AmpF/STR YFiler PCR Amplification Kit (Applied Biosystems) to amplify 17 Y-STRs. All reactions were performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). For best performance of the AmpF/STR Identifiler™ PCR Amplification Kit (Applied Biosystems), the optimal amount of initial genomic DNA is 0.5 to 1.25 ng. For samples containing >50 pg/ $\mu$ l DNA, PCR was performed according to the manufacturer's instructions [27], but bovine serum albumin (BSA; Sigma; final concentration 40 ng/ $\mu$ l) was added, and a double amount of AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. For samples containing <50 pg/ $\mu$ l DNA, in addition to adding BSA and a double amount of polymerase, the number of cycles was also increased from 28 to 31, and the extension step within cycles was prolonged to 2 min. The PCR reaction was performed with at most 13  $\mu$ l DNA. Simultaneously with the forensic samples, we amplified the positive control (AmpF/STR Control DNA 9947A, Applied Biosystems) and negative PCR and extraction controls. For best performance of the PowerPlex 16 System (Promega), the optimal amount of initial genomic DNA is 0.5 to 1 ng. For samples containing >28 pg/ $\mu$ l DNA, PCR was performed according to the manufacturer's instructions [28], but 2.75  $\mu$ l of PowerPlex 16 Primer Pair Mix (Promega) was added, and a double amount of AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. The final extension was prolonged from 30 to 45 min. For samples with a concentration <28 pg/ $\mu$ l, in addition to the measures described above, the number of cycles was increased from 32 to 34, and the extension step within cycles was prolonged to 90 s. The PCR reaction was performed with at most 18  $\mu$ l DNA. Simultaneously with the forensic samples, we amplified the positive control (Control DNA 9947A, Promega) and negative PCR and extraction controls. For best performance of the AmpF/STR MiniFiler™ PCR Amplification Kit (Applied Biosystems), the optimal amount of initial genomic DNA is 0.5 to 0.75 ng. For samples containing >50 pg/ $\mu$ l DNA, PCR was performed according to the manufacturer's instructions [29], but BSA (Sigma; final concentration 40 ng/ $\mu$ l) was added, and an additional 0.5  $\mu$ l of AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. For samples with the concentration <50 pg/ $\mu$ l, in addition to the measures described above, the number of cycles was increased from 30 to 33, and the extension step within cycles was prolonged to

2 min. The PCR reaction was performed with at most 13  $\mu$ l DNA. Simultaneously with the forensic samples, we amplified the positive control (AmpF/STR Control DNA 007, Applied Biosystems) and negative PCR and extraction controls. For best performance of the PowerPlex ESX 17 System (Promega), the optimal amount of initial genomic DNA is 0.5 ng. For samples containing  $>29$  pg/ $\mu$ l DNA, PCR was performed according to the manufacturer's instructions [30]. For samples with a concentration  $<29$  pg/ $\mu$ l, the number of cycles was increased from 30 to 33, and 6.5  $\mu$ l of PowerPlex ESX 17 5x Master Mix (Promega) was added. The PCR reaction was performed with at most 16  $\mu$ l DNA. Simultaneously with the forensic samples, we amplified the positive control (Control DNA 9947A, Promega) and negative PCR and extraction controls. For best performance of the AmpF/STR Yfiler™ PCR Amplification Kit (Applied Biosystems), the optimal amount of initial genomic DNA is 0.5 to 1 ng. For samples containing  $>50$  pg/ $\mu$ l DNA, PCR was performed according to the manufacturer's instructions [31], but BSA (Sigma; final concentration 40 ng/ $\mu$ l) was added, and 1.2  $\mu$ l AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used. For samples with a concentration  $<50$  pg/ $\mu$ l, in addition to the measures described above, the number of cycles was increased from 30 to 33, and the extension step within cycles was prolonged to 2 min. The PCR reaction was performed with at most 13  $\mu$ l DNA. Simultaneously with the forensic samples, we amplified the AmpF/STR Control DNA 9947A (Applied Biosystems) and AmpF/STR Control DNA 007 (Applied Biosystems), and the negative PCR and extraction controls.

Fluorescent-labeled products of the amplification kits were separated with an automatic ABI PRISM™ 3130 Genetic Analyzer (Applied Biosystems) using the 3130 Performance Optimized Polymer 4 (Applied Biosystems); the GeneScan-500 LIZ (Applied Biosystems) internal size standard with the kits AmpF/STR Identifiler, AmpF/STR MiniFiler, and AmpF/STR Yfiler PCR Amplification Kit (Applied Biosystems); ILS 600 (Promega) internal size standard with the PowerPlex 16 System (Promega) kit; and CC5 500 (Promega) internal size standard with the PowerPlex ESX 17 System (Promega) kit. Genetic profiles were determined using Data Collection v 3.0 and GeneMapper ID v 3.2 (Applied Biosystems) computer software.

In reference persons, typing of autosomal DNA was performed using the AmpF/STR Identifiler™ PCR Amplification Kit (Applied Biosystems); when statistical analysis did not reveal a PP of 99.9%, the PowerPlex 16 System (Promega) amplification kit and PowerPlex ESX 17 System (Promega) were used to obtain the profiles on loci Penta E, Penta D, D10S1248, D1S1656, D22S1045, D2S441, D12S391, and SE33. For the maternal relatives, mtDNA haplotypes were also obtained, and for the paternal relatives, Y-STR haplotypes using the AmpF/STR Yfiler PCR Amplification Kit (Applied Biosystems). In persons

from the elimination database, in addition to autosomal DNA typing using the AmpF/STR Identifiler™ PCR Amplification Kit (Applied Biosystems), typing of mtDNA was performed, and for males, also typing of Y-STRs using the AmpF/STR Yfiler PCR Amplification Kit (Applied Biosystems).

#### MtDNA sequencing

The two hypervariable regions HVI and HVII of the mtDNA were amplified by PCR in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The primers F15997/R16401 for HVI and F29/R408 for HVII [32] were used. PCR was carried out in a 25- $\mu$ l reaction mixture following Zupanič Pajnič et al. [33], but for the bone samples, 0.8  $\mu$ l of AmpliTaq Gold DNA Polymerase (Applied Biosystems) was used, BSA (Sigma; final concentration 50 ng/ $\mu$ l) was added, and the number of cycles was increased to 38. Prior to sequencing, the PCR products were purified using Centricon 100 spin dialysis columns (Millipore Corporation) following the manufacturer's recommendations. Sequencing reactions were performed in a Biometra UNO Thermoblock in both orientations in order to verify the accuracy of base-calling. In cases of length heteroplasmy in the poly-C strand, the polymorphisms behind the C-stretch in the forward sequencing reaction and before the C-stretch in the reverse reaction were confirmed by repeating amplification and sequencing reactions according to the recommendations [15]. Following Bandelt and Parson [34] in cases of heteroplasmic length variants, the dominant variants were reported. The primers used for sequencing the PCR products were the same as for the amplification. Sequencing reactions were carried out using 6  $\mu$ l ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, v 1.1 (Applied Biosystems), 8  $\mu$ l purified PCR product as a template, 2  $\mu$ l 5  $\mu$ M sequencing primer, and 4  $\mu$ l sterile distilled water for each sample. The sequencing conditions and products purification have been described by Zupanič Pajnič et al. [33]. Then, 20  $\mu$ l of Hi-Di™ formamide (Applied Biosystems) was added to 15 to 20  $\mu$ l of purified sequencing product, heat denatured, and snap cooled on ice. Automated DNA sequencing was carried out on an ABI PRISM™ 3130 Genetic Analyser (Applied Biosystems) using the 3130 Performance Optimized Polymer POP 4 (Applied Biosystems) and Data Collection v 3.0 Software (Applied Biosystems). The denatured samples were electrokinetically injected for 10 s at 1.2 kV into a 36-cm capillary array. Electrophoresis was run at 15 kV and 60°C with the UltraSeq36 POP4 sequencing module. The analysis of mtDNA sequencing data was performed using AB DNA Sequencing Analysis Software v 5.2 (Applied Biosystems). The sequences were aligned and compared with the Anderson sequence [35] from 16030 to 16381 for the HVI region and from 55 to 388 for the HVII region using BioEdit software.



## Statistical analysis

Genetic profiles obtained from the bones and reference samples were compared, and estimation of potential familial relationships was performed. The calculation of LR and posterior probabilities (PP) was performed with DNA VIEW software v.28.48 and 29.03 [36], mainly using allele frequencies of the Slovenian population [37, 38] and prior probability 1/89 [39]. LRs for chromosome Y-haplotypes and mtDNA haplotypes were calculated based on haplotype frequencies in various databases [40]. The counting method was used to estimate the haplotype frequencies, and the Balding and Nichols [41] correction for errors in sampling was considered [17, 42]. The reference database YHRD [43] and EMPOP [44] were used to determine the Y-chromosome and mtDNA haplotype frequencies. In the YHRD database, we used European metapopulation with 5,342 haplotypes, and in the EMPOP database, we used west Eurasian populations with 4,476 haplotypes. Whenever an agreement of autosomal genetic profiles and mtDNA haplotypes was noted between the bone and a relative, the product rule was used to estimate a combined LR [45]. The same was applied when an agreement was noted between autosomal genetic profiles and Y-STR haplotypes [42]. Following recommendations [5, 39, 46], the prior probability was set based on the number of victims reported (the recorded list of victims in the Konfin I mass grave), and a recommended PP (for kinship) of 99.9% was used (an LR of at least  $8.8 \times 10^4$  needed to be reached) with the goal of high confidence of correct identification of victims in the mass grave.

The genetic profiles obtained for the bones were compared to the profiles of persons included in the elimination database to monitor possible contamination of bone samples with modern DNA.

## Results and discussion

For DNA isolation from bones, Nagy et al. [47] used a BioRobot M48 (Qiagen). Our laboratory used a Biorobot EZ1 (Qiagen) for the same purpose. Montpetit et al. [48], Valgren et al. [49], and Kishore et al. [50] obtained DNA from various casework samples with a Biorobot EZ1 (Qiagen), for which they found a high purification efficacy. Prior to extraction, we decalcified the bone. After 72 h of decalcification, precipitate with incompletely decalcified bone powder was usually obtained.

Quantification of nuclear DNA resulted in determination of over 8 pg DNA/ $\mu$ l of isolate in all bones, with the exception of two femurs (Table 1). We detected 8 to 16 pg DNA/ $\mu$ l of isolate in six bones only (100 to 200 pg DNA was amplified in PCR), whereas in 76 bones (90%), we detected more than 16 pg DNA/ $\mu$ l of isolate, which

means that the initial amount of DNA in the PCR exceeded 200 pg DNA, the limit for low copy number PCR [51, 52]. In six bones in which the initial amount of DNA in the PCR did not exceed 200 pg, the autosomal and Y-STR loci were amplified in parallel, and only duplicated alleles were reported for comparison with the reference samples. In 28 of the 76 bones in which the initial amount of DNA in the PCR exceeded 200 pg, discrepancies were noted between the AmpF/STR Identifier™ PCR Amplification Kit (Applied Biosystems) and PowerPlex 16 System (Promega) for the shared loci. The heterozygote call for one locus in one amplification kit and an apparent homozygous call in the other amplification kit were noted 53 times, and heterozygote calls were interpreted.

The typing of autosomal and Y-STR loci was successful in 82 of the 84 bones, which represents a 98% success rate. Complete autosomal genetic profiles (17 STR loci and amelogenin) were obtained from 59 bones, partial profiles with one or two STR loci missing were obtained from 17 bones (primarily longer loci Penta E, Penta D, or D2S1338 were missing), and in the remaining six bones, 11 to 15 loci were amplified (Table 1). Complete Y-STR profiles (17 STR loci) were obtained from 41 bones, partial profiles with one or two missing Y-STR loci from 15 bones, in 16 bones, ten to 14 loci were amplified, and in the remaining ten bones, six to nine Y-STR loci were amplified (Table 1). The typing of the mtDNA HVI region was successful in 80 of the 84 bones, which represents a 95% success rate, and the typing of mtDNA HVII region was successful in 82 of the 84 bones, which represents a 98% success rate (Table 1). Palo et al. [4] used mtDNA analysis to identify skeletal remains of Finnish soldiers killed in the Second World War in the former Soviet Union and found the success rate of typing for long bones to be 96% in the HVI region and 91% in the HVII region. Of the 80 bones in the HVI region, eight (10%) had length heteroplasmy, and of the 82 bones in the HVII region, 23 (28%) had length heteroplasmy.

Of the 88 victims in the Konfin I mass grave, we managed to trace living relatives of 36 victims; they served for comparison with genetic profiles of the bones from the mass grave. Of the 84 bones found, we matched 28 bones with relatives and identified 28 victims of the massacre. Fourteen victims were identified by comparison to living sisters: we compared autosomal STRs and mtDNA haplotypes. In autosomal STRs, the values for LR ranged between  $1.9 \times 10^2$  and  $4.1 \times 10^9$ , and in mtDNA haplotypes between  $7.5 \times 10^1$  and  $2.2 \times 10^3$ , whereas the values for the combined LR ranged between  $4.2 \times 10^5$  and  $9.2 \times 10^{12}$  (PP ranged between 99.97% and >99.999999%). In addition, a maternal-line cousin served for comparison of autosomal STRs and mtDNA haplotypes, which helped identify one victim. The calculated values for combined LR and PP were  $1.3 \times 10^5$  and 99.92%, respectively. In addition,

**Table 1** Maximal nuclear DNA quantity (Quantifiler™ Human DNA Quantification Kit, Applied Biosystems), expressed in pg DNA/μl of isolate; efficiency of autosomal DNA typing (AmpF/STR Identifier™ PCR Amplification Kit (Applied Biosystems) and PowerPlex 16 System,

Promega), expressed as the number of successfully typed autosomal short tandem repeats (STRs); efficiency of Y-STR typing, expressed as the number of successfully typed Y-STRs; and efficiency of mtDNA typing (HVI and HVII) of the bones found in the Konfin I grave

Bone sample	Maximum quantity (pg/μl)	Autosomal STR loci	Y-STR loci	mtDNA
Femur A ant	221	18/18	17/17	HVI, HVII
Femur B ant	32	18/18	7/17	HVI, HVII
Femur C ant	60	17/18	17/17	HVI, HVII
Femur D ant	37	18/18	17/17	HVI, HVII
Femur E ant	70	18/18	17/17	HVI, HVII
Femur F ant	50	18/18	14/17	HVI, HVII
Femur G ant	40	17/18	12/17	HVI, HVII
Femur H ant	106	18/18	17/17	HVI, HVII
Femur I ant	26	17/18	17/17	HVI, HVII
R Femur 43 pat	237	18/18	17/17	HVI, HVII
R Femur 45 pat	46	17/18	14/17	HVI, HVII
R Femur 49 pat	116	18/18	17/17	HVI, HVII
R Femur 51 pat	56	18/18	17/17	HVI, HVII
R Femur 1	62	18/18	16/17	HVI, HVII
R Femur 2	10	13/18	12/17	
R Femur 3	43	18/18	16/17	HVI, HVII
R Femur 4	21	17/18	16/17	HVI, HVII
R Femur 5	18	16/18	14/17	HVI, HVII
R Femur 6	10	14/18	7/17	HVII
R Femur 7	24	18/18	17/17	HVI, HVII
R Femur 8	69	18/18	16/17	HVI, HVII
R Femur 9	17	18/18	16/17	HVI, HVII
R Femur 10	46	18/18	16/17	HVI, HVII
R Femur 11	114	18/18	17/17	HVI, HVII
R Femur 12	120	18/18	17/17	HVI, HVII
R Femur 13	35	18/18	17/17	HVI, HVII
R Femur 14	60	16/18	13/17	HVI, HVII
R Femur 15	100	18/18	17/17	HVI, HVII
R Femur 16	51	18/18	8/17	HVI, HVII
R Femur 17	110	18/18	17/17	HVI, HVII
R Femur 18	1,000	18/18	17/17	HVI, HVII
R Femur 19	270	18/18	17/17	HVI, HVII
R Femur 20	15	11/18	14/17	HVI, HVII
R Femur 21	142	18/18	17/17	HVI, HVII
R Femur 22	111	18/18	17/17	HVI, HVII
R Femur 23	130	18/18	17/17	HVI, HVII
R Femur 24	75	18/18	17/17	HVI, HVII
R Femur 25	36	18/18	17/17	HVI, HVII
R Femur 26	39	16/18	15/17	HVI, HVII
R Femur 27	150	18/18	16/17	HVI, HVII
R Femur 28	41	18/18	17/17	HVI, HVII
R Femur 29	48	15/18	6/17	HVI, HVII
R Femur 30	64	18/18	17/17	HVI, HVII
R Femur 31	17	16/18	13/17	HVI, HVII
R Femur 32	42	18/18	17/17	HVI, HVII
R Femur 33	100	18/18	17/17	HVI, HVII

**Table 1** (continued)

Bone sample	Maximum quantity (pg/ $\mu$ l)	Autosomal STR loci	Y-STR loci	mtDNA
R Femur 34	62	18/18	16/17	HVI, HVII
R Femur 35	64	18/18	10/17	HVI, HVII
R Femur 36	77	16/18	16/17	HVI, HVII
R Femur 37	280	18/18	17/17	HVI, HVII
R Femur 38	130	18/18	17/17	HVI, HVII
R Femur 39	150	18/18	17/17	HVI, HVII
R Femur 40	30	17/18	17/17	
R Femur 41	50	18/18	17/17	HVI, HVII
R Femur 42	43	17/18	12/17	HVI, HVII
R Femur 43	8	14/18	14/17	HVI, HVII
R Femur 44	26	17/18	17/17	HVI, HVII
R Femur 45	57	18/18	17/17	HVI, HVII
R Femur 46	47	24/24	7/17	HVI, HVII
R Femur 47	77	18/18	17/17	HVII
R Femur 48	100	18/18	17/17	HVI, HVII
R Femur 49	101	18/18	10/17	HVI, HVII
R Femur 50	90	18/18	9/17	HVI, HVII
R Femur 51	47	18/18	7/17	HVI, HVII
R Femur 52	18	18/18	17/17	HVI, HVII
R Femur 53	91	18/18	8/17	HVI, HVII
R Femur 54	14	14/18	16/17	HVI, HVII
R Femur 55	120	18/18	10/17	HVI, HVII
R Femur 56	109	18/18	17/17	HVI, HVII
R Femur 57	73	18/18	10/17	HVI, HVII
R Femur 58	48	17/18	17/17	HVI, HVII
R Femur 59	47	18/18	9/17	HVI, HVII
R Femur 60	24	18/18	16/17	HVI, HVII
R Femur 61	250	18/18	17/17	HVI, HVII
R Femur 62	33	18/18	14/17	HVI, HVII
R Femur 63	17	17/18	16/17	HVI, HVII
R Femur 64	18	18/18	15/17	HVI, HVII
R Femur 65	4	0/18	0/17	HVI, HVII
R Femur 66	36	18/18	17/17	HVI, HVII
R Femur 67	34	18/18	17/17	HVI, HVII
R Femur 68	5	0/18	0/17	HVI, HVII
R Femur 69	93	18/18	7/17	HVI, HVII
R Femur 70	15	22/24	11/17	HVI, HVII
R Femur 71	51	17/18	16/17	HVI, HVII

a maternal-line nephew served for comparison of autosomal STRs and mtDNA haplotypes that helped match one victim; after analyzing additional six STR loci, the statistical analysis showed high confidence of correct identification: the combined LR was  $8.9 \times 10^4$  and PP, 99.9%. Six victims were identified by comparison to living brothers; we compared autosomal STRs, mtDNA haplotypes, and Y-STR haplotypes. In autosomal STRs, the values for LR ranged between  $2.3 \times 10^2$  and  $3 \times 10^5$ , and in

mtDNA haplotypes, between  $5.6 \times 10^2$  and  $2.2 \times 10^3$ ; in Y-STR haplotypes, the values for LR ranged between  $5.9 \times 10^2$  and  $2.7 \times 10^3$ , the values for combined LR (autosomal DNA and mtDNA) ranged between  $1.3 \times 10^5$  and  $2.4 \times 10^8$  (PP ranged between 99.92% and 99.99996%), and the values for combined LR (autosomal DNA and Y-STRs) ranged between  $6.2 \times 10^5$  and  $7.3 \times 10^8$  (PP ranged between 99.98% and 99.99998%). One victim was identified by comparison to a living son; we compared autosomal STRs

and Y-STR haplotypes. In autosomal STRs, the value for LR was  $9.2 \times 10^4$ , in the Y-STR haplotype, the value for LR was  $2.7 \times 10^3$ , and the value for combined LR (autosomal DNA and Y-STRs) was  $2.5 \times 10^8$  (PP was 99.99996%). One victim was identified by comparison to a living daughter and wife; we were able to compare autosomal STRs only. The value for LR was  $3.7 \times 10^6$  and for PP, 99.997%. The last four victims were matched to living daughters; in these cases, we were able to compare autosomal STRs only, because no appropriate reference persons were available for the comparison of mtDNA haplotypes and Y-STR haplotypes. After the analysis of additional six STR loci for one victim, the statistical analysis showed high confidence of correct identification for all four victims with daughters as reference persons. The LR values ranged between  $2.5 \times 10^5$  and  $5.9 \times 10^5$ , and PP ranged between 99.96% and 99.98% (Tables 2 and 3).

We managed to obtain nuclear DNA from bones over 60 years old for successful STR typing. Of the total 36 possible identifications (of the 88 victims, reference

samples were obtained for 36 massacre victims only), we matched 28 victims to living relatives with high confidence of correct identification (PP ranged from 99.9% to more than 99.999999%). Similar to the experience of Irwin et al. [6], our experience shows that a combination of a higher number of genetic markers provides extremely high LRs that support the hypothesis that individuals' bones are related to the family references, rather than unrelated individuals. If we had analyzed only autosomal STR loci, we would have been able to identify only 12 victims with a high confidence of correct identification. Therefore, we deem it necessary to also include Y-STRs and mtDNA analyses and close as well as distant relatives of the maternal and paternal lines in the identification of Second World War victims. Of the 84 bones, 56 could not be matched to living relatives and, therefore, could not be identified. Their genetic profiles are waiting for the possibility of identification after new reference samples are obtained.

In spite of the complete list of 88 victims taken from the Central Prison on the night of 24 June 1945, we were not

**Table 2** Likelihood ratio (LR) and posterior probability (assuming 1/89 as the prior probability) for autosomal DNA (n-short tandem repeat (STR)), LR for mtDNA, and LR for Y-STRs, for the identified victims found in the Konfin I grave

Bone	Reference	LR <sub>(n-STR)</sub>	PP <sub>(n-STR)</sub>	LR <sub>(mtDNA)</sub>	LR <sub>(Y-STR)</sub>
Femur D ant <sup>a</sup>	Sister	$4.1 \times 10^9$	99.999998%	$2.2 \times 10^3$	
R Femur 11 <sup>a</sup>	Sister	$4.8 \times 10^6$	99.998%	$2.2 \times 10^3$	
Femur E ant	Sister	$1.8 \times 10^4$	99.5%	$2.2 \times 10^3$	
R Femur 43 pat	Sister	$6.8 \times 10^2$	87.3%	$1.5 \times 10^3$	
R Femur 66	Sister	$8.0 \times 10^3$	98.8%	$2.2 \times 10^3$	
R Femur 9	Sister	$1.4 \times 10^3$	93.4%	$2.2 \times 10^3$	
Femur B ant	Sister	$3.9 \times 10^4$	99.7%	$1.7 \times 10^2$	
R Femur 20	Sister	$1.9 \times 10^2$	65.0%	$2.2 \times 10^3$	
R Femur 26	Sister	$2.6 \times 10^3$	96.3%	$2.2 \times 10^3$	
R Femur 30	Sister	$6.7 \times 10^3$	98.7%	$7.5 \times 10^1$	
R Femur 42	Sister	$3.7 \times 10^3$	97.4%	$2.2 \times 10^3$	
R Femur 44	Sister	$1.6 \times 10^5$	99.94%	$2.2 \times 10^3$	
R Femur 52	Sister	$5.7 \times 10^5$	99.98%	$1.5 \times 10^2$	
R Femur 61	Sister	$1.9 \times 10^4$	99.5%	$2.2 \times 10^3$	
R Femur 34	Cousin	$5.8 \times 10^1$	37.0%	$2.2 \times 10^3$	
R Femur 46	Nephew	$4.0 \times 10^1$	28.8%	$2.2 \times 10^3$	
R Femur 49 pat	Brother	$2.3 \times 10^4$	99.6%	$2.2 \times 10^3$	$2.7 \times 10^3$
Femur G ant	Brother	$2.6 \times 10^3$	96.3%	$2.2 \times 10^3$	$5.9 \times 10^2$
R Femur 14	Brother	$2.3 \times 10^2$	70.0%	$5.6 \times 10^2$	$2.7 \times 10^3$
R Femur 18	Brother	$2.7 \times 10^5$	99.96%	$9.0 \times 10^2$	$2.7 \times 10^3$
R Femur 19	Brother	$3.0 \times 10^5$	99.97%	$7.5 \times 10^2$	$7.6 \times 10^2$
R Femur 43	Brother	$3.8 \times 10^3$	97.5%	$1.5 \times 10^3$	$2.7 \times 10^3$
R Femur 40	Son	$9.2 \times 10^4$	99.9%		$2.7 \times 10^3$
R Femur 23	Daughter and wife	$3.7 \times 10^6$	99.997%		
R Femur 41	Daughter	$5.9 \times 10^5$	99.98%		
R Femur 48	Daughter	$3.8 \times 10^5$	99.97%		
R Femur 53	Daughter	$2.5 \times 10^5$	99.96%		
R Femur 70	Daughter	$4.6 \times 10^5$	99.98%		

In reference sample, kinship with the victim is indicated

<sup>a</sup> Femur D ant and R femur 11 are brothers



**Table 3** Combined likelihood ratio and posterior probability (assuming 1/89 as the prior probability) for identified victims found in the Konfin I mass grave

Bone	Reference	LR <sub>(n-STR x mtDNA)</sub>	PP <sub>(n-STR x mtDNA)</sub>	LR <sub>(n-STR x Y-STR)</sub>	PP <sub>(n-STR x Y-STR)</sub>
Femur D ant <sup>a</sup>	Sister	$9.2 \times 10^{12}$	>99.999999%		
R Femur 11 <sup>a</sup>	Sister	$1.1 \times 10^{10}$	>99.999999%		
Femur E ant	Sister	$4.0 \times 10^7$	99.9998%		
R Femur 43 pat	Sister	$1.0 \times 10^6$	99.99%		
R Femur 66	Sister	$1.8 \times 10^7$	99.9995%		
R Femur 9	Sister	$3.2 \times 10^6$	99.997%		
Femur B ant	Sister	$6.7 \times 10^6$	99.999%		
R Femur 20	Sister	$4.2 \times 10^5$	99.97%		
R Femur 26	Sister	$5.9 \times 10^6$	99.998%		
R Femur 30	Sister	$5.0 \times 10^5$	99.98%		
R Femur 42	Sister	$8.4 \times 10^6$	99.999%		
R Femur 44	Sister	$3.7 \times 10^8$	99.99997%		
R Femur 52	Sister	$8.5 \times 10^7$	99.9999%		
R Femur 61	Sister	$4.2 \times 10^7$	99.9998%		
R Femur 34	Cousin	$1.3 \times 10^5$	99.92%		
R Femur 46	Nephew	$8.9 \times 10^4$	99.9%		
R Femur 49 pat	Brother	$5.1 \times 10^7$	99.9998%	$6.2 \times 10^7$	99.9998%
Femur G ant	Brother	$5.8 \times 10^6$	99.998%	$1.5 \times 10^6$	99.994%
R Femur 14	Brother	$1.3 \times 10^5$	99.92%	$6.2 \times 10^5$	99.98%
R Femur 18	Brother	$2.4 \times 10^8$	99.99996%	$7.3 \times 10^8$	99.99998%
R Femur 19	Brother	$2.2 \times 10^8$	99.99996%	$2.3 \times 10^8$	99.99996%
R Femur 43	Brother	$5.7 \times 10^6$	99.998%	$1.0 \times 10^7$	99.999%
R Femur 40	Son			$2.5 \times 10^8$	99.99996%

In the reference sample, kinship with the victim is indicated

<sup>a</sup> Femur D ant and R femur 11 are brothers

able to make identifications for all 36 victims for which family reference samples were received. We were able to make identifications for 28 of them. The other eight victims for whom references were available failed to match. There is another mass grave Konfin II only 20 m away from the mass grave Konfin I. It is known that the massacre in Konfin II happened only 2 days after the massacre in Konfin I. On the list of victims taken from the Central Prison on the night of 26 June 1945, there are 48 persons. The skeletal remains were not excavated from the mass grave Konfin II yet. We assume that among the skeletal remains from mass grave Konfin II, the victims from the list of mass grave Konfin I would be found.

In the process of identifying victims in the Konfin I mass grave, we minimized the possibility of contamination during genetic investigations. The authenticity of genetic profiles of bones was confirmed by clean isolation and amplification-negative controls for nuclear DNA, identical genetic profiles obtained using the AmpF/STR Identifiler™ PCR Amplification Kit (Applied Biosystems) and PowerPlex 16 System (Promega), and mismatch of genetic profiles of bones with persons from the elimination database. Because

of the high detection sensitivity of mtDNA analysis, low levels of exogenous DNA contamination were observed in extraction-negative controls and PCR-negative controls. The level of contamination was lower in the PCR-negative controls than in the extraction-negative controls. The haplotype of two extraction-negative controls matched the analyst, but no haplotype of the bone samples yielded the same sequence.

Identifying the victims in the Konfin I mass grave establishes a basis for further molecular genetic investigations of postwar mass graves in Slovenia when the possibility arises. We should stress the fact that mass graves with lists of victims are rare. The mass grave Konfin I represents such a rarity as it was possible to make a list of victims on a basis of archived documents. The methods of DNA extraction and amplification described here have proved to be highly efficient because we obtained 0.8 to 100 ng DNA/g of bones and complete genetic profiles of autosomal DNA, Y-STR haplotypes, and of mtDNA haplotypes. It should also be noted that the environment where these bones were preserved may favor DNA preservation. On the other hand, we obtained similar results

(successful typing of nuclear DNA and mtDNA) from two mass graves not located in karst caves: in identifying three victims in the grave at Mount Storžič [8, 9] and DNA typing of 25 victims in the Bodovlje Gorge (Bodoveljska grapa) mass grave (data not published yet).

DNA extraction proved effective from relatively small 0.5-g bone samples. The identity of the genetic profiles of bone specimens was verified by amplification of STRs with two different amplification kits. For degraded samples, we additionally used the amplification kit for mini-STRs. In two cases, in which statistical analysis were too low after amplification of STRs with the kits AmpF/STR Identifier (Applied Biosystems) and PowerPlex 16 System (Promega), the PowerPlex ESX 17 System (Promega) amplification kit was used to reach the LR of  $8.8 \times 10^4$ . When the genetic profiles of bones matched living relatives, the recommended PP of 99.9% was higher in all identifications, indicating that a sufficient number of genetic markers were investigated in identifying skeletal remains.

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