Skeletal Remains from World War II Mass Grave: from Discovery to Identification

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Aim To present the process of identification of skeletal remains from a mass grave found on a Dalmatian mountain-range in 2005, which allegedly contained the remains of civilians from Herzegovina killed in the World War II, including a group of 8 Franciscan monks.

Methods Excavation of the site in Dalmatian hinterland, near the village of Zagvozd, was accomplished according to archeological procedures. Anthropological analysis was performed to estimate sex, age at death, and height of the individuals, as well as pathological and traumatic changes of the bones. Due to the lack of ante-mortem data, DNA typing using Y-chromosome was performed. DNA was isolated from bones and teeth samples using standard phenol/chloroform/isoamyl alcohol extraction. Two Y-chromosome short tandem repeats (STR) systems were used for DNA quantification and amplification. Typing of polymerase chain reaction (PCR) products was performed on an ABI Prism 310 Genetic Analyzer. PCR typing results were matched with results from DNA analysis of samples collected from the relatives of supposed victims – blood samples from the living relatives and bone samples collected during further exhumation of died parents or relatives of the supposed victims.

Results The remains contained 18 almost complete skeletons, with considerable post-mortal damage. All remains were men, mainly middle-aged, with gunshot wounds to the head. DNA analysis and cross-matching of the results with relatives' data resulted in three positive identifications using the Y-chromosomal short tandem repeat (Y-STR) systems. All of the positively identified remains belonged to the Franciscan friars allegedly killed in Herzegovina and buried at the analyzed site.

Conclusion Our analysis of remains from a mass grave from the World War II confirmed the value of patrilineal lineage based on Y-STRs, even when missing persons had left no offspring, as was the case with Franciscan monks. Although this report is primarily focused on the identification of remains from a mass grave, it also emphasizes the role of forensic approach in documenting human right violations.

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In investigation of human rights violations, forensic specialists are often faced with identification of skeletal remains from mass graves. This is always a difficult task, especially when a lot of time has passed since the burial. The primary focus of such investigations is to determine the identity of the victims and return the remains to their families, in accordance with the Geneva conventions (1). As a rule, a positive identification is achieved by comparing pre-mortem data on missing persons with corresponding findings on the skeletons. These data include general anthropological variables, unique bone pathological and traumatological traits such as bone diseases, deformations, and injuries, as well as medical and dental histories. Determination of sex, age, height, and individuals' characteristics is critical for achieving identification. Life histories and descriptions by witnesses may also prove useful in the process of individualization, especially data on victims' clothing and personal belongings, state at the time of death, and manner of death (2-4). The possibilities to obtain such information for war victims are diminished due to long period between the death and recovery of the skeleton, negative effect on the memory of witnesses, the quality of preserved distinctive data, and the fact that mass graves contain skeletons from homogenous groups, such as young men in military clothes (5,6). The identification process is further aggravated by the fact that lists of possible decedents do not exist or are uncertain (5,6). In such cases, the available pre-mortem data are usually not sufficient, so identification and DNA typing techniques may be the only solution. DNA technology, including both short tandem repeat (STR) analysis and mitochondrial DNA (mtDNA) analysis, was already confirmed as a method of choice in the identification of missing person in 1991-1995 war in Croatia (2,3).

This report presents the identification process of skeletal remains from a mass grave, allegedly from the World War II. The identification process used Y-chromosomal short tandem repeat (Y-STR) DNA profiling as a single method of identification from bone specimens from the World War II.

Material and methods

Location

The samples for DNA analysis were taken from 18 skeletons from a mass grave in the village of Zagvozd, near the town of Imotski in the Dalmatian hinterland in Croatia, exhumed in April 2005.

According to the survivors' testimonies, after partisans had captured the Franciscan monastery in the town of Široki Brijeg, Bosnia and Herzegovina, in February 1945, 8 friars were killed somewhere in the region of Dalmatian mountain range on their way to Split in Croatia (7).

In the 1970s, there were allegations that 19 civilians were killed in the village of Zagvozd in 1945, including 8 friars from neighboring Herzegovina. Allegedly, they were buried near the house where they had been tortured. After a few days, the remains were removed to the field named Đokina sward, about a hundred meters from the village. Intensive investigation and data collection at the possible site of their execution and interment started in 1990. It was initiated by the Franciscan Province of Herzegovina and the families of the victims, as well as by the people of Zagvozd who wanted that friars' bones be buried in dignity (7). These activities started more than 50 years after the alleged incident, because Yugoslavian authorities after the World War II preferred to keep such events secret (7). In April 2005, experimental excavation started on Đokina sward and, after the first bones had been discovered, the police and district attorney were informed.

Exhumation and anthropological examination

The pit had a surface of 4 × 3.10 m and depth of 0.32 m. The soil above and around the postmortal remains was removed, exposing a number of articulated skeletons lying on the back or

on the face in an extended position. The bones were partly commingled because about 15 bodies were placed in the pit next to each other in 2 lines, facing each other with their legs, while 3 bodies were in the middle, laid down above them (Figure 1). One skeleton was found without the head, while other heads were fractured in many fragments. Two pairs of disarticulated long bones of the hands were found separately from the bodies. Almost all skeletons were bound with a wire around their necks, arms, or legs. The arms of 2 of them were tied on the back. No clothes and footwear were found, except few buttons and some remains of a textile material. Artifacts discovered included a razor, scissors, pencil, spoon, comb, ring, coins, remains of rosaries and several bullets, and empty cartridges for high velocity rifles. All relevant data were recorded, mapped, measured, photographed, and described, along with the information on the bones and all the artifacts found. After that, the bones were removed, placed in containers, and transported for further analysis to the Department of Forensic Medicine, Split University Hospital. The associated artifacts were brought to the Museum of Croatian Archeological Monuments in Split for conservatory proceedings. After all of the obvious remains had been removed, the entire area under the bodies was examined for possible small artifacts and bone fragments. Samples of earth were collected from beneath and around skeletons for chemical analysis.

Figure 1. Human skeletons exposed in mass grave after the superficial layer of soil had been removed.

In the autopsy room, the material was cleaned with water and soft brushes, dried, and partially reconstructed. Despite the fact that the skeletons were considerably damaged postmortally, they were examined to determine sex, age, and stature. Sex was estimated by examining the skeletal features of the skull, pelvis, and long bones (8,9). Age estimation was performed by examining the changes of the pubic symphysis using the Suchey-Brooks method (10). Antemortem stature was determined by measuring the long bones and the results were compared with the formulae and tables of Trotter and Glesser (11). The remains were then analyzed in more detail for signs of disease, injury, or skeletal anomalies.

After dentition was charted, several intact, healthy teeth were removed from each skull and subjected to DNA testing, together with samples of bones (femora) that seemed to be well preserved.

At the same time, ante-mortem descriptions of the missing friars were obtained as well as blood samples for DNA analysis from their living relatives (brothers, sisters, or nephews). Soon it became clear that these samples were not sufficient. Moreover, as it was alleged that remains of friars executed in the wider region of Herzegovina in 1944-1945 could be among the excavated skeletons, further collection of blood samples of living relatives was performed, as well as two additional exhumations of their dead parents. The total number of analyzed specimens from relatives was 45, among them 8 blood specimens and 37 teeth and bone samples.

DNA isolation

After bone surfaces were cleaned by abrasion with a grinding tip and sandpaper, the bone was crushed into small fragments and stored in sterile polypropylene tubes at -20°C until analysis. Further bone preparation and DNA extraction were done as described by Alonso et al (12). DNA from blood and bloodstain reference samples of

living relatives was isolated by standard Chelex 100 protocols (13).

DNA quantification

Data was collected using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis was performed with ABI Prism 7000 Sequence Detector Software (SDS), version 1.0 (Applied Biosystems) to generate an individual standard curve for each experiment and calculate DNA amount from each unknown sample. Human genomic DNA 9947 at 200 ng/μL concentrations (Applied Biosystems) was used as a DNA standard. We used QuantifilerTM human DNA quantification kit (Applied Biosystems). The quantification assay was performed in a total volume of 25 μL, containing 2 μL of DNA extract, Quantifiler human primer mix, and Quantifiler PCR reaction mix, with thermal cycling conditions according to the manufacturer's protocols (14). All reactions without templates served as negative controls.

DNA amplification and typing

PCR amplification was performed on Perkin-Elmer Thermal Cycler 9600, using the PowerPlex® Y System (Promega Corporation, Madison, WI, USA) and AmpFlSTR Yfiler PCR Amplification kit (Applied Biosystems) (15,16). The amount of DNA used for individual samples for both kits was from 100 pg to 1 ng.

Typing of PCR products were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with Data Collection Software. Electropherogram data were analyzed with GeneScan® Software and GeneMapper® ID software, version 3.2 (Promega). The internal standard was Liz-500 (Applied Biosystems).

Analysis of typing results

DNA profiles from bones and teeth were analyzed and compared with DNA profiles of living and dead relatives. DNA genotypes from the living relatives were obtained by analyzing the DNA isolated from blood and bloodstains, while those from the dead relatives were obtained by analyzing the DNA isolated from teeth and bone. The database was kept in the Microsoft Access 2000 (Microsoft, Seattle, WA, USA). Microsoft Excel 2000 (Microsoft, Seattle, WA, USA) was used for statistical calculation. Calculation for statistical probability of biological relationship was performed according to standard protocols (17-19).

Results

Morphological investigation revealed that the remains belonged to 18 adult male victims, mostly middle-aged.

All bones, but especially small and thin ones, as well as the edges of long bones, had considerable post-mortal damage. Consequently, the estimation of ante-mortem stature was made only on the basis of one or two long bones. In a single case stature could not be determined.

The bones revealed no pathological or degenerative changes. Only in a single case a sign of ante-mortem trauma was found as a healed fracture of the humerus. Gunshot injuries were present in 11 individuals – 10 of them had gunshot wounds to the head (Figure 2), mainly in the occipital region, while a single individual had an injury of the femur. Four persons had two gunshot injuries (head plus lower extremities). The reasons for the fracture and fragmentation of all other skulls and of long bones in 2 cases could not be reliably determined. The bones of forearms were tied with wire in 6 cases (Figure 3).

The types of analyzed samples, number of DNA isolations, and success of DNA amplification are presented in Tables 1 and 2. DNA was extracted from teeth and bone samples of 18 analyzed skeletons, as well as from blood samples of living relatives of 7 missing friars. In each case of teeth and bone samples, several DNA isolations were performed (Table 1). The concentration of isolated DNA was in the range from 14.4 pg/

Figure 2. Entrance gunshot wound of the head.

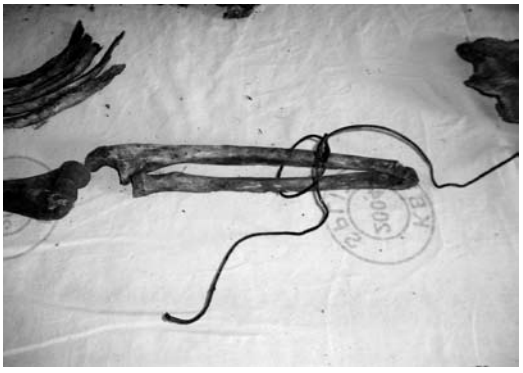

Figure 3. Forearm bones tied with a wire.

μL to 2.5 ng/μL. Moreover, DNA was extracted from one blood sample collected subsequently, and from 19 samples of teeth and bones taken during 2 additional exhumations of missing persons' dead parents (Table 2).

By using AmpFlSTR Yfiler PCR Amplification kit, 3 matches between missing persons and their living/dead relatives were obtained and thus 3 bodies were positively identified (Table 3). Using haplotypes in the general Croatian and European population database the probability of founding a man with same genotipe as Person 1, 2 and 3, was calculated (Table 4).

Discussion

The main goal of mass grave exhumation and examination of skeletal remains was the identification and determination of cause and manner of death. Allegedly, a group of 19 civilians was killed in 1945 and (re)buried after several days in a common grave. Although the region of their alleged first burial ground was investigated, no ar-

Table 1. Types of analyzed samples, number of DNA isolations and success of DNA amplification with PowerPlex Y System and AmpFlSTR Yfiler PCR Amplification kit

Case No.	Types of references samples	No. of DNA isolations	Success of DNA amplification	
			PowerPlex® Y System (12 loci)	AmpFlSTR Yfiler (17 loci)
1	teeth	3	–*	16/17
2	teeth	2	–	12/17
3	teeth	2	–	17/17
4	teeth	3	12/12	17/17
5	femur and teeth	5	10/12	12/17
6	teeth	3	–	17/17
7	teeth	4	–	12/17
8	femur and teeth	6	12/12	17/17
9	teeth	5	12/12	17/17
10	teeth	3	10/12	17/17
11	teeth	2	12/12	17/17
12	teeth	3	11/12	11/17
13	teeth	5	12/12	12/17
14	teeth	4	–	17/17
15	teeth	3	–	17/17
16	teeth	3	–	17/17
17	teeth	4	–	17/17
18	teeth	3	5/12	–
19	blood	1	12/12	17/17
20	blood	1	12/12	17/17
21	blood	1	12/12	17/17
22	blood	1	12/12	17/17
23	blood	1	12/12	17/17
24	blood	1	12/12	17/17
25	blood	1	12/12	17/17

*unsuccessful DNA amplification

Table 2. Types of analyzed samples, number of DNA isolations and success of DNA amplification with AmpFlSTR Yfiler PCR Amplification kit

Sample No.	Types of references samples	Number of DNA isolations	Success of DNA amplification (17 loci)
1	femur and teeth	5	3/17
2	femur and teeth	9	17/17
3	femur and teeth	3	–*
4	femur and teeth	4	17/17
5	femur and teeth	3	16/17
6	femur and teeth	4	8/17
7	femur and teeth	3	15/17
8	femur and teeth	3	15/17
9	femur	2	17/17
10	femur and teeth	2	–
11	blood	1	17/17
12	femur	3	17/17
13	femur	4	17/17
14	femur and teeth	3	17/17
15	femur and teeth	3	–
16	femur and teeth	3	17/17
17	femur	2	17/17
18	femur	4	–
19	femur	4	–
20	femur	4	–

*unsuccessful DNA amplification.

Table 3. Y-chromosome short tandem repeat (STR) genotypes of the skeletal remains of three missing persons and their presumptive relatives (nephews' blood and skeletal remains of dead brothers)

	Case 1			Case 2		Case 3	
Locus	nephew's 1 blood	missing person NN 1	nephew's 2 blood	missing person NN 2	brother's skeletal remains	missing person NN 3	brother's skeletal remains
DYS391	11	11	11	10	10	11	11
DYS389 I	13	13	13	13	13	13	13
DYS439	13	13	13	11	11	13	13
DYS389 II	31	31	31	29	29	31	31
DYS438	10	10	10	11	11	10	10
DYS437	15	15	15	14	14	15	15
DYS19	15	15	15	16	16	14	14
DYS392	11	11	11	11	11	11	11
DYS393	13	13	13	13	13	13	13
DYS390	24	24	24	25	25	24	24
DYS385	14;15	14;15	14;15	11;14	11;14	14;15	14;15

Table 4. Statistical calculations of probability of finding a man with the same genotype as Person 1, 2, and 3 in population.

Probability	Person 1	Person 2	Person 3
P1*	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}
P2†	2.09×10^{-3}	3.80×10^{-3}	2.09×10^{-3}

*Statistical calculations of probability according to the Croatian unpublished population data (n=167 haplotypes).
†Statistical calculations according to the eight loci from European database on *http://www.yhrd.org* (n=51 253 haplotypes).

cheological evidence was found, suggesting that the bodies had been removed to the grave analyzed in this study. Using morphological and anthropological analyses, remains were shown to contain bones of 18 adult male victims. Considerable post-mortal damage and changes of the bones compromised the possibility to estimate the precise age and stature of the individuals.

Gunshot injuries, likely caused by assault rifles, were identified in 11 out of 18 cases. We could not exclude the possibility that fractures and fragmentations of bones in uncertain cases were the result of gunshot injuries. Although it is not possible to make conclusions whether these injuries were inflicted before or after death, the anatomical distribution of the injuries, bullet trajectories through the skulls, as well as the fact that no clothes were found and that all victims were bound with a wire, strongly suggest that the victims were prisoners rather than soldiers killed in confrontation between two armed groups (20-22).

There was no ante-mortal information to match the post-mortal data obtained by standard forensic identification techniques, and the application of molecular methods in identification process seemed to be an imperative. We used Y-STR systems for several reasons as follows: all skeletal remains were male, some of them allegedly did not have any offspring, the presumptive parents were dead, and the living relatives – brothers, sisters, or nephews were not available in the sufficient number. STR systems located in the non-recombining region of the Y chromosome are widely used in forensic science for the identification of male individuals (18). The Y chromosome is passed down through generation from father to son, and does not change a lot between generations. Thus, it can be used to track parental lineage to see if the men in question are related through their fathers. This report confirmed the value of patrilineal lineage based on Y chromosome STRs, which is the only applicable method of DNA analysis in some circumstances. Some other studies have already shown that Y chromosome could provide important information if there are difficulties in identifying lineages from a specific male (23,24). On the other hand, our work clearly showed the importance of other DNA identification methods and their application in case work, such as mitochondrial DNA. However, the possibilities for the use of these methods for identification are often limited, especially in situations like the case presented, where ante-mortem data are completely absent. Moreover, in cases similar to this one, the samples are often of poor quality, which hinders the

DNA extraction, subsequent amplification, and final identification (22).

In our study, DNA amplification was performed by PowerPlex® Y System first (12 loci) and then repeated by new, more informative AmpFlSTR Yfiler PCR Amplification kit (17 loci). In both systems, the results were not completely sufficient, and in some cases no profiles or partial profiles only were obtained. DNA degradation or DNA polymerase inhibitions was the most likely explanation for unsuccessful amplification of some of the loci. Problems that forensic scientists most often face while working with DNA extracted from bones and teeth samples recovered from mass graves are limited DNA quantity, DNA degradation, contamination, and postmortem changes (3,12,25). The presence of inhibitor(s) may also prevent amplification. Inhibition is an especially significant problem when DNA is extracted from old and ancient material (25,26). One of the potential inhibitors is humic acid. DNA extraction from soil always results in co-extraction of other soil components, mainly humic acid or other humic substances, which negatively interfere with DNA detecting processes (26-28). The soil from the site described in this study was collected; its composition and possible PCR inhibition is still under analysis.

We successfully identified 3 persons out of 18 in the grave. One of them was a friar captured in a hydro-electric power station in Široki Brijeg in 1945. He sustained a gunshot wound of the parietal region of the head. Two other persons were also friars, allegedly captured in 1945 and killed somewhere near Ljubuški, Bosnia and Herzegovina. One had a gunshot wound of the occipital region of the head, while no signs of trauma were found in the other. These results confirm the presumption that there were more than 8 friars in the mass grave. Also, they confirm that our decision to collect the samples for DNA analysis from more relatives, living and dead, requiring 2 additional exhumations, was correct. We hope that our experience and data will be of value in further identifications of skeletal remains from other alleged graves in this part of Croatia and Bosnia and Herzegovina.

The ultimate goal of recoveries is to positively identify the remains and return them to their families. However, personal identification of war victims has many aspects, from ethical to humanitarian and medico-legal. In this context, the excavation of graves, examination of their contents, and analysis the post-mortal remains has another additional purpose – to collect forensic evidence that would permit prosecution of those responsible for the creation of the mass grave, crimes against humanity, and international human rights abuses (4,21,29). Proper and systematic exhumation of mass graves and post-mortem examination could demonstrate what happened and when, as well as help discover those who are accountable. These answers are essential for promoting reconciliation and justice, give the rights to the dead, and provide moral and emotional satisfaction to the living.

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