Slovenian Medical Journal

Molecular genetic aspects of ancient DNA analyses

Molekularnogenetski vidiki preiskav starodavne DNA

Irena Zupanič Pajnič

Institute of Forensic Medicine, Faculty of medicine, University of Ljubljana, Ljubljana, Slovenia

Correspondence/ Korespondenca:
Irena Zupanič Pajnič, e: irena.zupanic.pajnic@gmail.com

Key words:
archaeogenetics; human skeletal remains; damaged DNA; contamination

Ključne besede:
arheogenetika; človeški skeletni ostanki; poškodovana DNA; kontaminacija

Received: 29. 1. 2019
Accepted: 14. 8. 2019

Abstract

This review article presents molecular genetic aspects of human ancient DNA analyses and use of molecular genetic methods for study of DNA obtained from human archaeological biological materials. In archaeological biological materials, skeletal remains (bones and teeth) are often the only source of ancient DNA and we will focus on these tissues. From the literature reviewed, we will summarise which skeletal elements are most suitable for the investigation of ancient DNA and how to extract the DNA from them. The nature and preservation of ancient DNA will be described as well. However, low amount and degradation of ancient DNA causes several problems, especially when working with ancient human samples that may be contaminated with modern human DNA. To minimise the risk of contamination, several standard precautions are usually adopted and the authenticity of ancient DNA checked. We will pay special attention to these measures. The genetic markers most frequently examined in archaeogenetics and the advantages of new, high-performing sequencing techniques for the development and study of ancient DNA will be described. Using new techniques that may help us retrieve data of better quality and quantity, we can investigate more degraded DNA and thus older archaeological biological materials, thereby obtaining huge amounts of data that require the involvement of experts in the field of bioinformatics. The paper will be completed by the presentation of ancient DNA analyses performed in Slovenia.

Izvleček

Prispevek na pregleden način opisuje molekularnogenetske vidike preiskav človeške starodavne DNA in uporabo molekularnogenetskih metod za preučevanje DNA, pridobljene iz človeških arheoloških bioloških materialov. V arheoloških bioloških materialih so pogosto edini vir starodavne DNA skeletni ostanki (kosti in zobje), zato se bomo osredinili na ta tkiva. Iz pregledane literature bomo povzeli, kateri skeletni elementi so najprimernejši za preiskave starodavne DNA in kako iz njih pridobimo DNA. Prav tako bomo razložili naravo starodavne DNA in njeno ohranjenost. V arheoloških bioloških materialih je zelo malo DNA in je močno poškodovana. Zato se zlasti pri delu s starodavnimi človeškimi vzorci pojavijo težave, povezane s kontaminiranjem s sodobno DNA človeka. Za zmanjšanje tega tveganja je potrebno upoštevati več standardnih previdnostnih ukrepov in preverjati avtentičnost starodavne DNA. V prispevku bomo tem ukrepom namenili posebno pozornost. Opisali bomo genetske označevalce, ki jih v arheogenetiki najpogosteje preiskujemo, in prednosti novih, visoko zmogljivih tehnik sekvenciranja za razvoj in preučevanje starodavne DNA. Danes lahko z njimi preiskujemo bolj razgrajeno DNA in s tem starejše arheološke biološke materiale. Tako pridobivamo ogromne količine kakovostnih podatkov, ki zahtevajo vključevanje strokovnjakov na področju bioinformatike. Prispevek bomo zaključili s predstavitvijo preiskav starodavne DNA v Sloveniji.

Cite as/Citirajte kot: Zupanič Pajnič I. Molecular genetic aspects of ancient DNA analyses. Zdrav Vestn. 2020;89(3–4):171–89.

DOI: https://doi.org/10.6016/ZdravVestn.2923

1 Introduction

When genetic tests use DNA isolated from poorly preserved, sometimes centuries-old or millennia-old remains of organisms, we are talking about ancient DNA (aDNA). The time limit between ancient - archaeological and relatively modern - forensic human remains varies between countries. Most countries label as archeological anything that is older than 50 to 100 years (1). The definition also depends on other circumstances, such as the possibility of identifying persons and thus returning the remains to the family. If the person responsible for the death is still alive and criminal proceedings can be initiated against him or her, the remains are also considered forensic. According to Bouwman (2), ancient DNA is the DNA that is older than 70 years after the death of a person or living being. The beginnings of ancient DNA investigations date back to 1984, and the field experienced rapid development a few years later with the discovery of the polymerase chain reaction (PCR), which made it possible to study very small amounts and target-specific sections of DNA in archaeological biological material, and has been further strengthened by the development of newer, high-performance Next Generation Sequencing (NGS) techniques (3). Archaeogenetics is an interdisciplinary science in which the synthesis and understanding of all collected data (both archaeological and genetic) requires the cooperation of experts in various fields. By investigating ancient DNA, we answer archaeological questions, so in the absence of cooperation between different experts, genetic data on ancient DNA cannot be interpreted meaningfully. With the participation of archaeologists, paleozoologists, geneticists, bioinformatics scientists and others, we can use archaeogenetics to answer some social and cultural questions of human history and gain insight into the mechanisms of evolution of organisms. Thus, DNA obtained from archaeological remains offers us many opportunities to study ancient populations, their migrations, diseases, genetic changes due to diet (e.g., lactose intolerance) and specific genetic adaptations to the environment and certain diseases (e.g., malaria) (2,4). For example, studies of genetic diversity in modern populations point to the African ancestors of all modern humans (5). However, ancient DNA is highly degraded, so problems associated with contamination with modern DNA of people involved in investigations or who have been in contact with skeletal remains arise, especially when working with ancient human samples (6). In order to reduce the risk of erroneous results due to the possibility of contamination with modern DNA, several standard precautions should be taken in the field of ancient DNA investigations to prevent contamination and verify the authenticity of ancient DNA (7). A part of the articles cited in the literature section were reviewed by Marcel Obal (8) in his master's thesis and are summarized in this review article.

2 Influences of Various Factors on the Preservation of Ancient DNA

Archaeological skeletal remains contain very little DNA, which is severely degraded. The success of genetic testing is further limited by PCR inhibitors, which

are often present in old skeletons (9,10). The failure of investigations may also be influenced by high exposure to modern DNA contamination (11). The harmful effects of various environmental factors strongly affect the preservation of old skeletal remains, making it difficult to obtain undamaged and uncontaminated DNA from them. After the death of the organism, the cellular balance is disturbed. DNA damage and fragmentation occur, which makes the ratio between the number of fragments of a particular length and their length inversely proportional; the longest DNA fragments are preserved in the least amount (3,12). Examination of DNA isolated from remains of different ages found at different locations proves that DNA preservation is not only affected by the time elapsed since the death of the organism, but above all by the environment in which the organism was located after death. Geological and chemical properties of the soil and the presence of salt, exposure to radiation, pH, access of oxygen and moisture, the presence of microorganisms and temperature are the main environmental factors that affect preservation (13-15). In addition to fragmentation, these factors can also lead to cross-linking of chains as well as changes and deletions in the nucleotide sequence (13). The most suitable conditions for good DNA preservation are low exposure to ultraviolet (UV) radiation, rapid drying of mortal remains, low humidity, high salt concentration, slightly basic or neutral pH, low humic acid content, absence of microorganisms and especially low temperature, which is crucial (12). Samples of similar age stored at low temperatures are better preserved than those exposed to higher temperatures. The quantity and quality of DNA in ancient samples is also influenced by the handling of skeletons after excavation (16). Samples stored for longer periods in museum collections, usually at room temperature, give poorer typing results than freshly excavated samples (16). However, DNA preservation is also influenced by individual-specific factors such as race, gender, age and type of skeletal elements (17).

3 Composition and Course of Bone and Tooth Decomposition

The first isolations of ancient DNA were made from soft tissue remains, as researchers assumed that they contained the most DNA, similar to living beings. Later, they tried to isolate ancient DNA from bones as well. Successful isolation in 1989 showed that skeletal remains contained more DNA than preserved soft tissues (18). Due to their structure, bones and teeth are preserved the longest and best, so they are usually the best and often the only source of DNA. At the macrostructural level, soft tissues are physically more accessible to microorganisms and other environmental factors, which makes their decomposition faster, while bones are physically more resistant and therefore better preserved (13,19).

Understanding the composition and process of bone and tooth decomposition helps us choose suitable samples to obtain and study DNA in old skeletal remains, so let's take a look at bone and tooth structure! The anatomical parts of the tooth are the crown, the neck and the root. The outer layer of the crown is covered with enamel, which is the hardest tissue in the human body and is almost entirely of mineral origin and contains no cells.

The root of the tooth is covered with cementum, which is a mineralized tissue composed of hydroxyapatite, collagen, and other noncollagenous proteins (15). Cell-free cementum is located in the cervical part of the tooth root. The cementum, which contains cells trapped in the intercellular space, is located in the apical part of the tooth root and is a good source of DNA (20). At the border between the crown and the root - in the area called the tooth neck - enamel and cementum meet, and below them is the dentin that protects the dental pulp. The dentin and pulp form

the bulk of the tooth and, in contrast to the enamel, are rich in cells; they are mostly odontoblasts and fibroblasts. The dentin consists of hydroxyapatite, type I collagen, and water (15). Dental pulp is well vascularized and innervated and represents a rich source of DNA. Teeth with larger pulp and teeth with more roots are the best source of DNA as they contain more pulp cells and also have more dental cementum compared to teeth with a single root. According to the recommendations, the most suitable teeth are molars, if there are none, premolars are recommended for use (15). Macroscopically, the bone tissue is divided into compact and spongy bone. The compact bone tissue makes up the outer part of the ends of the long bones (epiphysis) and flat bones as well as the middle part of the long bones (diaphysis). Spongiosis is found inside the flat bones and at the ends of the long bones. Due to the fragility of the spongy bone, it needs an outer protective layer of compact bone (13). Microscopically bone is made up of cells and intercellular space. The intercellular space consists of an organic and an inorganic part. The organic part is mostly type I collagen and some other proteins and glycoproteins, while the inorganic part is mostly hydroxyapatite, composed of calcium and phosphate ions, and bicarbonate, magnesium, potassium, and sodium ions are also present (13). Calcium and phosphate ions form hydroxyapatite crystals, which make up the plates lying along the collagen fibres. The relation between collagen fibres and crystals ensures bone strength and resilience (13). Taphonomic processes that affect the preservation of DNA in skeletal tissues have already been described in the previous chapter (the time elapsed since the death of the organism, environmental factors and individually specific factors). How quickly and in what way environmental factors will affect changes in bones and teeth after death, however, is greatly influenced by porosity. The size of the pores in bone tissue and their interrelationship determine how water, microorganisms, and other particles will pass into and out of it (19). The collapse of bone cells and soft tissues in blood vessels makes the bone more porous. Due to the increase in porosity over time, fungi and bacteria present in the soil and cyanobacteria present in the water can more easily penetrate the tissue and make it even more porous, thus reducing its chances of survival (13). Conversely, there may also be a reduction in porosity due to permineralization, which gradually leads to fossilization, resulting in better bone preservation (19). The mechanisms of the degradation process and the location of DNA retention in bone tissue have not yet been fully investigated. Kemp and Smith are proponents of the hypothesis that DNA stability and its preservation in ancient skeletal remains are enabled by the DNA binding to hydroxyapatite, which represents the major bone mass (21). At the ultrastructural level, the slow degradation of the DNA molecule and protection against enzymatic processes in skeletal residues are thought to be influenced by the binding of its negatively charged phosphate groups to hydroxyl groups of hydroxyapatite, as confirmed by the fact that less DNA is retained with increased degradation of hydroxyapatite and the DNA molecule is thought to bind to collagen - DNA extraction from bone powder is therefore possible from the hydroxyapatite and collagen fraction (12,13,22).

Collagen and hydroxyapatite together form a tight structure that prevents the collagenases to be invaded by microorganisms due to small pores (23).

The preservation and amount of DNA do not always coincide with the macroscopic condition of the bone, as it may appear morphologically in poor condition, but in some cases, it is still possible to extract a sufficient amount of well-preserved DNA from it. In contrast, the condition at the microscopic level tells us much more about the preservation and amount of DNA. Investigation of the structure of medieval human bones by

transmission electron microscopy and immunohistochemical methods as well as the DNA content and its preservation in these bones showed a connection between the structure and the DNA content and its preservation. Well-preserved DNA is found in crystal aggregates, it's associated with a very well-preserved microstructure with little demineralization, preserved lamellae, high collagen content and a more compact appearance in transmission electron microscopy where intact osteons and a well-organized bone matrix with collagen and osteocalcin content are visible (22).

In teeth, the pulp is well vascularized and innervated and represents a rich source of DNA, but may be present in limited quantities or even absent in old teeth, while the root of the tooth contains about ten times more DNA than the crown, as the latter is mostly composed of cell-free tooth enamel. If enamel is used in sampling in combination with other dental tissues, the presence of minerals can interfere with extraction and inhibit DNA amplification in the PCR (15). After the death of an individual, teeth are subject to largely the same factors as bones, but they are more resistant to these factors and therefore a more appropriate choice for genetic testing, as they are better protected due to both tissue structure and jaw position. All of this provides additional defence against environmental influences and physical damage that could accelerate tissue breakdown. Investigations of forensic skeletal remains have shown that the most favourable medium in which teeth can be located from the death of an individual to the finding is air. Slightly worse results are given by teeth that were buried in the ground, and the worst by teeth that were in water (15). Dental tissues decompose slowly even in extreme conditions, as they are resistant to extreme temperatures and less susceptible to hydrolysis (12). At the same time, similar to bones, teeth are also protected by collagen. Mineralized collagen is much more resistant than the non-mineralized and can therefore be preserved in bones, in the absence of enzymatic degradation, for hundreds or even thousands of years (23). Water, pH, and porosity of mineralized tissue are factors influencing the solubility of hydroxyapatite; water allows the dissolution of mineral ions, and solubility increases with the lowering of pH. Alternately dry and humid environments and environments with constant water flow are more harmful than constantly humid environments (15,19,23). It is also necessary to consider whether the tooth fell out after the death of the individual or not, as the alveolar bone helps reduce the possibility of contamination (15).

4 Use of Various Skeletal Elements for Investigations of Ancient DNA

It is not always possible to sample a large number of skeletal elements for genetic investigations of skeletons from archaeological sites. Skeletons are often incomplete and are at the same time an important part of cultural heritage. Genetic tests are destructive, as a piece of bone or tooth is ground into powder, and thus present a severe encroachment on cultural heritage. Due to its destructive nature and interference with a non-renewable source of cultural heritage, genetic testing must be thoroughly justified by its contribution to both, the field of genetic testing and the field of cultural heritage. For ancient DNA investigations, we need to select the most suitable skeletal element and some alternatives that would also allow for a successful investigation.

According to current recommendations for sampling forensic skeletons, compact bones, especially long leg bones (femur and tibia) and teeth, are the most suitable for isolating and analysing DNA from skeletons, while flat and spongy bones, such as skull, vertebrae and ribs, are less suitable, with areas of compact bone being the most suitable for sam-

pling (24-27). Mundorff et al. (17,28) in recent studies of forensic skeletal remains demonstrate that long, compact bones may not be the (only) best choice for DNA analysis. They find that the results of analysis of the patella, foot bones and phalanges are comparable or even better than the analysis of femur and tibia. They find that the amount of nuclear DNA per unit mass of bone is much higher in small spongy bones, which have not been typically sampled and used for genetic testing so far, compared to compact bones (17). Confirmation of this is also some other forensic research, which speaks of very successful DNA analyses obtained from such bones, and even recommends the use of phalanges as a replacement for the femur for easier sampling and good results (29,30). Small spongy bones are easier to sample, as we do not need a saw to get samples, which reduces the possibility of contamination with modern DNA, and at the same time sampling these bones is faster and more efficient. The reasons for the greater amount and better preserved DNA in this type of bone are not yet known. Andronowski et al. (29) found, through a study of bone microstructure, that despite the large number of osteocyte lacunae in compact bone, there was no connection with the amount of DNA per unit mass of the sample. However, with the help of synchrotron radiation microcomputed tomography, they observed the remains of invisible soft tissues between the trabeculae of the spongy bone and hypothesized that the soft tissue remains contained bone marrow, periosteum, and endosteum cells, which was thought to have an effect on the greater amount of DNA in the spongy bone (29). Also, contrary to established recommendations for sampling forensic skeletons, recent research suggests that for genetic testing of skeletalized remains, it is best to sample dental cementum in the root area (20) rather than the entire tooth.

In investigations of archaeological skeletons, a great deal of research focuses on comparing different skeletal elements with the middle part of the temporal bone - the petrous part, which is one of the hardest bones in the human body (31,32). Pinhasi et al. (33) found that petrous part of the temporal bone is the most suitable skeletal element for sampling archaeological skeletal remains (33). Gamba et al. (34) concluded by comparing the amount of DNA in the petrous part of the temporal bone and teeth that the amount of endogenous DNA in the petrous part of the temporal bone was greater than that in the teeth, but in the latter, they did not sample only the dental cementum (34). Meanwhile, Hansen et al. (32), by examining both elements (dental cementum and petrous part of the temporal bone) obtained from the same skeleton, which differ in age and the environment in which they were after death, find that well-preserved dental cementum may contain the same or even a larger amount of DNA than the petrous part of the temporal bone, but in case of poor preservation of teeth (brittle teeth, without cementum), they recommend the use of the petrous part of the temporal bone. According to their research, the latter is more resistant or better protected in poor preservation conditions compared to dental cementum, so DNA is preserved in it longer (32). Similar findings are made by Pilli et al. (31) who, by comparing the petrous part of the temporal bone, femurs, and teeth of an individual skeleton, a total of thirteen skeletons from the 6th to 7th centuries, demonstrate that DNA is better preserved in the petrous part of the temporal bone compared to femur and teeth. They conclude that this is caused by the high bone density in the petrous part of the temporal bone, which increases resistance and reduces the damage and breakdown of DNA that would be caused by bacteria after the death of the organism (31).

5 Genetic Markers in Ancient DNA Investigations

Only about 5% of the total DNA molecule are exons, i.e., those parts that rep-

resent the sequences that encode proteins. The rest of the DNA molecule are noncoding regions called introns, of which role, although they make up the majority, is not yet fully known (35). In evolutionary processes, introns are exposed to slightly different processes than exons; mutations can occur in both coding and noncoding regions of DNA, but are not necessarily phenotypically expressed in the latter. But they still cause a high degree of polymorphism in them, as mutations are less controlled here than in the coding regions. Thus, for example, we find hypervariable loci on autosomes and sex chromosomes, which we use as markers to differentiate individuals in noncoding regions, as these regions are much more individually specific due to the high degree of polymorphism (35). There are three types of such polymorphisms in the human genome: minisatellites, microsatellites, or short tandem repeats (STRs), and single nucleotide polymorphisms (SNPs) (35). Of these genetic markers, STR loci on autosomes and the Y chromosome and SNP markers are mainly used in archaeogenetics. Microsatellites are regions of DNA that consist of successive repeating homologous units (basic motifs). The high degree of polymorphism of microsatellite loci is due to the different number of repetitions of the basic motifs, which is why we speak of length polymorphisms (35). In SNP markers, there are several possible variants of bases in the genome at specific sites; there is thus a difference between alleles only in one nucleotide. It is the most common form of polymorphism in the genome, occurring in the bi-, - or tetra-allelic form (35). Due to their size (STR loci of 100 bp to 400 bp and SNP markers of up to 150 bp), STR loci and SNP markers are the most suitable for use in investigations of highly degraded ancient DNA, as DNA amplification in the PCR is more successful when shorter sections are multiplied (36). Codominantly inherited autosomal microsatellites are located on any of the 22 pairs of autosomes, i.e., chromosomes that are not responsible for sex determination. Like autosomes and the X chromosome, the Y chromosome also contains microsatellites, but there are fewer of them on the Y chromosome due to its small size (12). The Y chromosome is not involved in recombination, so genetic testing of the Y chromosome microsatellites can follow the paternal line, as all offspring of the same father have an identical Y chromosome haplotype. The section of the DNA molecule inherited from only one parent is called the haplotype.

DNA molecules are not only found in the cell nucleus, but are also found in the mitochondria - the organelles responsible for producing cellular energy. The mtDNA is 16,569 bp long and has a fully defined nucleotide sequence (37). Due to the extraordinary polymorphism, the noncoding control region of mtDNA is of particular interest for investigations of ancient DNA (38).

Human cells contain numerous copies of the mitochondrial genome, which gives, compared to only two copies of the nuclear genome, a much greater chance of extracting mtDNA from old, poorly preserved biological samples (39). Each cell contains approximately 500 mitochondria, and each mitochondria contains 5 to 10 mtDNA molecules (40). In the investigation of ancient DNA, the advantage of using mtDNA over nuclear DNA is also that it is preserved for a longer time, as it is protected from exonuclease degradation by its circular conformation and mitochondrial envelope (40). Due to the listed properties of mtDNA, biological samples of which nuclear DNA typing is not successful can be investigated with mtDNA polymorphisms. MtDNA is inherited from the mother and is transmitted unchanged to all maternal offspring regardless of gender, so we use its nucleotide sequences as markers for maternal pedigrees, which must be taken into account when choosing a reference or comparative sample in identifying historical figures. Individuals with identical mtDNA nucleotide se-

quences have a common female ancestor (39), which forms the basis for the identification of biological patterns by mtDNA typing. The mitochondrial genome is therefore very useful for determining the identity of old remains and studying genealogical relationships, as it can also be used as a reference sample for maternal relatives several generations away (38).

Ancient DNA is usually degraded into 100 to 500 bp long fragments, and the bases are altered due to molecular damage (depurination and deamination) (18,41). Understanding these processes and their effects on ancient DNA is essential for the correct selection of target sequences, which must be short, as this is the only way we can obtain genetic information from ancient DNA and avoid misinterpretation of results. In the past, genetic testing of ancient DNA has been based primarily on mtDNA testing (42). Recently, however, some research groups (including ours) have reported successful typing of nuclear DNA obtained from ancient skeletons (43-46).

Autosomal nuclear DNA typing is most appropriate for identification purposes, as it is individually specific and gives us information about kinship by both parents (47). We investigate the length polymorphisms of the STR regions or SNP polymorphisms, as they are extremely variable, individually specific due to recombination, and have a high power of differentiation that allows reliable identification of the individual (12,43,48). When typing of STR nuclear regions is not possible due to strong DNA degradation, SNP markers can also be used. Recent developments in NGS technology have many advantages in DNA tests derived from skeletal remains compared to previously used capillary electrophoresis technology (49). With NGS technology, highly degraded skeletal remains can also be identified using SNP identification markers (50) of which amplified fragment lengths are below 150 bp.

In identifying historical figures and other individuals where close relatives who would be most appropriate for the autosomal polymorphisms investigation using STR and SNP markers, are no longer available. Instead, we can use distant relatives in the maternal or paternal line, including linear genetic markers of mtDNA and the Y chromosome in the investigation. The Y chromosome and mtDNA allow us to trace the paternal and maternal line (the Y chromosome is inherited in unchanged form from father to sons, and mtDNA is also transmitted in unchanged form to all offspring, regardless of gender). Matching Y chromosome haplotypes indicates a common paternal ancestry and mtDNA on the maternal side.

Uniparental inheritance of mtDNA and Y chromosome does not result in recombination, except in the short, terminal region of the Y chromosome, which recombines with the X chromosome (51). In identifying historical figures, paternal or maternal relatives that are several generations away can be used as reference samples with the Y chromosome or mtDNA (52-54). If distant relatives are not available to identify historical figures and other individuals, DNA phenotyping can be used (55). This is an area that has developed recently and allows us to predict the external visual characteristics of an individual from a DNA molecule, e.g., eye colour and hair colour (56) and biogeographical or ancestral origin (57). This is how we make a facial composition. The analyses of the Y-STR, Y-SNP markers and the mtDNA control regions, as well as the identification of haplogroups through which phylogeographic origin can be determined, have until recently been the main methods for determining ancestral origin, as these markers are geographically highly differentiated (58) and by increasing the phylogenetic resolution of the Y chromosome, researchers have recently demonstrated additional informativeness in Western European populations as well (59,60). However, investigations of markers on the Y chromosome and mtDNA only give us information about the paternal

and maternal lines, as they are not subject to recombination. They show only a partial picture of the origin, especially in cases of complex genealogy of the individual. In contrast, the ancestry informative markers (AIM), which are widely distributed across autosomes, provide a more complete picture of ancestral origin and are used to determine the most likely biogeographical origin or origin of a population, to which the individual belongs. Today, they represent the main markers for researching the ancestral origin of an individual (61). AIMs are genetic polymorphisms, mostly single-nucleotide polymorphisms - SNPs, which show large differences in allele frequencies between different ethnic groups and can be genetically distinguished from each other on this basis (62). The genetic origin is inferred from a comparison of the genetic diversity of the investigated sample with the diversity patterns in modern populations. In addition to detecting the genealogy of an individual, AIMs also play an important role in identifying missing persons and victims of mass disasters (63,64). Compared to STR markers, AIMs are very short and can be used to successfully genotype the samples obtained from highly degraded skeletal remains with low DNA content (65).

6 Prevention of Contamination and Verification of the Authenticity of Ancient DNA

Old skeletal remains contain very little or highly degraded DNA; therefore, archaeological skeletons are highly susceptible to contamination with modern DNA (21). The correct approach to the excavation of skeletal remains, anthropological processing and storage of this kind of biological material is crucial for the success of genetic investigations. Improper procedures in handling skeletal remains and their improper storage can lead to contamination and degradation of endogenous bone and tooth DNA. This results in erroneous results of molecular genetic analyses or failed analysis (41). After the excavation, cleaning, anthropological examination and storage of skeletal remains, three things must be taken into account from the point of view of further genetic investigations.

First! When excavating, the use of protective equipment (especially clean gloves, face masks and protective clothing) is mandatory. Its use is also necessary in anthropological analysis (additional disinfection of the work surface and instruments is required). Second! When cleaning skeletal remains, it is necessary to take into account the fact that washing skeletal remains before storing them reduces the pH and salt concentration in the bones, which negatively affects the preservation of DNA (16). Third! It is very important to store skeletal remains after excavation in suitable conditions. According to Fulton (66), the most suitable way for long-term storage of old skeletal remains depends on the environmental conditions in which the samples were collected at the time of finding or excavation. If the sample was frozen at the time of finding, it is best to maintain this temperature for storage. If the sample was found at room temperature, it should be stored in a cool, dry environment and not frozen, especially if several freeze/thaw cycles are foreseen. In general, avoiding environmental conditions that are known to affect DNA damage is critical to sample maintenance. Ideal is a cool, dry, temperature-stable environment (66). DNA is susceptible to damage due to repetitive freezing and thawing cycles and should therefore be thawed as infrequently as possible (67). Heat, freezing/thawing and moisture must be avoided (66).

Prior to genetic testing, surface contamination due to improper handling (gripping skeletal remains without protective gloves) is most common. Contamination of the deeper layers can also occur, depending on the porosity and preservation of the skeletalized remains (6,68). Surface contamination in the genetic laboratory

is removed by various methods. Among them, the most common are rinsing in water, detergent, acid, ethanol or bleach, UV irradiation, physical removal of the surface in the case of bones and, lastly, obtaining DNA from material taken from the inside of the bone or tooth. To effectively remove surface contamination a different combination of the listed techniques is usually used. Bones in laboratories are most often cleaned mechanically and chemically, while in the case of teeth mechanical cleaning is replaced by UV irradiation. This way, the amount of contaminating DNA present and the amount of inhibitors are reduced (69). The bones are cleaned by physically removing the surface using a grinder and then washed with detergent, water and ethanol. We remove the surface layer of bone to reduce contamination that occurs due to direct handling of the bones prior to genetic testing.

However, contamination of ancient DNA with modern human DNA can also occur during genetic testing, usually due to improper handling of samples without the use of protective equipment (gloves, surgical masks, protective caps, protective coats, protective shoe covers), but contamination may also be due to the presence of exogenous DNA in reagents, on materials, or in the air where DNA binds to aerosols (70). Ancient DNA is highly degraded and very limited in quantity, so the possibility of its contamination with modern DNA is very high (41,70). When working with ancient DNA, it is crucial in the laboratory to prevent and detect contamination with modern DNA and to follow the criteria for verifying the identity of ancient DNA (41,69). There must be an inverse relationship between the amplification efficiency and the length of the multiplied products in ancient DNA, indicating the degradation and damage of ancient DNA (41).

The identity of ancient sequences can also be verified by a pattern of DNA molecule damage and mutations characteristic of ancient DNA (41). In order to avoid laboratory contamination as much as possible, bone and tooth samples should be processed and DNA extracted from them in a separate laboratory, which is exclusively intended for treating old skeletal remains. The premises for processing skeletal samples, DNA extraction and preparation of PCR reactions must be strictly separated from the premises where the so-called "post-PCR" procedures are performed (69). The laboratory must be equipped with chambers with Hepa filters that prevent the entry of contaminated DNA. During the extraction process, protective clothing should be worn, and laboratory surfaces should be bleached and UV irradiated (41). To identify the possible source of contamination, it is important to establish an elimination database of genetic profiles of all persons who have handled samples since excavation and through further analyses in order to confirm the correctness or authenticity of genetic profiles obtained from the studied samples. Whenever possible, the same genetic markers as for ancient DNA should be typed in laboratory workers, museum staff, anthropologists, and archaeologists who have come into contact with skeletal remains. In addition, it is important to analyse the negative control at the same time as each batch of samples, and the negative control should also be included in each PCR to check and identify the possible cause of contamination of any of the previously used reagents and materials (41). DNA must be extracted from the same sample at least twice and an identical DNA sequence must be obtained by typing both extracts (41).

7 Obtaining Ancient DNA From Skeletal Remains

Highly efficient extraction methods are the basis for researching and obtaining any genetic data from ancient biological materials, as sufficient amounts of DNA of sufficient quality need to be obtained for a successful genetic investigation (10,14). As recent studies have shown, complete

demineralization is most appropriate for obtaining DNA from old skeletal remains, as it allows larger amounts of DNA to be obtained from archaeological samples (71-73). When isolating DNA, care must be taken to avoid exposing the samples to high temperatures, strong detergents, or treatment with other aggressive processes, as this prevents further degradation of the ancient DNA (69). For the most successful demineralization, it is crucial to grind the bones and teeth into as fine a powder as possible to allow as large a sample surface as possible to come into contact with the chelation solution (69). Grinding is carried out using a homogenizer and liquid nitrogen; with the latter we cool the samples and metal chambers for grinding and thus prevent overheating. The decalcification process with 0.5 M EDTA allows the separation of bone cells from bone mass (74). EDTA strongly binds calcium and thus allows demineralization (69). For complete demineralization of 1 g of bone or tooth powder 15 ml of 0.5 M EDTA is required (75). Extraction buffer for lysis, proteinase K (endolytic serine protease, which cleaves proteins into individual amino acids), and DTT (reductant, which cleaves the disulfide bond between cysteine residues in the protein) are added to the precipitate obtained after demineralization. After incubation, the extracted DNA lysate is obtained. Many PCR inhibitors are present in old skeletons, so the extracted DNA must be purified. In bone samples excavated from the soil, the most common inhibitors of the PCR are humic and flavinic acid and calcium chloride (76).

PCR inhibitors prevent the binding of polymerase to the DNA strand and should be removed as much as possible for efficient amplification in the PCR. DNA is purified using a Biorobot EZ1 (Qiagen) device, which is based on the binding of DNA molecules to the surface of silicon-coated magnetic particles; binding takes place in the presence of chaotropic salts (77). The device separates the magnetic particles with bound DNA molecules from the rest of the lysate with a magnet, and then the magnetic particles are washed and the DNA eluted with water or buffer (77). This method of purification is very effective with ancient DNA. The presence of PCR inhibitors in the extracted bone DNA can be checked by qPCR, which allows simultaneous quantification of nuclear DNA, male DNA, determination of the degree of DNA degradation and the presence of PCR inhibitors in isolates (45). Given that skeletal remains from archaeological sites are exposed to adverse environmental influences, strong DNA degradation can be expected. In such samples, it is crucial to assess the amount and quality of DNA available for genetic testing. To assess the degree of DNA degradation at the same locus, we multiply two fragments of different lengths. The ratio between the amount of short and long fragment allows us to assess the quality of DNA through the calculation of the degradation index. The qPCR thus identifies bone samples that would be more successfully examined with alternative genotyping markers, e.g., with SNP markers, than with conventional typing of STR loci (45).

For successful investigations of ancient DNA, in addition to advanced DNA extraction and purification techniques, we need to use amplification kits that have greater tolerance to PCR inhibitors and are more sensitive and robust (78). Such are the kits that have been on the market for the last few years. Older kits that are still used are less effective at amplifying ancient DNA.

8 Examples of Ancient DNA Investigation in Slovenia

In 2007, the Institute of Forensic Medicine completely renovated the premises of the Laboratory for Molecular Genetics and acquired a separate laboratory, which is exclusively intended for the treatment of old skeletal remains. We also recently acquired a new laboratory for the preparation

of samples for massive parallel sequencing - the technology we have equipped ourselves with. According to the recommendations described for investigating ancient DNA by Rohland and Hofreiter (69), in order to avoid laboratory contamination, bone and tooth samples need to be processed and DNA extracted from them in a separate laboratory intended exclusively for the treatment of old skeletal remains. The premises for processing skeletal samples, DNA extraction and preparation of PCRs must be strictly separated from the premises where the so-called "post-PCR" procedures are performed (69). All investigations of archaeological skeletons are performed in accordance with the recommendations for investigating ancient DNA, which were described in the chapter *Prevention of Contamination and Verification of the Authenticity of Ancient DNA*. In 2004, we began investigating old forensic skeletons (especially from the Second World War period), initially investing a lot of time in optimizing the extraction process. The experience gained in genetic research of skeletal remains from Slovenian post-war mass graves is of great help in developing methods for molecular genetic investigations of much older skeletal remains from archaeological sites.

Thus, in 2011, we performed a molecular genetic investigation of skeletons from the 17th-century Auersperg Chapel (44). In 2017, we obtained a research project funded by the Slovenian Research Agency (J3–8214 - *Determination of the Most Appropriate Skeletal Elements for Molecular Genetic Identification of Old Human Remains*), the main goal of which is to re-evaluate current global recommendations for selecting long leg bones and teeth for DNA typing and based on the obtained results to change the recommended strategy of sampling skeletal elements for genetic identification of skeletal remains. According to previous studies, DNA is preserved the longest and best in long bones, especially the femurs and teeth (24-27) (in archaeological skeletons, in the petrous part of the temporal bone) (33), so according to current recommendations for genetic identification of skeletal remains, long leg bones (femurs) and teeth (24-27) (in the case of archeological skeletons, the petrous part of the temporal bone) (33) are sampled. Recent studies on relatively fresh skeletons have shown that small bones of the hands and feet (17,28-30) would be more suitable for genetic identification, so in the case of old skeletal remains, the project investigates whether other skeletal elements than femurs and teeth (in archaeological skeletons, the petrous part of the temporal bone) are more suitable for the genetic identification of an individual. Small bones of hand and feet are easier to sample because we do not need a saw to get samples, which reduces the possibility of contamination with modern DNA. To compare the success of obtaining DNA from different skeletal elements, the project uses skeletons from the period of the Second World War and skeletons from various archaeological sites. Skeletons from the period of the Second World War were investigated by Marcel Obal (8) in his master's thesis entitled "Different Skeletal Elements as a Source of DNA for Genetic Identification of Second World War Victims". In three skeletons from the post-war mass graves, he processed 56 skeletal elements per skeleton and obtained the largest amounts of DNA from the bones of feet and hands. Skeletons from post-war mass graves and archaeological sites serve as models of poorly preserved skeletal remains, and the results of research will be able to be applied to routine forensic cases of molecular genetic identifications of skeletons. The research project is based on the need for rapid and efficient sampling of skeletal elements and the successful acquisition of nuclear genetic profiles to identify missing persons and victims of mass disasters. As part of the project, an agreement was reached with the Public Institute of the Republic of Slovenia for the Protection of Cultural Heritage to obtain skeletal remains from Slovenian archaeo-

logical sites, with archaeologists sampling about 20 skeletal elements from 17 skeletons, especially from hands and feet (and for comparison, the femur, the petrous part of the temporal bone and teeth). As part of the ongoing research project, we performed the faculty Prešeren assignment, entitled *Determination of Suitability of Small Bones of the Hands and Feet for Molecular Genetic Typing of Old Skeletal Remains*, which was awarded the Faculty of Medicine Prešeren Award for 2017 (79). Archaeological skeletons were also included in a study to determine the possibility of using reagents for quantitative PCR and to determine the amount and degradation of DNA to predict the success of nuclear microsatellite typing (45). We were also interested in whether we could use genetic methods to determine the colour of hair and eyes on an old skeleton (80). Each of these studies will be described in more detail below.

In 2009, archaeologists excavated five 17th-century skeletons at a market in Ljubljana in the side chapel of the Franciscan monastery church, which is known to have been Auersperg's tomb.

At the foot of one of the skeletons they found a metal vessel with a heart that had the name Ferdinand II. and the years of birth and death (1655-1706) engraved in it. In 2011, at the Institute of Forensic Medicine, we performed a molecular genetic study of skeletons from the Auersperg Chapel on behalf of the City Museum of Ljubljana (44). The skeletons were poorly preserved, the bones disintegrated into small pieces. Only in two skeletons were fragments of the femurs and teeth preserved, and in the others, we used parts of skulls for molecular genetic examination. We were able to obtain nuclear DNA from the more than 300-year-old teeth of skeleton 4 for successful typing of STR markers of autosomal DNA and Y chromosome. We were able to obtain a complete male genetic profile of nuclear autosomal DNA, an almost complete Y chromosome haplotype that allows us to trace the paternal line and comparison to living offspring on the paternal side, and an mtDNA haplotype that allows us to track the maternal line and comparison to still living offspring on the maternal side. The obtained genetic profiles did not match the profiles of the persons from the elimination database. We have demonstrated an inverse relationship between the amplification efficiency and the length of the amplified products, which confirms their authenticity (44). In order to be able to identify the skeleton for which we obtained the genetic profiles, the profiles should be compared with the still living direct offspring in the paternal or maternal lineage. Unfortunately, we have not yet received comparative family samples. It is very important that investigations of ancient DNA are well planned so that they can actually be carried out to completion. As part of the Prešeren assignment entitled *Determination of Suitability of Small Bones of the Hands and Feet for Molecular Genetic Typing of Old Skeletal Remains*, we conducted a pilot study in 2017, which included 13 skeletons from various Slovenian archaeological sites. The oldest skeleton was from the 3rd century, the youngest from the 18th century. For each archaeological skeleton, 6 skeletal elements (temporal bone, femur, tooth, hand bone, foot bone, and phalanx) were included in the investigation, from which ancient DNA was obtained, quantified, and typed with autosomal STR markers. The obtained genetic profiles were evaluated on the basis of the number of successfully multiplied STR markers. By statistical processing, we found that the tiny bones of the palms and soles of the feet are also suitable for genetic investigations of ancient skeletal remains. The amount of preserved ancient DNA from the temporal bones, teeth, and femurs was comparable to that from the tiny bones of the terminal parts of the limbs. The same was true for the success of STR marker typing, which was comparable between the investigated skeletal elements. We found that for genetic testing of

ancient skeletons, it would make sense to sample feet bones, palm bones, and phalanges in addition to the temporal bones, femurs, and teeth (79). The obtained genetic profiles did not match the profiles of the persons from the elimination database. We have demonstrated an inversely proportional relationship between the amplification efficiency and the length of the amplified products, which confirms their authenticity (79).

By using the qPCR technique and determining the amount of DNA and its degradation, we managed to predict the success of genetic investigations of nuclear STR markers in ancient skeletal remains and skeletons from the period of the Second World War (45). It is very important for ancient DNA to obtain as much information as possible about isolated DNA, as we are dealing with severely damaged molecules whose investigations of STR markers are difficult and expensive. Therefore, before the process of typing STR markers, it makes sense to obtain information on the quality of DNA in addition to the amount of isolated DNA.

For this purpose, we used the latest, highly informative qPCR kit PowerQuant System (Promega). With it, we determined the amount of total nuclear DNA, the amount of male DNA that allows us to determine sex, and the degree of DNA degradation that tells us how long the fragments are in the isolate. The obtained data were used to evaluate the success of the STR marker typing. The purpose of the investigation was to examine whether the PowerQuant quantification kit can be used as a screening method for the successful typing of STR markers in old skeletons and thus to predict the successful typing of STR loci. We found that using this kit, which is significantly cheaper than STR amplification kits, we can predict the success of STR typing in old bones (45).

By phenotyping DNA, which makes it possible to predict the external visual properties of an individual from a DNA molecule, we were able to determine eye and hair colour in 60 skeletons from the Second World War period (80). The SNP markers of the HIrisPlex system were investigated and in all but one skeleton, despite the age of the skeletal remains, we successfully typed the extracted DNA and obtained information on the eye and hair colour and demonstrated their potential to be applied in identifying World War II victims (80). An investigation is underway in which we will determine the colour of the eyes and hair for a large number of ancient skeletons, which are between 200 and 1,700 years old, using NGS technology.

9 Conclusion

In this article, we focused on molecular genetic investigations of ancient DNA derived from human skeletal remains and found that, according to previous studies in archaeogenetics, it is best to sample the petrous part of the temporal bone (33) and store the samples in a cool and dry environment (66). Because there is very little DNA in archaeological biological materials and it is severely damaged, problems associated with contamination with modern human DNA occur, especially when working with ancient human samples. Therefore, in order to reduce the risk of contamination, it is necessary to consider a number of standard precautions and to verify the authenticity of ancient DNA (69). The field of investigation of ancient DNA by genetic methods has made a major breakthrough in recent years using NGS technology, as the new technology can be used to investigate more degraded DNA and thus older archaeological biological materials. We see great potential in the use of the new technology for further investigations of ancient DNA in our country (abroad, these investigations have been underway for some time in top laboratories), as they will allow us to investigate quantitatively extremely modest and degraded samples. We will be able to use SNP identification markers whose length

does not exceed 150 bp (50) to identify and establish kinship relationships. With NGS technology, we will be able to investigate the phenotypic markers of SNP, with which we will be able to determine the colour of hair and eyes (56), but also the biogeographical or ancestral origin (57) of archaeological skeletons. Phenotypic and biogeographic markers of SNPs are also significantly shorter and promise good results in investigations of degraded samples of ancient DNA (56,57). With NGS technology, we will investigate the SNP markers of the Y chromosome and mtDNA of ancient skeletons much faster and easier, and determine the haplogroups through which we can determine phylogeographic origin, as these markers are geographically highly differentiated (58). Due to the closed systems, these investigations will be less susceptible to contamination and therefore more suitable for analysing human ancient DNA. In mtDNA, we will be able to investigate not only the control region but also the entire mitochondrial genome (81).

Molecular genetic investigations of ancient DNA are in their infancy in Slovenia. Some of the investigations carried out are encouraging, but a faster development of this field will certainly require the interest and work of a larger number of researchers and sufficient funding for expensive investigations. It will also be necessary in the archeological, conservation and museum professions to draw up precise guidelines for the handling of human remains when there are possibilities for ancient DNA investigations of excavated archaeological samples and to carefully plan meaningful investigations. Archaeogenetics is an interdisciplinary science in which the synthesis and understanding of all collected data requires the cooperation of experts in various fields. Archaeogenetics thus today requires close collaboration between molecular genetics, bioinformatics, archeology, and paleozoology (49). Due to the great importance of a connection and cooperation of different disciplines, it would be most appropriate to obtain sources of funding for interdisciplinary projects, which would, with the cooperation of various institutions, meaningfully link the results of different disciplines and lead to a comprehensive interpretation of the results.

10 Acknowledgment

I would like to thank Matija Črešnar (Department of Archeology, Faculty of Arts, University of Ljubljana and the Public Institute for the Protection of Cultural Heritage) and Tamara Leskovar (Department of Archeology, Faculty of Arts, University of Ljubljana) for obtaining permits and sampling archaeological skeletons. Živa Geršak (Faculty of Medicine, University of Ljubljana) for research work within the research done within the students' Prešeren research work (*Determination of Suitability of Small Bones of the Hands and Feet for Molecular Genetic Typing of Old Skeletal Remains*) and Barbara Gornjak Pogorelc and Katja Vodopivec Mohorčič for assisting in molecular genetic testing of ancient DNA in the Laboratory for Molecular Genetics, Institute of Forensic Medicine, Faculty of Medicine, University of Ljubljana. Special thanks go to Marcel Obala for his examination of skeletons from the period of the Second World War and a detailed and extensive review of the relevant literature, which I partially summarize in the review article. I would also like to thank the Commission of the Government of the Republic of Slovenia for resolving the issues of hidden mass graves for the excavations of victims of the Second World War.

Part of the studies described in the review article was co-financed by the Slovenian Research Agency (ARRS) from the state budget (project, *Determination of the Most Appropriate Skeletal Elements for Molecular Genetic Identification of Old Human Remains*, No. J3–8214 and the program, *Metabolic and Congenital Factors of Reproductive Health, Childbirth II*, No. P3–0124).

References

1. Márquez-Grant N, Webster H, Truesdell J, Fibiger L. Physical anthropology and osteoarchaeology in Europe: History, current trends and challenges. Int J Osteoarchaeol. 2016;26(6):1078-88. DOI: 10.1002/oa.2520
2. Bouwman A, Rühli F. Archaeogenetics in evolutionary medicine. J Mol Med (Berl). 2016;94(9):971-7. DOI: 10.1007/s00109-016-1438-8 PMID: 27289479
3. Baum DA, Futuyma DJ, Hoekstra HE, Lenski RE, Moore JA, Peichel CL, et al. The Princeton Guide to Evolution. Princeton: Princeton University Press; 2013. pp. 477-9.
4. Hagelberg E, Quevedo S, Turbon D, Clegg JB. DNA from ancient Easter Islanders. Nature. 1994;369(6475):25-6. DOI: 10.1038/369025a0 PMID: 8164735
5. Ingman M, Kaessmann H, Pääbo S, Gyllensten U. Mitochondrial genome variation and the origin of modern humans. Nature. 2000;408(6813):708-13. DOI: 10.1038/35047064 PMID: 11130070
6. Sampietro ML, Gilbert MT, Lao O, Caramelli D, Lari M, Bertranpetit J, et al. Tracking down human contamination in ancient human teeth. Mol Biol Evol. 2006;23(9):1801-7. DOI: 10.1093/molbev/msl047 PMID: 16809622
7. Cooper A, Poinar HN. Ancient DNA: do it right or not at all. Science. 2000;289(5482):1139. DOI: 10.1126/science.289.5482.1139b PMID: 10970224
8. Obal M. Uporaba različnih skeletnih elementov za genetsko identifikacijo žrtev druge svetovne vojne. [Master's Thesis]. Ljubljana: M. Obal; 2018.
9. Ziętkiewicz E, Witt M, Daca P, Zebracka-Gala J, Goniewicz M, Jarząb B, et al. Current genetic methodologies in the identification of disaster victims and in forensic analysis. J Appl Genet. 2012;53(1):41-60. DOI: 10.1007/s13353-011-0068-7 PMID: 22002120
10. Irwin JA, Just RS, Loreille OM, Parsons TJ. Characterization of a modified amplification approach for improved STR recovery from severely degraded skeletal elements. Forensic Sci Int Genet. 2012;6(5):578-87. DOI: 10.1016/j.fsigen.2012.01.010 PMID: 22402195
11. Anderung C, Persson P, Bouwman A, Elburg R, Götherström A. Fishing for ancient DNA. Forensic Sci Int Genet. 2008;2(2):104-7. DOI: 10.1016/j.fsigen.2007.09.004 PMID: 19083805
12. Zupanič Pajnič I. Molekularnogenetska identifikacija skeletnih ostankov. Med Razgl. 2013;52:213-34.
13. Campos PF, Craig OE, Turner-Walker G, Peacock E, Willerslev E, Gilbert MT. DNA in ancient bone - where is it located and how should we extract it? Ann Anat. 2012;194(1):7-16. DOI: 10.1016/j.aanat.2011.07.003 PMID: 21855309
14. Putkonen MT, Palo JU, Cano JM, Hedman M, Sajantila A. Factors affecting the STR amplification success in poorly preserved bone samples. Investig Genet. 2010;1(1):9. DOI: 10.1186/2041-2223-1-9 PMID: 21092342
15. Higgins D, Austin JJ. Teeth as a source of DNA for forensic identification of human remains: a review. Sci Justice. 2013;53(4):433-41. DOI: 10.1016/j.scijus.2013.06.001 PMID: 24188345
16. Pruvost M, Schwarz R, Correia VB, Champlot S, Braguier S, Morel N, et al. Freshly excavated fossil bones are best for amplification of ancient DNA. Proc Natl Acad Sci USA. 2007;104(3):739-44. DOI: 10.1073/pnas.0610257104 PMID: 17210911
17. Mundorff A, Davoren JM. Examination of DNA yield rates for different skeletal elements at increasing post mortem intervals. Forensic Sci Int Genet. 2014;8(1):55-63. DOI: 10.1016/j.fsigen.2013.08.001 PMID: 24315589
18. Hofreiter M, Serre D, Poinar HN, Kuch M, Pääbo S. Ancient DNA. Nat Rev Genet. 2001;2(5):353-9. DOI: 10.1038/35072071 PMID: 11331901
19. Kendall C, Høier Eriksen AM, Kontopoulos I, Collins MJ, Turner-Walker G. Diagenesis of archaeological bone and tooth. Paleogeogr Palaeocl. 2018;491:21-37. DOI: 10.1016/j.palaeo.2017.11.041
20. Mansour H, Krebs O, Sperhake JP, Augustin C, Koehne T, Amling M, et al. Cementum as a source of DNA in challenging forensic cases. J Forensic Leg Med. 2018;54:76-81. DOI: 10.1016/j.jflm.2017.12.015 PMID: 29328966
21. Kemp BM, Smith DG. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. Forensic Sci Int. 2005;154(1):53-61. DOI: 10.1016/j.forsciint.2004.11.017 PMID: 16182949
22. Coulson-Thomas YM, Norton AL, Coulson-Thomas VJ, Florencio-Silva R, Ali N, Elmrghni S, et al. DNA and bone structure preservation in medieval human skeletons. Forensic Sci Int. 2015;251:186-94. DOI: 10.1016/j.forsciint.2015.04.005 PMID: 25912776
23. Turner-Walker G. The chemical and microbial degradation of bones and teeth. In: Pinhasi R, Mays S: Advances in Human Palaeopathology. New York: John wiley and Sons; 2008. pp. 1-29.
24. Siriboonpiputtana T, Rinthachai T, Shotivaranon J, Peonim V, Rerkamnuaychoke B. Forensic genetic analysis of bone remain samples. Forensic Sci Int. 2018;284:167-75. DOI: 10.1016/j.forsciint.2017.12.045 PMID: 29408726

25. Miloš A, Selmanović A, Smajlović L, Huel RL, Katzmarzyk C, Rizvić A, et al. Success rates of nuclear short tandem repeat typing from different skeletal elements. Croat Med J. 2007;48(4):486-93. PMID: 17696303
26. Prinz M, Carracedo A, Mayr WR, Morling N, Parsons TJ, Sajantila A, et al.; International Society for Forensic Genetics. DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI). Forensic Sci Int Genet. 2007;1(1):3-12. DOI: 10.1016/j.fsigen.2006.10.003 PMID: 19083722
27. Edson SM, Ross JP, Coble MD, Parsons TJ, Barritt SM. Naming the Dead - Confronting the Realities of Rapid Identification of Degraded Skeletal Remains. Forensic Sci Rev. 2004;16(1):63-90. PMID: 26256813
28. Mundorff AZ, Bartelink EJ, Mar-Cash E. DNA preservation in skeletal elements from the World Trade Center disaster: recommendations for mass fatality management. J Forensic Sci. 2009;54(4):739-45. DOI: 10.1111/j.1556-4029.2009.01045.x PMID: 19486445
29. Andronowski JM, Mundorff AZ, Pratt IV, Davoren JM, Cooper DM. Evaluating differential nuclear DNA yield rates and osteocyte numbers among human bone tissue types: A synchrotron radiation micro-CT approach. Forensic Sci Int Genet. 2017;28:211-8. DOI: 10.1016/j.fsigen.2017.03.002 PMID: 28315820
30. Hines DZ, Vennemeyer M, Amory S, Huel RL, Hanson I, Katzmarzyk C, et al. Prioritizing sampling of bone and teeth for DNA analysis in commingled cases. In: Adams BJ, Byrd JE, eds. Commingled Human Remains: Methods in Recovery, Analysis, and Identification. Oxford: Elsevier Science; 2014. pp. 275-305. DOI: 10.1016/B978-0-12-405889-7.00013-7
31. Pilli E, Vai S, Caruso MG, D'Errico G, Berti A, Caramelli D. Neither femur nor tooth: petrous bone for identifying archaeological bone samples via forensic approach. Forensic Sci Int. 2018;283:144-9. DOI: 10.1016/j.forsciint.2017.12.023 PMID: 29301114
32. Hansen HB, Damgaard PB, Margaryan A, Stenderup J, Lynnerup N, Willerslev E, et al. Comparing Ancient DNA Preservation in Petrous Bone and Tooth Cementum. PLoS One. 2017;12(1):e0170940. DOI: 10.1371/journal.pone.0170940 PMID: 28129388
33. Pinhasi R, Fernandes D, Sirak K, Novak M, Connell S, Alpaslan-Roodenberg S, et al. Optimal ancient DNA yields from the inner ear part of the human petrous bone. PLoS One. 2015;10(6):e0129102.
34. Gamba C, Jones ER, Teasdale MD, McLaughlin RL, Gonzalez-Fortes G, Mattiangeli V, et al. Genome flux and stasis in a five millennium transect of European prehistory. Nat Commun. 2014;5(1):5257. DOI: 10.1038/ncomms6257 PMID: 25334030
35. Jamieson A, Bader S. A guide to forensic DNA profiling. New Yrok: Wiley; 2016. pp. 5-6, 15-8, 32-4, 37-8, 108-9, 115-6.
36. Butler JM. Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers. 2nd edition. Amsterdam: Elsevier; 2005. pp. 86-90.
37. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981;290(5806):457-65. DOI: 10.1038/290457a0 PMID: 7219534
38. Fisher DL, Holland MM, Mitchell L, Sledzik PS, Wilcox AW, Wadhams M, et al. Extraction, evaluation, and amplification of DNA from decalcified and undecalcified United States Civil War bone. J Forensic Sci. 1993;38(1):60-8. DOI: 10.1520/JFS13376J PMID: 8426158
39. Holland MM, Fisher DL, Mitchell LG, Rodriquez WC, Canik JJ, Merril CR, et al. Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam War. J Forensic Sci. 1993;38(3):542-53. DOI: 10.1520/JFS13439J PMID: 8515208
40. Hopwood AJ, Mannucci A, Sullivan KM. DNA typing from human faeces. Int J Legal Med. 1996;108(5):237-43. DOI: 10.1007/BF01369817 PMID: 8721422
41. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, et al. Genetic analyses from ancient DNA. Annu Rev Genet. 2004;38(1):645-79. DOI: 10.1146/annurev.genet.37.110801.143214 PMID: 15568989
42. Palo JU, Hedman M, Söderholm N, Sajantila A. Repatriation and identification of the Finnish World War II soldiers. Croat Med J. 2007;48(4):528-35. PMID: 17696308
43. Zupanič Pajnič I, Gornjak Pogorelc B, Balažic J. Molecular genetic identification of skeletal remains from the Second World War Konfin I mass grave in Slovenia. Int J Legal Med. 2010;124(4):307-17. DOI: 10.1007/s00414-010-0431-y PMID: 20217112
44. Zupanič Pajnič I. Molecular genetic analyses of 300-year old skeletons from Auersperg tomb. Zdrav Vestn. 2013;82:796-808.
45. Zupanič Pajnič I, Zupanc T, Balažic J, Geršak ŽM, Stojković O, Skadrić I, et al. Prediction of autosomal STR typing success in ancient and Second World War bone samples. Forensic Sci Int Genet. 2017;27:17-26. DOI: 10.1016/j.fsigen.2016.11.004 PMID: 27907810
46. Zupanič Pajnič I, Petaros A, Balažic J, Geršak K. Searching for the mother missed since the Second World War. J Forensic Leg Med. 2016;44:138-42. DOI: 10.1016/j.jflm.2016.10.015 PMID: 27810583
47. Cattaneo C, Craig OE, James NT, Sokol RJ. Comparison of three DNA extraction methods on bone and blood stains up to 43 years old and amplification of three different gene sequences. J Forensic Sci. 1997;42(6):1126-35. DOI: 10.1520/JFS14273J PMID: 9397557

48. Pilli E, Boccone S, Agostino A, Virgili A, D'Errico G, Lari M, et al. From unknown to known: identification of the remains at the mausoleum of fosse Ardeatine. Sci Justice. 2018;58(6):469-78. DOI: 10.1016/j.scijus.2018.05.007 PMID: 30446077
49. Knapp M, Lalueza-Fox C, Hofreiter M. Re-inventing ancient human DNA. Investig Genet. 2015;6(1):4. DOI: 10.1186/s13323-015-0020-4 PMID: 25937886
50. Mehta B, Daniel R, Phillips C, McNevin D. Forensically relevant SNaPshot ® assays for human DNA SNP analysis: a review. Int J Legal Med. 2017;131(1):21-37. DOI: 10.1007/s00414-016-1490-5 PMID: 27841004
51. Balding D. Weight-of-evidence for Forensic DNA Profiles. West Sussex, England: John Wiley & Sons; 2005. pp. 50-1. DOI: 10.1002/9780470867693
52. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, et al. Identification of the remains of the Romanov family by DNA analysis. Nat Genet. 1994;6(2):130-5. DOI: 10.1038/ng0294-130 PMID: 8162066
53. Jehaes E, Toprak K, Vanderheyden N, Pfeiffer H, Cassiman JJ, Brinkmann B, et al. Pitfalls in the analysis of mitochondrial DNA from ancient specimens and the consequences for forensic DNA analysis: the historical case of the putative heart of Louis XVII. Int J Legal Med. 2001;115(3):135-41. DOI: 10.1007/s004140100221 PMID: 11775015
54. King TE, Fortes GG, Balaresque P, Thomas MG, Balding D, Maisano Delser P, et al. Identification of the remains of King Richard III. Nat Commun. 2014;5(1):5631. DOI: 10.1038/ncomms6631 PMID: 25463651
55. Kayser M, de Knijff P. Improving human forensics through advances in genetics, genomics and molecular biology. Nat Rev Genet. 2011;12(3):179-92. DOI: 10.1038/nrg2952 PMID: 21331090
56. Walsh S, Liu F, Wollstein A, Kovatsi L, Ralf A, Kosiniak-Kamysz A, et al. The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. Forensic Sci Int Genet. 2013;7(1):98-115. DOI: 10.1016/j.fsigen.2012.07.005 PMID: 22917817
57. Phillips C, Prieto L, Fondevila M, Salas A, Gómez-Tato A, Alvarez-Dios J, et al. Ancestry analysis in the 11-M Madrid bomb attack investigation. PLoS One. 2009;4(8):e6583. DOI: 10.1371/journal.pone.0006583 PMID: 19668368
58. Karafet TM, Mendez FL, Meilerman MB, Underhill PA, Zegura SL, Hammer MF. New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. Genome Res. 2008;18(5):830-8. DOI: 10.1101/gr.7172008 PMID: 18385274
59. Larmuseau MH, Vanderheyden N, Van Geystelen A, van Oven M, Kayser M, Decorte R. Increasing phylogenetic resolution still informative for Y chromosomal studies on West-European populations. Forensic Sci Int Genet. 2014;9:179-85. DOI: 10.1016/j.fsigen.2013.04.002 PMID: 23683810
60. Larmuseau MH, Otten GP, Decorte R, Van Damme P, Moisse M. Defining Y-SNP variation among the Flemish population (Western Europe) by full genome sequencing. Forensic Sci Int Genet. 2017;31:e12-6. DOI: 10.1016/j.fsigen.2017.10.008 PMID: 29089250
61. Kosoy R, Nassir R, Tian C, White PA, Butler LM, Silva G, et al. Ancestry informative marker sets for determining continental origin and admixture proportions in common populations in America. Hum Mutat. 2009;30(1):69-78. DOI: 10.1002/humu.20822 PMID: 18683858
62. Espregueira Themudo G, Smidt Mogensen H, Børsting C, Morling N. Frequencies of HID-ion ampliseq ancestry panel markers among greenlanders. Forensic Sci Int Genet. 2016;24:60-4. DOI: 10.1016/j.fsigen.2016.06.001 PMID: 27326551
63. Phillips C, Salas A, Sánchez JJ, Fondevila M, Gómez-Tato A, Alvarez-Dios J, et al.; SNPforID Consortium. Inferring ancestral origin using a single multiplex assay of ancestry-informative marker SNPs. Forensic Sci Int Genet. 2007;1(3-4):273-80. DOI: 10.1016/j.fsigen.2007.06.008 PMID: 19083773
64. Kidd KK, Speed WC, Pakstis AJ, Furtado MR, Fang R, Madbouly A, et al. Progress toward an efficient panel of SNPs for ancestry inference. Forensic Sci Int Genet. 2014;10:23-32. DOI: 10.1016/j.fsigen.2014.01.002 PMID: 24508742
65. Silvia AL, Shugarts N, Smith J. A preliminary assessment of the ForenSeq™ FGx System: next generation sequencing of an STR and SNP multiplex. Int J Legal Med. 2017;131(1):73-86. DOI: 10.1007/s00414-016-1457-6 PMID: 27785563
66. Fulton LT. Ancient DNA - Methods and Protocols: Setting up an ancient DNA laboratory. New Jersey: Humana Press Inc; 2012. pp. 1-11.
67. Lindahl T. Instability and decay of the primary structure of DNA. Nature. 1993;362(6422):709-15. DOI: 10.1038/362709a0 PMID: 8469282
68. Salamon M, Tuross N, Arensburg B, Weiner S. Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc Natl Acad Sci USA. 2005;102(39):13783-8. DOI: 10.1073/pnas.0503718102 PMID: 16162675
69. Rohland N, Hofreiter M. Ancient DNA extraction from bones and teeth. Nat Protoc. 2007;2(7):1756-62. DOI: 10.1038/nprot.2007.247 PMID: 17641642
70. Graham EA. DNA reviews: ancient DNA. Forensic Sci Med Pathol. 2007;3(3):221-5. DOI: 10.1007/s12024-007-9009-5 PMID: 25869168

71. Jakubowska J, Maciejewska A, Pawłowski R. Comparison of three methods of DNA extraction from human bones with different degrees of degradation. Int J Legal Med. 2012;126(1):173-8. DOI: 10.1007/s00414-011-0590-5 PMID: 21717154
72. Amory S, Huel R, Bilić A, Loreille O, Parsons TJ. Automatable full demineralization DNA extraction procedure from degraded skeletal remains. Forensic Sci Int Genet. 2012;6(3):398-406. DOI: 10.1016/j.fsigen.2011.08.004 PMID: 21885362
73. Zupanič Pajnič I, Debska M, Gornjak Pogorelc B, Vodopivec Mohorčič K, Balažic J, Zupanc T, et al. Highly efficient automated extraction of DNA from old and contemporary skeletal remains. J Forensic Leg Med. 2016;37:78-86. DOI: 10.1016/j.jflm.2015.11.001 PMID: 26615474
74. Bender K, Schneider PM, Rittner C. Application of mtDNA sequence analysis in forensic casework for the identification of human remains. Forensic Sci Int. 2000;113(1-3):103-7. DOI: 10.1016/S0379-0738(00)00223-1 PMID: 10978609
75. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parsons TJ. High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet. 2007;1(2):191-5. DOI: 10.1016/j.fsigen.2007.02.006 PMID: 19083754
76. Ewing MM, Thompson JM, McLaren RS, Purpero VM, Thomas KJ, Dobrowski PA, et al. Human DNA quantification and sample quality assessment: developmental validation of the PowerQuant(®) system. Forensic Sci Int Genet. 2016;23:166-77. DOI: 10.1016/j.fsigen.2016.04.007 PMID: 27206225
77. Zupanič Pajnič I. Extraction of DNA from Human Skeletal Material. In: Goodwin W. Forensic DNA Typing Protocols. New york: Humana Press; 2016. pp. 89-108.
78. Zupanič Pajnič I, Gornjak Pogorelc B, Balažic J, Zupanc T, Štefanič B. Highly efficient nuclear DNA typing of the World War II skeletal remains using three new autosomal short tandem repeat amplification kits with the extended European Standard Set of loci. Croat Med J. 2012;53(1):17-23. DOI: 10.3325/cmj.2012.53.17 PMID: 22351574
79. Geršak ŽM. Določitev primernosti drobnih kosti nog in rok za molekularno genetsko tipizacijo starih skeletnih ostankov. Ljubljana: ŽM. Geršak; 2017.
80. Chaitanya L, Pajnič IZ, Walsh S, Balažic J, Zupanc T, Kayser M. Bringing colour back after 70 years: predicting eye and hair colour from skeletal remains of World War II victims using the HIrisPlex system. Forensic Sci Int Genet. 2017;26:48-57. DOI: 10.1016/j.fsigen.2016.10.004 PMID: 27780108
81. Strobl C, Eduardoff M, Bus MM, Allen M, Parson W. Evaluation of the precision ID whole MtDNA genome panel for forensic analyses. Forensic Sci Int Genet. 2018;35:21-5. DOI: 10.1016/j.fsigen.2018.03.013 PMID: 29626805