DNA Identification of Skeletal Remains from the World War II Mass Graves Uncovered in Slovenia

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Aim To present the joint effort of three institutions in the identification of human remains from the World War II found in two mass graves in the area of Škofja Loka, Slovenia.

Methods The remains of 27 individuals were found in two small and closely located mass graves. The DNA was isolated from bone and teeth samples using either standard phenol/chloroform alcohol extraction or optimized Qiagen DNA extraction procedure. Some recovered samples required the employment of additional DNA purification methods, such as N-buthanol treatment. Quantifiler™ Human DNA Quantification Kit was used for DNA quantification. PowerPlex 16 kit was used to simultaneously amplify 15 short tandem repeat (STR) loci. Matching probabilities were estimated using the DNA View program.

Results Out of all processed samples, 15 remains were fully profiled at all 15 STR loci. The other 12 profiles were partial. The least successful profile included 13 loci. Also, 69 referent samples (buccal swabs) from potential living relatives were collected and profiled. Comparison of victims' profile against referent samples database resulted in 4 strong matches. In addition, 5 other profiles were matched to certain referent samples with lower probability.

Conclusion Our results show that more than 6 decades after the end of the World War II, DNA analysis may significantly contribute to the identification of the remains from that period. Additional analysis of Y-STRs and mitochondrial DNA (mtDNA) markers will be performed in the second phase of the identification project.

DNA profiling has become the core method for forensic human, animal, and plant identification. The primary value of this procedure has significantly increased over the last fifteen years due to the introduction of short tandem repeat (STR) loci in routine paternity testing, as well as forensic and mass disaster human identification (1). Data obtained by DNA typing are highly reliable and can be used as a powerful tool for producing valuable results (2).

Various procedures, such as identification of the remains by direct facial recognition by a living person, by fingerprint, dental analysis, identification of special features (eg, scars, tattoos), recognition of clothing and belongings, autopsy findings, analysis of skeletal remains by forensic anthropologists (estimation of the species, sex, age, race, stature, and period of time since death), reconstruction of facial features from the skull, hair comparison, and DNA analysis could all be used, with more or less success, to identify human remains (3). The selection of appropriate procedure and its usefulness depends on the circumstances and the state of the examined remains. Unfortunately, in war circumstances, with significant number of remains buried in a single mass grave, identification is much more difficult (4).

Considering the temporal gap of 60 years, DNA analysis seems to be the only viable approach for the identification of victim remains from the end of the World War II. While working with bones and teeth, forensic scientists are usually confronted with many problems, such as insufficient quantity of DNA, high level of DNA degradation, and the presence of polymerase chain reaction (PCR) inhibitors. Sixty years long deposition of samples in humid soil could significantly enhance the influence of all potential adverse factors. Therefore, careful optimization of all the stages of the procedures employed in the analysis of this type of samples is obligatory (5). The mission of DNA identification of victims from the recent armed conflicts in Bosnia and Herzegovina (6,7), Croatia (3), and Kosovo (6) solved a multitude of difficulties up to then unseen in the DNA analysis of skeletal remains. Now, the same team of scientists was involved in a new challenge – DNA identification of skeletal remains from the World War II mass graves located in this part of Europe.

Slovenia is located at the intersection between Central and South-Eastern Europe, a region with a very turbulent recent history. Wars that took place in this area during the 20th century, including the World War II, left behind a significant number of dead and missing persons. There are no precise official data on the number of missing persons in this country, but the approximate number amounts up to tens of thousands. This estimate is additionally complicated by several post-war incidents and mass executions made by Yugoslav communists, which were hidden and denied by official communist authorities for almost 50 years (8). One of the most atrocious post-war incidents was "Croats' Way of the Cross," when tens of thousands of people were mass executed. Official estimates range from 45 000 to 200 000 victims (8). Recently, regional democratic governments put in significant efforts to identify individuals discovered in several mass graves scattered throughout the region. In order to address numerous requests of missing people's relatives, the municipality of Škofja Loka commissioned the DNA analysis of skeletal remains and reference samples. Due to the state of decomposition of the remains, application of standard methods for human identification was insufficient and DNA identification was requested. Here, we report the first results of these analyses.

Materials and methods

Handling of skeletal remains

Mortal remains of 27 persons were recovered from two small collective graves uncovered at the location of Lovrenska Grapa, in the vicinity

of the Slovenian town of Škofja Loka (Figure 1) during the last week of October 2006. The larger of the two mass graves contained skeletal remains of 20 persons (Figure 2) and the smaller contained 7 bodies. The graves were situated one next to another, in a wood, close to a spring (Figure 3). According to reliable testimonies of surviving witnesses, the larger of the two graves contains the bodies of Slovenian home guardsmen shot by Partisans in the spring of 1945. The smaller grave most probably contains the remains of 7 German war prisoners, who buried the Slovenians and dug out another grave where they themselves were buried.

Figure 1. Position of Škofja Loka area.

Figure 2. Skeletal remains from the bigger mass grave.

Figure 3. Location of both mass graves.

The remains were recovered and processed by local archeologists and anthropologists. Samples for DNA analysis (femoral fragments and teeth) were collected and labeled. After that, bone fragments from all 27 bodies and teeth from 18 of them were transported to the Laboratory of Forensic Genetics, at the Institute for Genetic Engineering and Biotechnology, University of Sarajevo. Upon arrival at Institute for Genetic Engineering and Biotechnology, the samples were assigned case numbers (Figure 4) and the relevant information was entered into the Chain of Custody forms. Until further processing, the samples were stored at -80°C.

Figure 4. Labeled samples upon arrival to the laboratory.

For each bone sample, the entire exterior was sanded clean to remove potential contaminants. All bone surfaces were cleaned from remnant soft tissue and soil traces using grinding stone attached to Dremel® rotary tool (Dremel Multifunktionswerkzeuge, Leinfelden-Echterdingen, Germany). Then, the samples were successively washed in mild detergent, 5% bleach, sterile distilled water, and 100% ethanol, and air-dried. Thoroughly dried samples were pulverized us-

ing a sterilized Waring® blender (Waring Products, Torrington, CT, USA) and powder transferred to sterile 15-mL conical polypropylene tubes. The same procedure, except for sanding phase, was applied to the teeth. Double extractions were performed for each sample, following the previously described phenol-chloroform (9) and optimized Qiagen (Qiagen GmbH, Hilden, Germany) procedures (Internal Commission on Missing Persons Bone DNA extraction protocol).

Centricon-100® centrifugal filter units (Millipore, Billerica, MA, USA) were used for DNA purification and concentration. The same samples required additional purification step with n-butanol (10). The concentrates were transferred to 1.5-mL microcentrifuge tubes and diluted with DNA-free double distilled H_2O to the final volume of 50-100 μL.

DNA concentration was determined using Quantifiler Human DNA Quantification Kit (Applied Biosystems Foster City, CA, USA) as described previously (11). The reaction was carried out in AB 7300 Real-Time PCR System (Applied Biosystems) according to the manufacturer's recommendations.

The PowerPlex 16 kit (Promega Corp., Madison, WI, USA) was used to simultaneously amplify 15 STR loci as follows: D3S1358, TH01, D21S11, D18S51, Penta E, D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D, vWA, D8S1179, TPOX, FGA, as well as the gender determination locus Amelogenin. Amplification was carried out as described previously (12). The total volume of each reaction was 25μL. The PCR amplification was carried out in PE Gene Amp PCR System Thermal Cycler (Applied Biosystems), according to the manufacturer's recommendations. In samples with small amount of DNA, the number of cycles was increased to 32 and elongation time extended to 90 seconds.

Electrophoresis of the amplification products was preformed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The raw data were compiled and analyzed using accessory software, 310 Data Collection Software and GeneMapperTM 3.2 (Applied Biosystems). Numerical allele designations of the profiles were obtained by processing with Powertyper16 Macro (Promega Corp.).

Handling of referent samples

Referent samples (69 buccal swabs) from potential living relatives were collected, recorded, and preliminarily labeled by local DNA experts in Slovenia. Most of the donors are still living in Slovenia, but some of the relatives sent their samples from abroad, even from the USA. Buccal swabs were used as the DNA source. Internal Institute for Genetic Engineering and Biotechnology collection kit was used for collection. Dried and labeled samples were transported to the Institute for Genetic Engineering and Biotechnology laboratory. Upon arrival, the samples were relabeled and the relevant information entered into the Chain of Custody forms. The samples were stored at -80°C until DNA extraction employing Qiagen Dnaeasy™ Tissue Kit (13).

PowerPlex® 16 kit (Promega Corp.) was used for further analysis. Similar amounts of DNA were used in all PCR reactions. Amplification was carried out as described previously (12). The total reaction volume was 25μl. PCR amplification was carried out in PE Gene Amp PCR System Thermal Cycler (Applied Biosystems), according to the manufacturer's recommendations. Electrophoresis of the amplification products was performed on ABI PRISM 377 Sequencer (Applied Biosystems), in 5% bis-acrilamide gel (Long Ranger® Single Packs, Cambrex Bio Science, Rockland, ME, USA). Raw data were compiled and analyzed using the accessory software: ABI PRISM® Data Collection Software and Gene Scan®. Numerical allele designations of the profiles were obtained by processing with Powertyper16 Macro.

Statistical analysis

Comparative analysis of DNA profiles obtained from skeletal remains and referent samples, estimation of potential familiar relationships, calculation of paternity, and sibling indexes, as well as calculation of matching probability were performed in DNA-View program, created by Charles H Brenner (14).

Results

Samples (13 bones and 14 teeth) from 27 skeletal remains were processed. Preliminary quantification of the extracted DNA defined the samples that required additional purification by n-buthanol procedure, and those were quantified again. The quantification results showed that the mean concentration varied between 2.13×10^{-3}ng/μL and 5.20×10^{-1}ng/μL, for samples for which DNA profiles were obtained. Based on the quantification results, extended PCR procedure (32 cycles with elongation time extended to 90 seconds) was recommended for 12 samples.

The characteristics and procedures employed for all the obtained remains STR profiles are presented in Table 1. The number of detected loci varied from 13 to 16 per profile. PP16 STR profiling was least successful for bone samples BS0016 and BS0025. Partial profiles of these samples consisted of alleles detected across 13 STR loci. Finally, 15 samples were profiled at all 15 STR loci. Other 12 profiles were partially generated. Also, 69 referent buccal swabs collected from relatives of persons missing in that area were successfully profiled.

Comparison of victims' profile against referent samples database resulted in 4 strong matches. In addition, 5 other profiles were matched to certain referent samples with lower probability.

Discussion

This study demonstrated that the experience gathered over the last almost fifteen years through the identification projects of missing persons in Bosnia and Herzegovina and Croatia could be successfully used to identify skeletal

Table 1. Summary of DNA analysis of skeletal remains' samples for submitted DNA profiles

Body code	Sample code	Sample type	DNA extraction procedure	Additional n-butanol precipitation	Mean qantity	PCR protocol	Number of loci with detected alleles	Positive identification
LG-06/D1	BS0001	tooth	Qiagen	+	6.65×10^{-2}	Standard	16/16	–
LG-06/D2	BS0002	tooth	phenol-chloroform	–	2.35×10^{-2}	Extended	16/16	–
LG-06/D3	BS0003	tooth	phenol-chloroform	–	2.48×10^{-2}	Standard	15/16	–
LG-06/D4	BS0004	tooth	Qiagen	+	2.75×10^{-2}	Standard	15/16	–
LG-06/D5	BS0005	tooth	phenol-chloroform	–	4.15×10^{-2}	Standard	15/16	–
LG-06/D6	BS0006	tooth	Qiagen	+	4.21×10^{-2}	Standard	16/16	–
LG-06/D7	BS0007	tooth	Qiagen	–	7.11×10^{-2}	Standard	16/16	–
LG-06/D8	BS0008	tooth	Qiagen	–	2.97×10^{-2}	Standard	16/16	–
LG-06/D9	BS0009	tooth	phenol-chloroform	–	7.95×10^{-3}	Extended	16/16	–
LG-06/D10	BS0010	tooth	phenol-chloroform	–	6.06×10^{-1}	Standard	16/16	+
LG-06/D11	BS0011	bone	Qiagen	+	1.03×10^{-2}	Extended	16/16	+
LG-06/D12	BS0012	tooth	Qiagen	–	1.02×10^{-1}	Standard	16/16	–
LG-06/D13	BS0013	tooth	phenol-chloroform	–	5.09×10^{-2}	Standard	16/16	–
LG-06/D14	BS0014	tooth	Qiagen	+	5.29×10^{-2}	Standard	15/16	–
LG-06/D15	BS0015	bone	Qiagen	+	1.76×10^{-2}	Extended	14/16	–
LG-06/D16	BS0016	bone	Qiagen	–	2.51×10^{-2}	Extended	13/16	–
LG-06/D17	BS0017	bone	phenol-chloroform	+	3.11×10^{-3}	Extended	15/16	–
LG-06/D18	BS0018	tooth	Qiagen	–	2.26×10^{-2}	Extended	16/16	+
LG-06/D19	BS0019	bone	Qiagen	+	1.32×10^{-2}	Extended	14/16	+
LG-06/D20	BS0020	bone	Qiagen	–	2.97×10^{-2}	Standard	16/16	–
LG-06/1	BS0021	bone	phenol-chloroform	–	7.46×10^{-3}	Extended	16/16	–
LG-06/2	BS0022	bone	phenol-chloroform	–	1.55×10^{-2}	Extended	16/16	–
LG-06/3	BS0023	bone	phenol-chloroform	+	2.13×10^{-3}	Extended	14/16	–
LG-06/4	BS0024	bone	phenol-chloroform	–	7.96×10^{-2}	Standard	16/16	–
LG-06/5	BS0025	bone	phenol-chloroform	+	5.57×10^{-3}	Extended	13/16	–
LG-06/6	BS0026	bone	Qiagen	–	4.27×10^{-2}	Standard	14/16	–
LG-2005/0	BS0027	bone	Qiagen	–	5.20×10^{-1}	Standard	14/16	–

remains not only 10-15 years old, but also much older remains. It showed that current protocols and the procedures optimized for relatively fresh bones and teeth could be used, without significant modifications, in the analysis of much older samples.

More than 6 decades after the World War II, DNA analysis has become almost the ultimate solution for identification of the remains from that period. Fortunately, technologies available to forensic genetics expand the area of possibilities almost daily. The introduction of the most recent methods allows forensic science community to achieve results that were unimaginable just a few years ago. Genetic typing through the analysis of STR loci is the most successful approach to forensic DNA profiling since its early beginnings (15,16) and, over the last two decades, it has become a method of choice for the identification of human remains. DNA analysis by means of PCR as a tool for identification of the missing was not possible before the late 1980s (17). Most of these identifications were achieved by mtDNA analysis (16).

Nowadays, application of each new procedure, such as optimized DNA extraction phenol-chlorophorm (8), optimized Qiagen, or some other mixture of different (18) protocols for bone samples, or different approach to the bone powdering (19) increases the possibility for the nuclear DNA profiling of degraded skeletal remains. The most recent concept of miniSTR kits (20) will almost certainly upgrade the analysis of ancient DNA from old bones and teeth.

These first results could be used as a model for further DNA identification of human remains exhumed from the World War II mass graves that are, unfortunately, scattered all over Europe. Thus, we might be able to bring closure to families who may have been waiting for decades for answers about their missing (17). Many of these relatives are aged which makes it more urgent to solve these cases.

We succeeded in identifying four persons and provide closure to their families, as well as enabled them to provide their loved ones a dignified resting place. Furthermore, these results may reinstate hope for other families that they would also find their missing relatives and get answers to the long-standing questions. Additional analysis of Y-STRs and mtDNA markers will be performed in the second phase of the identification project.

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