Rom J Leg Med [28] 40-49 [2020]
DOI: 10.4323/rjlm.2020.40

GENETIC ANALYSIS OF SKELETAL REMAINS OF WAR VICTIMS

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Abstract: This article presents the genetic analysis of Second World War skeletal remains excavated from the Slovenian mass grave, where 43 prisoners were executed in 1943 according to the archived victims list. In 2013, two graves were found and at least 16 incomplete skeletons were exhumed. Thirty-nine bones were included in the genetic analyses. Family reference samples were collected for 10 victims from the list. Extracted DNA was quantified using the PowerQuant kit, and autosomal and Y-STR kits were used for STR typing. Up to 7 ng DNA/g of powder was acquired from the samples analyzed. We managed to obtain 15 unique genetic profiles, corresponding to 15 different individuals. Four genetic profile matches were ascertained, among which two cases were a match between a victim and a family reference (a son and daughter, respectively), and two cases a match between two respective victims, the latter highlighting the fact that some of the victims were related. On the archived victims list there were four pairs of brothers, and two pairs were proved through genetic analysis. The statistical analyses showed a high confidence of correct identification of two victims through family references, and among victims two pairs of brothers with posterior probability greater than 99.9%.

Key words: skeletal remains; war victims; missing person identification; STR typing.

INTRODUCTION

Naming the deceased of unknown identity in order to clarify unanswered questions regarding their fate and helping relatives bury the remains in a family grave is anything but a simple task, and such an undertaking can still present significant difficulty. The following factors are recognized as frequent challenges to overcome when attempting the identification of unknown human remains: dislocated body parts, hidden mass graves, relocation from a primary burial site and commingling of remains, insufficient information available about the event of death, a lengthy time elapsed from death until the discovery of victims' remains, and lack of ante-mortem data (Goodwin 2017). In the case of the Second World War, identifying living relatives is encumbered by the fact that a long period of time has elapsed since the deaths. In order to test relationship, autosomal STR profiles from skeletal remains are compared to those from presumed relatives in an attempt to establish similarities. When distant relatives from the maternal or paternal line are available, one option is to use lineage markers from mitochondrial DNA and the Y chromosome. Lineage markers have limited discriminatory power and they have to be combined with autosomal STR or other non-DNA evidence (Irwin *et al*. 2012). Skeletal remains, especially aged ones, are among the most challenging biological samples for forensic human identification analyses. Old bones may contain very low amounts of badly degraded DNA, and DNA typing's success is limited by PCR inhibitors (Zietkiewicz *et al*. 2012; Irwin *et al*. 2012). The great risk of contamination also limits how successful DNA typing will be (Amory *et al*. 2012).

Innumerable hidden mass graves have been unearthed in recent years throughout the world, a direct result of war, oppressive regimes, and human trafficking crimes. These particularly include cases connected to the Second World War, such as missing Norwegian soldiers buried in Russia (Morild *et al*. 2015) and mass graves found in Poland (Ossowski *et al*. 2013, 2016a), the military dictatorship in Argentina in the 1970s (Romanini *et al*. 2012), the Spanish Civil War (Baeta *et al*. 2015; Betancor *et al*. 2011; Rios *et al*. 2010, 2012), and more recent attempts to identify dead migrants' bodies on the Mediterranean coast of Italy (Olivieri *et al*. 2018; Bertoglio *et al*. 2019). The Slovenian Government

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Commission on Concealed Mass Graves has identified over 600 concealed mass graves from the war in the past 30 years (Ferenc 2008). The majority of the victims of these killings remain buried and unidentified. For most mass graves in Slovenia, no documents exist to base victim identification on. For some, it is possible to make a list of the victims based on archival material. Genetic identification of Second World War victims has been carried out for only a handful of mass graves in Slovenia, and attempts to identify the Mačkovec mass grave victims are described here.

MATERIALS AND METHODS

Historical background

In 1943, according to historical data (a preserved list of victims), 45 prisoners were transported from Ribnica and 43 executed at Mačkovec Hill; two of them were able to escape the killings. The corpses were not buried immediately, and they may have been exposed to human or animal activity prior to their burial. In 2008, mechanical sounding performed on more than 30 locations confirmed the existence of a hidden mass grave, which the locals marked with a cross after the war. Excavations in 2013 proved the existence of two graves at the location. The larger one, where the incomplete remains of at least 14 individuals were found, and the smaller one, where at least two incomplete individuals' remains were found. The living relatives of the victims that could be traced decided on a common grave and group burial, and so the purpose of the genetic analysis was to identify some of the victims and thus to prove that the hidden mass grave in fact contained some of the victims on the archived list of victims.

Bone sampling

The exhumed bones were physically damaged, abraded, porous, and fragmented. The poorly preserved nature of human remains dating back to the Second World War presents an obstacle in STR typing, resulting partial profiles, and consequently all the tibiae and femurs were analyzed in order to obtain as many full genetic profiles as possible. DNA analyses were performed on nine right femurs, eight left femurs, 10 right tibiae, 11 left tibiae, and one tibia with undetermined laterality, or a total of 39 bones. Small (5–8 cm long) compact cortical diaphysis fragments of each bone sample were cut and frozen at −20 °C until extracting the DNA.

Family references samples and elimination database samples

Buccal swab samples were collected for 13 family references for 10 different victims according to the victims list; for three of the victims, two family references were obtained. Presumed family relations with the deceased were sons (for four victims), daughters (for three victims), a sister (for one victim), nephews on the father's side (for three victims), and grandchildren on the father's side (for two victims). For close relatives, autosomal STRs are generally informative enough to confirm kinship, whereas for distant relatives on the father's side (nephews and grandchildren) Y-STR haplotypes can be used for determining a kinship match and increasing the kinship likelihood ratio. In the case of a sister, the possibility of family connections can be evaluated using autosomal STRs and mitochondrial DNA haplotypes.

In addition to family references, buccal swabs were collected from all individuals that had handled the remains, thus making it possible to determine the source of DNA in the event of contamination. All relatives and persons included in the elimination database signed an informed consent allowing analyses, and the research project was approved by the Medical Ethics Committee of the Republic of Slovenia (144/06/14).

DNA extraction

Specific precautions were followed, and the skeletal remains were handled under conditions for minimizing contamination (Parson *et al*. 2014; Rohland and Hofreiter 2007; Pääbo *et al*. 2004). Cleaning, grinding, decalcification, and purification of DNA from bones were performed following previously published procedure by Zupanič Pajnič. Each sample batch was accompanied by extraction-negative controls in order to ensure that the extraction plastics and reagents were clean and to monitor possible contamination events during DNA extraction procedure. Extraction of DNA from buccal swabs was performed using the EZ1 DNA Investigator Kit (Qiagen, Hilden, Germany, EU) according to instructions provided by the manufacturer (Qiagen 2014).

DNA quantification

The quantity and quality of the samples was performed using the PowerQuant System (Promega, Madison, WI, USA) in order to assess the amount of human DNA in the extracts, the presence of male DNA, possibly present inhibitors of PCR reaction, and the degree of DNA degradation. Degradation index

was estimated by amplification of two targets differing in length, one being short (autosomal-Auto), with the same data utilized to detect the amount of total human DNA, and the other being longer (degradation-Deg). The ratio between the Auto and Deg targets in the form of [Auto]/[Deg] is presented by a degradation index. Moreover, IPC shift was determined to detect PCR inhibitors. All PowerQuant amplifications were carried out in duplicate (Promega 2019). Raw data were obtained using the ABI 7500 Real-Time PCR System (Applied Biosystems-AB, Foster City, CA, USA), and the results analyzed by PowerQuant Analysis Tool software (Promega 2019). The minimum value for the IPC shift was set at 0.30, and the threshold was set at 2 for the [Auto]/[Deg] (Promega 2019). Duplicate amplifications of the positive and negative control reactions were carried out along with the bone extracts.

DNA typing

To test the family relationship between family reference samples obtained and victims excavated, autosomal and Y-chromosomal STRs were analyzed. Genetic profiles were determined after duplicate PCR amplification using autosomal and Y-STR kits. Genotyping of 15 autosomal STRs and amelogenin was performed using the AmpFlSTR NGM Kit (AB). STR typing of Y-chromosomal STRs was performed using the PowerPlex Y System (Promega) for all bones and, in addition, the PowerPlex Y23 System (Promega) for bones that matched family references or another victim excavated from the Mačkovec mass grave. Both Y-STR kits contain the same twelve markers, whereas the PowerPlex Y23 contains eleven additional STRs. The amplification protocols and the thermal cycling conditions for all PCR reactions were followed according to the manufacturer's instructions (AB 2009; Promega 2012, 2014). The Nexus Master Cycler (Eppendorf, Hamburg, Germany, EU) was used for DNA amplification. The maximum volume of extracts (10 µl) was used for amplification of all bone samples using the NGM kit and up to 1 ng of DNA was used for amplification using both PowerPlex kits. Simultaneously with bone samples, negative PCR and extraction controls were amplified using the maximum volume of extracts. The PCR products were separated on an ABI PRISM™ 3130 Genetic Analyzer (AB) using the 3130 POP 4 (AB). The genetic profiles were determined using Data Collection v 3.0 and GeneMapper ID v 3.2 (AB) software with a peak amplitude threshold of 50 RFU for all dyes. The consensus autosomal genetic profiles were determined after duplicate PCR amplification. STR typing was also carried out for persons included in the elimination database and for reference samples using the NGM kit (AB) and the PowerPlex Y System kit (Promega) according to the manufacturer's instructions.

Statistical analysis

Genetic profiles obtained from bones and family references were compared, and the possibility of family connections was evaluated. Statistical computations—namely likelihood ratios (LR) or paternity and sibling indexes and posterior probabilities (PP)—were made using DNA VIEW software version 37.15 (Brenner 2007) and allele frequency data for the Slovenian population. Moreover, the eastern European metapopulation from the YHRD database (Willuwiet and Roewer 2007), in which 1,460 eastern European Y-STR haplotypes were found, was used to calculate frequencies and likelihood ratios for Y chromosome haplotypes. If a match between a victim and a relative was found in both autosomal and Y-STR profiles, the product rule was applied to determine a combined LR (Walsh *et al.* 2008). Given the number of victims reported missing and written on the archived list of victims for the Mačkovec killing, a prior probability of 1/44 was set and an advised PP (for kinship) of 99.9% was used to correctly identify the victims with high confidence (Biesecker *et al.* 2005; Brenner and Weir 2003; Prinz *et al.* 2007).

RESULTS

DNA quantification

Table 1 shows the results of DNA quantification determined with the PowerQuant (Promega). Because DNA was suspended in 50 µl and 0.5 g of bone powder was used for extraction, up to 7 ng DNA/g of powder was acquired from analyzed samples. The lowest quantity detected was 0.0008 ng/µl of extract, and the highest was 0.07 ng/µl of extract (Table 1). No inhibition was detected in any of the samples analyzed because no IPC shift values exceeded 0.30 (Table 1), suggesting that all of the PCR inhibitors were eliminated during extraction and purification with the EZ1 Biorobot system. Developmental validation of the PowerQuant consistently detected concentrations of human DNA as low as 0.5 pg/µl (Ewing *et al.* 2016) and DNA quantities below 0.5 pg/µl are indicated in Table 1 as <0.0005. We measured DNA quantities above 0.5 pg/µl DNA in all bone samples amplifying the Auto target (Table 1). For two samples, the Deg

target was not amplified, and 15 samples showed DNA quantification values for the Deg target lower than 0.5 pg/µl (Table 1). Samples analyzed showed different levels of DNA degradation, ranging from slightly to severely degraded. Values for degradation index ranged between 2.48 and 74.61, and for two samples it was not possible to calculate the [Auto]/[Deg] ratio because the Deg target was not detected (Table 1). It was possible to confirm the presence of male DNA in the samples by amplifying the Y chromosomal target. Y target was amplified in all bones (Table 1). Male sex obtained with amplification of the PowerQuant Y target was confirmed with amplification of the amelogenin included in NGM kit (all bones generated

Table 1. Characteristics, DNA quantity and quality (Auto, Deg, and Y target, IPC shift, and [Auto]/[Deg] ratio), and efficiency of 16 autosomal loci STR/amelogenin typing with the NGM kit (Applied Biosystems) of 39 DNA samples extracted from bones of Second World War victims excavated from the Mačkovec mass grave (sg = small grave, lg = large grave). The concentration of Auto, Deg, and Y targets are expressed in ng DNA/µl of extract and DNA quantities below 0.5 pg/µl DNA are indicated as < 0.0005. The efficiency of NGM typing is expressed as the number of loci with complete results, number of loci with partial results (dropout of one of the two alleles present at a particular locus), and number of loci with complete locus dropouts. The number of drop-ins is recorded as well

Sample	DNA Quantity Auto target [ng/µl]*	DNA Quantity Deg target [ng/µl]*	DNA Quantity Y target [ng/µl]*	IPC Shift*	[Auto]/[Deg] ratio*	Autosomal STR summary results #
R Femur 1 (sg)	0.0233	/	0.0112	-0.09	Undet.	16/0/0
L Femur 1 (sg)	0.0343	0.0012	0.0155	-0.41	28.33	16/0/0
R Femur 1 (lg)	0.0032	<0.0005(0.0002)	0.0023	-0.54	15.27	16/0/0
R Femur 2 (lg)	0.0058	0.0023	0.0033	-0.5	2.48	16/0/0
R Femur 3 (lg)	0.0027	0.0006	0.0021	-0.45	4.74	16/0/0
R Femur 4 (lg)	0.0053	0.0013	0.0021	-0.52	4.02	16/0/0
L Femur 1 (lg)	0.0058	0.0019	0.0034	-0.86	2.98	16/0/0
L Femur 2 (lg)	0.0053	0.0021	0.0035	-0.5	2.58	15/1/0 (drop-in 1x)
L Femur 3 (lg)	0.0158	0.0039	0.0113	-0.9	4.05	15/1/0
L Femur 4 (lg)	0.0074	0.0007	0.0027	-0.57	10.86	16/0/0
L Femur 5 (lg)	0.0045	0.0008	0.0035	-0.14	5.48	16/0/0
L Femur 6 (lg)	0.0042	0.0006	0.0019	-0.57	6.55	16/0/0
R Femur 5 (lg)	0.0082	<0.0005(0.0004)	0.0038	-0.49	22.61	16/0/0
R Femur 6 (lg)	0.0032	/	0.0016	-0.38	Undet.	5/5/6
R Femur 7 (lg)	0.0107	0.0009	0.0056	-0.64	12.56	16/0/0
R Femur 8 (lg)	0.0294	0.0051	0.0145	-0.65	5.8	16/0/0
L Femur 7 (lg)	0.0101	0.0021	0.0070	-0.76	4.87	16/0/0
L Tibia 1 (sg)	0.0144	0.0023	0.0080	-0.1	6.19	16/0/0
R Tibia 1 (lg)	0.0037	0.0007	0.0017	-0.66	5.26	13/2/1 (drop-in 2x)
R Tibia 2 (lg)	0.0119	<0.0005(0.0004)	0.0076	-0.41	28.37	13/3/0
R Tibia 3 (lg)	0.0071	<0.0005(0.0004)	0.0032	-0.43	18.78	13/1/2 (drop-in 2x)
R Tibia 4 (lg)	0.0035	<0.0005(0.0002)	0.0014	-0.39	16.06	10/3/3 (drop-in 1x)
R Tibia 5 (lg)	0.0033	<0.0005(0.0002)	0.0018	-0.45	14.54	14/2/0
R Tibia 6 (lg)	0.0700	0.0010	0.0394	-0.14	71.21	14/2/0
R Tibia 7 (lg)	0.0013	<0.0005(0.0002)	0.0007	-0.60	5.51	13/2/1
R Tibia 8 (lg)	0.0061	0.0015	0.0042	-0.31	4.18	16/0/0
R Tibia 9 (lg)	0.0048	<0.0005(0.0004)	0.0020	-0.74	12.18	14/2/0
R Tibia 10 (lg)	0.0279	0.0015	0.0132	-0.57	18.62	16/0/0
L Tibia 1 (lg)	0.0100	0.0006	0.0061	-0.05	16.06	16/0/0
L Tibia 2 (lg)	0.0025	<0.0005(0.0003)	0.0008	-0.49	9.54	5/3/8
L Tibia 3 (lg)	0.0090	0.0017	0.0054	-0.24	5.54	16/0/0
L Tibia 4 (lg)	0.0231	<0.0005(0.0003)	0.0072	-1.02	74.61	14/1/1 (drop-in 1x)
L Tibia 5 (lg)	0.0130	0.001	0.0046	-0.6	13.08	16/0/0
L Tibia 6 (lg)	0.0012	<0.0005(0.0003)	0.0008	-0.78	4.72	5/3/8
L Tibia 7 (lg)	0.0030	<0.0005(0.0002)	0.0018	-0.63	15.32	12/4/0
L Tibia 8 (lg)	0.0057	<0.0005(0.0004)	0.0025	-0.80	14.26	16/0/0
L Tibia 9 (lg)	0.0038	0.0006	0.0182	-0.07	60.47	14/2/0
L Tibia 10 (lg)	0.0008	<0.0005(0.0003)	0.0006	-0.46	3.12	16/0/0 (drop-in 1x)
R/L Tibia (lg)	0.0011	<0.0005(0.0003)	0.0006	-0.93	3.82	11/2/3 (drop-in 1x)

*From PowerQuant System, # 16 autosomal STRs using the NGM amplification kit (Applied Biosystems): number of loci with complete results / number of loci with partial results / number of loci with complete locus drop-outs.

a male profile) and Y-STR typing (Supplementary Tables 1, 2, and 3).

DNA typing

In Table 1, the autosomal STR summary results column describes typing success by means of comparing the consensus STR profile of a particular bone sample to the consensus STR profile of the victim that the bone belongs to and determined from different skeletal elements typed from the same skeleton (number of completely successful typed loci / number of partly successful typed loci (dropout of one of the two alleles present at a particular locus) / number of loci where a complete dropout occurred). From 22 of the 39 samples (56%), a complete 16-locus STR/amelogenin profile was obtained (in sample L tibia 10-lg one allele drop-in was observed), with the remaining 17 samples revealing partial STR profiles. Fifteen loci were successfully genotyped in two samples, 14 in five samples, 13 in four samples and 12, 11, and 10 in one sample, respectively. In three samples, only five loci were successfully typed (Table 1). Allele and locus dropouts and drop-ins appeared correlated with low DNA quantities. In most of the samples in which the PowerQuant Deg target was not detected or was below 0.5 pg/µl, partial profiles were generated, including three of the least successfully typed bones (L tibia 2-lg, L tibia 6-lg and R femur 6-lg), for which only 5 loci were typed without dropouts (Table 1).

DNA profiles obtained from 39 bones analyzed revealed 15 unique autosomal profiles corresponding to 15 victims. Supplementary Table 1 lists consensus autosomal STR profiles, and Supplementary Table 2 lists consensus Y-chromosomal STR haplotypes of bone samples analyzed from the Mačkovec mass grave and family reference samples (only reference samples that matched bone profiles). The consensus profiles obtained with the NGM kit (AB) for autosomal STRs and with the Power Plex Y System (Promega) for Y-chromosomal STRs were determined from different skeletal elements typed from the same skeleton.

Out of 15 unique profiles, 14 were found among femurs and tibias excavated from the large grave and one unique profile was generated from femurs and tibiae excavated from the small grave. For five victims, profiles of both femurs and tibias matched, for two

Supplementary Table 1. Consensus autosomal STR profiles of bone samples analyzed from the Mačkovec mass grave and family reference samples (only reference samples that matched bone profiles) obtained with the NGM kit (Applied Biosystems). The consensus profiles were determined from different skeletal elements typed from the same skeleton

Bone/tooth sample	D10S1248	vWA	D16S539	D2S1338	AMEL.	D8S1179	D21S11	D18S51	D22S1045	D19S433	TH01	FGA	D2S441	D3S1358	D1S1656	D12S391
R FEM 1 (sg), L FEM 1 (sg), L TIB 1 (sg)	13/16	16/18	11/14	17/19	X/Y	10/14	31.2/32.2	13/14	15/16	13/13	7/8	19/22	13/15	15/18	14/17	17/24
R FEM 1 (lg), R TIB 4 (lg), L TIB 6 (lg)	13/16	16/18	11/12	18/25	X/Y	13/13	29/31	15/16	15/15	13/14	8/9.3	20/24	11/15	14/19	12/17.3	17/21
R FEM 2 (lg), L FEM 1 (lg), R TIB 8 (lg), L TIB 3 (lg)	15/15	15/18	11/11	17/20	X/Y	10/13	28/30	14/16	12/17	14/15	9.3/9.3	19/21	11/14	18/18	12/15.3	18/24
R FEM 3 (lg), L FEM 4 (lg), R TIB 3 (lg), L TIB 2 (lg),	15/15	14/17	11/12	17/20	X/Y	11/14	28/30	14/16	12/14	14/15	6/9.3	19/22	11/14	18/18	12/16	18/25
R FEM 4 (lg), L FEM 5 (lg), R TIB 5 (lg), L TIB 5 (lg)	14/15	15/16	12/13	17/25	X/Y	14/15	28/29	13/14	16/16	14/14	7/9.3	21/22	14/14	14/16	16/16.3	21/25
L FEM 2 (lg), R TIB 1 (lg)	13/15	17/17	12/13	16/24	X/Y	12/13	29/30.2	21/22	11/16	14/14	7/9.3	21/25	14/15	15/17	14/17	18/18
L FEM 3 (lg), R/L TIB (lg)	14/15	19/20	8/13	12/13	X/Y	12/14	31/32.2	12/15	14/16	12/13	6/9	20/23	10/11	15/17	11/12	18.3/22
R FEM 6 (lg), L FEM 6 (lg), R TIB 9 (lg), L TIB 4 (lg)	13/16	16/17	11/13	17/17	X/Y	13/15	30/32.2	11/17	11/15	13/16.2	9.3/9.3	18/19	11/14	16/17	12/15	18/18
R FEM 5 (lg)	13/14	15/17	10/11	17/17	X/Y	9/11	28/29	11/18	11/16	15/15	6/9	20/21	11/14	16/17	13/16.3	21/22
R FEM 7 (lg)	13/14	16/17	12/12	24/24	X/Y	10/15	28/32.2	15/15	11/16	13/14	9/9.3	23/24	11/14	14/17	14/16.3	20/22
R FEM 8 (lg), L FEM 7 (lg), R TIB 2 (lg), L TIB 7 (lg)	13/16	16/18	12/14	16/20	X/Y	10/14	31.2/32.2	13/16	16/16	13/13	6/8	20/22	11/13	16/16	17/17.3	17/24
R TIB 10 (lg), L TIB 9 (lg)	13/14	16/17	10/12	16/20	X/Y	12/13	29/29.2	12/14	11/17	14/14	7/9.3	21/22	10/11	15/16	16/18.3	19/19
R TIB 7 (lg), L TIB 1 (lg)	14/16	16/18	10/12	16/20	X/Y	10/11	28/31.2	15/16	11/12	13/15	9/9.3	21/22	11.3/13	16/17	17.3/19.3	18/18
R TIB 6 (lg), L TIB 8 (lg)	15/16	16/16	11/13	17/20	X/Y	12/13	27/33.2	13/14	15/16	12/12	9.3/9.3	20/25	10/12	15/18	12/14	18/18
L TIB 10 (lg)	14/16	14/18	9/11	17/20	X/Y	13/14	31.2/31.2	14.2/16	15/16	12/14	8/9.3	24.2/25	11/14	16/17	16/17	18/21
Family reference 2 (son)	15/17	16/18	11/13	17/19	X/Y	10/12	32.2/33.2	14/14	15/16	12/13	6/9.3	19/20	12/14	15/15	14/15	18/20
Family reference 12 (daughter)	14/14	16/18	9/12	16/20	X/X	11/14	31/31.2	15/16	11/16	14/15	7/9.3	21/22	11/11.3	17/17	11/17.3	18/22

Supplementary Table 2. Consensus Y-chromosomal STR haplotypes of bone samples analyzed from the Mačkovec mass grave and family reference sample (only the reference sample that matched bone haplotypes) obtained with the Power Plex Y System (Promega). The consensus profiles were determined from different skeletal elements typed from the same skeleton

Bone/tooth sample	DYS391	DYS389I	DYS439	DYS389II	DYS438	DYS437	DYS19	DYS392	DYS393	DYS390	DYS385
R FEM 1 (sg), L FEM 1 (sg), L TIB 1 (sg)	11	14	10	31	11	14	16	11	13	25	11/14
R FEM 1 (lg), R TIB 4 (lg), L TIB 6 (lg)	10	13	11	30	9	14	15	13	13	24	14/14
R FEM 2 (lg), L FEM 1 (lg), R TIB 8 (lg), L TIB 3 (lg)	10	13	12	29	10	14	13	11	13	24	16/18
R FEM 3 (lg), L FEM 4 (lg), R TIB 3 (lg), L TIB 2 (lg)	10	13	12	29	10	-	13	11	13	24	16/18
R FEM 4 (lg), L FEM 5 (lg), R TIB 5 (lg), L TIB 5 (lg)	11	13	12	31	10	15	16	11	13	24	15/15
L FEM 2 (lg), R TIB 1 (lg)	10	13	10	30	11	14	16	11	13	25	11/14
L FEM 3 (lg), R/L TIB (lg)	10	14	11	30	10	14	16	11	14	23	12/15
R FEM 6 (lg), L FEM 6 (lg), R TIB 9 (lg), L TIB 4 (lg)	10	13	11	30	9	14	15	13	13	24	14/16
R FEM 5 (lg)	11	13	12	32	10	14	16	11	13	24	14/14
R FEM 7 (lg)	10	13	10	30	11	14	16	11	13	25	11/12
R FEM 8 (lg), L FEM 7 (lg), R TIB 2 (lg), L TIB 7 (lg)	11	14	10	31	11	14	16	11	13	25	11/14
R TIB 10 (lg), L TIB 9 (lg)	10	13	10	30	11	14	15	11	13	25	11/14
R TIB 7 (lg), L TIB 1 (lg)	11	13	13	33	10	15	17	11	13	24	14/15
R TIB 6 (lg), L TIB 8 (lg)	10	12	10	28	10	16	15	11	13	22	13/14
L TIB 10 (lg)	11	13	11	-	11	14	15	11	-	-	-
Family reference 2 (son)	10	12	10	28	10	16	15	11	13	22	13/14

Supplementary Table 3. Matched consensus Y-chromosomal STR haplotypes of bone samples analyzed from the Mačkovec mass grave and the Y-chromosomal STR haplotype of the family reference sample (only the reference sample that matched bone haplotypes is shown) obtained with the Power Plex Y23 (Promega). The consensus profiles were determined from different skeletal elements typed from the same skeleton

Vzorec	DYS576	DYS389I	DYS448	DYS389II	DYS19	DYS391	DYS481	DYS549	DYS533	DYS438	DYS437	DYS570	DYS635	DYS390	DYS439	DYS392	DYS643	DYS393	DYS458	DYS385	DYS456	YGATAH4
R TIB 6 (lg) , L TIB 8 (lg) Family reference 2 (son)	16	12	21	28	15	10	25	12	11	10	16	18	22	22	10	11	12	13	14	13, 14	13	11
L TIB 1 (sg), R FEM 1 (sg), L FEM 1 (sg) R FEM 8 (lg), L FEM 7 (lg), L TIB 2 (lg), L TIB 7 (lg)	17	14	19	31	16	11	23	12	12	11	14	20	23	25	10	11	11	13	15	11, 14	16	12
R FEM2 (lg), L FEM 1 (lg), R TIB 8 (lg), L TIB 3 (lg) R FEM3 (lg), L FEM 4 (lg), L TIB 2 (lg), R TIB 3 (lg)	16	13	21	29	13	10	22	12	12	10	14	19	23	24	12	11	12	13	16	16, 18	15	12

victims identical profiles were obtained from three bones, for another five victims a match was found in two bones analyzed, and for the last three victims the genetic profile was generated from a single bone. Bones with matching profiles belonging to a specific victim are shown in Table 2.

Unique genetic profiles were compared to family references to identify victims. Supplementary Table 3 shows matched consensus Y-chromosomal STR haplotypes of bone samples analyzed from the Mačkovec mass grave and the Y-chromosomal STR haplotype of the family reference sample (only the reference sample that matched bone haplotypes). Y-chromosomal haplotypes were obtained with the PowerPlex Y23 (Promega) and the consensus profiles were determined from different skeletal elements typed from the same skeleton. One match was observed between a victim and a family reference (a son). Additionally, in two pairs of victims a Y-STR haplotype match was observed, suggesting paternal lineage kinship among those respective pairs (Supplementary Tables 2 and 3). For one of the victims, a sister was used

as a family reference. Because autosomal STR analysis did not confirm a brother–sister kinship pair, mtDNA typing was not performed.

Statistical analysis

Table 3 summarizes matching sample pairs, the LR and PP for autosomal DNA (n-STR) and LR for Y-STRs, LR combined (LRc), and PP combined (PPc) for autosomal and Y-STRs (n-STR × Y-STR) for the identified victims found in the Mačkovec mass grave. The PP were calculated assuming 1/44 as the prior probability (see Methods). In the reference column of Table 3, the family relationship between the victim and family reference person and between victims is shown, and in the bone column all the bones that generated identical genetic profiles and thus belong to the same victim are listed.

Four genetic profile matches were ascertained, among which two cases were a match between a victim and a family reference (son and daughter, respectively), and two cases a match between two respective victims, the latter highlighting the fact that some of the victims were related. On the archived victims list there were four pairs of brothers. For R TIB 6 and L TIB 8 from the large grave (victim 14 in Table 2, both tibiae generated identical genetic profiles, except that R TIB 6 showed allelic drop-out in two systems, while full profile was obtained from L TIB 8), a potential family relationship with a son (reference sample 2, Supplementary Tables 1, 2, and 3) was calculated, whereas for R TIB 7 and L TIB 1 from the large grave (victim 13 in Table 2, both tibiae generated identical genetic profiles) a potential family relationship with a daughter (person 12, Supplementary Table 1) was calculated. In the autosomal STRs, the values for LR expressed as the paternity index (PI) ranged between 2.9×10^5 and $1.6 \times$

Table 2. Bones that belong to the same victims as determined by genetic profiles obtained with autosomal and Y-STR typing (sg = small grave, lg = large grave)

Victim	Bone 1	Bone 2	Bone 3	Bone 4
1	R FEM 1 (sg)	L FEM 1 (sg)	L TIB 1 (sg)	
2	R FEM 1 (lg)	L TIB 6 (lg)	R TIB 4 (lg)	
3	R FEM 2 (lg)	L FEM 1 (lg)	R TIB 8 (lg)	L TIB 3 (lg)
4	R FEM 3 (lg)	L FEM 4 (lg)	L TIB 2 (lg)	R TIB 3 (lg)
5	R FEM 4 (lg)	L FEM 5 (lg)	R TIB 5 (lg)	L TIB 5 (lg)
6	L FEM 2 (lg)	R TIB 1 (lg)		
7	L FEM 3 (lg)	R/L TIB (lg)		
8	L FEM 6 (lg)	R FEM 6 (lg)	R TIB 9 (lg)	L TIB 4 (lg)
9	R FEM 5 (lg)			
10	R FEM 7 (lg)			
11	R FEM 8 (lg)	L TIB 7 (lg)	L FEM 7 (lg)	R TIB 2 (lg)
12	R TIB 10 (lg)	L TIB 9 (lg)		
13	R TIB 7 (lg)	L TIB 1 (lg)		
14	R TIB 6 (lg)	L TIB 8 (lg)		
15	L TIB 10 (lg)			

Table 3. LR and PP (assuming 1/44 as the prior probability) for autosomal DNA (n-STR) and LR for Y-STRs, LR combined (LRc), and PP combined (PPc) for autosomal and Y-STRs (n-STR × Y-STR, assuming 1/44 as the prior probability) for the identified victims found in the Mačkovec mass grave. In the reference sample, kinship with the victim and between victims is indicated, and in the bone sample all the bones that generated identical genetic profiles and thus belong to the same victim are listed (sg = small grave, lg = large grave)

Bone	Reference	LR (n-STR)	PP (n-STR)	LR (Y-STR)	LRc (n-STR x Y-STR)	PPc (n-STR x Y-STR)
R TIB 6 (lg) **L TIB 8 (lg)**	son	1.6×10^6	99.997%	1.5×10^3	2.3×10^9	99.999998%
R TIB 7 (lg) **L TIB 1(lg)**	daughter	2.9×10^5	99.983%			
L TIB 1 (sg) **R FEM 1 (sg)** **L FEM 1 (sg)**	brother[1]	5.9×10^6	99.9992%			
R FEM 2 (lg) **L FEM 1 (lg)** **R TIB 8 (lg)** **L TIB 3 (lg)**	brother[2]	1.7×10^5	99.972%			

[1]brother victim R FEM 8 (lg), L FEM 7 (lg), R TIB 2 (lg), L TIB 7 (lg); [2]brother victim R FEM 3 (lg), L FEM 4 (lg), L TIB 2 (lg), R TIB 3 (lg).

10^6 (PP ranged between 99.983% and 99.997%) for the victim identified through comparison with a daughter and for the victim identified through comparison with a son, respectively, considering a prior probability of 1/44 (Table 3). By comparing autosomal STRs and Y-STRs in identifying the victim through comparison with a living son, an LR value of 1.5×10^3 for the Y-STR haplotype was calculated using the YHRD database and the LRc was estimated to be 2.3×10^9 and a PPc of 99.999998%, again considering a prior probability of 1/44 (Table 3). By comparing Y-STR haplotypes, we found two additional matches among four victims, suggesting paternal lineage kinship among those pairs. After a close inspection of the victims list and their surnames and birthplaces, we concluded that there were four pairs of brothers among the victims, and we managed to confirm kinship for two of those pairs with DNA analysis. Bone samples belonging to the first of the two pairs of brothers are L TIB 1, R FEM 1, and L FEM 1 from the small grave (victim 1 in Table 2, all bones generated identical genetic profiles) and R FEM 8, L FEM 7, R TIB 2, and L TIB 7 from the large grave (victim 11 in Table 2, all bones generated identical genetic profiles). The second pair of brothers was determined by DNA typing of the following bone samples: R FEM 2, L FEM 1, R TIB 8, and L TIB 3 from the large grave (victim 3 in Table 2, all bones generated identical genetic profiles) and R FEM 3, L FEM 4, L TIB 2, and R TIB 3 from the large grave (victim 4 in Table 2, all bones generated identical genetic profiles). LR expressed as a sibling index was estimated to be 5.9×10^6 for the first pair of brothers and 1.7×10^5 for the second pair of brother victims. By taking into account a prior probability of 1/44, a PP of 99.9992% and 99.972%, respectively, was obtained. The PP was higher than 99.9% for both pairs of brother victims and already shows a high confidence of correct identification (Table 3). However, Y-STR typing was performed as well, and matching Y-haplotypes confirmed common paternal lineage. Unfortunately, we were unable to confirm the identity of the two pairs of brothers because there were no living relatives available to serve as family references. Further attempts have been made to find living relatives of these victims, but they have not been successful.

In extracting, quantifying, and STR typing the skeletal remains, the possibility of contamination during DNA analysis was minimized. The results do not indicate contamination because no Auto, Deg, or Y targets were detected by the PowerQuant kit and no genetic profiles were generated from negative controls using the autosomal and Y-STR kits. Identical genetic profiles were acquired when using duplicate amplifications and two different amplification kits, and when analyzing various skeletal elements of the same skeleton. In addition, genetic profiles of the bones did not match any person from the elimination database, and therefore we ruled out any possible DNA contamination.

DISCUSSION

Although 70 years have passed since the end of the Second World War, identification of Slovenian victims is still relevant because a large number of individuals killed during that time are still missing and have not been identified. In identifying victims of the Second World War, we face many problems that limit the success of identification. Finding relatives after 75 years to serve as family references is a complex task and only relatives relating to 10 cases of the missing were possible to use for identification of the Mačkovec mass grave victims. DNA extraction efficiency and the removal of potential PCR inhibitors is crucial in successful DNA analysis of old skeletal remains (Watherston *et al*. 2018). Various studies have shown that total demineralization greatly enhances DNA yield (Amory *et al*. 2012). Demineralization is better and faster with very small pieces of powder, and generation of fine powder maximizes the surface area of the sample that will eventually contact the chelating solution and produces higher DNA yields (Rohland and Hofreiter 2007). Purification of DNA using magnetic beads provides a very efficient DNA binding capacity, and removal of inhibitors makes possible maximum recovery of DNA (Kishore *et al*. 2006). For extracting genomic DNA from the Mačkovec mass grave bones, a highly efficient extraction protocol that comprises generation of fine powder, complete demineralization, and efficient purification with an EZ1 device was used. Since Second World War skeletal remains typically contain small amounts of DNA and drop-outs might occur because of stochastic effects (Gill *et al*. 2000), duplicate PCR amplifications were performed to determine the consensus profiles of the bones analyzed, and duplicate alleles were interpreted. All of the above led to successful autosomal and Y-STR typing of the Mačkovec mass grave bones and allowed the identification of victims exhumed from the hidden mass grave. After typing of 39 bones, 15 unique genetic profiles were obtained. Successfully obtained unique full consensus autosomal and Y-chromosomal genetic profiles make possible comparison with living relatives; two victims

were identified and statistical parameters indicated an association with a family member (a daughter and son, respectively). In the first case, in which a daughter was used for the family reference, only autosomal STRs were used for kinship analysis and confirmation of family association. In second case, in which a son was used for family reference, the victim was identified by a combination of autosomal and Y-STR analysis. The archived victims list included four pairs of brothers and kinship for two of those pairs was confirmed with DNA analysis, but not the identity because there were no living relatives available to serve as family references. The two main reasons for the low number of victims identified are a lack of family references covering all victims and the fact that not all of the victims that were listed on the archived victims list were found in the hidden mass grave. The living relatives of the victims decided on a common grave and group burial, and so the purpose of the genetic analysis was accomplished: namely, to identify some of the victims and prove that the hidden Mačkovec mass grave did in fact contain some of the victims listed on the archived victims list. However, as in some previous cases (Morild *et al*. 2015; Ossowski *et al*. 2016b, 2017), it was proven again that forensic science might bring closure to families who lost their relatives in the Second World War and had been searching for the missing for many years.

Conflict of interest

Author declare that she has no conflict of interest.

Acknowledgement

We would like to thank Anja Krek, Vesna Kovačič and Marcel Obal for their help and to the Government Commission on Concealed Mass Graves of the Republic of Slovenia for its support in excavations of Second World War victims. This study was partially financially supported by the Slovenian Research Agency (project "Determination of the most appropriate skeletal elements for molecular genetic identification of aged human remains" J3-8214).

References

1. Applied Biosystems. AmpFlSTR NGMTM PCR Amplification Kit User Guide. 2009. Foster City, CA.
2. Baeta M, Núñez C, Cardoso S, Palencia-Madrid L, Herrasti L, Etxeberria F, de Pancorbo MM. Digging up the recent Spanish memory: genetic identification of human remains from mass graves of the Spanish Civil War and posterior dictatorship. Forensic Sci Int Genet. 2015;19:272-279.
3. Bertoglio B, Grignani P, Di Simone P, Polizzi N, De Angelis D, Cattaneo C, Iadicicco A, Fattorini P, Presciuttini S, Previderè C. Disaster victim identification by kinship analysis: the Lampedusa October 3rd, 2013 shipwreck. Forensic Sci Int Genet. 2019; DOI: https://doi.org/10.1016/j.fsigen.2019.102156
4. Betancor E, Fregel R, Almeida M, Suárez NM, Pestano J. DNA typing for the identification of eight victims of Spanish Civil War reprisals in the Canary Islands: The case of "the Fuencaliente thirteen" mass graves (Fuencaliente, La Palma). Forensic Sci Int Genet Suppl Ser. 2011;3(1):e301-302.
5. Biesecker LG, Bailey-Wilson JE, Ballantyne J, Baum H, Bieber FR, Brenner C, Budowle B, Butler JM, Carmody G, Conneally PM, Duceman B, Eisenberg A, Forman L, Kidd KK, Leclair B, Niezgoda S, Parsons TJ, Pugh E, Shaler R, Sherry ST, Sozer A, Walsh A. Epidemiology. DNA identifications after the 9/11 World Trade Center attack. Science. 2005;310:1122-1123.
6. Brenner CH. DNA-VIEW 2007 User Guide. 2007, Oakland, CA.
7. Brenner CH, Weir BS. Issues and strategies in the DNA identifiaction of World Trade Center victims. Theoret Popul Biol. 2003;63:173-178.
8. Ewing MM, Thompson JM, McLaren RS, Purpero VM, Thomas KJ, Dobrowski PA, DeGroot GA, Romsos EL, Storts DR. Human DNA quantification and sample assessment: Developmental validation of the PowerQuant system. Forensic Sci Int Genet. 2016;23:166-177.
9. Ferenc M. Topografija evidentiranih grobišč (Topography of documented mass graves). In: Dežman J (ed) Poročilo Komisije Vlade Republike Slovenije za reševanje vprašanj prikritih grobišč 2005-2008. Družina, Ljubljana, 2008:7-27.
10. Gill P, Whitaker J, Flaxman C, Brown N, Buckleton J. An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. Forensic Sci Int. 2000;112:17-40.
11. Goodwin WH. The use of forensic DNA analysis in humanitarian forensic action: The development of a set of international standards. Forensic Sci Int. 2017;278:221-227.
12. Irwin JA, Just RS, Loreille OM, Parsons TJ. Characterization of a modified amplification approach for improved STR recovery from severely degraded skeletal elements. Forensic Sci Int Genet. 2012;6:578-587.
13. Kishore R, Hardy WR, Anderson VJ, Sanchez NA, Buoncristiani MR. Optimization of DNA extraction from low-yield and degraded samples using the biorobot EZ1 and biorobot M48. J Forensic Sci. 2006;51:1055-1061.
14. Prinz M, Carracedo A, Mayr WR, Morling N, Parsons TJ, Sajantila A, Scheithauer R, Schmitter H, Schneider PM; International Society for Forensic Genetics. DNA Commission of the International Society for Forensic Genetics (ISFG): Recommendations regarding the role of forensic genetics for disaster victim identification (DVI). Forensic Sci Int Genet. 2007;1(1):3-12.
15. Morild I, Hamre SS, Huel R, Parsons TJ. Identification of Missing Norwegian World War II Soldiers, in Karelia Russia. J Forensic Sci. 2015;60(4):1104-1110.
16. Rohland N, Hofreiter M. Ancient DNA extraction from bones and teeth, Nat. Protoc. 2007;2:1756.
17. Olivieri L, Mazzarelli D, Bertoglio B, De Angelis D, Previderè C, Grignani P, Cappella A, Presciuttini S, Bertuglia C, Di Simone P, Polizzi N, Iadicicco A, Piscitelli V, Cattaneo C. Challenges in the identification of dead migrants in the Mediterranean: The case study of the Lampedusa shipwreck of October 3rd 2013. Forensic Sci Int. 2018;285:121-128.
18. Ossowski A, Diepenbroek M, Kupiec T, Bykowska-Witowska M, Zielińska G, Dembińska T, Ciechanowicz A. Genetic Identification of Communist Crimes' Victims (1944–1956) Based on the Analysis of One of Many Mass Graves Discovered on the Powazki Military Cemetery in Warsaw, Poland. J Forensic Sci. 2016;61(6):1450-1455.
19. Ossowski A, Diepenbroek M, Zwolski M, Falis A, Wróbel M, Bykowska-Witowska M, Zielińska G, Szargut M, Kupiec T. A case study of an unknown mass grave - Hostages killed 70 years ago by a Nazi firing squad identified thanks to genetics. Forensic Sci Int. 2017;278:173-176.
20. Ossowski A, Kuś M, Brzeziński P, Prüffer J, Piątek J, Zielińska G, Bykowska M, Jałowińska K, Torgaszev A, Skoryukov A, Parafiniuk M. Example of human individual identification from World War II gravesite. Forensic Sci Int. 2013;233(1-3):179-192.

21. Ossowski A, Kuś M, Kupiec T, Bykowska M, Zielińska G, Jasiński ME, March AL. The Polish Genetic Database of Victims of Totalitarianisms. Forensic Sci Int. 2016a;258:41-49.
22. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M. Genetic Analyses from Ancient DNA. Annu Rev Genet. 2004;38:645-679.
23. Parson W, Gusmão L, Hares DR, Irwin JA, Mayr WR, Morling N, Pokorak E, Prinz M, Salas A, Schneider PM, Parsons TJ. DNA Commission of the International Society for Forensic Genetics: Revised and extended guidelines for mitochondrial DNA typing. Forensic Sci Int Genet. 2014;13:134-142.
24. Promega Corporation. PowerQuant System Technical Manual. 2019, Madison, WI.
25. Promega Corporation. PowerPlex Y System Technical Manual. 2012, Madison, WI.
26. Promega Corporation. PowerPlex Y23 System Technical Manual. 2014, Madison, WI.
27. Qiagen Companies. EZ1 DNA Investigator Handbook. 2014, Hilden.
28. Ríos L, García-Rubio A, Martínez B, Alonso A, Puente J. Identification process in mass graves from the Spanish Civil War II. Forensic Sci Int. 2012;219(1-3):e4-9.
29. Ríos L, Ovejero JI, Prieto JP. Identification process in mass graves from the Spanish Civil War I. Forensic Sci Int. 2010;199(1-3):e27-36.
30. Romanini C, Catelli ML, Borosky A, Pereira R, Romero M, Salado Puerto M, Phillips C, Fondevila M, Freire A, Santos C, Carracedo A, Lareu MV, Gusmao L, Vullo CM. Typing short amplicon binary polymorphisms: supplementary SNP and Indel genetic information in the analysis of highly degraded skeletal remains. Forensic Sci Int Genet. 2012;6:469-476.
31. Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains, Forensic Sci. Int. Genet. 2012;6:398-406.
32. Thèves C, Cabot E, Bouakaze C, Chevet P, Crubézy É, Balaresque P. About 42% of 154 remains from the "Battle of Le Mans", France (1793) belong to women and children: Morphological and genetic evidence. Forensic Sci Int. 2016;261:30-36.
33. Walsh B, Redd AJ, Hammer MF. Joint match probabilities for Y chromosomal and autosomal markers. Forensic Sci Int. 2008;174:234-238.
34. Watherston J, McNevin D, Gahan ME, Bruce D, Ward J. Current and emerging tools for the recovery of genetic information from post mortem samples: New directions for disaster victim identification. Forensic Sci Int Genet. 2018;37:270-282.
35. Willuwiet S, Roewer L. Y chromosome haplotype reference database (YHRD): Update. Forensic Sci Int Genet. 2007;1:83-87.
36. Ziętkiewicz E, Witt M, Daca P, Zebracka-Gala J, Goniewicz M, Jarząb B, Witt M. Current genetic methodologies in the identification of disaster victims and in forensic analysis. J Appl Genet. 2012;53:41-60.