



# Analyses of Second World War Skeletal Remains Using a Forensic Approach

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## Abstract

This chapter presents analyses of skeletal remains from World War II using a forensic approach. Aged bones are challenging samples of biological material for DNA typing because their DNA content is very low and greatly degraded. The exceptional risk of contamination and the presence of inhibitors further limit the success of DNA typing. Because the DNA content is so limited, aged skeletons are exposed to contamination by people involved in excavation, anthropological analyses, and genetic testing. To prevent and track potential contamination by contemporary DNA, a number of standard precautions are used and they are described. The composition of bones and teeth and their degradation process is discussed. In addition to morphological structure, special attention is paid to factors affecting the preservation of DNA in old bones and teeth. Based on the literature reviewed and some analyses performed, the chapter summarizes which skeletal elements are most suitable for investigating World War II skeletal remains. It discusses how to clean and grind bone and tooth samples, how DNA can be extracted from the powder obtained, and how DNA quality and quantity can be determined for extracts using real-time quantification. The genetic markers most frequently examined in aged DNA and the advantages of new, high-performing sequencing techniques for the development and study of aged DNA are described. Using innovative methods that may help in retrieving higher-quality and increased data makes it possible to investigate more degraded DNA. Storing samples is especially important in laboratories engaged in forensic genetics. Efficient long-term bone storage is necessary to guarantee sample stability across time so that new markers as well as new technologies can be used for future retesting. The results of some aged bone sample storage studies are

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presented. The chapter concludes with a presentation of World War II victim identifications performed in Slovenia.

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**Keywords**

Disaster victim identification · World War II skeletal remains · Degraded DNA · DNA typing

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## 8.1 Introduction

In forensic investigations, unknown remains of human bones must often be identified. If the identity of the remains cannot be confirmed by standard forensic methods, it is possible to use the teeth and bones to perform molecular genetic identification. This has proven to be a very successful method for identifying missing persons, victims of mass disasters, and victims of wartime conflicts (Biesecker et al. 2005; Prinz et al. 2007; Zupanič Pajnič 2013b; Hartman et al. 2011; Montellius and Lindblom 2012; Ossowski et al. 2013; Morild et al. 2015). Among biological samples, skeletal remains are some of the most challenging for DNA typing because the condition of the skeletal remains is often not conducive for recovering DNA (Burger et al. 1999). Aged bones have a very low content of DNA that is badly degraded, and the success of DNA typing is further limited by the presence of inhibitors of PCR (Zietkiewicz et al. 2012; Irwin et al. 2012). A high contamination risk also constrains successful DNA typing (Anderung et al. 2008).

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## 8.2 Composition of Bones and Teeth and Their Degradation

The amount of DNA in old skeletal remains and its quality depends on the preservation of tissues that are subjected to various environmental factors after death (Iwamura et al. 2004). In a living cell, special mechanisms take care of repairing damaged DNA. DNA repair mechanisms do not function after the death of the organism because the cellular balance is broken. Due to exposure to bacteria, fungi, and nucleases, DNA damage continues to occur, resulting in the survival of very fragmented DNA (Baum et al. 2013). Understanding the composition of bones and teeth and their degradation process is a key factor in determining DNA content and plays an important role in selecting suitable samples for obtaining endogenous DNA.

Teeth are anatomically divided into the crown, neck, and root. The outer layer of the crown is covered with enamel, which is almost entirely of mineral origin and does not contain cells. The tooth root is covered with cementum, which is a mineralized tissue consisting of hydroxyapatite, collagen, and other non-collagen proteins (Higgins and Austin 2013). Cell-free cementum is located in the neck of the tooth root. Cementum containing cells is located in the apical part of the tooth root and is a good source of DNA (Mansour et al. 2018). In the dental neck, enamel and

cementum come into contact. Below both of them, dentine protects the tooth pulp. The dentine and pulp comprise the majority of the tooth and, unlike the enamel, are rich in cells (mostly odontoblasts and fibroblasts). The dentine consists of hydroxyapatite, collagen type I, and water (Higgins and Austin 2013). The quantity of DNA recoverable from a particular tooth and from one group of teeth to another greatly varies; the amount of DNA isolated and its quality are also affected by dental pathologies, dental procedures that were carried out, how much time has gone by since the tooth was extracted until the DNA was isolated, and the age of the donor (Schwartz et al. 1991). Dental pulp is a good source of DNA. The quantity of DNA is related to the volume of the tooth pulp and the kind of tooth. Teeth with larger pulp and multi-root teeth are the best source of DNA because they contain many pulp cells and have more tooth cementum compared to single-root teeth. According to the recommendations, molars contain the most DNA and are the best for DNA analyses, followed by premolars (Higgins and Austin 2013). The best teeth for isolating DNA are as follows: first of all, molars, premolars, canines, and incisors that have not undergone endodontic treatment, and then molars, premolars, canines, and incisors that have been treated.

Bones are macroscopically divided into compact and spongy bone. Compact bone can be found in the outer part of the ends of the long bones (epiphysis), the outer part of the flat bones, and the middle part of the long bones (diaphysis). Spongy bone can be found inside the flat bones and inside of the ends of the long bones. Because of the fragility of spongy bone, it needs an outer protective layer of compact bone (Camposa et al. 2012). Microscopically, bone consists of cells and an extracellular matrix made up of an organic part and an inorganic part. The organic part mostly consists of type I collagen plus some other proteins and glycoproteins, and the inorganic part mostly consists of hydroxyapatite composed of calcium and phosphate ions (Camposa et al. 2012). Following death, the DNA is maintained much better in teeth and bones than it is in soft tissue (Iwamura et al. 2004; Camposa et al. 2012; Kendall et al. 2018). The slow degradation of DNA and the protection against enzymatic processes in the skeletal remains is influenced by the binding of negatively charged phosphate groups to hydroxyapatite hydroxyl groups, and the DNA molecule is bound to collagen as well (Camposa et al. 2012; Coulson-Thomas et al. 2015). Collagen and hydroxyapatite together form a tight structure that, due to small pores, prevents collagenases of microorganisms from breaking into the structure (Turner-Walker 2008). The size of the pores and their interconnection determine how water, microorganisms, and other particles pass through and out of the structure (Kendall et al. 2018). The environment that skeletal remains are located in from death until they are found greatly impacts the degradation of bones and teeth. The decay of bone cells and the soft tissue in the blood vessels make the bone more porous. Bone porosity determines the speed and manner in which environmental factors affect bone changes. Fungi and bacteria present in the soil and cyanobacteria present in the water can easily break into the bone tissues due to increased porosity with time, making the bone tissue even more porous (Camposa et al. 2012). In contrast, the environmental impact can also reduce porosity due to permineralization, which gradually leads to fossilization, making the bone tissue

better preserved (Kendall et al. 2018). The pores in the teeth are smaller than in bone, and therefore the DNA in teeth is preserved better than in bones. Thus, it is possible for DNA to persist for centuries or even millennia (Turner-Walker 2008).

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### 8.3 Factors Affecting the Preservation of DNA in Skeletal Remains

Analysis of aged skeletal remains found at various locations proves that the preservation of DNA is affected not only by the postmortem interval, but primarily by the environment the remains are subjected to. Factors affecting DNA preservation in skeletal remains include the soil's humidity, temperature, geochemical properties, and pH as well as microorganisms in the soil (Höss et al. 1996; Poinar et al. 1996; Putkonen et al. 2010; Higgins and Austin 2013). The most important factors for preserving DNA are the soil temperature and its humidity, and environments that are very stable with minimal yearly temperature or humidity fluctuation are favorable for preserving DNA. Wet warm environments dramatically reduce the amount of DNA because of extensive damage and fragmentation (Smith et al. 2003). The presence of water, the pH of the environment, and mineralized tissue's porosity are factors that influence the solubility of hydroxyapatite. Water helps dissolve mineral ions, and a decrease in pH increases solubility. Alternating dry and humid environments and environments with continual water flow are more harmful to DNA preservation than a continuously wet environment (Turner-Walker 2008; Higgins and Austin 2013; Kendall et al. 2018). There also exist factors particular to individuals affecting the preservation of DNA; these include race, age, sex, age, and the type of skeletal elements (Camposa et al. 2012; Romanini et al. 2012; Mundorff and Davoren 2014).

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### 8.4 Sampling Strategy and DNA Typing of Skeletal Remains

The assessment of bone and tooth suitability for genetic analysis is difficult, and investigations are costly and often unsuccessful. Consequently, much recent research focuses on finding the most appropriate skeletal elements that would allow the acquisition of well-preserved DNA for genetic typing (Hansen et al. 2017; Pilli et al. 2018). Several studies of using skeletal remains for nuclear DNA typing determined that teeth yield the best typing results, followed by femurs and tibias (Edson et al. 2004; Miloš et al. 2007; Misner et al. 2009). These bones are not always available for use in sampling and genetic testing. In the study to identify victims of the World Trade Center attack, DNA was extracted from small bones, including patellae, foot phalanges, and metatarsals; these produced rates that were comparable to both femurs and tibias (Mundorff et al. 2009). Despite the wide spread conviction that dense (i.e., cortical) bone contains DNA that is better preserved than in spongy (i.e., cancellous) bone, a few researchers have reported that commonly overlooked small skeletal types of the feet and hands have been even more useful than weight-bearing long bones. Among them, Mundorff et al. (2009) and Mundorff

and Davoren (2014) found that metatarsals, metacarpals, and phalanges as well as patellae were very similar to or even better than the femurs and tibias in the DNA that they yield. It is easy to sample such bones with a disposable scalpel, thereby reducing potential contamination of DNA. Recent studies of teeth show that, for genetic typing of skeletal remains, it is most appropriate to sample tooth cementum in the region of the tooth root and not whole teeth (Mansour et al. 2018). A study performed by Pilli et al. (2018) compares various skeletal elements with the middle section of the petrous bone (the temporal bone), which is among the toughest human bones (Hansen et al. 2017). Pinhasi et al. (2015) determined that the petrous bone is the most suitable skeletal part for sampling ancient human skeletons. For identifying which skeletal elements yield superior results in DNA preservation in 70-year-old skeletons, different skeletal element types were sampled from three World War II skeletons. The goal was to optimize the sampling process for identifying victims of World War II (Obal et al. 2019). With the help of measurements such as the quantity and quality of DNA (measured using qPCR, in which short and long fragments were targeted and degradation rates determined), and autosomal STR typing success, an effort was made to determine the best kinds of skeletal elements for identifying the identity of the victims. The best parameters for assessing skeletal types that show superior preservation of DNA were the DNA quantity and number of STR loci that were typed successfully. Among 56 different elements, 15 elements in all three skeletons yielded a full profile. Among the skeletal types containing the most DNA on average in all three skeletons studied were mostly bones of the hand and the feet and metatarsal and metacarpal bones exhibited the highest yield. Among 15 bones yielding full genetic profiles in the three skeletons, 14 also contained the greatest average DNA amounts except for tibias, for which the average yield of DNA did not correspond to the success of profiling because the amount was lower (Obal et al. 2019). Our second study was performed on ancient bones. The aim was to establish if acquiring sufficient DNA quantities and then profiling the autosomal STR markers would succeed for small bones of the feet (i.e., metatarsal bones) and of the hands (i.e., phalanges and metacarpal bones) from ancient skeletons in comparison to skeletal types suggested by current recommendations (i.e., petrous portion of temporal bones, femurs, and teeth). Therefore, 48 samples of bone from eight skeletons (six eighteenth-century skeletons and two third-century skeletons) were acquired from five Slovenian archeological locations. The study sampled six skeletal types from each skeleton (molar, temporal bone, femur, metacarpal bone, proximal phalanx from the hand, and metatarsal bone). Careful precautions were adhered to for preventing any contamination. The greatest yields were identified in temporal bones, and the lowest were in femurs. The degree of success for STR typing was evaluated based on the number of loci that were successfully typed, and the study confirmed a robust correlation between the quantity of DNA extracted and the degree of success for typing STRs. The results indicate that it would be appropriate to involve metacarpal and metatarsal bones in the sampling strategies for identification even in the study of ancient bones (Geršak et al. 2019). Based on recent studies performed by various research groups (including this author's research on World War II and ancient skeletons), the present recommendations for preferentially testing long bones

taken from the legs must be re-evaluated, and in the future there should be changes to the sampling strategy used by laboratories that type bone samples.

Recent forensic studies have focused on identifying which skeletal types offer superior DNA preservation, but there has been a lack of focus on measuring variation within particular bone type (Barta et al. 2014; Higgins et al. 2015; Alberti et al. 2018). As early as 2004, Pääbo noticed that yields of aged DNA might vary among extracts of different samples taken from the same bone, even when the same extraction method is used (Pääbo et al. 2004). It is necessary to focus sampling efforts on sections of bone that will result in maximum recovery of genetic material, and so it is of great interest to explore the variation in DNA content within a bone in the future. DNA content variation within a bone was explored by measuring nuclear DNA quantity and quality (using qPCR in which short and long fragments were targeted and degradation rates determined) in World War II metatarsal and metacarpal bones (data not published yet). To exclude the influence of taphonomic issues (postmortem interval and the environment that the remains were subjected to), 213 bones from a single World War II mass grave were examined to ensure that the skeletons had decomposed for the same duration and under the same conditions. From each bone, DNA was extracted from the compact diaphysis and from spongy epiphyses. A statistical analysis indicated that DNA yields differed significantly in extracts from the diaphysis and extracts obtained from the epiphyses of the same bones in metacarpals and metatarsals. A higher difference in DNA yield between the diaphysis and epiphyses was found in metacarpals in comparison to metatarsals. On average, 12 times more DNA was obtained from metatarsal epiphyses than the diaphysis, and 26 times more from metacarpal epiphyses than the diaphysis. It was determined that the best place to sample from within metatarsal and metacarpal bones from World War II is spongy epiphyses (data not published yet).

#### **8.4.1 Contamination Monitoring and Its Prevention**

Preventing and detecting contamination by contemporary or modern DNA is essential in forensic DNA examinations of aged remains. Modern DNA contamination can occur during excavation, as well as anthropological and molecular genetic investigations. Because of the small DNA quantities extracted from old teeth and bones, it is difficult to distinguish between endogenous DNA and much more prevalent modern DNA. Studies of old skeletal remains in forensic laboratories should be guided by experience in analysis of ancient DNA, which usually deals with significantly older samples, for which problems caused by inhibition, degradation, and contamination consequently usually increase (Pääbo et al. 2004; Gilbert et al. 2005; Rohland and Hofreiter 2007). Recommendations for preventing contamination and criteria that confirm the authenticity of the genetic profiles are therefore used. In order to ascertain the authentic character of bone and tooth genetic profiles, an elimination database is always created that contains the genetic profiles of every person that came in contact with the skeletal remains. To prevent contamination in the laboratory as much as possible, it is necessary to handle samples and carry out

DNA extraction in dedicated laboratory premises where post-PCR work has never been carried out (Pääbo 1990). The laboratory may further be equipped with Hepa-filtered hoods to ensure that no DNA has entered the facility. All of the work for extraction should be carried out using protective garments, the workspace should be cleaned using bleach, and UV lights should be used to irradiate it (Pääbo 1989). Testing of an extraction-negative control and PCR-negative controls must be carried out in parallel with bone DNA typing to detect any contamination that has been introduced from reagents at the time of the extraction and amplification procedure. Excavators, anthropologists, and laboratory researchers (included in the elimination database) should be typed for the same genetic markers whenever possible, and their genetic profiles should not match the profiles obtained from bones. Each specimen should be used to prepare multiple extracts, and these should produce identical DNA profiles (Pääbo et al. 2004). It is necessary to quantify the number of amplifiable DNA molecules. An inverse correlation must exist between the size of the amplification product and amplification efficiency, which reflects damage and degradation in the aged DNA template (Pääbo et al. 2004).

To reduce the contamination possibility to a minimum at the author's laboratory, the extraction procedure is performed in a room exclusively designed to process World War II remains. Mechanical cleaning is carried out in a closed cytostatic C-(MaxPro)<sup>3</sup>-130 (IskraPio) safety cabinet. To reduce cross-contamination to a minimum, a maximum of eight bone or tooth samples are processed during each extraction batch, and a blank control is included with each batch. DNA quantification is performed to test whether an inverse correlation exists between the size of the amplification product and the amplification efficiency. Cleaning in detergent, ethanol, and bi-distilled water, plus UV-light irradiation are carried out to minimize contamination of surfaces from prior handling. The extraction process includes extraction-negative controls to check their cleanliness. Cleanliness of amplification-negative controls is checked as well. Analyses of tooth and bone sample are separated physically and time-wise from sample analyses for the elimination database and family reference samples. Duplicate analysis is performed for all bone and tooth samples, extraction-negative controls, and negative template controls. DNA is extracted from teeth and bones a minimum of two times or from different skeletal types from the same person to verify the genotyping results, and results that are reproducible are utilized for interpretation. No contamination of old bone and tooth DNA samples was detected in the laboratory because strict precautions to prevent contamination are followed.

#### **8.4.2 Bone and Tooth Sample Preparation**

When directly handled and washed, the sample surface seems to be contaminated, as well as (to various degrees, depending on porosity and preservation), bone and tooth interiors (Gilbert et al. 2005; Salamon et al. 2005; Sampierto et al. 2006). Because of this, bone and tooth samples are purified and decontaminated in the laboratory. Samples of bone are cleaned chemically and mechanically, and they are irradiated



with UV light, and the samples from teeth are chemically cleaned and then subjected to UV-light irradiation prior to being ground into a powder. No procedures for removing contamination occurring during excavation or storage of remains, or during collection are 100% efficient, but cleaning may enhance the ratio between endogenous DNA and contaminating DNA, and it may help reduce the quantity of inhibitors that are introduced into the extraction (Rohland and Hofreiter 2007). The samples of bone are cleaned by physically removing the surface with a rotary sanding device, and they washed successively in detergent, sterile bi-distilled water, and ethanol. An approximately 1–3 mm layer is stripped from the bone surface through sanding to reduce any contamination caused by prior handling. Skeletal remains are cleaned in a MC 3 (IskraPio) closed microbiological safety cabinet located in premises exclusively designed for handling old skeletal remains. Between processing each sample, all of the tools used to drill, cut, and grind the bones are cleaned. They are washed with water, bleach (6% sodium hypochlorite) or DNA Away™ (Molecular BioProducts) and sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), followed by 80% ethanol. After this, all of the material is sterilized and undergoes UV-irradiation a minimum of overnight, or as long as 72 h. Warming bones may cause endogenous DNA to degrade (Alonso et al. 2001). To keep the bones from warming while drilling and cutting take place, abrasion and cutting are performed at a lower speed, and the bone is frequently cooled in liquid nitrogen. A factor that affects DNA extract quality is the quality of bone powder used for the extraction procedure. Very fine bone powder must be obtained for extraction of a sufficient amount of DNA from skeletal remains. Very fine-grained powder yields improved and more rapid demineralization, and the generation of fine powder maximizes the sample surface area for eventual contact with the chelating solution (Rohland and Hofreiter 2007). Rohland and Hofreiter (2007) compared samples ground into a fine powder with a freezer mill versus samples that were ground into pieces measuring a few millimeters in diameter with a mortar and pestle; they determined that higher yields were obtained from the fine powder. The performance of a Bead Beater MillMix 20 tissue homogenizer (Tehtnica–Domel, Železniki, Slovenia) was tested for grinding the human bone samples on 60 World War II femurs and tibias, and it was found to be very successful in the recovery of high-quality powder from skeletal remains from World War II (Zupanič Pajnič 2017). Grinding is performed in grinding vials made of metal using metal balls that are cooled with liquid nitrogen before use. Samples of teeth and bones are cooled with liquid nitrogen before grinding as well to avoid overheating during powdering. Grinding is performed at 30 Hz for 1 min. Pulverization of skeletal remains is performed in premises exclusively designed for handling old skeletal remains. The grinding vials must be fully cleaned before they are used again. Between samples, they are washed with water, and then bleach (6% sodium hypochlorite) or DNA Away™ (Molecular BioProducts), followed by sterile bi-distilled water (Sartorius-Stedim Biotech or Millipore), and finally 80% ethanol. In the end, the vials undergo sterilization and UV irradiation a minimum of overnight or as long as 72 h, plus another 30 min before they are used. There must be an adequate number of grinding vials for preparing more than one sample each day.



### 8.4.3 Extraction of DNA

Harmful effects due to different environmental factors impact the ability to obtain uncontaminated intact DNA from aged bones, and so it is very important to take proper steps to guarantee that the highest possible DNA yields are obtained when analysing poor-condition skeletal remains. The DNA extraction method that is used as key for the quantity and quality of the DNA acquired, and it has a great effect on STR typing success (Putkonen et al. 2010; Irwin et al. 2012). Optimization of extraction methods is necessary for obtaining DNA from old skeletons. Highly efficient extraction methods are the basis for studying and obtaining any genetic data from old bones in forensic investigations. The most recent studies indicate that total demineralization is the optimum method for extracting DNA from old bones (Jakubowska et al. 2012; Amory et al. 2012) because total demineralization greatly increases the share of full profiles, and this correlates with better quality of DNA. The DNA extraction method that was developed at the author's laboratory for acquiring DNA of high quality from remains of World War II skeletons was developed based on a total demineralization process, and it has proved effective from as little as 0.5 g of powder from tooth or bone (Zupanič Pajnič et al. 2016a; Zupanič Pajnič 2016). Analysis was performed on 111 teeth and bones taken from World War II mass graves for evaluation of this method (Zupanič Pajnič 2011), as well as on 54 samples of skeletal remains from World War II (Zupanc et al. 2013). No undigested tooth or bone powder remained after the demineralization and lysis stages because complete decalcification was applied with ethylenediaminetetraacetic acid ( $\text{Na}_2\text{EDTA}$ ).  $\text{Na}_2\text{EDTA}$  is a powerful chelator that can bind metallic ions like calcium in the tooth or bone powder, making it possible to remove it. High quantities of  $\text{Na}_2\text{EDTA}$  are needed to dissolve some of the hydroxyapatite matrix that is specific to tooth and bone samples (Rohland and Hofreiter 2007). To achieve complete demineralization, 15 mL of 0.5 M  $\text{Na}_2\text{EDTA}$  per gram of tooth or bone powder is required. In theory, this amount of  $\text{Na}_2\text{EDTA}$  can only bind the quantity of calcium in 1 g of tooth or bone powder (Loreille et al. 2007). To totally demineralize 0.5 g of tooth or bone powder, 10 mL of  $\text{Na}_2\text{EDTA}$  was used in the extraction protocol. After decalcification, an extraction buffer, proteinase K, and DTT are mixed in to the precipitate, and this is incubated for 2–3 h at 56 °C. Alongside calcium chloride, humic acids are general PCR inhibitors that are often encountered in samples bone taken from soil (Krenke et al. 2008). When PCR inhibitors are present, this can create imbalances or the loss of peaks at particular STR loci, and this may produce a genetic profile that is incomplete. The purification of DNA using an organic extraction method (commonly used in forensic laboratories) is performed using chloroform and phenol. They are both dangerous, and processing using them should always be carried out with a vented fume hood. Because they are toxic, using other efficient methods to purify DNA is much safer. The purification of DNA using magnetic beads and robotic devices was included in the extraction protocol (Zupanič Pajnič et al. 2016a; Zupanič Pajnič 2016) and no inhibition was detected. High efficiency of magnetic particles in DNA purification and PCR inhibitor removal was also confirmed in some other studies that were not conducted on skeletal remains but

on casework samples (Nagy et al. 2005; Montpetit et al. 2005; Kishore et al. 2006; Valgren et al. 2008). The purification procedure using automated devices does not use aggressive organic solvents such as phenol or chloroform, devices are easy to use, and it is not necessary to prepare reagents (all of the reagents except DTT are available in the kits). The purification procedure is automated and it requires only 20–30 min to complete. The entire process of extraction is performed in a large filter tip for single use, and the remainder of the extraction reagents is also safely put in a cartridge for one-time use. As a result, manual pipetting is not necessary and the extraction process is less prone to contamination. Purification based on magnetic particles can be modified for robotic machines from different suppliers or can be carried out by hand. DNA from skeletal material from World War II was successfully purified using Biorobot EZ1 (Qiagen) and the AutoMate Express Instrument (TFS). It is believed that the established extraction methods (Zupanič Pajnič et al. 2016a, Zupanič Pajnič 2016) will make a contribution to the options for automated DNA extraction from skeletal remains, thus streamlining procedures for extracting high-quality DNA from skeletal material in forensic laboratories. In addition, commercial kits for automated DNA extraction from bone samples will make possible test and result standardization in forensic laboratories, will increase tooth and bone sample throughput, and will minimize the possibility of human error resulting in sample mixing.

#### 8.4.4 Real-Time Quantification

In compromised bone samples, it is vital to examine the quality and quantity of human DNA that is available for genetic analysis. To obtain this information, the DNA extracts from the bone samples should be quantified by multiplex real-time or quantitative PCR (qPCR). New, highly informative multiplex qPCR kits that quantify total human nuclear DNA, male DNA, the level of DNA degradation, and PCR inhibitor presence all at the same time are used by the author's laboratory to determine the DNA quality and quantity from old tooth and bone samples. The multicopy targets that new kits use offer a much lower detection level than the single-copy assays used in the past. Samples of World War II skeletal remains from mass graves were subjected to extreme environmental conditions, which resulted in extensive degradation of DNA. For assessing the extent of degradation of DNA, two regions with different lengths from the same autosomal multicopy locus are amplified using new qPCR kits. The ratio of the relative amounts of short autosomal (Auto) and long degradation (Deg) amplicons provides an evaluation of DNA quality through a quantitative estimate of the extent of DNA degradation (i.e., the [Auto]/[Deg] ratio) for each sample. It was determined that the DNA extracted from the skeletons recovered from World War II Slovenian mass graves is highly degraded with an [Auto]/[Deg] ratio or degradation index much higher than 2 (Zupanič Pajnič et al. 2017). In addition to determining the concentration of DNA, new kits also detect the Internal Positive Control (IPC), which is part of each amplification reaction, and its amplification is utilized for detecting inhibitors

found in the sample. A quantification screening method that is both effective and able to identify DNA samples that do not result in useful short tandem repeat (STR) profiles is greatly desired in forensic genetic laboratories because STR typing has a strong impact on the costs and time connected with DNA profile development. Preservation of the sample to allow single nucleotide polymorphism (SNP) or mitochondrial DNA (mtDNA) analyses is also essential. The great sensitivity of the new qPCR assays could be applied to identify DNA samples with quantities below the STR reaction sensitivity and thus for avoiding analysing samples that are less likely to be amplified, resulting in less expenditure of money and time. When testing the qPCR PowerQuant assay (Promega) on 60 World War II bone samples, it was wondered whether the kit is capable of predicting downstream STR typing success and whether it could be used to screen for autosomal STR typing success. STR profiles that were full or partial but useful were only produced from bone extracts in which short autosomal (Auto) PowerQuant targets and long degradation (Deg) ones were detected. It was determined that, after PowerQuant quantification, STR typing of old bones should be carried out only when both the Auto and Deg targets are simultaneously detected. The PowerQuant kit can identify bone DNA samples that will not produce useful STR profiles and that would be analysed with greater success using other genetic markers like SNPs and mtDNA (Zupanič Pajnič et al. 2017).

#### 8.4.5 Genetic Markers

DNA obtained from aged skeletons has deteriorated to a small size on average, usually between 100 and 500 bp, and bases become altered due to molecular damage (Pääbo 1989; Hofreiter et al. 2001). Hydrolytic and oxidative changes are most probable to damage DNA as time passes. Oxidative changes result in modifications of bases, whereas damage due to hydrolytic changes causes the deamination of bases as well as depyrimidination and depurination. Both of these mechanisms diminish the size and number of fragments that PCR can amplify (Keyser-Tracqui and Ludes 2005). Knowledge of these processes and the effects they have on DNA aids researchers in using appropriate assays (targeting of short amplicons) in order to retrieve DNA from aged skeletons. Sex determination was the first study performed on skeletal remains (Fattorini et al. 1988). Formerly, mitochondrial DNA tests were regularly used to forensically identify old skeletal remains (Anslinger et al. 2001; Palo et al. 2007). Recently, some research groups, including the author's own, have described successful nuclear DNA typing from old skeletal remains (Lee et al. 2010; Zupanič Pajnič et al. 2010, 2016b, 2017; Zupanič Pajnič 2013a). Autosomal nuclear DNA is usually typed for missing person identification because it is specific to individuals due to recombination and offers kinship information about both parents. In autosomal DNA, STRs or SNPs are investigated because their high discriminatory power allows reliable identification of the individual (Zupanič Pajnič et al. 2012; Zupanič Pajnič 2013a; Quatrehomme et al. 2019; Pilli et al. 2018). Close relatives are the most suitable for analysing autosomal STRs or SNPs. When close relatives

are not available for identification, it is possible to use distant relatives on the paternal and maternal sides and lineage markers from mtDNA or from the Y chromosome analysed. The Y chromosome passes in unchanged form from fathers to sons, and mtDNA from mothers to all offspring, regardless of sex, and they make it possible to trace the paternal and maternal lines. When even distant relatives are not available for identification, externally visible characteristics of skeletal remains—biogeographical ancestry, or BGA (Phillips et al. 2009; Hollard et al. 2017), eye color (Walsh et al. 2011), and color of the hair (Walsh et al. 2013) are possible to predict through the recent establishment of DNA phenotyping in forensics (Kayser and de Knijff 2011; Kayser 2015).

The HirisPlex system for predicting parallel color of eyes and hair from DNA recently showed successful retrieval of eye and hair color information from World War II skeletal remains recovered from Slovenian mass graves using capillary electrophoresis technology for separation of phenotypic SNPs and its possible use for identifying missing persons and disaster victims (Chaitanya et al. 2017). The HirisPlex system was applied to 49 samples of DNA acquired from the teeth and bones of World War II victims, and all 49 of the samples resulted in complete HirisPlex profiles except for one MC1R DNA marker (N29insA) that was absent in 83.7% of the samples (Chaitanya et al. 2017).

Until recently, the chief methods for ancestry inference were Y-chromosome STR/SNP and mtDNA control region and mtDNA SNP analyses because they are geographically highly differentiated (Karafet et al. 2008). By increasing the phylogenetic resolution of Y chromosome, researchers recently proved additional informativeness in western European populations (Larmuseau et al. 2014, 2017; Ralf et al. 2015). Analysis of Y-chromosomal and mtDNA markers offers useful information for studying paternal and maternal lineages. Nonetheless, they are not subjected to recombination, and they show only an incomplete picture of an individual ancestry, particularly in the case of complex admixture within a particular individual's genealogy. In contrast, autosomal ancestry-informative markers (AIMs) or ancestry SNPs that have a broad distribution through the autosomes offer a fuller image of overall ancestry and they have utility for identifying an individual's most likely BGA or population of origin, and they have become the main markers for investigating individual ancestry (Kosoy et al. 2009; Tasker et al. 2017). AIMs are classified as genetic polymorphisms; they are mainly SNPs with significant allele frequency divergence between ethnic groups to better characterize genetic differences between them (Espregueira et al. 2016). It is possible to infer genetic ancestry by comparing the genetic diversity of a sample with variation patterns in contemporary populations. In addition to tracing genealogies of individuals, AIMs can play an important role in identifying missing persons and the victims of large-scale disasters (Phillips et al. 2007; Kidd et al. 2014; Puente et al. 2016) and in BGA prediction in crime case work (Pereira et al. 2017; Tasker et al. 2017; Hollard et al. 2017). In comparison to STRs, they are very short and can be used for genotyping difficult forensic samples from badly degraded skeletal remains with minimal amounts of DNA (Romanini et al. 2015; Silvia et al. 2017; Al-Asfi et al. 2018). By genetic typing of STRs and SNPs found on Y chromosome and

sequencing of mtDNA control region and mtDNA coding region SNPs to determine Y-chromosomal and mtDNA haplogroups, and investigations of AIMs, the BGA of World War II victims' skeletons could be determined in the future, and thus their possible ethnic background. In addition, various databases, such as YHRD (the Y-Chromosome STR Haplotype Reference Database) and also the EMPOP (the European Mitochondrial DNA Population Database) can be used to search for matching haplotypes and determination of BGA in the case of a match. There are over 150,000 haplotypes of different populations from across the world in the YHRD database (Willuwiet and Roewer 2007). Because the Y chromosome is inherited on the paternal line, the matching of haplotypes indicates a common ancestral paternal line. By searching for ancestry information using the YHRD database, it is possible to obtain information about matching Y haplotypes and which population they belong to. The same can be applied to analyses of the mtDNA control region. MtDNA is inherited on the maternal line, and the matching of haplotypes indicates a common ancestral maternal line. By using the EMPOP database (Parson and Dür 2007), which contains more than 34,000 haplotypes of different populations from all over the world, it is possible to obtain information about matching mtDNA haplotypes and which population they belong to. Recently, Polish researchers performed genetic analyses of early World War II Sobibór death camp victims. Even though they could not identify them through phylogenetic analyses, they determined that the skeletal remains, originally believed to originate from victims of Russian–Polish conflicts, actually seem to belong to Jews. This is the first solid evidence of Holocaust crimes (Diepenbroek et al. 2018), and it shows the importance of forensic genetics for aiding historical research.

In highly degraded skeletal remains, particularly old ones, STR typing is often not possible. Advances made recently in massively parallel sequencing (MPS), also known as next-generation sequencing (NGS), offers strong advantages over technologies used previously for analysing DNA recovered from human remains (Knapp et al. 2015). With NGS technology, identification of highly degraded skeletal remains could be achieved using identity SNPs (Mehta et al. 2017), which can be used because they are much shorter than STRs. Because of the high copy number per individual cell, mitochondrial DNA analysis is a key DNA detection method used by forensic scientists when old skeletal remains have to be identified. Traditional Sanger sequencing using capillary electrophoresis is time-consuming and cost-prohibitive, and NGS technology provides many advantages. To predict eye and hair color, phenotypic SNPs can be analysed with NGS technology and AIMs to provide BGA information.

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## 8.5 Sample Storage

Storage of samples is of great importance at laboratories engaged in forensic genetics because only high-quality storage makes it possible to successfully recover DNA from a small amount of badly degraded DNA. The quality and quantity of DNA in specimens of aged skeletal remains is influenced by post-excavation treatment, and

samples that have been stored for years in collections at museums, generally at room temperature, exhibit a poorer amplification success rate in comparison to samples that have been freshly excavated (Pruvost et al. 2007). According to Malmstrom (2007), freezing skeletal remains is a preferred method to ensure optimum preservation of DNA. The main reason for freezing samples is to keep DNA degradation to a minimum, preventing the loss of cell integrity and maintaining the quantity and quality of genomic DNA (Lee et al. 2010). Previous studies indicate that there is a reduction in DNA yield with refrigerated liquid DNA (Lee et al. 2010), with temperature changes during freezing and thawing of the samples (Lee et al. 2010; McElderry et al. 2011), and in samples kept in microcentrifuge tubes (Gaillard and Strauss 2000). In addition, spontaneous decay has been reported with progressive molecular damage to DNA (Lindahl 1993). When identification of missing persons based on skeletal remains has been completed, the bone samples typically undergo long-term storage at  $-20^{\circ}\text{C}$  for possible future retesting (Ballou and Stolorow 2013).

Bone samples from World War II can yield low-quantity and low-quality DNA, and it is necessary to perform duplicate analyses of various genetic markers to identify World War II victims. No DNA extract generally remains after analyses, and efficient bone storage is required to guarantee sample stability over time for possible retesting making use of new markers and technologies. Following molecular genetic analysis of World War II victims from Slovenia, small femur fragments are routinely kept at  $-20^{\circ}\text{C}$ . Some authors have observed lower DNA recovery from frozen liquid DNA extracts (Anchordoquyn and Molina 2007; Lee et al. 2010; Hubel et al. 2014), and a study was performed on World War II femurs frozen for 10 years to investigate how freezing bone samples impacts DNA preservation (Friš et al. 2019). To achieve this goal, the quantity of DNA acquired from 57 femurs of World War II victims in a 2009 study (data in Zupanič Pajnič et al. 2010) was compared with the DNA quantities acquired from the identical bones after 10 years in a freezer using the same extraction method and quantification kit. The sample of bone used in this study was adjacent to the one that was used in 2009. The bones were all stored in their original form for 10 years at a temperature of  $-20^{\circ}\text{C}$ . Up to 100 ng DNA/g of powdered bone was acquired from femurs in 2009 and up to 31 ng DNA/g of powdered bone from the same femurs studied after lengthy storage. Statistical analysis demonstrated a marked difference in DNA yield from extracts acquired from World War II bones in 2009 and from extracts acquired from the same bones kept at  $-20^{\circ}\text{C}$  after 10 years, with a greater amount of DNA extracted from bones in 2009 compared to 2019. As described for frozen liquid DNA extracts (Lee et al. 2010; Pokines 2016), reduced DNA recovery was also confirmed for frozen samples of bone, with a marked decrease in yield of DNA after being frozen for 10 years (Friš et al. 2019). The findings showed a reduced DNA recovery for frozen samples of bone.

The second study on storing World War II bone samples focused on comparing the yield of DNA obtained from fragments of bone and powdered bone stored in a freezer (Grđina et al. 2019). Generally not all of the bone powder prepared with a grinder is used for extraction, and because the powdering procedure is

time-consuming it was asked whether storing powdered bone would be useful for future analysis. Hummel (2003) suggests that DNA extraction should take place as soon as possible after homogenizing of bone samples, and long-term bone powder storage could lead to degradation of DNA (even at a low temperature). Researchers concur that storing samples of bone is crucial because preservation of DNA primarily depends on the environmental conditions (Lindahl 1993). Hummel (2003) states that powdering the bones increases the surface area that is exposed and as a result oxidative damage to DNA. Considering this, the aim of the study was to investigate DNA yield in World War II fragments of bone and compare this to the DNA yield from powdered bone stored under identical conditions for 10 years at a temperature of  $-20^{\circ}\text{C}$ . The samples that were analysed had remained following molecular genetic identification carried out in 2009 for 88 Slovenian victims buried in the World War II Konfin Shaft 1 Mass Grave (Zupanič Pajnič et al. 2010). The amount of DNA acquired from 57 femur fragments was compared to the amount of DNA acquired from powdered bone from the same femurs that had been frozen for the same length of time. DNA was extracted from a bone fragment using a piece sampled next to the one used in 2009. This involved working with old DNA, and so every precaution was considered to avoid possible contamination. Statistical analyses showed a difference significant at the 0.05 level in the DNA yield when comparing bones fragments and powdered bone stored at  $-20^{\circ}\text{C}$  for 10 years, and the findings showed there is a greater amount of DNA in powdered bone than in fragments of bone. Because the powdering procedure is time-consuming, it is recommended that, in addition to storing the bone fragment, the powdered bone also be stored for future analyses (Grđina et al. 2019). The results show that lengthy storage of bone powder does not necessarily correspond to a lower quantity of DNA that is extracted from frozen powdered bone when compared to a bone fragment that is frozen, and it is suggested that powdered bone left over from the initial extraction of DNA should be stored long term for investigations in the future along with fragments of bone.

Efforts in forensic research with DNA have focused on the development of innovative methods for long-term storage of samples because today's standard is suboptimal (Lee et al. 2010). Multiple factors have to be considered. Humidity is important because dry storage with a solid matrix inhibits molecular-level movement, and chemical reactions are thus less likely (Pokines 2016). However, when moisture is introduced into the sample again or temperature variations occur, it is again possible for chemical reactions to occur (Pokines 2016). The temperature that the sample is maintained at also impacts the preservation of DNA, and changes in the crystallinity values of the bone mineral are visible after a single freezing, and the decrease in DNA yield is greater after each cycle of freezing and thawing (McElderry et al. 2011). DNA is damaged by repeated cycles of freezing and thawing, and it should therefore be defrosted as rarely as possible (Lindahl 1993). Initial contamination, the effects of light, seal efficiency, and intrinsic bone factors, such as its type and size, also have to be considered (Lee et al. 2010). Fulton (2012) states that the best protocol for lengthy storage of aged specimens varies and that it depends on the way the specimens were collected. Samples that were frozen when



collected should be maintained at that temperature. Samples that were collected at room temperature ought to be stored in a cool, dry environment with a stable temperature, but they might not benefit from freezing, especially if several cycles of freezing and thawing are expected (Fulton 2012). More studies are needed to improve the understanding of forensic DNA sample methodologies for long-term storage and how they affect the quantity of DNA.

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## 8.6 Identification of World War II Victims in Slovenia

Even though seven decades have elapsed since the end of World War II, identification of World War II victims remains relevant because many individuals killed at that time are still missing and have not been identified. Discovering remains dating from World War II is a common occurrence in Slovenia. The Slovenian Government Commission on Concealed Mass Graves has recorded more than 600 hidden mass graves from World War II over the last quarter century (Ferenc 2008). These killings that took place in World War II, amounting to almost 100,000 victims, represent one of the largest-scale losses of life in the modern history of Slovenia. Most victims of killings during and after the war remain buried and are still unidentified. The victims did not have court trials and they were not convicted of crimes. There exist no documents for most mass graves in Slovenia that victim identification could be based on. For some of them, a list of the victims can be made based on archives. Even for those, it is difficult to find living relatives because such a long time has elapsed since the massacres during World War II. The author's team has been conducting genetic investigations of victims of World War II from only a few mass graves, where a list of the Slovenian victims was found in archives. Some of the victims have been identified because putative relatives (close and/or distant) were available for identification (Zupanič Pajnič 2008; Zupanič Pajnič et al. 2010, 2018a, b). A 99.9% recommended posterior probability was applied, with the aim of high confidence in correctly identifying the individuals (Brenner and Weir 2003; Biesecker et al. 2005; Prinz et al. 2007). By combining various genetic markers and involving both close and distant relatives, a sufficiently high statistical probability of positively identifying the victims from mass graves was achieved, even if it was possible to trace only distant relatives of the victims. This proved that by combining many genetic markers it is possible to identify victims of World War II despite an absence of close relatives. Relatives of identified victims have attained certainty about the disappearance of their loved ones after over seven decades, and they have buried the identified skeletal remains in family graves. Genetic identifications have helped reveal information about historical events in Slovenia immediately after World War II. Recent phylogenetic analysis of Polish World War II death camp victims and the establishment of their Jewish origin shows the great importance of forensic genetics for aiding historical research even when it is not possible to identify victims by name (Diepenbroek et al. 2018).

For traceability of possible contamination, for each mass grave an elimination database was created. The database included genetic profiles for the nuclear DNA

(autosomal and Y-chromosomal STRs) and mtDNA of every person that had had contact with the skeletal remains. When identifying victims of the World War II mass graves, the chance of contamination occurring during genetic investigation was minimized. The authenticity of bones' genetic profiles was checked through clean isolation and amplification-negative controls used for nuclear DNA, identical genetic profiles acquired using different PCR amplification kits, and mismatch of genetic profiles for bones with people included in the elimination database.

### 8.6.1 Identification of the Konfin Shaft 1 Mass Grave Victims

In 2008/2009, one-third of the remains of 88 Slovenian victims from the Konfin Shaft 1 Mass Grave (*Grobišče Brezno pri Konfinu 1*) were identified (Zupanič Pajnič et al. 2010). Victims were taken from prison on the night of 24 June 1945 and transported to the place of execution. The victims included 40 men that were wounded (patients that had been taken from the hospital in Ljubljana and moved to the central prison of the OZNA (secret police) 2 weeks before their execution), and 48 that had been selected from the prisoners. These men did not have court trials and they were not convicted of any crimes. Their corpses were dumped into a 45-m-deep cave and the opening was dynamited. The remains were not covered with soil, which would have held the skeletons in their original positions. Precipitation runoff flowed unimpeded into the cave, and its bottom, measuring 20 m<sup>2</sup>, was covered completely with a layer of bone sand mud 2 m thick. Living relatives were traced for 36 of the victims. Eighty-four right femurs were analysed and the genetic profiles were compared to genetic material from living relatives. Y-chromosome haplotypes and autosomal genetic profiles were acquired from 98% of the skeletal remains, and mtDNA haplotypes from 95% of the remains for the HVI region and from 97% of the remains for the HVII region. The genetic profiles for nuclear DNA and mtDNA were established for the reference persons. When comparing genetic profiles, 28 out of the 84 bones analysed were matched with relatives still living (siblings, children, nephews, or cousins) and the statistical analysis showed a high degree of confidence for correct identification of all of the 28 victims (PP ranged from 99.9% to more than 99.999999%). Nuclear DNA was obtained from skeletal remains over 70 years old, and STR typing was successful. This shows that combining a large number of genetic markers offers very high likelihood ratios, supporting the hypothesis that the bones of individuals are related to the family references instead of unrelated individuals. If the analysis had involved only autosomal STR loci, only 12 victims could have been identified with high confidence that the identification was correct. It is therefore also deemed necessary to include Y-STRs and mtDNA analyses, and also to include both close and distant relatives on both the maternal and paternal lines when identifying World War II victims. Identification of the remains in the Konfin Shaft 1 Mass Grave established a basis for future molecular genetic investigations of Slovenian post-war mass graves when the opportunity arose. It should be stressed that mass graves are rarely associated with lists of their victims. The Konfin

Shaft 1 Mass Grave is such a rare case because it was possible to compile a list of its victims based on archival documents. The methods for extracting and amplifying DNA that were used for identifying the Konfin Shaft 1 Mass Grave victims proved very efficient because full genetic profiles were generated for autosomal DNA, mtDNA haplotypes, and Y-STR haplotypes (Zupanič Pajnič et al. 2010).

### 8.6.2 Identification of the Storžič Victims

In 1944, below Mount Storžič, at an elevation of 1200 m, five men were taken to a killing site. One of them fell into a shaft during an attempt to escape, and the other four were killed and their bodies were buried in a hidden mass grave. This grave was found in 2007 and four skeletons were excavated. One victim was identified based on an anthropological study, and the other three were identified by genetic methods. Identification was performed by analysing excavated femurs and teeth and comparing the genetic profiles obtained with the living relatives' genetic material (Zupanič Pajnič 2008). Autosomal genetic profiles, Y-chromosome haplotypes, and mtDNA haplotypes, were acquired from all of the tooth and bone and from reference persons. When comparing the genetic profiles, all the teeth and bones matched with living relatives (for the first victim the reference person was a daughter, for the second victim the reference person was a niece on the maternal line, and for the third victim the reference persons were a son and a niece on the maternal line). The statistical analysis exhibited a high confidence that identification was correct with posterior probabilities (PP) that ranged from 99.9999% to 99.999999%, speaking in favor of the positive identification of the victims.

### 8.6.3 Identifying a Cranium from Bohinj

During World War II, a young woman was executed in Bohinj and she left behind a 1-year-old daughter. Four decades ago, a skeleton was excavated from an individual grave in the Bohinj area and only the cranium from the grave was kept at the Little War Museum (*Mali vojni muzej*). The burial site conformed to accounts from witnesses, but initial anthropological and morphological screening was unable to identify the sex of the remains. In 2015, genetic analysis was performed on the cranium to determine whether it was from the mother of the presumed daughter serving as a family reference. The genetic analysis was conducted alongside a more careful anthropological analysis of the cranium. The genetic examination was carried out on two molars and a petrous portion of the temporal bone, and the results acquired from the teeth corroborated the results from the temporal bone. Nuclear DNA for STR typing was obtained from the temporal bone and from the left second molar. A complete autosomal genetic profile, which included amelogenin locus, indicated that the cranium was from a male, and analyses of Y-STRs provided further confirmation of this. The same conclusions were reached following an anthropological analysis, which indicated that the cranium had come from a very

young Caucasoid male. The male sex of the cranium excluded the possibility that it had belonged to the mother of the female reference used for comparison (Zupanič Pajnič et al. 2016b).

#### 8.6.4 Identification of the Mače Victims

In 2015, genetic identification of a Slovenian banker and industrialist was performed using autosomal and Y-STR markers (Zupanič Pajnič et al. 2018a). He was killed along with his wife (she was the first Yugoslav female pilot) in January of 1944 close to his residence (Strmol Castle). The couple came from well-known and wealthy Slovenian families, who were part of the pre-war elite in Slovenia. The concealed grave with the skeletal remains of the husband and wife was discovered in 2015 in Mače, and only the incomplete remains of a male skeleton and a female skeleton were recovered. A piece of the skull, ribs, and vertebrae were absent from the male's skeleton, and of the female's skeleton the only parts found were the skull lacking the lower jaw and a foot. It is presumed that animals carried the bones away from the shallow grave. Because of the lengthy timespan since the World War II massacres, finding living relatives for identifying victims in such mass graves is difficult. Living relatives could be found only for the husband (two nephews and a niece on the paternal side), and, because his wife had no children, it was not possible to genetically identify her. From the poorly preserved and incomplete remains of the skeletons, genetic typing was performed on the female skeleton's right third molar (skeleton B) and on the male skeleton's left second molar, right tibia, and right femur (skeleton A). The sexes of the two skeletons were confirmed using amelogenin and Y-STR typing. Skeleton A's left second molar, right tibia, and right femur yielded identical profiles. Full autosomal and Y-STR profiles allowed identification of the male skeleton through a comparison to family references. Y-STRs confirmed the relationships between selected males (an uncle and nephews). The prior probability was established on the basis of the number of victims that were reported, and the study used a recommended PP (for kinship) of 99.9% with the aim of high confidence for correctly identifying the mass grave victim. The product rule was applied in order to estimate a combined LR for autosomal and Y-STRs, and the results of statistical analysis indicated a high confidence for correct identification with a PP of 99.997%. After over seven decades, the couple's skeletal remains were returned to their living relatives, and the relatives buried them in a family grave (Zupanič Pajnič et al. 2018a).

#### 8.6.5 Identification of the Mačkovec Victims

In 1943, according to historical data, 43 prisoners were executed at Mačkovec Hill; two others were able to escape the killings. Bones lying in the forest next to the road were buried in subsequent years; although they could have had been exposed to wild animals prior to their burial. The number of excavated skeletons thus differed from

the number of victims presented in historical documents. Excavations in recent years have proven the existence of two hidden mass graves at the location in question. The first one is designated “Mačkovec, large grave,” where the remains of 14 different individuals buried in a shallow grave were found, and the second one “Mačkovec, small grave,” where two individuals’ remains were found. The human remains were originally covered only with fallen leaves and branches. Consequently, the skeletal remains discovered were poorly preserved, being exposed to harmful environmental factors. In 2013, the author’s team was asked to perform genetic identification of victims whose skeletal remains had been excavated from the Mačkovec Mass Grave. Anthropological study determined how many victims there were based on the number of ulnas. However, all the tibias and femurs were included in the genetic analyses because genetic profiling from these delivers far greater success than those from ulnas. All the tibias and femurs were analysed to obtain as many unique genetic profiles as possible. Moreover, the poorly preserved nature of remains dating back to World War II presents an obstacle in STR typing, resulting in allele dropouts and partial profiles, and therefore more skeletal elements should be genotyped to determine a genetic profile. DNA analysis was performed on nine right femurs, eight left femurs, ten right tibias, 11 left tibias, and one tibia with undetermined laterality, or a total of 39 bones. Buccal swab samples were collected for 13 family references for ten 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). An anthropological report for the larger of the two graves described eight right and seven left femurs, and genetic profile matches were found for five pairs of femurs. Furthermore, out of ten right and ten left tibias and one tibia without determined laterality found in the larger grave, as described by anthropology analysis, it was possible to match nine pairs according to genetic profiles. Left and right femurs, as determined by an anthropologist, and a tibia, all of which were found in the smaller grave, had matching genetic profiles and thus belong to the same victim. Four different genetic profile matches were ascertained, among which two cases were a match between a victim and a family reference, and two cases a match between two respective victims, the latter highlighting the fact that some of the victims were related. The product rule was applied in order to estimate a combined LR for autosomal and Y-STRs, and a high confidence of correct identification was demonstrated through statistical analyses with PP greater than 99.9% for all of the victims identified (data not published yet).

### **8.6.6 Identification of the Babna Gora Victims**

In 2016, the victims of the single largest massacre of a family in Slovenia were genetically identified (Zupanič Pajnič et al. 2018b, 2020). Ten people from the same family were killed in World War II: nine of them in 1942 and one immediately after the war. During the massacre in 1942, seven members were killed in the forest and

their bodies were hidden in a mass grave. Soon after these seven people were killed, the mother and grandmother of the children that had survived were murdered at their home, and the two of them were buried in the local cemetery. Two children, a 4-year-old girl and her 3-year-old brother, were the only ones who survived the massacre. Besides these two children, an uncle also survived the killings in 1942, but he was murdered immediately after World War II. A total of ten people from the same family were killed during World War II. Exhumation of the remains started in March 2015, but archeologists exhumed only three female skeletons, which were incomplete. Twenty meters from this site, relatives later found bones, and the burial location of at least three males was excavated in August 2016. The grave excavated in 2015 was labeled a female grave, and the one excavated in 2016 was labeled a male grave. The family members were buried in shallow graves in the woods, and the skeletons that were exhumed were incomplete at both sites. Aside from teeth in one mandible, no teeth were found. The reason why the skeletons were incomplete could be because the graves were very shallow, making it very probably that wild animals scattered the victims' remains in the woods. The remains underwent taphonomic processes after deposition, which resulted in a poor state of preservation. The bones that were exhumed were abraded, otherwise damaged, and porous, without features that would permit anthropologists to macroscopically determine their laterality. The graves were positioned along a creek in the woods, and so water exposure caused diagenetic and degradation processes. For the first skeleton recovered from the female grave, the only parts found were a piece of the skull without teeth, part of a femur, and part of a tibia. For the second, the pieces recovered were part of the skull, a segment of spine, a humerus, and the right leg bones. The skull preserved three mandibular teeth: the left and right second molars and also the left first premolar. For the third skeleton in the female grave, only part of the skull lacking teeth, a piece of the spine, and a tibia were discovered. In the male grave, only individual bones (including the diaphysis from six femurs) were recovered by archeologists, and the number of individuals buried there was at least three. Following the removal of the skeletal remains from the two graves, the author's team was requested to carry out genetic identification by comparing findings with two living relatives (the aforementioned daughter and son). Altogether, analysis was made of 12 bones and teeth, and these were compared with the two living relatives. Seven bones and one molar yielded nuclear DNA for successful STR typing. The female grave yielded autosomal profiles from only one of the skeletons, and autosomal profiles were obtained from five of the six femurs in the male grave. The relationships among the males were further confirmed through analyses of Y-STRs and STR profiles made it possible to identify four members of the family; an aunt from the female grave, and from the male grave two uncles plus the father of the living children, who served as family references. The product rule was applied to calculate a combined likelihood ratio for autosomal and Y-STRs, and high confidence of having made a correct identification was demonstrated through statistical analyses, with PP greater than 99.9% for three of the four victims identified. For identification of the aunt, the PP determined following capillary electrophoresis STR typing was not high enough. To achieve a better PP, we used the next-generation

sequencing Precision ID GlobalFiler NGS STR Panel (TFS) and, following an analysis of additional autosomal STR loci, our statistical analysis indicated a PP exceeding 99.9%, showing that a sufficient number of genetic markers had been studied to identify the aunt's skeletal remains (Zupanič Pajnič et al. 2019). After over seven decades, the remains of the victims were returned to the two children that had survived, and they laid their relatives to rest in the family grave (Zupanič Pajnič et al. 2020).

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