

Recommendations regarding the genetic analysis of human remains in the context of repatriation

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Abstract

Genetic analysis is known to be applied in many scientific fields. Within the repatriation of human remains, it could also potentially play a role. Although genetic analysis has its benefits, it also has its limitations, particularly when dealing with human remains from which only ancient DNA can be recovered and analyzed. As stakeholders with different competences and interests are involved in the decision-making process of repatriation cases, it is important to provide comprehensible comprehensive information in advance. This document aims to provide information regarding the possibilities and challenges of DNA typing and interpretation within the scope of the repatriation of human remains.

The relevance of genetic analysis in each particular repatriation case should be considered and discussed with all stakeholders before the start of the DNA analysis process. Genetic analysis can interfere in the repatriation process and should not be considered as a stand-alone technique. A strictly biological approach would ignore the complexity of identity and could undermine family histories. A multidisciplinary approach is always required. The interpretation of the obtained DNA results should thus be considered in the light of primary information (e.g. historical records, other analytical data, archaeological findings), if available. Moreover, the impact of DNA sampling on the human remains should be assessed. As results can be surprising and challenge previous assumptions, the possible outcome of genetic analysis and interpretation should also be clarified in advance. So even though it could be technically feasible, the implementation of genetic analysis in repatriation cases could be limited by the possible ethical, social and political outcome of the investigation.

Genetic analysis can thus have a place in certain repatriation cases of human remains; however, its application should be widely discussed between different parties involved in the repatriation process. This document can serve as a guidance in the decision-making process and during the procedure of genetic analysis.

Keywords: genetic analysis, human remains, ancient DNA, repatriation, recommendations

Samenvatting

Genetische analyse wordt toegepast in verschillende wetenschappelijke vakgebieden. Ook tijdens de procedure van repatriëring van menselijke resten kan het potentieel een rol spelen. Maar ook al kent genetische analyse voordelen, het heeft ook zijn limieten, in het bijzonder wanneer van menselijke resten enkel 'ancient' DNA kan worden bekomen en geanalyseerd. Gezien belanghebbenden met verschillende competenties en interesses zijn betrokken bij de procedures van repatriëring, is het belangrijk om voorafgaandelijk begrijpelijke informatie te verstrekken. Dit document tracht informatie betreffende de mogelijkheden en beperkingen van DNA-analyse en interpretatie te geven in het kader van de repatriëring van menselijke resten.

De relevantie van genetische analyse moet in elke aanvraag tot repatriëring worden overwogen en besproken met alle stakeholders vooraleer een DNA-analyse kan worden opgestart. Genetische analyse kan toegepast worden in dit proces, maar mag niet als een losstaande techniek worden beschouwd. Een louter biologische aanpak zou de complexiteit van identiteit negeren en zou familiegeschiedenissen kunnen ondermijnen. Hierdoor is een multidisciplinaire aanpak altijd vereist. Verkregen DNA-resultaten moeten dus steeds geïnterpreteerd worden in de context van primaire informatie (vb. Historische gegevens, andere analytische data, archeologische bevindingen), indien aanwezig. Bijkomend, de impact van staalname voor DNA-analyses op menselijke resten moet worden beoordeeld. Aangezien de resultaten van genetische analyse verrassend kunnen zijn en voorafgaande veronderstellingen in vraag kunnen stellen, moeten de mogelijke resultaten en de interpretatie op voorhand verduidelijkt worden. Ook al zou het technisch mogelijk zijn, de toepassing van genetische analyse in repatriëring kan gelimiteerd worden door mogelijke ethische, sociale en politieke gevolgen van het onderzoek.

Genetische analyse kan dus een plaats hebben in bepaalde aanvragen tot repatriëring van menselijke resten. Echter, de toepassing van genetische analyse moet uitgebreid besproken worden tussen de verschillende partijen die betrokken zijn bij de repatriëringprocedure. Dit document kan dienen als een leidraad in deze discussie en gedurende de genetische analyse.

Trefwoorden: genetische analyse, menselijke resten, ancient DNA, repatriëring, aanbevelingen

Résumé

L'analyse génétique est connue pour être appliquée dans de nombreux domaines scientifiques. Dans le cadre du rapatriement de restes humains, elle pourrait aussi potentiellement jouer un rôle. Bien que l'analyse génétique ait ses avantages, elle a aussi ses limites, notamment lorsqu'il s'agit de restes humains dont seul l'ADN ancien peut être récupéré et analysé. Étant donné que les parties prenantes qui sont concernées par le rapatriement ont des compétences et des intérêts différents, il est important que des informations compréhensibles soient fournies préalablement. Ce document s'efforce d'informer sur les possibilités et limitations concernant les analyses ADN et l'interprétation dans le cadre du rapatriement de restes humains.

Avant le début du processus d'analyse de l'ADN, la pertinence de l'analyse génétique doit être considérée et discutée avec toutes les parties prenantes. L'analyse génétique peut intervenir dans ce processus, mais ne peut pas être considérée comme une technique autonome. Une approche strictement biologique ne tiendrait pas compte de la complexité de l'identité et pourrait compromettre les histoires familiales. Une approche pluridisciplinaire est toujours nécessaire. L'interprétation des résultats obtenus par l'ADN doit donc être envisagée à la lumière d'informations primaires (par exemple, des documents historiques, d'autres données analytiques, des découvertes archéologiques), si disponibles. En outre, il convient d'évaluer l'impact du prélèvement d'ADN sur les restes humains. Étant donné que les résultats peuvent être surprenants et remettre en cause des hypothèses antérieures, il convient également de clarifier à l'avance les résultats possibles des analyses génétiques et de son interprétation. Même si elle est techniquement réalisable, la mise en œuvre de l'analyse génétique dans le cas de rapatriement peut être limitée par les résultats éthiques, sociaux et politiques possibles de l'enquête.

L'analyse génétique peut donc avoir sa place dans certains cas de rapatriement de restes humains. Son application doit néanmoins être largement discutée entre les différentes parties impliquées dans le processus de rapatriement. Ce document peut servir comme un guide dans le processus décisionnel et pendant la procédure d'analyse génétique.

Mots-clés : analyse génétique, restes humains, ADN ancien, rapatriement, recommandations

1. DNA DEGRADATION AND CONSERVATION

Human remains present in Federal Scientific Institutions (FSIs) and other public and private collections date back several decades to centuries. As DNA degrades over time, these human remains usually contain low quantities of (highly) degraded DNA, often referred to as ancient DNA (aDNA) (Allentoft *et al.*, 2012; Pääbo *et al.*, 2004). After death, DNA degrades as a result of chemical processes affecting it and the discontinuation of enzymatic repair processes maintaining the genome integrity in living cells (Dabney *et al.*, 2013b; Pääbo *et al.*, 2004). DNA degrading factors involve endogenous enzymes and environmental microorganisms and invertebrates as well as hydrolytic and oxidative processes (Dabney *et al.*, 2013b; Orlando *et al.*, 2021; Pääbo *et al.*, 2004; Raffone *et al.*, 2021; Uysal-Yoca *et al.*, 2021). Post-mortem degradation, causing DNA fragmentation and modification of bases, is one of the main factors complicating DNA analysis. Degraded DNA is usually fragmented into pieces of 40-500 bp in size because of strand breaks (Pääbo, 1989). In addition, the length of amplified DNA is also limited by modifications blocking DNA polymerases during PCR (Dabney *et al.*, 2013b; Gilbert *et al.*, 2007a; Pääbo *et al.*, 2004). Moreover, other types of modifications result

in nucleotide misincorporations during amplification because of nucleotide transformations. Degradation reactions modifying cytosines are most common, resulting in cytosine (C) to thymine (T) and guanine (G) to adenine (A) substitutions (Briggs *et al.*, 2007). C to T and G to A misincorporations are predominantly represented at the 5' and 3' ends of aDNA fragments.

Environmental factors can influence the state of DNA preservation in skeletal remains (Raffone *et al.*, 2021; Uysal-Yoca *et al.*, 2021). These factors include temperature, pH and humidity. High temperatures increase DNA degradation rates, whereas low temperatures slow down chemical reactions responsible for degradation. Therefore, samples originating from hot climates are particularly prone to DNA damage. Besides high temperature, humidity also causes deterioration of DNA. Moreover, it favors the penetration and growth of microorganisms because of the porosity of bone allowing for moisture accumulation in the pores. Thus, humidity induces DNA degradation as well as environmental contamination by microorganisms. Additionally, when human remains are in direct contact with soil, soil acidity influences DNA preservation. Low pH values damage DNA, whilst DNA is more stable at neutral to slightly alkaline conditions.

Human remains should be stored in optimal conditions to prevent further endogenous DNA degradation and microbial growth. Consequently, it is recommended to store the human remains in a dry, cold and clean place. Fixation in formaldehyde solutions, frequently applied to soft tissues and organs for preservation purposes, must be avoided. Treatment with formaldehyde complicates DNA extraction as well as amplification (Gilbert *et al.*, 2007b; Zavala *et al.*, 2022). In addition, conservation conditions should not conflict with other scientific investigations (e.g. morphological analyses, isotopic investigation). All recommendations regarding conservation of human remains are summarized in Tab. 1.

DNA degradation occurs in both nuclear DNA (nucDNA) and mitochondrial DNA (mtDNA); however mtDNA is more resistant to degradation than nucDNA because of its smaller and circular appearance. Moreover, one cell contains multiple copies of mtDNA, whilst only one copy of nucDNA. MtDNA is thus abundant compared to nucDNA.

In order to obtain reliable results, protocols accounting for the expectation of low amounts of degraded DNA should be implemented.

- ▶ The storage facility of human remains should be dry, cold and clean.
- ▶ Fixation in formaldehyde solutions must be avoided.
- ▶ Conservation conditions should not conflict with other scientific investigations

Tab. 1 – Recommendations - Conservation

2. CONTAMINATION MANAGEMENT

Besides dealing with limited amounts of degraded DNA, contamination of the human remains with exogenous DNA complicates the analysis even more (Korlević *et al.*, 2015; Uysal-Yoca *et al.*, 2021). As DNA contamination interferes with downstream analysis and interpretation, controlling and preventing further DNA contamination is of utmost importance.

Common sources of contaminating DNA are of human origin and microorganisms originating from the environment (Llamas *et al.*, 2017; Uysal-Yoca *et al.*, 2021). The contamination of human DNA can occur from individuals handling or examining human remains without taking precautions. The resultant contemporary human DNA contamination will lead to false positive results and the possible reporting of the contamination as authentic DNA. DNA contamination can also arise whilst handling multiple human remains, resulting in cross contamination between the substrates. Microbial DNA contamination, however, occurs from environmental microorganisms entering bones and teeth during decomposition (Korlević *et al.*, 2015; Llamas *et al.*, 2017). DNA extracts generated from bones or teeth can contain up to 99 % of co-extracted microbial DNA molecules.

Preventive measures should be implemented during storage of the human remains in FSIs and other public and private institutions. Firstly, precautions against further contamination with contemporary human DNA should be taken when handling the stored remains. It is advised to always wear protective gear during manipulation which include disposable gloves, a hairnet and a surgical mask (Llamas *et al.*, 2017; Uysal-Yoca *et al.*, 2021). Other protective equipment such as arm sleeves, clean protective suits or gowns are also highly recommended. Secondly, to avoid cross contamination, changing disposable gloves between handling different specimens is recommended (Knapp *et al.*, 2012; Llamas *et al.*, 2017). It is also suggested to store the remains per individual to prevent sample mix up.

The control of contamination continues when processing the human remains and samples in dedicated DNA laboratories. Laboratories suited for working with ancient DNA are equipped to avoid the introduction of contaminating DNA to samples or specimens. Standard set up requirements for these facilities include separation between ancient DNA extraction, PCR preparation and post-PCR working areas as carry-over of PCR amplification products is one of the main issues in DNA analysis (Knapp *et al.*, 2012; Llamas *et al.*, 2017). Moreover, spatial isolation between different activities within the ancient DNA lab to prevent cross contamination is also recommended (Knapp *et al.*, 2012). Ideally, preparation of human remains, sampling, DNA extraction and PCR set up are performed in different rooms

or hoods. Laboratory personnel entering and working in these dedicated rooms must always wear protective clothing (Knapp *et al.*, 2012; Llamas *et al.*, 2017). Laboratory clothing such as protective overalls or gowns, face masks, hairnets and regularly changed gloves help to avoid introducing exogenous human DNA into the working areas. However, contemporary human DNA contamination is not only caused by direct contact between individuals and the human remains or samples (Edson & Christensen, 2013). Contamination by indirect transfer from contaminated equipment, consumables or surfaces is also possible. Standard cleaning protocols for surfaces and equipment should therefore be in place (Knapp *et al.*, 2012; Llamas *et al.*, 2017). Regular chemical cleaning using for example DNA degrading detergents or bleach followed by short wavelength ultraviolet (UV-C) irradiation is recommended. Moreover, the use of certified DNase free reagents, clean laboratory equipment and sterilized consumables also controls the introduction of contaminating DNA molecules. Furthermore, samples originating from human remains must never be processed together with reference samples originating for example from potential relatives of the human remains. As reference samples contain high amounts of contemporary human DNA, parallel analysis could lead to contamination of ancient samples. Ancient samples and other contemporary casework samples should also be separated during analysis whenever possible. Lastly, traceability, the ability to trace back all laboratory steps, is of utmost importance. All completed steps, from the receipt of the human remains until the reporting of results, should be described in detail. For instance, a detailed description of all procedures with timing and documentation of all involved laboratory personnel per analysis step, used consumables and products should be recorded (Alpaslan-Roodenberg *et al.*, 2021). Traceability not only allows for the monitoring of possible introduction of contamination, but also ensures quality control during laboratory procedures.

The creation of elimination databases allows for the monitoring of contamination during sample handling by comparing the DNA profiles obtained from the human remains and the profiles recorded in the database (Edson & Christensen, 2013; Pajnič *et al.*, 2016). All laboratory staff employed at the dedicated laboratory responsible for the analysis of human remains should be included in an elimination database.

An exclusion database containing profiles of museum staff could also be created. Because of their intense contact with the human remains during manipulation, the risk of them contaminating the substrates is elevated. However, this database is only useful when records of all people who handle the human remains are being kept (Llamas *et al.*, 2017). Museum personnel as well as laboratory staff participating in an elimination database should be well informed about the database and its application. It is therefore important that staff give their permission through an informed consent. Even though both elimination databases can identify modern human DNA contamination, not all modern human contamination will be detected as there are no records or DNA profiles available of all individuals who handled the remains in the past. For example, even though in most cases there are no records available about the excavation, it would be useful to complete the database with DNA profiles of individuals who excavated the human remains. All recommendations regarding contamination management are summarized in Tab. 2.

Other contamination measures that can be taken during specific steps of the analysis are discussed per subject below.

3. SAMPLING OF THE HUMAN REMAINS

In the context of repatriation, the only available biological tissues are usually bones or teeth. Exceptionally, hair, dried skin or nails are also available as a potential source of DNA. Successful recovery of DNA from these challenging human remains starts with targeted sampling of DNA-rich biological material. Furthermore, the choice of sampling location is also influenced by the potential contamination of the human remains under investigation. Lastly, awareness of the destructive nature of genetic analysis is important and can influence the selection of a substrate for sampling as well.

Differences in initial DNA quantity and DNA preservation contribute to varying DNA concentrations among various human remains. Regarding bones, DNA is generally best preserved in dense cortical bone (Adams & Byrd, 2014). Sampling of skeletal human remains should therefore aim to target areas containing the densest cortical bone available (Edson *et al.*, 2004). Samples generated from the midsection of weight-bearing long bones

Recommendations for FSIs and other public and private institutions

- ▶ Protective clothing such as face masks, hairnets and disposable gloves should be worn whenever handling human remains. Arm sleeves and protective suits or gowns are also highly recommended.
- ▶ Disposable gloves should be changed in between handling two different specimens.
- ▶ Human remains originating from one individual should be stored separately from other individuals.
- ▶ An elimination database containing DNA profiles of the museum personnel is optional.

Recommendations for dedicated DNA laboratories

- ▶ Pre-PCR working areas should be separated from post-PCR rooms.
- ▶ Spatial isolation between different pre-PCR activities is recommended.
- ▶ Personnel entering and working in the laboratory should wear protective clothing including a protective suit or gown, regularly changed disposable gloves and a hairnet.
- ▶ Surfaces and equipment should be cleaned chemically followed by UV-C irradiation.
- ▶ Certified DNase free reagents, clean laboratory equipment and sterilized consumables should be used.
- ▶ Separation of ancient samples and reference samples during analysis is required.
- ▶ Traceability allows for contamination management as well as quality control.
- ▶ Comparison between DNA profiles obtained from a substrate and an elimination database containing DNA profiles of all laboratory staff should be performed regularly.

Tab. 2 – Recommendations - Contamination management

(femur and tibia) for example perform better than all other long bones (Adams & Byrd, 2014; Milos *et al.*, 2007). Femora and tibiae diaphysis should therefore be prioritized over other long bones whenever possible. However, the densest bone of the human body is the petrous portion of the temporal bone which is located inside the cranial vault at the base of the skull (Frisch *et al.*, 1998; Hansen *et al.*, 2017; Pinhasi *et al.*, 2015). The petrous bone or pars petrosa consists of the sensory organs of the inner ear protected by a bony labyrinth, termed the otic capsule. Because of its high density and resistance to damage, petrous bones have been shown to generate high endogenous DNA yields, thereby outperforming other bones (Edson *et al.*, 2004; Gamba *et al.*, 2014). Density, however, is not equally distributed along the petrous bone. Hence, when sampling the human cranium it is recommended to aim for the most dense part of the petrous bone,

namely the osseous inner ear containing the cochlea (Pinhasi *et al.*, 2015).

Apart from bones, teeth can also be an optimal source of DNA (Adams & Byrd, 2014; Gamba *et al.*, 2014; Hansen *et al.*, 2017; Higgins *et al.*, 2015; Milos *et al.*, 2007). Different tissues can be distinguished within the human teeth: enamel, dentin, pulp and cementum. DNA content and preservation varies across these different regions of human teeth (Adler *et al.*, 2011; Higgins *et al.*, 2015). Sampling of human teeth should therefore prioritize tooth tissue containing sufficient amounts of DNA known for preserving DNA well, namely the outer layer of the root, the cementum (Adler *et al.*, 2011; Damgaard *et al.*, 2015; Higgins *et al.*, 2015). Targeting this cementum layer in ancient teeth has been proven to generate higher endogenous DNA yields compared to samples originating from other DNA-containing dental tissues, the inner dentin layer and pulp. Even though

cementum is the preferred target, dentin often also contains sufficient amounts of endogenous DNA (Adler *et al.*, 2011; Higgins *et al.*, 2015).

Both petrous bone and tooth cementum contain higher levels of endogenous DNA than any other available ancient human remain (Gamba *et al.*, 2014; Hansen *et al.*, 2017). Although other body parts could also be successful, these substrates are preferred when DNA analysis is performed and should be preferentially sampled when possible. Comparison of DNA preservation in tooth cementum and petrous bone from the same individual shows petrous bone provides higher overall endogenous DNA contents, regardless of preservation conditions (Hansen *et al.*, 2017). Nevertheless, petrous bone does not always outperform tooth cementum. Teeth with good molecular preservation, which roughly corresponds with macroscopic preservation, perform on average just as well as petrous bones. Good visual tooth preservation is characterized by roots with thick, hard and compact cementum layers. Conversely, poorly preserved teeth displaying brittle and chalky roots often show lower success rates than the petrous bones of the same individual.

Macroscopic preservation is only a rough indication of molecular preservation. Thus, even if a skeletal human remain does not look degraded, it is still possible that DNA analysis is unsuccessful because of poor molecular preservation. However, except for genetic analysis, there are no other screening methods available to properly assess DNA preservation to date (Bollongino *et al.*, 2008). Visual inspection of all available human remains prior to selecting the substrate for sampling is therefore recommended. Examples of indicators for good macroscopic preservation of bones are smooth and intact surfaces, strong, non-brittle edges and dense or compact looking bone tissue (Llamas *et al.*, 2017).

In addition to attempting to target substrates containing sufficient amounts of endogenous DNA, potential surface contamination of substrates by exogenous human DNA should also be considered. DNA contamination from previous handling cannot be overcome despite all precautions that should be taken when storing and handling human remains. Hence, exogenous human DNA contamination rates should be minimized (Llamas *et al.*, 2017). Human remains with a lower risk of contamination are

therefore preferred substrates. For example, the endocranial location of the petrous pyramid in intact skulls protects it from contaminating modern human DNA. Also, teeth situated in jaw sockets are preferred because of the jawbone encapsulating and thereby protecting the roots. Furthermore, sample locations that allow for adequate decontamination without loss of endogenous DNA are also appropriate. Decontamination protocols can include the irradiation of all sample sides with UV-C light, mechanical and/or chemical decontamination methods (Llamas *et al.*, 2017). Mechanical decontamination involves the physical removal of the outermost layer of the skeletal human remains using a sanding disc, a drill bit or a sand blaster (Llamas *et al.*, 2017; Rohland *et al.*, 2018). Alternatively, human remains can be chemically cleaned using diluted bleach (Corrêa *et al.*, 2021; Llamas *et al.*, 2017). Bleach treatment, however, not only excludes potential DNA contamination, it also damages the targeted endogenous DNA (Orlando *et al.*, 2021).

As mentioned before, sampling for DNA analysis is a destructive research method. Sampling can cause severe damage to the sampled human remains and the collected samples are consumed during the analysis. Hence, besides DNA content and contamination rates, the impact of DNA sampling should also be considered when selecting the substrate for sampling. For example, each skull contains only two petrous bones and, in some cases, just one is available. Sampling of the petrous part can result in irreplaceable damage to a precious cranium or result in loss of additional information (Hansen *et al.*, 2017). Teeth can be a valid alternative, in particular because of their larger quantity in individuals. Conversely, tooth sampling can be just as damaging, especially when only a few teeth are preserved or when teeth within jaws are forcefully removed. Thus, even though petrous bone preserves DNA extremely well and teeth are shown to provide high levels of endogenous DNA, other substrates should be considered as well. However, sampling methods for other skeletal human remains can also be destructive, including drilling or cutting and powdering the bones (Harney *et al.*, 2021). Moreover, analysis of these human remains is less likely to succeed due to the lower endogenous DNA content.

Regardless of the human remains used, as small as possible sample sizes should be taken using protocols as minimally destructive

as possible, thereby attempting to preserve the morphological traits of the sampled bones or teeth (Alpaslan-Roodenberg *et al.*, 2021; Prendergast and Sawchuk, 2018; Sirak & Sedig, 2019). Sample size depends on the DNA concentration, tissue density and subsequent DNA preservation of the human remains. Human remains with high DNA content for instance, allow for smaller sample sizes. Minimally invasive protocols for teeth involve only sampling the roots, without compromising the morphologically important tooth crowns (Cobb, 2002; Damgaard *et al.*, 2015; Harney *et al.*, 2021). Tooth cementum can be sampled using the protocol described by Damgaard *et al.* (2015) for instance. It involves separating the root from the crown followed by the removal of as much inner dentin from the root as possible. A hollow root enriched with cementum is eventually obtained and powdered. After root sampling, it is possible to place the tooth crown back into its jaw socket (Llamas *et al.*, 2017). This protocol however results in the loss of an entire root. In some cases, for example when isolated teeth are the only available biological material, loss of roots can still be too destructive. Therefore, a protocol without complete destruction of the root can be implemented (Harney *et al.*, 2021). This protocol involves the direct exposure of tooth roots to an incubation buffer without prior cutting or drilling. Teeth handled using this protocol thus remain completely intact, aside from sample discoloration. Dentin can also be sampled by removing the crown followed by drilling into the dentin or directly into the tooth (Alakoç & Aka, 2009; Cobb, 2002; Rudbeck *et al.*, 2005). Regarding petrous bones, choosing a disarticulated one is less destructive than sampling the petrous bone of a complete or reconstructed skull. Several methods for isolated petrous bones have been described before, including locating the otic capsule via bone removal or cutting the petrous in half and the subsequent bone powdering of the cochlea (Hansen *et al.*, 2017; Pinhasi *et al.*, 2019). Nevertheless, these methods significantly compromise the morphology of the petrous bone. Alternatively, generating bone samples of the cochlea can be performed by drilling a hole into the internal auditory meatus or into any part of the petrous portion giving direct access to the cochlea (Pinhasi *et al.*, 2019). However, even though the external characteristics of the petrous part remain largely preserved, these methods do not allow visualization of the cochlea which may result in less accurate sampling of the cochlea. In the case of a complete or reconstructed

cranium, it is not advisable to remove the temporal bone to access the petrous pyramid as this would result in major structural damage (Pinhasi *et al.*, 2019; Sirak *et al.*, 2017). Instead, a cranial base drilling method for sampling of the cochlea should be applied (Sirak *et al.*, 2017). This sampling protocol minimizes structural damage to the cranium whilst allowing access to the otic capsule by drilling from the cranial base. However, targeting the cochlea with the cranial base drilling method is also less accurate, compromising the quality of the bone samples. For all other types of skeletal human remains, it is possible to collect bone samples directly from the bone without cutting the bone into smaller fragments. By drilling straight into the bone surface, bone powder or bone chips can be obtained which creates a small hole in the sampled human remain (Mckinnon & Higgins, 2021; Rohland *et al.*, 2018). This method thus results in only limited destruction compared to protocols involving cutting the bone substrates prior to bone powdering.

Apart from the tooth incubation method described by Damgaard *et al.* (2015), all other sampling methods require drilling or milling to prepare powder or chips before DNA extraction. These methods, however, can damage the targeted DNA as drilling or milling with high speed generates heat (Adler *et al.*, 2011). Sampling of human skeletal remains should therefore always be done with low speed. Responsible treatment of human remains also includes reducing the need of additional sampling if supplementary analysis is required (Alpaslan-Roodenberg *et al.*, 2021). This can include the short-term preservation of residual sampled material. Preservation of material should always be discussed with and approved by all parties involved in the return of the human remains. These preserved samples may only be used for the purpose of repatriation and should be disposed of as soon as the process is completed.

In conclusion, there is not just one set of guidelines that applies to human remains. Above all other recommendations, it is advised to discuss the sampling of the human remains with all parties concerned on a case-by-case basis (Alpaslan-Roodenberg *et al.*, 2021). All elements addressed above must be carefully evaluated using all available information provided by experts in the field. A well-considered and supported strategy should be discussed and planned prior to sampling.

In all cases, sampling must be conducted by experts trained in DNA sampling techniques of bones and teeth as inexpert handling can result in severe damage of the human remain under investigation (Alpaslan-Roodenberg *et al.*, 2021; Prendergast & Sawchuk, 2018; Sirak & Sedig, 2019). It may also result in inaccurate targeting of the desired substrate leading to suboptimal results. Furthermore, as previously mentioned, the dedicated staff who are sampling human remains should also be trained in contamination control. Contamination management before, during and after the sampling of human remains is of utmost importance to limit the introduction of additional contemporary human DNA and cross contamination. All recommendations regarding sampling of human remains are summarized in Tab. 3.

4. DNA EXTRACTION

DNA extraction subsequent to sampling of the human remains of choice is aimed at retrieving the available DNA. These skeletal substrates often contain highly degraded DNA present in limited amounts. Thus, DNA extraction methods for human skeletal remains should be highly efficient and be able to maximize the recovery of primarily short DNA fragments (Rohland & Hofreiter, 2007a, 2007b; Xavier *et al.*, 2021). As bones and teeth often contain large amounts of molecules inhibiting subsequent PCR amplification, extraction protocols should also aim to minimize the co-extraction of these inhibitors.

In general, protocols for DNA extraction from samples originating from bones and teeth consist of three steps (Xavier *et al.*, 2021). First, the tissue and cell structures are chemically broken by incubation in a lysis buffer. This induces DNA release from the substrate into the lysis buffer. Most lysis buffers used for incubation of human skeletal remains contain ethylene diamine tetra-acetic acid (EDTA) (Loreille *et al.*, 2007). EDTA is able to bind certain chemical structures, such as calcium ions. One of the primary components of bone and tooth tissue, the inorganic mineral hydroxyapatite, is composed of several calcium compounds. EDTA thus demineralizes bones and teeth in incubation by binding these calcium ions, thereby releasing DNA molecules interacting with hydroxyapatite. Complete demineralization of bone or tooth tissue has been proven to be a very efficient protocol for

DNA extraction from skeletal human remains (Desmyter & De Greef, 2008; Jakubowska *et al.*, 2012; Loreille *et al.*, 2007). Besides hydroxyapatite, bone and teeth tissue are mainly composed of a protein called collagen (Loreille *et al.*, 2007). The protease proteinase K, which is able to digest proteins present in bones and teeth, is therefore added to the lysis buffer (Rohland & Hofreiter, 2007a). Furthermore, some studies showed that there is no improvement when using solutions with addition of other chemicals (Desmyter & De Greef, 2008; Rohland & Hofreiter, 2007a). It is therefore recommended to use a lysis buffer consisting of EDTA and proteinase K. Both bone powder and chips as well as teeth roots can be incubated using this lysis buffer.

The lysis buffer enriched with DNA is then supplemented with a highly concentrated salt-binding buffer, promoting the purification of the DNA by binding it to silica particles. For instance, DNA binding can be performed by adding silica-coated paramagnetic beads to the solution (Rohland *et al.*, 2018). The implementation of magnetic beads for DNA binding enables automation using specialized laboratory systems. This purification method, however, can only be performed in smaller volumes. Alternatively, a DNA purification protocol based on silica-spin columns combined with extension reservoirs has a higher volume capacity (Dabney *et al.*, 2013a; Rohland *et al.*, 2018). Moreover, silica-spin columns allow for pooling of multiple lysates in a single column, thereby increasing the total amount of endogenous DNA after purification (Xavier *et al.*, 2021).

The implemented purification protocol should enable the retrieval of short DNA fragments. Dabney *et al.* (2013a) for example developed a DNA purification technique that enables the retrieval of fragments as short as 35 bp by optimizing the binding buffer (Dabney *et al.*, 2013a; Rohland *et al.*, 2018). It is recommended to apply this protocol or any other protocol optimized for highly degraded skeletal samples to ancient human remains (Glocke & Meyer, 2017; Loreille *et al.*, 2007; Xavier *et al.*, 2021).

Lastly, co-extraction of inhibitors is minimized and adsorbed DNA is desalted by several wash steps with an ethanol-based solution after which DNA is eluted into a buffer with a low concentration of salt.

General recommendations

- ▶ The sampling strategy is case dependent and should be discussed with all parties concerned.
- ▶ Sampling must always be done by experts trained in contamination control and DNA sampling techniques of bones and teeth.
- ▶ Three parameters will influence the choice of sampling location: DNA content, contamination rate and destruction by sampling. All parameters should be evaluated thoroughly.

Recommendations regarding DNA content

- ▶ Sampling should aim to target DNA-rich substrates.
- ▶ Macroscopic preservation of bones and teeth should be evaluated visually. Macroscopically well-preserved substrates often yield higher amounts of DNA.
- ▶ DNA in bones is best preserved in dense cortical bones. The osseous inner ear part of the petrous bone is the densest bone of the human body, followed by weight-bearing long bones.
- ▶ DNA in teeth is best preserved in the outer layer of teeth, the cementum.
- ▶ Both petrous bones and teeth contain higher amounts of DNA than any other substrate. Petrous bone only outperforms teeth with poor macroscopic and molecular preservation.

Recommendations regarding contamination rates

- ▶ Human remains with a lower contamination risk are preferred substrates.
- ▶ Sample locations allowing for adequate decontamination without loss of endogenous DNA are also appropriate.
- ▶ Decontamination protocols of outer surfaces of skeletal human remains should consist of irradiation with UV-C light, mechanical cleaning and/or chemical cleaning.

Recommendations regarding sample destruction

- ▶ The destructive nature of DNA analysis should always be considered prior to sampling. Sample sizes should be as small as possible.
- ▶ The applied sampling protocol should be as minimally invasive as possible, aiming to preserve the morphological traits of the substrate.
- ▶ Teeth sampling protocols should only target the root.
- ▶ The sampling of isolated petrous bones is less invasive than the sampling of petrous bones in situ.
- ▶ Petrous bones located in complete or reconstructed crania should be sampled using the cranial base drilling method.
- ▶ Samples from all other skeletal human remains can be obtained by drilling straight into the bone surface to obtain bone powder or chips.
- ▶ Sampling by drilling or milling should always be done with low speed.
- ▶ Residual sampled material can be stored during the process of repatriation to reduce the need for additional sampling. Samples may only be stored if all stakeholders are in agreement.

Tab. 3 – Recommendations - Sampling

Eluted DNA is stable over a long period of time, especially when stored frozen. As downstream protocols only consume a part of the extraction product, DNA extracts are available for multiple analyses. Proper conservation of extraction products throughout the process of repatriation thus limits the need for additional sampling (Alpaslan-Roodenberg *et al.*, 2021). When agreed by all stakeholders, preservation of extraction products is recommended during the procedure. These molecular products may only be used for the purposes of repatriation.

Pretreatment of the sampled substrates for the removal of DNA contamination can be implemented prior to incubation in a lysis buffer (Damgaard *et al.*, 2015; Korlević *et al.*, 2015). Brief chemical or enzymatic treatment in a predigestion buffer removes a fraction of the contaminating DNA. Solutions can contain sodium hypochlorite, also known as bleach, sodium phosphate or EDTA and proteinase K. During this pretreatment step, a part of the exogenous DNA present on the surface of the substrate will be released into the predigestion buffer, whilst endogenous DNA is largely protected within the structure of bone or tooth tissue. This results in an increased proportion of endogenous DNA content. However, treatment

of samples in a predigestion buffer also adversely affects endogenous DNA by destroying part of it (Korlević *et al.*, 2015). Thus, digestion of samples prior to DNA extraction increases the percentage of endogenous DNA but lowers the overall endogenous DNA yield.

To monitor for contaminating DNA originating from chemicals, cross contamination or laboratory personnel during extraction protocols, it is recommended to include blank controls in every extraction lot (Llamas *et al.*, 2017). Blank or negative controls contain lysis buffer with no sample added and are processed in parallel with the samples using the same procedure, consumables and reagents (Rohland & Hofreiter, 2007b). All recommendations regarding DNA extraction are summarized in Tab. 4.

5. DNA PROFILING

A DNA extraction protocol aimed at the retrieval of as many degraded DNA fragments as possible should be accompanied with a DNA profiling protocol suited for low amounts of degraded DNA to obtain informative genetic data. DNA profiling can include Polymerase Chain Reaction (PCR)-based methods as well as Next-Generation Sequencing (NGS) or

General recommendations

- ▶ Samples originating from bones or teeth should be incubated in a lysis buffer consisting of EDTA and proteinase K.
- ▶ The lysis buffer should be supplemented with a highly concentrated salt optimized for the binding of short DNA fragments to silica particles.
- ▶ DNA binding can either be performed by adding silica-coated paramagnetic particles to the solution or by using silica-spin columns.
- ▶ Several wash steps for the desalting of DNA and removal of co-extracted inhibitors should be implemented using an ethanol-based solution.
- ▶ Elution of DNA is performed by adding a buffer with a low salt concentration.
- ▶ When agreed by all stakeholders, extraction products should be preserved for the purpose of supplemental analysis within the context of repatriation.

Recommendations regarding contamination management

- ▶ Brief chemical or enzymatic predigestion can be performed to reduce or remove exogenous DNA present on samples.
- ▶ Include negative controls in the extraction series to monitor contamination.

Tab 4 – Recommendations - DNA extraction

Massively Parallel Sequencing (MPS) protocols (Vai *et al.*, 2020), or a combination of both (Elwick *et al.*, 2023).

DNA profiling using PCR is based on the binding of primers to DNA fragments to produce a sufficient number of amplicons. These amplicons are then separated based on their length using capillary electrophoresis or sequenced by Sanger-based sequencing. The targeted fragments in aDNA samples are often of subamplicon size and contain DNA damage that can block amplification, causing partial or no amplification by PCR. Primers allowing for the amplification of shorter fragments are therefore designed; however, application of these mini-primers is more time-consuming and sample-consuming (Berger & Parson, 2009; Butler *et al.*, 2003; Kim *et al.*, 2013). Moreover, along with other technical issues, mini-amplicon sizes can still exceed fragment size present in aDNA samples.

Unlike PCR-based approaches, some NGS protocols do not rely on binding of primers directly to DNA fragments (Orlando *et al.*, 2015; Vai *et al.*, 2020). These protocols involve converting extracted samples into DNA libraries instead (Gansauge & Meyer, 2013; Orlando *et al.*, 2015; Vai *et al.*, 2020). Short DNA segments called adapters are ligated to both ends of the fragments during library preparation. These adaptors serve as binding sites for primers during PCR, enabling the amplification of DNA libraries. Amplified DNA libraries are then sequenced using NGS platforms. Hence, even very short fragments, which cannot be analyzed using regular PCR methods, can be recovered by NGS protocols. Sample-specific index sequences can be attached to or embedded in these adaptors (Kircher *et al.*, 2012; Meyer & Kircher, 2010). Index sequences or barcodes allow tracing back to the source of each DNA fragment when multiple libraries are pooled and processed in parallel. It is recommended to introduce indexes into both adapters or to process samples completely separate to avoid cross contamination and sample misidentification (Gansauge & Meyer, 2013; Kircher *et al.*, 2012).

Specific protocols have been developed for the preparation of DNA libraries originating from aDNA samples (Vai *et al.*, 2020). For example, Briggs *et al.* (2012) and Meyer *et al.* (2010) described a protocol producing DNA libraries containing double-stranded

fragments. Another double-stranded DNA library construction protocol for aDNA was described by Bentley *et al.* (2008). Both protocols are less suited for samples containing extremely degraded DNA present in only trace amounts (Bennett *et al.*, 2014; Vai *et al.*, 2020). Subsequently, the protocol proposed by Gansauge *et al.* (2013; 2020) was developed for these highly degraded DNA samples (Vai *et al.*, 2020). This protocol allows for the recovery of highly damaged DNA molecules as well as very short molecules which are frequently lost during double-stranded library methods (Gansauge & Meyer, 2013). However, single-stranded library preparation of ancient human remains does not always outperform double-stranded protocols (Bennett *et al.*, 2014; Wales *et al.*, 2015). The latter are often sufficient for the processing of samples with moderate to good preservation (Rohland *et al.*, 2018). Moreover, single-stranded DNA library preparation is more expensive and time-consuming than the double-stranded methods. It is therefore recommended that a preliminary screening with a double-stranded DNA library building protocol is performed (Wales *et al.*, 2015). Only samples originating from heavily degraded human remains could be analyzed directly using a single-stranded method.

Amplification of all DNA fragments converted into library molecules ensures the survival of the DNA library (Gansauge & Meyer, 2013). Amplified DNA libraries are thus available for multiple analyses, thereby minimizing the need for additional sampling. Conservation of the prepared DNA libraries throughout the process of repatriation is therefore advised (Alpaslan-Roodenberg *et al.*, 2021). DNA libraries should only be preserved in agreement with all parties involved and may only be used for the purposes of repatriation.

As mentioned before, most ancient human remains are characterized by low amounts of endogenous DNA embedded within high levels of contaminating microbial DNA (Llamas *et al.*, 2017; Orlando *et al.*, 2015; Rohland *et al.*, 2015). DNA libraries originating from these human remains would contain large fractions of exogenous DNA fragments, hindering subsequent sequencing protocols. In these samples, endogenous fragments can be enriched relative to other DNA molecules by in-solution hybridization capture (Gorden *et al.*, 2021; Orlando *et al.*, 2015). This target enrichment improves the accessibility to the

DNA molecules of interest during sequencing. Enrichment strategies can be implemented during library preparation by selectively incorporating damaged DNA molecules into libraries, or after library preparation by targeting regions of interest. These targets include for example mtDNA, sets of Single Nucleotide Polymorphisms (SNPs), chromosomes or whole genomes. Recent advancements even have enabled the target enrichment of DNA extracts containing extremely low endogenous DNA content (Suchan *et al.*, 2021). PCR-based methods are less prone to microbial DNA contamination as primers for PCR are human genome specific; however, even though these primers only sporadically bind to microbial DNA, the possible occurrence of micro-organism based artefacts should be considered during the evaluation of Short Tandem Repeat (STR) profiles (Smajlović-Skenderagić *et al.*, 2021).

Contrary to PCR-based methods, amplification of DNA libraries is not limited by miscoding DNA damage. However, nucleotide transformations lead to nucleotide misincorporations during sequencing which results in incorrect DNA profiles (Rohland *et al.*, 2015; Vai *et al.*, 2020). The occurrence of these damage patterns can be reduced by treating DNA libraries with the enzymes uracil-DNA-glycosylase (UDG) and endonuclease VIII (Gansauge & Meyer, 2013; Rohland *et al.*, 2015; Vai *et al.*, 2020). As removal of damaged nucleotides reduces error rates, treatment with UDG and endonuclease VIII should be considered.

However, damage patterns typically present in aDNA samples - short fragments containing nucleotide changes predominantly at the 5' and 3' ends of the fragments - can also be useful for the validation of authenticity of the results as well as for the identification of modern human contamination (Briggs *et al.*, 2007; Loreille *et al.*, 2018; Vai *et al.*, 2020). Authentication of NGS data is mainly based on the presence of nucleotide changes (Briggs *et al.*, 2007). Therefore, it is advised to preserve at least a part of the transformed nucleotides to differentiate between authentic DNA sequences and contamination (Gansauge & Meyer, 2013; Orlando *et al.*, 2015; Rohland *et al.*, 2015; Vai *et al.*, 2020). For instance, DNA authentication can be performed on untreated test libraries produced from fractions of samples (Orlando *et al.*, 2015; Rohland *et al.*, 2015). The remaining fractions can be treated with UDG

and endonuclease VIII to produce additional damage-free DNA libraries. Another approach produces single DNA libraries partially treated to remove most damage, but preserving damage signals at the ends of the molecules (Rohland *et al.*, 2015). Lastly, untreated libraries can also be of particular interest, namely for highly contaminated samples combined with target enrichment of damaged molecules. Besides the observation of nucleotide misincorporations, authentication of NGS data is also based on heterozygosity levels in haploid markers (Krause *et al.*, 2010; Orlando *et al.*, 2021; Rasmussen *et al.*, 2010). Heterozygosity levels in mtDNA and the X-chromosome (only in males) are estimated by calculating consensus rates among multiple sequences.

Conversely, authentication of data obtained using PCR-based methods is more difficult. Even though the high allele variability of STR markers allows for the detection of multiple contributors which indicates the presence of modern human contamination, exogenous human DNA contamination cannot easily be excluded. Moreover, authentication of single source profiles is challenging, as it is mainly based on fragment size.

NGS can thus overcome most limitations encountered during PCR-based methods, making it the preferred platform for DNA profiling of human remains. Nevertheless, it is possible for PCR-based methods to also yield useable data, especially when analyzing mtDNA in well-preserved human remains (Vai *et al.*, 2020). Conversely, the implementation of NGS does not necessarily lead to successful generation of DNA profiles. For example, aDNA studies are often characterized by the screening of many samples in order to identify a subset of samples promising for further analysis (Rohland *et al.*, 2015).

The method of choice depends on the research question and thus the type of DNA data that needs to be generated. It is thus advised to define the research question prior to the analysis of the human remains (Alpaslan-Roodenberg *et al.*, 2021). Research questions should always be formulated in collaboration with all stakeholders.

Appropriate controls similar to controls in extraction protocols should always be included, regardless of the profiling approach (Gansauge & Meyer, 2013; Llamas *et al.*, 2017; Rohland

et al., 2018). Besides positive and negative controls, blank controls carried through the extraction protocol and all downstream steps should also be included. All recommendations regarding DNA profiling are summarized in

Tab. 5. Extraction blanks and negative controls monitor for contaminating DNA introduced during laboratory procedures. Additionally, sample-specific barcodes ligated to DNA fragments during the preparation of DNA libraries

General recommendations

- ▶ Research questions should always be formulated prior to the start of DNA analyses, in collaboration with all stakeholders.
- ▶ PCR-based methods or NGS protocols can be chosen dependent on the research question.

Recommendations regarding PCR-based methods

- ▶ PCR-based methods are of particular interest when analyzing mtDNA.
- ▶ Primers producing mini-amplicons are preferred over regular primers.

Recommendations regarding NGS protocols

- ▶ NGS is the preferred platform for DNA profiling of ancient human remains.
- ▶ Double-indexing of library fragments is recommended whenever samples are pooled or processed in parallel.
- ▶ Both double-stranded and single-stranded DNA libraries can be constructed. It is recommended to perform a preliminary screening with a double-stranded DNA library building protocol. Heavily degraded samples are preferably analyzed using a single-stranded DNA library.
- ▶ In agreement with all parties involved, DNA libraries should be preserved for the purpose of supplemental analysis within the context of repatriation.
- ▶ Target enrichment can improve the accessibility to the DNA molecules of interest during sequencing.
- ▶ Damage patterns characteristic for aDNA samples can be reduced by uracil-DNA-glycosylase (UDG) and endonuclease VIII treatment.

Recommendations regarding contamination management

- ▶ Authentication via NGS is based on the identification of nucleotide changes typically present in aDNA samples as well as on consensus rates in mtDNA and the X-chromosome (male-only).
- ▶ Authentication via PCR is based on the presence of very short DNA fragments, making it more difficult to authenticate obtained DNA data.
- ▶ Positive and negative controls should always be implemented during DNA profiling.
- ▶ Blank controls carried through the extraction protocol and all downstream steps should also be included.
- ▶ In NGS protocols, ligation of sample-specific barcodes or indexes to DNA fragments during library preparation can detect contamination in subsequent steps.

Tab. 5 – Recommendations - DNA profiling

for NGS allow for the detection of modern human DNA contamination as well as cross contamination in subsequent steps (Llamas *et al.*, 2017; Rohland *et al.*, 2015).

6. POSSIBILITIES AND LIMITATIONS OF GENETIC ANALYSIS

In the light of repatriation of human remains there are three types of genetic analyses that could be applied: sex indication, genetic kinship testing and inferring the genetic ancestry. Other applications of genetic analysis are not discussed as these are considered to be beyond the scope of the repatriation process.

6.1. Sex indication

Sex indication of human remains is an essential part of human identification. Of all the analyses discussed, indication of sex is the most frequently performed. However, it is important to recognize that chromosomal sex is only one element in how sex and gender are contextually and culturally defined (Fowler *et al.*, 2022).

Biological sex indication by DNA analysis is based on the fact that men, in contrast to women, have a Y chromosome. Therefore, Y chromosome-specific markers are highly useful in sex estimation of human skeletal remains (Kayser, 2017). Relying on a test that solely measures the presence of Y-chromosomal DNA in a human DNA sample, however, could lead to false sex indication (Jobling *et al.*, 2019). Indeed, if no Y-chromosomal DNA is detected this could be a proof of female origin, but it could also be due to the presence of too little and/or too degraded DNA.

The most popular marker for sex typing is amelogenin, a gene contributing to the tooth enamel formation. The amelogenin gene is present on both the X and Y chromosome with a variation in base pair size. The X chromosome, present in both men and women, acts as an internal control in sex testing. Amelogenin-based testing has been shown to be successful in sex indication of human remains (Alterauge *et al.*, 2021; Coble *et al.*, 2009; Rothe *et al.*, 2015).

Even though amelogenin-based testing is an effective method for sexing, problems due to different causes (e.g. population-specific

genetics, particular mutations) could be encountered (Dash *et al.*, 2020). Therefore, other Y-chromosomal markers have been developed to be used as an alternative to or in combination with amelogenin (Butler & Li, 2014).

Molecular methods that rely on particular sex-specific markers not only are compromised by allelic dropout but also by modern exogenous contamination (Mittnik *et al.*, 2016; Skoglund *et al.*, 2013). Biological sex indication using shotgun sequencing data can overcome these issues. Shotgun sequencing sexing based approaches rely on the ratios of the number of reads. For example, R_y indicates sex by considering the ratio of sequences mapped to the Y-chromosome and the sequences mapped to both sex chromosomes (Skoglund *et al.*, 2013). An alternative method calculates R_x , the number of alignments to the X-chromosome compared to the number of sequences originating from the autosomes (Mittnik *et al.*, 2016). Both methods have been applied successfully in the biological sexing of human remains (Loreille *et al.*, 2018).

Besides the technical issues, the sex of an individual is more complicated than the presence or absence of a Y chromosome. For example, the sex chromosomes (XX or XY) from individuals with differences or disorders of sex development (DSDs) can be contradictory to their anatomical sex (Ainsworth, 2015). As genetic sex not always equals a person's phenotype, the outcome of molecular sexing should thus be interpreted with care.

6.2. Genetic kinship testing

Genetic kinship testing evaluates the biological relationship between individuals. It is based on the fundamental principle of genetics that individuals who are closely related share DNA from their common ancestors. The more distant the relationship, the less autosomal DNA is shared. In theory, a parent and an offspring share 50 % of the autosomal DNA, which means that per generation the amount of shared autosomal DNA by descent is halved. For example, on average only 3.1 % is shared between an individual and their great-great-great-grandparent (5 generations). It is generally accepted that autosomal DNA testing is limited to investigating family relationships within the last five or six generations. Beyond that, genetic segments become too small and eventually disappear due to chance (Perego *et al.*, 2019; Phillips *et al.*,

2012; Wild, 2021). In addition, the amount of DNA that gets passed from parents to offspring can vary because of recombination resulting in new combinations of polymorphisms (Wild, 2021).

Within the scope of repatriation genetic kinship testing could assist in different purposes, like the identification of human remains or the evaluation of kinship scenarios between multiple human remains. But regardless of the scenario, the same requirements are applicable: (a) the number and type of genetic markers, (b) the *a priori* assumptions, (c) the tested relatives and (d) the genetic population data. For each case, the extent to which these principles are met will impact the outcome of the kinship test.

The most studied polymorphisms in the human DNA with respect to kinship testing are STRs and SNPs. The choice between autosomal STRs and SNPs depends on the availability of instrumentation, the fragmentation of DNA in the questioned human remains and the number of generations between the questioned individuals. The ubiquitous CE technology, limited in the number of genetic markers that can be analyzed in a single experiment, could be used for STR markers. These length polymorphism markers have a higher variability than SNPs, hence less markers in the DNA need to be analyzed when dealing with STRs instead of SNPs. In order to obtain the same informative power 5 times more SNPs are needed compared to STRs (Amorim & Pereira, 2005; Gill, 2001). However, the analysis of STR markers requires the presence of larger DNA fragments, which could be problematic when analyzing human remains mostly containing aDNA. Indeed, while STR analysis amplifies fragments up to 300 bp, the region of interest of SNPs is only a single nucleotide, implying the amplification of fragments to a minimum of 30 bp (Alteraue et al., 2021; Xavier et al., 2021). NGS technology allows many more SNPs to be analyzed from a single sample in a single experiment. These high density SNP data allow pairwise kinship testing beyond the first cousin (3 generations) limit of STR testing (Amorim et al., 2018; Kling et al., 2021b; Mitnik et al., 2019; Moltke et al., 2021; Ringbauer et al., 2024; Tillmar et al., 2021).

If the relationship of the questioned individuals spans more than 6 generations, only the analysis of the haploid lineage markers mtDNA and Y-chromosomal DNA could assist (King et al., 2014). Lineage markers, however, are less discriminating than autosomal markers. Also,

not all consenting relatives are appropriate for genetic relationship testing by lineage markers as both uniparental markers are only shared between individuals that belong to direct maternal or paternal lines. Consequently, associations between individuals that do not share the same maternal or paternal line could not be identified by analyzing these markers, even if they are closely related. Y-chromosomal haplotyping is further limited as it is only present in male individuals. The genetic information gained from haplotyping could be increased by analyzing relatives from both the paternal and maternal lineage of the questioned remains (Coble et al., 2009).

Kinship investigations could also be performed using a combination of autosomal and haploid markers (Ambers et al., 2014; Coble et al., 2009; Fowler et al., 2022; Irwin et al., 2007; Rothe et al., 2015). The application of autosomal markers does not exclude the use of lineage markers, and vice versa. In particular cases, the combined analysis of both marker types could increase the genetic information for kinship interpretation.

Besides the choice of autosomal marker (STR, SNP), whether or not in combination with haploid markers, other issues should also be considered. *A priori* assumptions about the identity of the questioned remains should be made through contextual information, including amongst others historical, archaeological and medical data (Alteraue et al., 2021).

The contextual data should result in a well-documented family pedigree. Depending on the availability of relatives, the pedigree should contain all generations between the questioned remains and their modern living descendants (Gill et al., 1994; King et al., 2014) or other deceased ancient relatives with known identities (Charlier et al., 2013; Larmuseau et al., 2014). If kinship between human remains is questioned, their genetic relationship should also be anticipated by contextual data, whether this results in a documented pedigree or not (Alteraue et al., 2021; Vai et al., 2020). If considering numerous generations back in time, everybody is mutually related (Kling et al., 2021a). It therefore only makes sense to define relatedness between individuals with respect to a pedigree.

Genetic kinship strictly denotes the biological relationship between individuals, not the social relationships. The interpretation of the

obtained DNA profiles with reference to the given pedigree should however consider the possibility that the social parent is not always the biological parent, even in cases where the necessary reliable national registers and birth certificates are available and accessible (Copeland, 2020). The number of false biological father-child relationships is expected to vary between populations (Greeff & Erasmus, 2015). Estimated rates vary from 1-2 % to 10 % in different studies (Kling *et al.*, 2021a). Nevertheless, the interpretation of the genetic data should avoid the pitfall of possible biological anomalies in family relationships and consider possible alternatives. Hence, a paternity mismatch should not influence the identification of the human remain in question (King *et al.*, 2014).

It is therefore good practice to involve multiple relatives of a deceased individual, if available. Logically, testing closer relatives should result in a lower chance for paternity mismatch as there are less paternity events in the given pedigree compared to more distantly related relatives. Moreover, as close relatives share more DNA compared to more distant relatives, it is advised to analyze the closest relative(s) of the human remains in question whenever possible. However, the chance to trace back the mismatching event increases when less paternity events are considered, which could result in privacy issues. The stakeholders should thus consider a policy about the management and communication of possible false biological paternity events in a given pedigree.

Increasing the number of tested relatives from a given pedigree is not only interesting to circumvent possible false biological paternity events in the given pedigree, but it will also determine the outcome of the identification in a positive way. In general, a more complete pedigree will result in a more reliable identification (Kling *et al.*, 2021a).

If autosomal STR data are generated, the information must be quantified performing statistical interpretation. Therefore, some genetic parameters, like allele frequencies of the population to which the studied individuals belong, should be known (Kling & Tillmar, 2019). When data for the given population are not available, kinship evaluation by STR data can be limited (Zvénigorosky *et al.*, 2020). Besides the lack of reliable allele frequencies, the possible genetic substructure of the given population by endogamy could also be an issue for statistical calculations (Alterauge *et al.*, 2021).

In contrast to STR data, high-density SNP data generated by NGS do not need these data as a measure of relatedness can be provided without any prior information.

If lineage markers are assessed the interpretation leads to in an exclusion when the haplotypes of the tested individuals do not match. In the case of matching haplotypes, the probability of a coincidental match instead of a match by kinship should be evaluated. This probability is assessed by determining the frequency of the particular haplotype in an appropriate database of the relevant population(s). High quality population databases for mtDNA and Y-chromosomal haplotypes (respectively EMPOP and YHRD, respectively) containing representative and quality-checked datasets have been established (Parson & Dür, 2007; Willuweit & Roewer, 2015). As both databases can show underrepresentation of particular regions compared to others, their appropriateness should be evaluated case by case (Göbel *et al.*, 2020).

Finally, to establish a final decision the obtained genetic data should be combined with other data and records, even including possible discordant elements (Alterauge *et al.*, 2021; Coble *et al.*, 2009; King *et al.*, 2014). After all, the outcome of genetic analysis should be considered along with other investigations in the decision-making process of repatriation cases. The stakeholders should balance the weight of both genetic and non-genetic data, which could be done in a statistical way (Egeland *et al.*, 2000; King *et al.*, 2014).

6.3. Inference of genetic ancestry

Some human remains currently present in Belgian FSIs and other public and private institutions have no recorded provenance. Their repatriation could be very difficult or even impossible if the identification of the appropriate communities is a requirement for return. Inferring the genetic ancestry of the human remains via DNA analysis could assist in this process by defining an association between an individual and a population.

Importantly, genetic ancestry solely concerns the geographical region(s) from which a person's biological ancestors originated. It does not correspond to concepts such as ethnicity or "race", that are defined by non-genetic

factors. For the same reason, biogeographical ancestry cannot be equated with language, religion, or other cultural or traditional manifestations (Schneider *et al.*, 2019).

Worldwide, genetic differences between human populations increase with geographical distance. Inferring biogeographical ancestry by DNA analysis is based on this genetic human population diversity. The contemporary human genetic population structure is a result of multiple events in the past, such as regional variation in selection (e.g. depigmentation in Europeans), genetic drift (the change in frequency of a genetic variant in the population due to random chance) and admixture (a sudden increase in gene flow between two differentiated populations) (Phillips, 2015). Following these events, the human genetic population structure evolves over time. This time-dependent character should be considered when studying ancient human remains in relation to their biogeographical ancestry (Wright *et al.*, 2018).

For the DNA-based inference of a person's biogeographical ancestry, specific genetic markers referred to as ancestry-informative markers (AIM) are analyzed. AIMs are markers observed solely in population groups from particular geographical regions or which are very common in one geographical region while rare in others (Phillips, 2015). As AIMs are located in autosomal DNA, Y-chromosomal DNA and mtDNA, they reflect the geographical origin of both parents, the paternal ancestors and the maternal ancestors, respectively.

Autosomal AIMs, mostly SNPs, are selected from population genetic studies around the world. They can be used to determine the biogeographical ancestry of a person at the level of the continental regions Europe, sub-Saharan Africa, East Asia, South Asia, Oceania, and the Americas (referring to the respective indigenous populations) (De la Puente *et al.*, 2021). Considerable information about the biogeographical provenance of an individual can only be obtained if reliable and appropriate population genetic data are used as a reference. Moreover, the geographical spectrum of ancestry of a tested individual can only be described if that spectrum of ancestry is well represented in the consulted population studies (Schneider *et al.*, 2019). Therefore, it is good practice to refer to the population genetic reference data set when reporting biogeographical ancestry.

By increasing the number of AIMs, which is possible with NGS whole genome and genome-wide sequencing, the possibility to determine ancestry at subcontinental level is enhanced. However, it is not always appropriate to define small populations confined to narrow regions. Besides the number of analyzed SNPs, the genetic substructure of a population can also influence the accuracy of the origin inference. This should be studied case by case.

The possibilities and limitations of DNA-based biogeographical origin inference at a subcontinental level within the scope of repatriation of ancient human remains is well illustrated by Wright *et al.* (2018). Their study evaluated the potential of autosomal, Y-chromosomal and mtDNA markers to identify the origin of unprovenanced ancient human remains from Aboriginal Australians. In this study, DNA data from both pre-European Aboriginal Australian human remains and contemporary Aboriginal Australians were studied. Pre-European DNA data were recovered from human remains from archaeological excavations of known burial sites or previously repatriated remains of known provenance. As a high degree of accuracy of provenance is required for contemporary Aboriginal Australians, only DNA data with a published geographic affiliation were used. Due to admixture, mainly from European colonization, Y-chromosomal DNA was not considered to be a good AIM. MtDNA alone was also not recommended as some mitochondrial haplotypes had no contemporary matches or they were geographically widespread, which resulted in an inconclusive interpretation. Autosomal DNA data, obtained by whole genome sequencing, revealed a substantial ancient population structure, based on three subpopulations. This ancient population structure shows strong genetic affinities between ancient pre-European and contemporary Aboriginal Australian genomes originating from the same geographical location. The authors state that their findings suggest that a similar approach could be used for the return of other indigenous human remains if the ancient population history is known and a contemporary database is available.

Despite the fact that, according to Wright (*et al.* 2018, p. 6), Y-chromosomal and mitochondrial markers were inappropriate markers to infer biogeographical origin of Aboriginal Australians, lineage markers have proven to be useful in other cases (e.g. Rothe *et al.*, 2015;

Ambers *et al.*, 2016). Ideally, a combination of markers, which increases the genetic information for inferring the biogeographical origin of an individual, should be used (Ambers *et al.*, 2016; Diepenbroek *et al.*, 2021; Rothe *et al.*, 2015). It is recommended to evaluate the appropriateness of AIMs case-specific considering the abovementioned genetic requirements as well as contextual information and data from other scientific disciplines (e.g. isotopes, anthropology). The combination of genetic and non-genetic data results in a multidisciplinary evaluation of the provenance of the questioned human remains.

7. ETHICAL MANAGEMENT

Working with human remains comes with great responsibility towards the deceased ancient individuals as well as to their present living descendants. Dedicated DNA laboratory staff and geneticists engaged in repatriation of human remains should be aware of the sensitive nature of the work they are conducting. Besides laboratory best practices, they should therefore also follow some other best practices and supplemental guidelines.

The most important task of DNA experts is providing information to all parties involved in the repatriation of human remains. As genetics is a challenging subject, most people do not know what to expect from DNA analysis. It is therefore essential to explain the possibilities as well as the limitations of DNA analysis (Alpaslan-Roodenberg *et al.*, 2021). The possible outcomes of genetic analysis and interpretation should also be communicated in advance, as results can differ from what was known or expected. All information must always be presented in a way that is comprehensible for a non-specialist audience. After genetic analysis, the results should be made available to all stakeholders. Results and interpretation should also be communicated in a transparent way allowing verification or critical re-examination of the results (Alpaslan-Roodenberg *et al.*, 2021; Larmuseau and Bodner, 2018).

As part of informing all stakeholders, a detailed plan should be prepared before the beginning of any DNA analysis. This plan should include the sampling strategy and the expected impact on the studied human remains. As aforementioned, the sampling site and approach

should always be defined in consultation with all stakeholders. Furthermore, it should involve a full description of all the techniques that will be used, from DNA extraction to DNA profiling, and the type of DNA data associated with DNA profiling techniques, that will be generated. All stakeholders should approve this plan prior to genetic analysis of human remains. Laboratory staff may only work within the scope of this research plan, any adjustments should always be consulted with those involved in the original plan.

Before proceeding to the genetic analysis of any type of biological sample, DNA experts must ensure that all regulations were followed. The stated investigations and treatment of obtained data may never be in conflict with the applicable (inter)national legal framework(s). Furthermore, members of associated communities should always be consulted first regarding destructive genetic analysis (Balter, 2017). Importantly, communities should not only be represented by individuals claiming to be descendants of the human remains, but also by the larger community (Curry, 2021). Communities should be involved in the genetic analysis as part of the group of stakeholders. Consulting indigenous communities is not the responsibility of DNA laboratories; however, DNA laboratories should verify if native people were consulted regarding these destructive analyses. Researchers should not proceed with the analysis of the human remains until communities show support. Consultation of possible associated communities is nearly often impossible in the case of unprovenanced remains. However, stakeholders should at least try their best to seek any information regarding possible provenance and associated communities of the human remains.

Human remains should always be treated with care and respect as they are precious and finite specimens (Alpaslan-Roodenberg *et al.*, 2021). This includes making strict agreements regarding transfer of the remains between those responsible for the human remains and the researchers. Transfer of human remains to the DNA laboratory should be recorded and a date for the return should be discussed. Human remains should never be in the stewardship of the laboratory for a long period of time, they should always be returned at the earliest possible time. Responsible treatment also includes minimizing the impact of sampling on human remains. Guidelines regarding minimally destructive sampling are covered in section 3. Sampling of the human remains. In addition,

the morphology of the examined human remains should be documented appropriately (e.g. photography, micro-CT scans, casts, 3D-scans which were taken) prior to sampling. Lastly, researchers must ensure that data generated from specimens in the context of repatriation are never exploited for other purposes or submitted to any (scientific) database (Curry, 2021; Wild, 2021). As genetic data contains a lot of information about an individual, DNA laboratories may only work within the strict

boundaries of the predetermined plan. Data storage and safeguarding should further be discussed with all stakeholders and can be incorporated in the research plan.

Regularly, it is required to obtain genetic profiles of living individuals for comparison. In these cases, it is important to provide sufficient information to and get permission from individuals serving as a reference through an informed consent. This informed consent

Recommendations regarding information sharing

- ▶ Communication should be adapted to a non-specialist audience.
- ▶ Possibilities, limitations and possible outcomes should be communicated to all stakeholders prior to genetic analysis.
- ▶ After genetic analysis, results should be made available to stakeholders.
- ▶ Communication should be transparent, allowing verification or re-examination of the results.
- ▶ A detailed plan containing the sampling strategy and estimated impact, a full description of the techniques that will be used and the type of DNA data that will be generated should be prepared and approved prior to genetic analysis.

Recommendations regarding responsible treatment of human remains

- ▶ Strict agreements regarding transfer of human remains between those responsible for the remains and laboratory staff must be made.
- ▶ The impact of sampling should be minimized.
- ▶ The morphology of the examined human remains should be documented appropriately before sampling.
- ▶ Data obtained from human remains may never be exploited for purposes other than repatriation.
- ▶ Data obtained from human remains that are to be repatriated may never be submitted to any (scientific) database.

Recommendations regarding other ethical issues

- ▶ Stated investigations and treatment of obtained data may never be in conflict with the applicable (inter)national legal framework(s).
- ▶ Members of communities associated with human remains should be involved in the genetic analysis of human remains as part of the group of stakeholders.
- ▶ Living individuals engaging in the genetic analysis of human remains by providing DNA samples should only agree to participate through an informed consent.
- ▶ Genetic information obtained from participants may never be exploited for other genetic studies.
- ▶ Genetic information obtained from participants may never be uploaded to any (scientific) database.
- ▶ Reference samples can be collected by a buccal swab.

Tab. 6 – Recommendations - Ethical management

should include information about how and when the genetic data will be used, clearly indicating the data will only be used within the scope of return of human remains. Genetic information obtained from participants may never be exploited for any larger genetic study (Wild, 2021). Moreover, DNA experts must ensure that genetic data is not uploaded to any (scientific) database (Schiermeier, 2021). Participating individuals should also be notified that the disclosure of their genetic data could reveal privacy issues, not only for the participants themselves, but also for their families and even communities. After obtaining informed consent, it is recommended to collect reference samples by means of a buccal swab because of its minimally invasive and easy performable nature. Reference samples should be analyzed separately from the human remains in time and space to avoid contamination. All recommendations regarding ethical management are summarized in Tab. 6.

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