UNIVERSIDADE D
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# GENOME-WIDE DNA ANALYSIS FROM BURNED PETROUS BONES USING aDNA EXTRACTING METHODS AND NEXT GENERATION SEQUENCING

## AN EXPERIMENTAL STUDY

**VOLUME 1**

**Dissertação no âmbito do Mestrado em Antropologia Forense orientada pelo a Professora Doutora Eugénia Cunha, co-orientada pelo Professor Doutor Ron Pinhasi e apresentada ao Departamento de Ciências da Vida da Universidade de Coimbra.**

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*Ashes denote that fire was;*
*Respect the grayest pile*
*For the departed creature's sake*
*That hovered there awhile.*

*Fire exists the first in light,*
*And then consolidates, —*
*Only the chemist can disclose*
*Into what carbonates.*

Fire by Emily Dickinson

# Table of Content

## Abstract

The identification of severely degraded human remains, such as burn victims and cremated remains from mass fatalities or crimes against humanity, present a particular challenge in Forensic Anthropology. The present study experimentally investigated how the combustion of human petrous bones affects the DNA present in the cochlea. In addition, the discolouration of the bones and the weight loss due to the exposure to heat were analysed. The petrous bones (n = 8, four of them ancient and four modern) were randomly assigned to a temperature between 145 °C and 500 °C, with a modern sample always following an ancient one, and heated to the selected temperature in an electric muffle-furnace with a thermostat. The cochlea was then extracted and milled into bone powder. DNA was extracted from the bone powder using the Dabney protocol (Dabney et al., 2013a) and sequenced using a *Next Generation Sequencing* method. The results show that although highly fragmented, valuable DNA material could be extracted from the samples up to a temperature of 500 °C. Furthermore, there is a proportional relationship between the intensity of the heat exposure and the weight loss of the samples. The surface colour of the bones also changes gradually to the heat intensity from pale brown to a light grey colour. Given the promising results of this pilot study, further research is needed to investigate appropriate DNA extraction and sequencing methods for severely degraded forensic samples to help facilitate the process towards positive identification of human remains from particularly challenging contexts.

*Keywords: Burnt bones, DNA, aDNA, Next Generation Sequencing, Fire death, Degraded DNA, Forensic identification*

## Resumo

A identificação de restos humanos severamente degradados, tais como vítimas carbonizadas e restos cremados de mortes de desastres em massa e crimes contra a humanidade, apresenta um desafio particular na antropologia forense. O presente estudo investiga experimentalmente como a combustão da parte petroseal do temporal humano afecta o ADN presente na cóclea. Além disso, foi analisada a descoloração dos ossos e a perda de peso devido à exposição ao calor. As porções petroseais (n = 8, quatro deles antigos e quatro modernos) foram aleatoriamente submetidos a uma temperatura entre 145 °C e 500 °C, com uma amostra moderna sempre a seguir a uma antiga, e aquecida à temperatura selecionada num forno eléctrico de mufla com um termóstato. A cóclea era então extraída e moída em pó de osso. O ADN foi extraído do pó ósseo usando o protocolo Dabney (Dabney et al., 2013a) e sequenciado usando um método de sequenciação da Next Generation. Os resultados mostram que embora altamente fragmentado, ainda pode ser extraído ADN valioso das amostras até uma temperatura de 500 °C. Além disso, existe uma relação proporcional entre a intensidade da exposição ao calor e a perda de peso das amostras. A cor da superfície dos ossos também muda gradualmente com a intensidade de calor de castanho pálido para uma cor cinzenta clara. Dados os resultados promissores deste estudo piloto, é necessária mais investigação para investigar métodos apropriados de extração e sequenciação de ADN para amostras forenses severamente degradadas para ajudar e facilitar o processo de identificação positiva de restos humanos de contextos particularmente desafiantes.



*Palavras-chave: Ossos queimados, ADN, aDNA, sequenciação Next Generation, morte por fogo, ADN degradado, identificação forense*

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# 1. Introduction

The victim identification is historically deeply rooted in Forensic Anthropology and is one of the key tasks of the discipline. During the identification process, Forensic Anthropologists usually create a biological profile and look for secondary identifiers such as medical findings and prostheses, among others. In this way, the number of possible individuals can at least be narrowed down, if not a positive identification can be achieved (Cunha & Cattaneo, 2017). But taphonomic factors can alter or destroy diagnostically relevant traits of a skeleton and thus limit the scope of Forensic Anthropologists. Besides individual factors such as body weight, age at death or sex, extrinsic factors have a major effect to the remains. Mann et al. refer to temperature, UV radiation, water, burial depth, acidity and clothing as physical factors, and carnivores, rodents, insects, bodily trauma, and plant roots as biological factors. Whereas temperature, water, and exposure tend to have the greatest effect on the skeleton (Mann et al., 1990).

Death and fire have always been a frequently witnessed duo. On the one hand, there are cases of natural disasters such as bushfires, forest fires or mass fatality events like plane crashes, but also accidents such as house fires or car fires in which people die in the flames. But fire can also be used as a weapon to kill a human being or to conceal the cause of death and make the existing physical evidence of a murder disappear. Last but not least, there is the context of war and crimes against humanity, where fire is used as a brutal weapon or as a means to cover up the executed crimes. The identification of fire victims is characterised by special constraints and limitations. The soft tissue may be completely burnt and even the skeleton may be fragmented. This often prevents visual identification, complicates the establishment of a biological profile, and even odontological identification, which otherwise would be considered the primary identifier, can be rendered impossible by exposure of the skull to very high temperatures. If feet and hands are lost, friction ridge analysis can also be excluded as the primary identifier of the victim (Pokines, 2013). Since, according to *INTERPOL*, positive identification is only possible if the post-mortem data entirely match the ante-mortem data, genetic analysis is often the only way to achieve positive identification in such cases, and this is only an option if there already exists a suspicion of identity and thus a comparison sample (Cunha & Cattaneo, 2017). In such cases, where positive identification is to be made by DNA analysis, the triage role, especially in mass fatalities and crimes against humanity, is the main task of Forensic Anthropology in the current distribution of responsibilities. By analysing important biological parameters, especially the estimation of sex, age at death, skeletal pathologies, skeletal anomalies, and variations, can be crucial indicators to narrow down the possible individuals and thus accelerate the identification process (de Boer et al., 2019).

However, the field of genetics has had a great influence on Forensic Anthropology in the last few years and due to the recent research successes, it's probable that the influence will continue to increase. This is not only about research in molecular genetics itself, such as the increasingly far-reaching understanding of methylation processes (Correia Dias et al., 2022; Correia Dias et al., 2020b; Hong et al., 2017; Huang et al., 2015) or the influence of ancient DNA research in forensics (Alias & Swaminathan, 2016; Gaudio et al., 2019; Gettings et al., 2015; Jäger et al., 2017; Xavier et al., 2021), but also about the

expansion of the necessary databases, e.g., the global DNA database for identifying missing Persons *I-Familia (INTERPOL, 2021)*. However, studies of DNA extraction and analysis in burnt bone have revealed a rather disillusioning reality of this technology. At a relatively early stage of combustion, the organic matrix of the bone is already attacked, and with it the DNA present in it (Imaizumi, 2015). However, the exact threshold of DNA survival under heat is still unclear and requires further research. In this thesis, an experimental study is conducted. It is intended to contribute to narrow this gap. The aim of this project is therefore to investigate the destruction behaviour of DNA in bones under controlled temperature. In order to recover the highest possible amount of DNA, an optimal sampling strategy will be worked out, and methods from the field of ancient DNA (aDNA) will be used for high-yield extraction and sequencing. Side effects such as colour changes or possible weight loss are also observed and documented. The results of this project are intended to contribute to reducing the barriers that exist in the identification of fire victims and to increase the chance of a positive identification through DNA analysis.

# 2. Heat induced alteration of the bone

Burnt bones pose a particular challenge for Forensic Anthropologists. Under heat, organic and inorganic components of the bone are altered or destroyed. The resulting profound changes in the bone, such as colour changes, shrinkage, weight loss and other deformations, lead to constrains regarding osteological measurements and morphological observations. These characteristics of burnt bone result from the interaction of the factors of temperature and exposure time. It appears that there is a general basic pattern in the thermal alteration of bones, but no specific correlation between the individual characteristics of burnt bones and the duration and intensity of the fire has yet been established. However, studies such as those by the research teams around Tim Thompson (Thompson & Ulguim, 2016; Thompson, 2004) or David Gonçalves (Gonçalves et al., 2013) are contributing to closing this gap as well. Since these three factors played a greater role than others in this thesis, the changes in surface colour, in weight and the pyrolysis of DNA are discussed in more detail in this chapter. Anything else would go beyond the scope of this thesis.

## 2.1 Colouration

As *Baby* pointed out in 1954, the recording and description of the surface colour of heat-damaged bones is crucial (Baby, 1954). To a certain extent, these colour changes can provide information about the temperature to which the remains were exposed (Baby, 1954; Bonucci & Graziani, 1975). When bones are heated, dehydration (100 °C – 600 °C) of the bone structure occurs first, followed by decomposition (300 °C – 800 °C), then inversion (500 °C – 1100 °C) and finally fusion of the matrix (700 + °C). It is mainly the decomposition of the bone structure that affects the surface colouration of the bone (Thompson, 2004). With increasing exposure to heat, the bone goes through a sequence of colours, from unburned very pale brown to reddish, dark brown colouring to black, light blueish, and grey and finally white (Devlin & Herrmann, 2015). Figure 1 shows the discolouration process under the influence of heat in bones, Figure 2 in teeth. The initial black colouration and the increasing brittleness of the bone are caused by the burning of organic materials such as collagen and carbon. Prolonged and/or more intense exposure to heat subsequently attack the crystalline structures of the bone matrix. This leads to the blueish, grey, and finally white colouring of the bone. Likewise, the deterioration of the crystalline bone matrix causes a general shrinkage of the bone parts (Devlin & Herrmann, 2015).

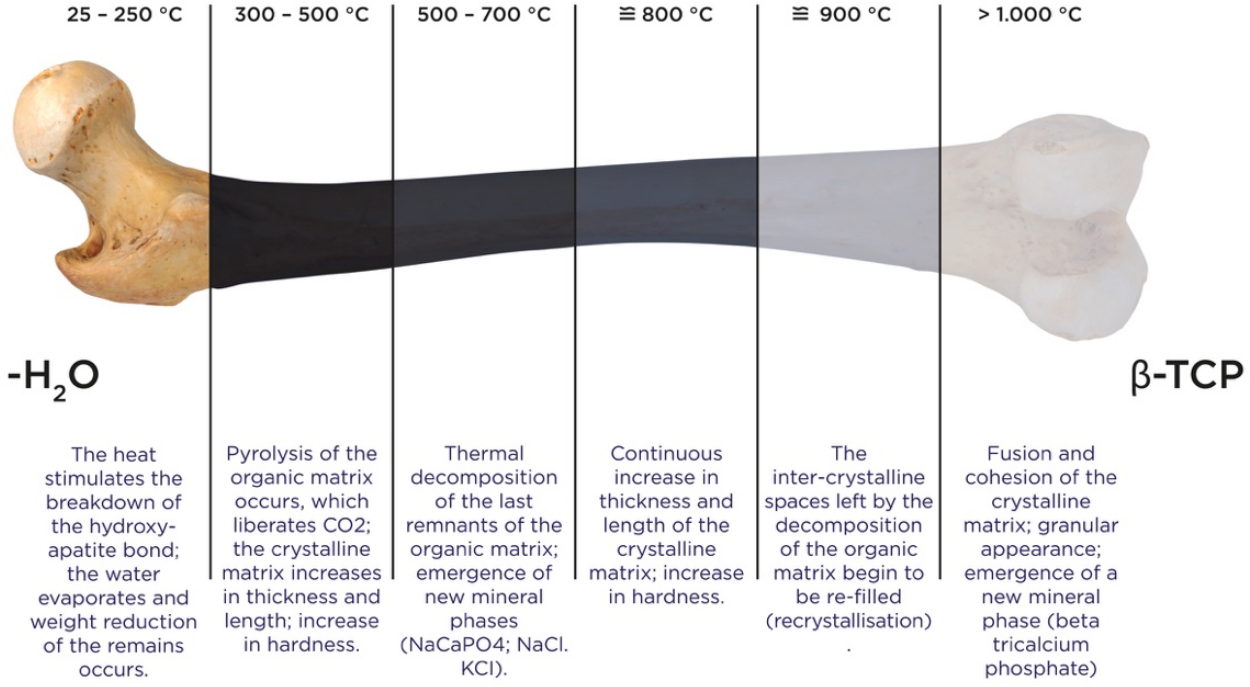



*Figure 1 Discolouration process of bones under the influence of heat and the associated biochemical changes. The image was originally published in Portuguese in Machado et. al, 2022.*

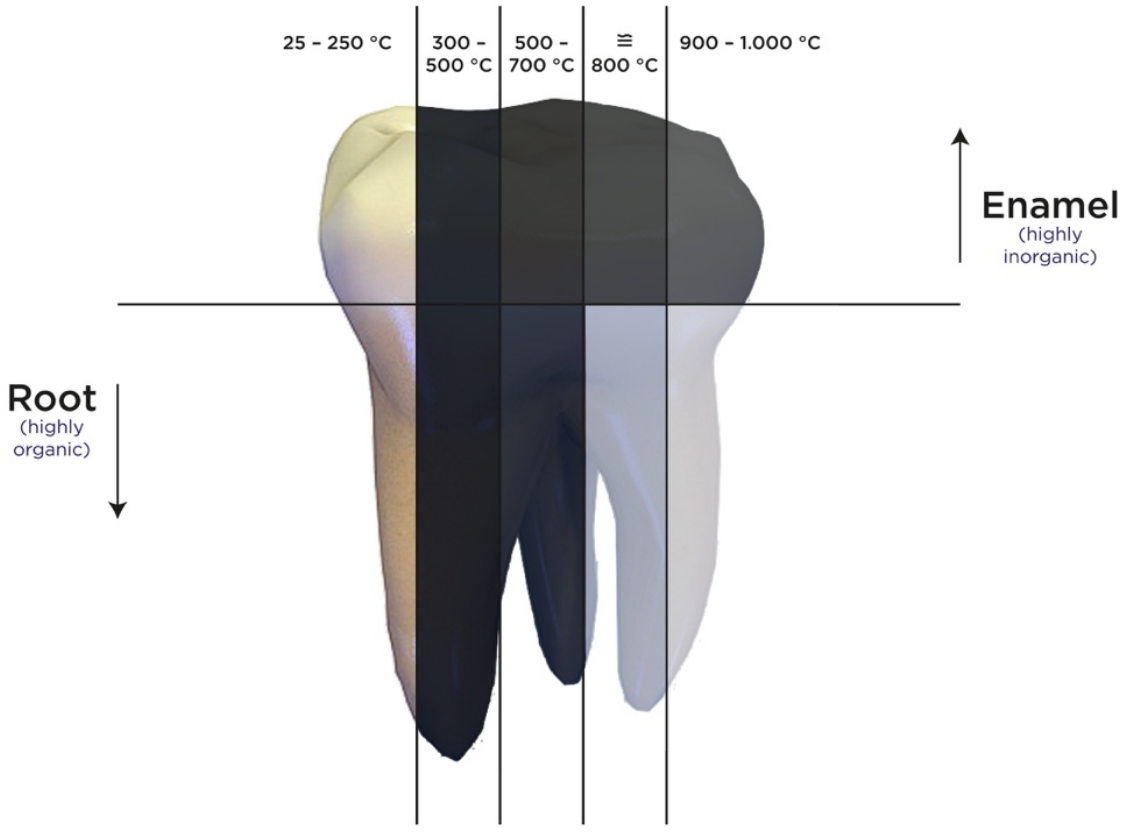



*Figure 2 Discolouration process of teeth under the influence of heat and the associated biochemical changes. The image was originally published in Portuguese in Machado et. al, 2022.*

## 2.2 Weight loss

The literature on weight loss in burnt bones is relatively scarce. There are a few studies from the US that were conducted between 1997 and 2011 (Bass & Jantz, 2004; Murad, 1998; Van Deest et al., 2011; Warren & Maples, 1997) and a recent study on Thai samples (Thamrong Chirachariyavej & Seetala Sanggarnjanavanich, 2006). Studies on European samples were published by Malinowski and collaborators in 1969 (Malinowski & Porawski, 1969) and Herrmann in the mid-seventies (Herrmann, 1976). The research team around Gonçalves published a study on Portuguese samples in 2013 (Gonçalves et al., 2013).

One finding that emerges from all the studies is that the weight loss of bone is mainly due to dehydration and decomposition of the heat-induced bone structure. In other words, weight loss is linked to the loss of water and the organic components of the bone. As with all other taphonomic changes, it makes a difference whether the bone was dry or fleshed when it was burnt. Since dry samples have already lost a significant amount of water and organic material due to the natural decay of the bone, the heat-induced weight loss is less than the one of fresh bone. In addition, the sex of the remains is also a significant factor. The Portuguese study additionally shows that age at death also affects the amount of weight loss in females (Gonçalves et al., 2013). Regarding temperature, and again referring to the four stages of heat-induced transformation in bone according to the studies of Tim Thompson, it should be said that heat-induced weight reduction usually occurs at a relatively low burn intensity (< 800 °C) (Thompson, 2004).

## 2.3 DNA and heat

The success rate in recovering DNA from burn victims is relatively low, which has a disadvantageous effect on the identification process in such forensic cases. In addition to the previously described, and further physical changes of the bone, there are also non-visible chemical changes due to burning and pyrolysis. The organic matrix of a bone is destroyed comparatively quickly by exposure to high temperatures, which also applies to the DNA embedded in it (Imaizumi, 2015). The first changes already occur at a combustion temperature of 100 °C – 600 °C (Thompson, 2004), but the exact threshold value for the survival of DNA under the influence of heat is still unclear (Imaizumi et al., 2014). What is certain, however, is that in the case of fire victims, severely degraded DNA, which is only present in a few copies, must be analysed. Highly efficient sampling and extraction methods are elementary for successful DNA recovery (Misner et al., 2009) with sampling either at crime or discovery sites or later during examination of the remains being a key task for Forensic Anthropologists.

Protocols for sampling, extraction, library preparation of aDNA are therefore particularly relevant for forensic genetic research in the field of fire victims, precisely because they aim to extract and amplify highly degraded, very short DNA fragments with an average of 30 – 70 base pairs (bp) (Gaudio et al., 2019).

## 3. State of the Art: Forensic DNA sampling vs. Ancient DNA sampling

Burn victims with severely charred bodies also often have severely degraded DNA, which makes genetic identification difficult, especially when focusing on *Short Tandem Repeats (STR)* analysis. However, interest in research in this area has increased in recent years and several studies have been published on the identification of burnt bones, examining different approaches from both forensic and aDNA analysis. Fredericks et al. (2012), and Fredericks et al. (2015) as well as Imaizumi et al. (2014) were successful in extracting DNA from burnt bovine compact bones at up to 250 °C in experimental studies using forensic DNA sampling methods. These studies showed that no more DNA could be extracted above 210 °C (Fredericks et al., 2012; Fredericks et al., 2015; Imaizumi et al., 2014). To analyse human DNA from samples from forensic casework Schwark et al. collected samples from 13 burnt cadavers and aimed to create an *STR* profile and sequence mitochondrial DNA (*mtDNA*). This study shows that while *STR* profiling is possible on well-preserved and semi-burnt bones, the results on severely burnt bones should be treated with caution as they may not be authentic, if *STR* profiling is successful at all (Schwark et al., 2011). Cattaneo et al. pioneered the field of controlled burning and DNA analysis. They burned compact human bones at 800 °C – 1.200 °C for 20 minutes and examined charred bones obtained from actual forensic cases. The amplification of 120 bp products of human mitochondrial DNA region V were examined, and it was found that none of these burnt samples contained amplifiable DNA (Cattaneo et al., 1999).

First attempts to work with ancient DNA sampling methods were made by Fondevila et al. in an experiment on a single charred femur. In their case report, the authors used mini-*STRs* and *Single Nucleotide Polymorphism (SNPs)* to confirm identity by DNA profile comparison. This is already an interesting application of ancient DNA methods in forensics, which has the potential to be expanded (Fondevila et al., 2008). However, in all the studies described, the researchers did not sample petrous bones, nor did they apply *Next Generation Sequencing (NGS).*

Studies focusing on petrous bone sampling have also mainly focused on sequence-tagged sites and *STR* typing to obtain a match between DNA profiles. In 2018, Kulstein et al. obtained eight samples from modern but highly degraded petrous bone and were able to recover a sufficient amount of DNA to sequence and *STR* profile using capillary electrophoresis. In addition, the researchers examined phenotyping and 54 biogeographic ancestry *SNPs* using *Massive Parallel Sequencing* on an *Illumina MiSeq*. However, six of the eight samples were from unidentified human remains and therefore could not be compared. The phenotyping results of the identified remains did not always match the actual appearance characteristics (Kulstein et al., 2018).

The studies described above show that *STR* analysis cannot provide reliable results in the presence of severely degraded DNA. Alverez-Cubero et al. and Gettings et al. therefore emphasise that the use of autosomal *SNPs* can be of great importance in forensic identification, as they allow DNA phenotyping. Moreover, *NGS* can be of advantage when using autosomal *SNPs* within forensics, as it makes parallel genotyping of a large number of *SNPs* possible (Alvarez-Cubero et al., 2017; Gettings et al., 2015), as shown in Gaudio's et al. study. Gaudio et al. successfully extracted a moderate to high percentage of

endogenous DNA from eight highly degraded, partially burnt crania. The DNA extraction was performed using the Dabney protocol (Dabney et al., 2013a; Gaudio et al., 2019), which is a silica-based extraction method that focuses on the isolation and recovery of short DNA fragments down to 35 bp (Dabney et al., 2013a). In recent years, such alternative extraction methods to the usual lysis protocols used in the field of forensic genetics, e.g. the total demineralisation Loreille protocol, which, in the case of severely destroyed samples and in combination with PCR-based amplification strategies, produced no or hardly usable results (Xavier et al., 2021). The Dabney protocol was combined with *Next Generation Sequencing* (*Illumia NextSeq500),* that allowed genotyping of the 627. 719 *SNP* position, indicating the geographic origin of the remains. The results were consistent with the seven remains identified (Gaudio et al., 2019). As stated earlier, the threshold for DNA survival in burnt remains is still unclear. However, this may change significantly with the application of optimal sampling methods and aDNA and *NGS* approaches. This shows that more research is much needed in this area.

## 4. DNA as Evidence: Possibilities and Risks of the Hyped Technology.

Despite the ground-breaking possibilities in criminal investigations and the identification processes, that have resulted from massive research in recent years, DNA profiling must also be viewed critically. Not only when it comes to searching for possible perpetrators in connection with a crime event, but also in connection with the identification of missing persons and human remains.

An important point is that DNA can never be used to draw conclusions about the course of events, which is particularly problematic in cases of murder and rape. In these cases, it cannot be ruled out that the DNA trace found was laid by everyday actions or by consensus (Murphy, 2007). The question of contamination must be treated just as critically. Contamination with an external source is a technical problem that can occur during both sample collection and laboratory analysis. Contamination occurs most frequently directly at crime scenes, during the recovery of corpses or human remains and especially when the sample in question is highly degraded DNA (Machado & Granja, 2020). To the extent that Forensic Anthropologists become more involved in the sampling of bone powder for DNA analysis in the future, and this is assumed in this thesis, they must always be aware of the high risk of contamination and adapt their working methods accordingly. At this point, however, it should be said that the more sensitive the analysis methods become, the more likely it is that contaminants will be detected. *Next Generation Sequencing*, for example, can determine the deamination pattern of a DNA sample, making it possible to distinguish a degenerate sample from a sample from someone who is still alive. However, the risk of cross contamination remains (Gettings et al., 2015).

But not only are analysis methods becoming more sensitive and thus laboratory results more meaningful, but also more domain specific software is being developed that uses probability-based genotyping. That means using statistics and algorithms to deal with complex DNA samples, as is the case with burn victims. For example, critical loci of a genome are modelled based on probabilities, which can help in phenotyping or the estimation of the geographical origin despite a very small amount of sample input. And even if one still cannot speak of unambiguous results and the approach remains one of probability, the scope for misinterpretation is becoming smaller and smaller with such computer-assisted modelling. However, such programmes must not be seen as a black box. Scientists and investigators, but also all those involved in the administration of justice, must know how to read, and interpret the results of such software (Butler, 2015).

However, it must also be said that even if everyone involved is aware of the limitations, no one can perceive without bias. The perception of each individual is always influenced by different ideas, habits and epistemic cultures of the respective social or professional group. This is of course also the case with DNA technologies. The analytical possibilities, but also the results derived from such analysis, are perceived differently by the individual actors within and outside the chain of custody. A critical role in this is played by the staging of forensic science as the hero of criminal investigations in various media, especially in television programmes. Through them, the myth of DNA profiling as an infallible and always objective all-rounder has been consolidated (Machado & Granja, 2020).This is referred to as the *CSI Effect*. This can lead to legal cases based on scientific evidence, preferably a DNA profile, being

perceived by outsiders, but also by judges or jurors, as easier to prove, safer and more reliable. Even if this is not true in practice (Huey, 2010). In some cases, investigators have even indicated that the public insists that certain investigative techniques, they have seen in various crime shows, are used. It is therefore understandable that investigating police agencies focus on cases where scientific evidence such as DNA profiles is available, especially when they are under-resourced. The chances of legal success are higher in such cases, recognition is greater, and this confirms the quality of their work (Murphy, 2007). The *CSI Effect* thus not only influences the general classification of DNA evidence as infallible but can even indirectly affect the work of the police. To what extent and what consequences can be expected should be the subject of future research. Finally, it should be noted that the knowledge gained from working with DNA is always based on comparison. Therefore, as long as there is no comparison sample, there can be no match (Cunha & Cattaneo, 2017).

All of this shows that it is important, especially with heavily decomposed or otherwise altered bodies, to draw on the expertise of Forensic Anthropology in the assessment of secondary identifiers, namely the biological profile and unique identification factors. This may not positively identify a person, but it can narrow down the number of possible individuals. Thus, to a certain extent, possible errors due to contamination can be counteracted. Also, if the findings match one or more missing persons, family members can be asked for comparison samples, which can lead to a positive identification in the next step.

# 5. Material and Methods

Since petrous bone shows the highest bone density in the skull and, due to its position inside the skull, it is fairly protected from external factors such as heat exposure and contamination, it increasingly coming to the forefront of current research as a strategically interesting sampling region, (Gamba et al., 2014; Gaudio et al., 2019; Pinhasi et al., 2015; Sirak et al., 2017). Therefore, it was decided to use eight human petrous bones as a sample set for the research. The sample ($n = 8$) is heated under controlled conditions between 100 °C and 500 °C, whereby the attainment of the temperature determines the period of combustion, i.e., once the desired temperature is reached, it is maintained for five minutes, then the cooling phase begins. The aim is to extract the cochlea, as it represents the densest bone part of the auditory capsule. The cochlea is processed into bone powder from which the DNA fragments are extracted. This method is combined with a *NGS* method, to analyse the quantity and quality of the recovered DNA.

## 5.1 Characterisation of the Sample

For highly decomposed or degraded human remains, a highly efficient sampling method is the key to optimal DNA recovery. When dealing with bone, both forensic genetics and aDNA research show that the density of the bone is an indication that the sample can potentially yield DNA, as these factors correlate positively. It is also important to select a region that is potentially more protected from external taphonomic factors as well as from contamination by foreign DNA and has as little microbial damage as possible. Therefore, the petrous part of the temporal bone is becoming more attractive as a target region for DNA extraction (Gamba et al., 2014; Pinhasi et al., 2015). It is positioned at the base of the skull between the sphenoid bone and the occipital bone and forms the endocranial part of the temporal bone. This area is therefore partially inside the skull and is thus exposed to a lesser degree to external taphonomic factors that accelerate the post-mortem degradation of organic material than, for example, the femur or teeth. Figure 3 also shows that even in the case of fire, the part of the temporal bone to which the petrous part is anchored inside the skull withstands heat for a relatively long time compared to other skeletal parts. In addition, the petrous bone is home to the densest bone part of the human body: a bony labyrinth consisting of the cochlea, the vestibule and the three semi-circular canals. In 2015, Pinhasi and collaborators found that the cochlea, within the petrous part, is the region with the highest potential for DNA recovery (Pinhasi et al., 2015).

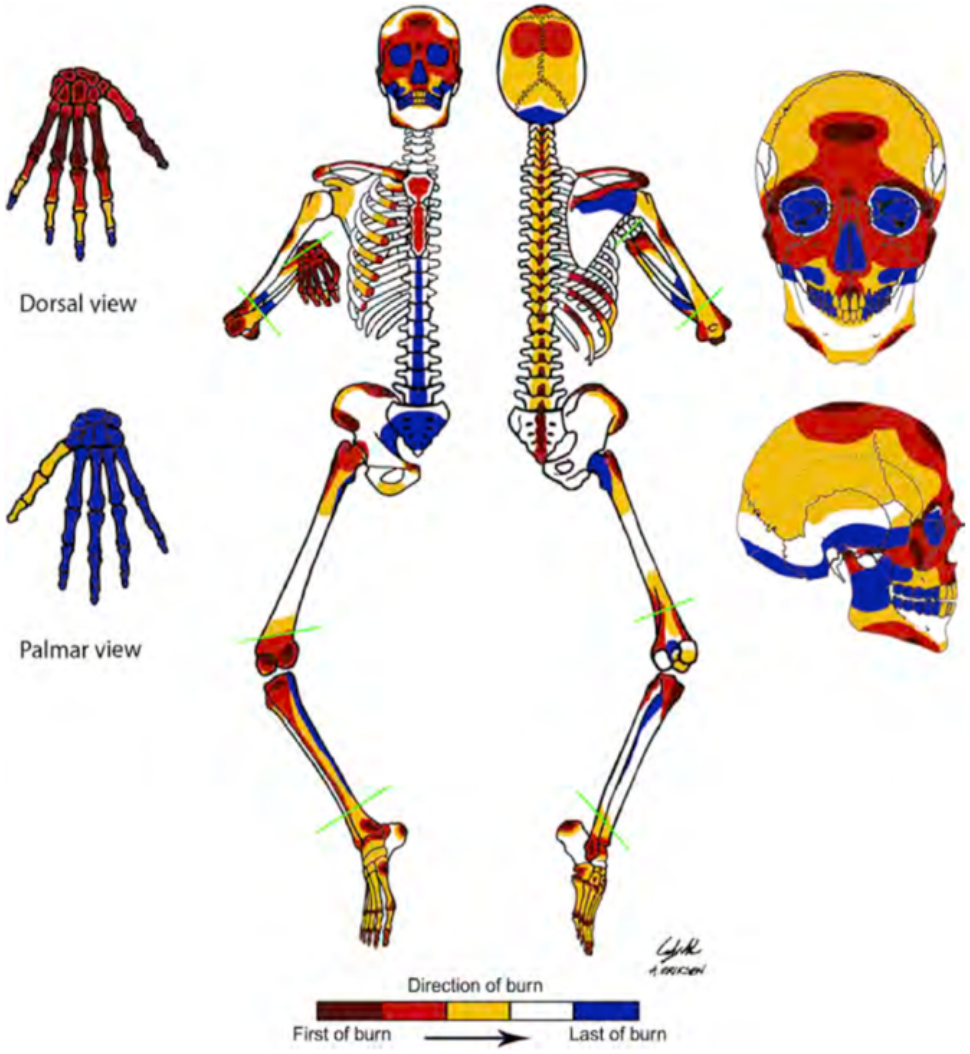
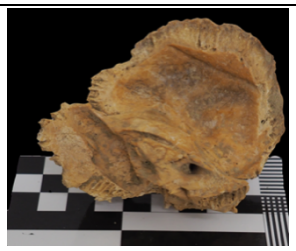
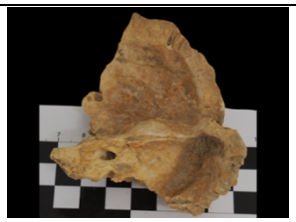
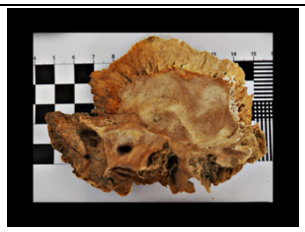
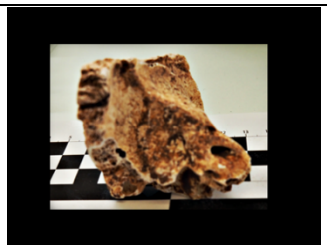
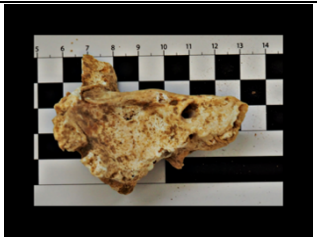
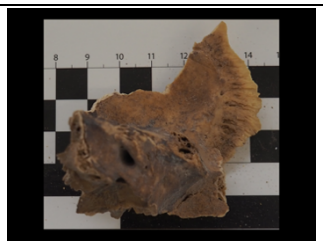
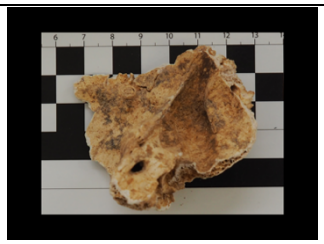



*Figure 3 Anterior and posterior view of the skeleton in fighting posture, with the first, secondary and last burn sites on the body marked in colour. The figure shows that the temporal bone in the area of the petrous bone withstands the heat for a relatively long time. The original image was published in Schmidt, C. W. et. al., 2015.*

A small sample size of $n = 8$ was chosen for this experiment, as there is strictly limited access to human petrous bones that can be made available for such an experiment. Four temporal bones come from an archaeological context from the *Avar-Slavic* period (circa 900 to 400 AD) and were recovered in Novi Sad, Serbia, in the *Bulevar Evrope.* These four samples were provided by the *Pinhasi Lab* for this experiment. The other four samples are petrous bones without the squama part coming from an unidentified modern context from the 1970s in Portugal, the exact time since death is unknown. All the ethical procedures were considered when choosing the sample.

*Table 1 Details and taphonomic alteration of the unburned sample*

| ***Ref. no.*** | | ***Context*** | ***Taphonomic details*** | ***Weight [g]*** |
|---|---|---|---|---|
| PB 1 |  left temporal bone | Avar-Slavic | Small cracks and weathering on the whole bone | 28.64 |
| PB 2 |  right temporal bone | Avar-Slavic | Light porosity and around the outer acoustic meatus; small cracks; weathering on the whole bone | 39.95 |
| PB 3 |  left temporal bone | Avar-Slavic | High porosity antero-distally; bone growth at the petrous pyramid; degrative bone activity on the outer squama; small cracks; weathering on the whole bone | 25.99 |
| PB 4 |  left temporal bone | Avar-Slavic | Long post-mortem crack at the outer squama posteriorly; high porosity distally; small cracks; weathering on the whole bone | 54.65 |
| PB 5 |  left petrous bone | Modern | Minimal soft tissue remains | 19.5 |
| PB 6 |  left petrous bone | Modern | Petrous part is open laterally; exposure of the otic capsule; minimal soft tissue remains | 18.1 |

| | | | | |
|---|---|---|---|---|
| PB 7 |  <br>right petrous bone | Modern | Minimal soft tissue remains at the lateral part of the petrous bone; porosity distally | 23.7 |
| PB 8 |  <br>right petrous bone | Modern | Light porosity at lateral part of the petrous bone; minimal soft tissue remains | 54.65 |

## 5.2 The Experiment

The sample (*n = 8*) is heated under controlled temperature conditions between 100 °C and 500 °C, whereby the attainment of the temperature determines the period of combustion, i.e., once the desired temperature is reached, it is maintained for five minutes, then the cooling phase begins. The aim is to extract the cochlea, as it represents the densest bone part of the auditory capsule. The cochlea is processed into bone powder from which the DNA fragments are extracted. This method is combined with a *Next Generation Sequencing (NGS)* method, to analyse the quantity and quality of the recovered DNA. *NGS* can bring the advantage of genome wide data in the forensic field, especially when working with severely degraded DNA where analysis of *Short Tandem Repeats (STRs)* may be unsuccessful, allowing parallel genotyping of a large number of autosomal *Single-Nucleotide Polymorphisms (SNPs)* (Alvarez-Cubero et al., 2017). Data to be analysed are endogenous DNA yields, sequence read lengths, DNA deamination frequencies, and chromosomal sex. All steps of the experiment were carried out by Evelyn Zelger herself. A PhD student from the *Department of Forensic Anthropology* at the *University of Coimbra* assisted in the combustion process, and the bioinformatic analysis was carried out with the help of Daniel Fernandes, PhD from the *Pinhasi Lab* at the *University of Vienna*. All the laboratory work was supervised by the *Pinhasi Lab* team in Vienna. The sequencing was carried out on behalf of *Pinhasi Lab* by *Bio Center Vienna*. Based on the State of the Art given in Chapter 3 and with the methods just summarised, the following results are expected:

1. It is expected that the cochlea will be a suitable sample for the recovery of usable DNA fragments.

2. It is expected that the colour of the bones will change during the combustion process as follows: no discernible colour changes when heated to 100 °C, pale yellow to yellow discolouration at 200 °C – 300 °C, dark brown discolouration between 300 °C – 350 °C, black to dark grey colour between 400 °C – 500 °C.

3. The Dabney protocol (Dabney et al., 2013a) is ideally suited for the extraction of short DNA fragments from highly degraded bones.

4. A high percentage of endogenous DNA is expected in samples heated at 100 °C – 250 °C (above 40 %), a moderate percentage at 300 °C – 350 °C (between 15 % – 40 %), a low percentage at 400 °C (below 15 %) and no usable DNA at the higher temperatures.

5. Less DNA is expected to be recovered from the archaeological samples than from the modern samples. This is already evident in the pre combustion state of the samples.

*5.2.1 The combustion*

The combustion process takes place in an electric muffle-furnace with a thermostat at the *Department of Life Science* of the *University of Coimbra*, where the bones are not burnt with fire, but merely heated. This already shows the first limitations of this experiment. Placing bones in a hot laboratory furnace never corresponds to the conditions to which bones are exposed in a fire, in terms of temperature and atmospheric composition. However, this cannot be prevented in such an experimental context and is therefore assumed.

Another limitation is the lack of any soft tissue, which significantly affects the combustion process in a real-life scenario and further protects the inner region of the skull bones from heat and the pyrolysis it induces. This is attempted to be counteracted by lower combustion temperatures. A fire in a forensic context is usually between 600 °C and 1,200 °C, but Gaudio et al., 2019 were able to determine through a colour comparison of temporal bones from three fire victims that the petrous part in these cases was exposed to a maximum temperature of 645 °C using the Shipman scale (Shipman et al., 1984), respectively 500 °C using the Ellingham scale (Ellingham et al., 2015) (Gaudio et al., 2019).

*Figure 4 Electric muffle furnace with thermostat at the Faculty of Life Sciences of the University of Coimbra. The bones were placed in the oven with the inner acoustic meatus facing upwards, as shown in the picture.*

For this experiment, the temperature of 500 °C is therefore defined as the maximum temperature, with 100 °C as the lowest value. Between the two lowest values of 100 °C and 200 °C, a 100 °C increase was chosen because studies (see Chapter 3) have shown that DNA extractions are possible in this combustion temperature range. From 200 °C, the temperature is increased in 50 °C steps. The three lowest temperatures 100 °C, 200 °C and 250 °C could not be maintained since the muffle-furnace exceeded all three temperatures (see Table 2).

*Table 2 Planned and effective combustion temperatures of the experiment*

| ***Ref. no.*** | ***Bone part*** | ***Combustion chamber*** | ***Combustion temp. (planned) [°C]*** | ***Combustion temp. (effective) [°C]*** |
|---|---|---|---|---|
| PB 6 | left petrous bone | Electric muffle-furnace with thermostat | 100 °C | 145 °C |
| PB 3 | left temporal | Electric muffle-furnace with thermostat | 200 °C | 220 °C |
| PB 8 | left petrous bone | Electric muffle-furnace with thermostat | 250 °C | 260 °C |
| PB 1 | left temporal | Electric muffle-furnace with thermostat | 300 °C | 300 °C |
| PB 5 | right petrous bone | Electric muffle-furnace with thermostat | 350 °C | 350 °C |
| PB 2 | right temporal | Electric muffle-furnace with thermostat | 400 °C | 400 °C |
| PB 7 | right petrous bone | Electric muffle-furnace with thermostat | 450 °C | 450 °C |
| PB 4 | left temporal | Electric muffle-furnace with thermostat | 500 °C | 500 °C |

When it comes to time, it is almost impossible to determine an average duration of a fire in a forensic context. If the fire is not artificially extinguished, the basic components of the fire, fuel, oxygen, heat, and a chemical oxidation, maintain the exothermic oxidation reaction by themselves and thus determine the duration of the burning (DeHaan, 2015).

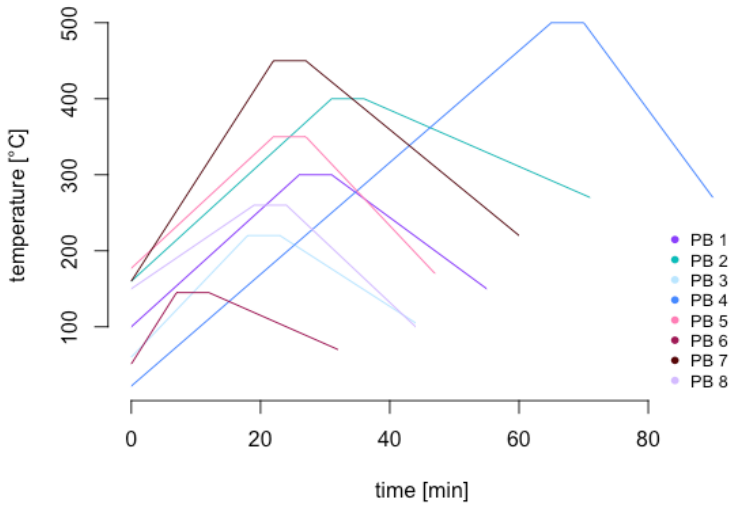



*Figure 5 Temperature curves of the individual samples during combustion*

Since there are no reference points in terms of time and this is a first study with petrous bone and aDNA extraction methods combined with *NGS* under controlled temperature conditions, temperature is treated as the main factor that determines the time of heat exposure.

The bone is placed in the oven with the inner acoustic meatus facing upwards, the oven is closed and heated to the desired temperature threshold. After the threshold is maintained for five minutes, the cooling phase begins. To prevent the bone from cracking due to a temperature shock, the first approximate 20 minutes or the time of the cooling phase to below 300 °C take place in the closed oven. The oven door is then opened a little and only opened completely after further 15 minutes. The burnt bone is placed on a wooden board covered with aluminium foil to cool completely overnight.

The bone samples are randomly assigned to a temperature, whereby an archaeological sample is always followed by a modern one. This allows us to see how the original preservation status of the bone at similar temperatures can affect the pyrolysis of DNA.

After the burning process, the samples are examined, focusing on the colour changes and the weight of the bones. Over the years, there have been several standardised descriptions of this discolouration pattern. The model used in this paper was developed by Shipman et al. in 1984. In it, the researchers systematically classified the surface colour of burnt bone using the *Munsell Soil Colour Charts* (Munsell, 1994) and defined five colour stages that can be assigned to a temperature range. Each stage is described by variations in hue, value and chroma and is divided into a dominant surface colour and several secondary colours.

*Table 3 Colour stages and their associated temperatures according to Shipman et al., 1984*

| | ***Temperature*** | ***Predominant colour*** | ***Minor colour*** |
|---|---|---|---|
| Stage I | 20 °C – 285 °C | Neutral white, pale yellow | Yellow, very pale brown |
| Stage II | 286 °C – 525 °C | Reddish brown | Very dark greyish brown, neutral dark grey, brown |
| Stage III | 526 °C – 625 °C | Neutral black | Medium blue, reddish yellow |
| Stage IV | 626 °C – 940 °C | Neutral white | Light blue, grey, light grey |
| Stage V | 940 °C + | Neutral white | Medium grey, reddish yellow |

*5.2.2 Bone powder sampling*

There are several methods for obtaining cochlear bone powder from the inner ear. Which method is used depends strongly on the condition and fragmentation of the skull. At this step the expertise of Anthropologists is needed because their assessment is essential for the outcome of the sampling on the one hand, but also for the integrity of the remains on the other. It is important to find a balance between the goal of sampling and the responsibility towards the dignity of the deceased.

The most reliable method, but also one of the most invasive, is the sandblasting protocol published in 2019 by the research team around Ron Pinhasi (Pinhasi et al., 2019). It should only be used if the petrous bone is already isolated and, at best, both individual's petrous bones are still present. First, the approximate location of the cochlea must be identified externally, which is slightly anterolateral to the internal auditory canal and medial to the eminence arcuata and semicircular canal. A sandblaster is then used to remove the various layers of bone until the cochlea becomes visible. It can be recognised by its round, detached shape, and its slightly yellowish colour. By making specific cuts around the cochlea, it can then be extracted and cleaned of dense bone remnants. Bone powder can then be generated from the clean isolated cochlea, ideally using a mixer mill. However, the cochlea can also be ground or crushed by hand.

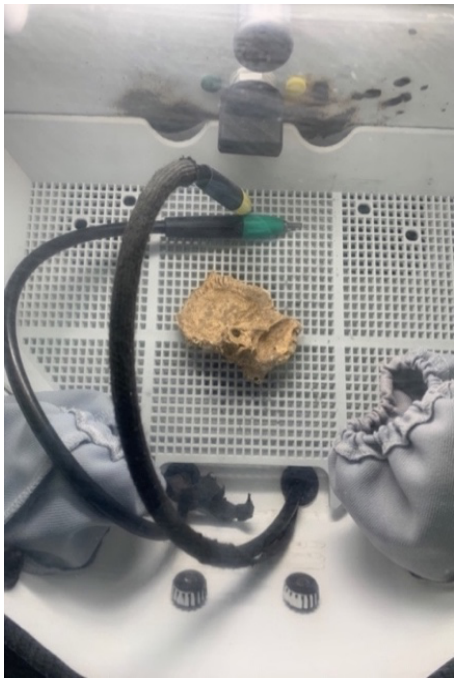


*Figure 6 Before the grinding process can start put the sample into a cross-linker for a total of 10 minutes on each side. Then, switch on the sandblaster and plugged in vacuum cleaner on and place the sample into the cleaned grinding hood.*

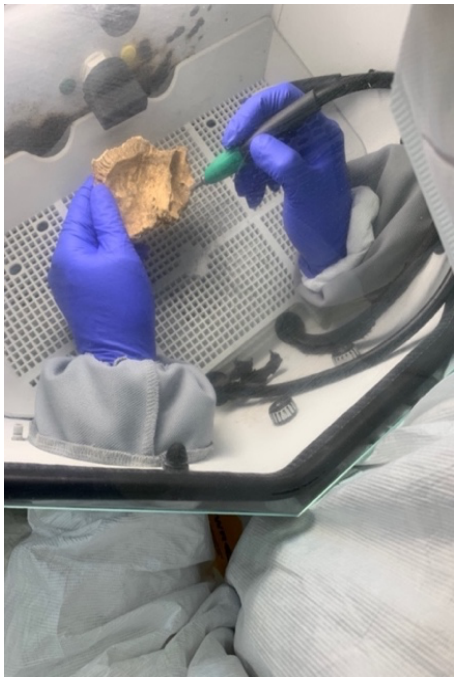


*Figure 7 First, clean the surface of the sample using the air blaster.*

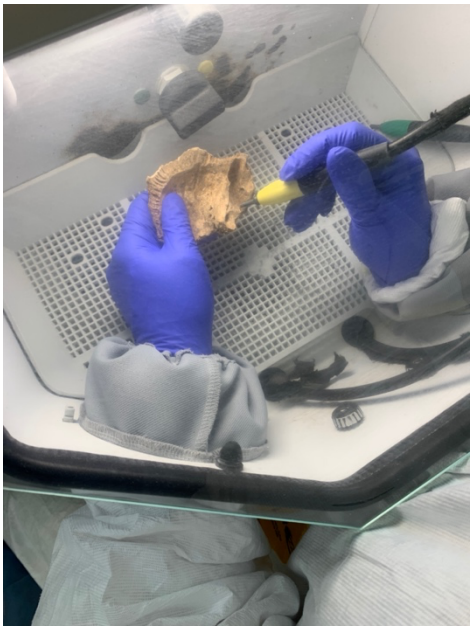


*Figure 8 Second, clean the surface of the sample using the sand blaster. Carefully remove the top layer of bone until the bone is clearly lighter in colour.*

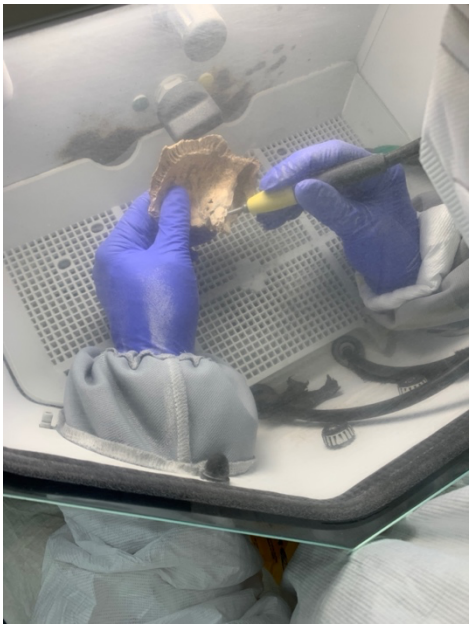


*Figure 9 Identify the approximate location of the cochlea from the outside. Then use the sand blaster to ablate the different layers of bone until the cochlea becomes visible.*

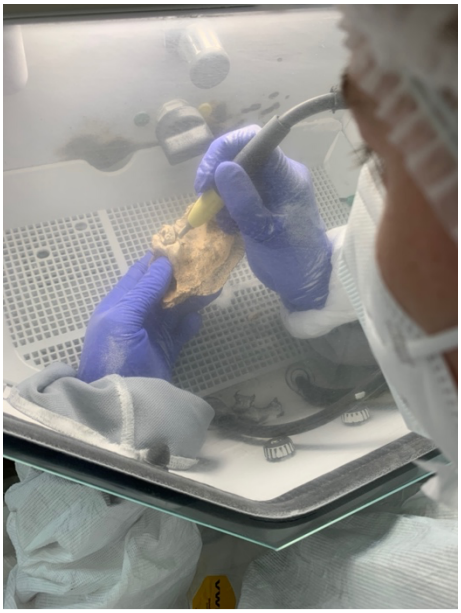


*Figure 10 Continue grinding until the cochlea can be isolated from the petrous bone. Clean the cochlea from the dens bone that is still attached to it using the sand blaster. Then clean the entire bone, the cochlea and the grinding hood from the sand that was not vacuumed out with the air blaster.*

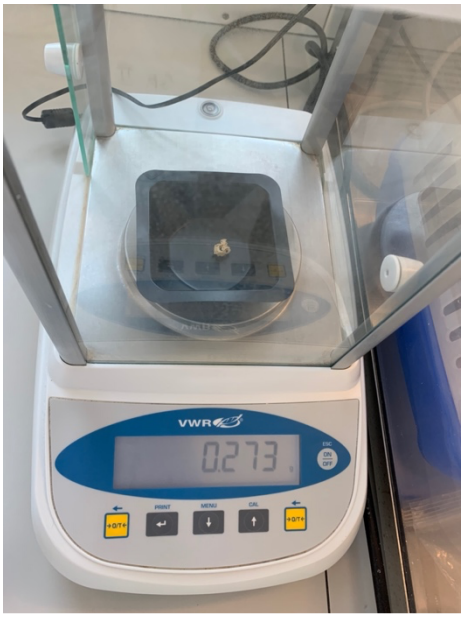



*Figure 11 Switch off the sand blaster and the vacuum cleaner, open the grinding hood and place the cochlea in a bleached collection boat. Weigh the cochlea and put it into the cross-linker in the collection boat for a total of 10 minutes on each side. After the time in the cross-linker has elapsed, construct the clean grinding jar: Place the plastic ring in the upper part and the ball in the lower part. Put the sample into the jar and close it. Fix the jar in the mixer mill and start it for 30 seconds. Then wait 10 minutes and start another 30 second session.*

*Figure 12 When the milling process is finished, remove the jar from the mixer mill. Carefully open the jar and roughly scrape off the upper part with a clean spatula. Collect the powder in the boat. Then carefully drop the ball that was previously in the lower part into the upper part of the jar and try to get as much powder off the ball as possible.*

*Figure 13 Pour most of the powder that is in the lower part of the jar into the boat and scrape the lower part thoroughly. Weigh the powder and store it in a DNA low bind tube in the refrigerator.*

In cases where a skull is complete or the petrous bone is still firmly embedded in the skull structure, the petrous bone should not be removed under any circumstances in order to keep the integrity of an entire bone, but a drilling method through the cranial base should be used. This applies to a large proportion of forensic cases, which is why this method is particularly interesting for the field of Forensic Anthropologists.

The skull is punctured from below through the bony ridge that separates the jugular foramen from the carotid canal at the base of the skull. First, the ridge is carefully eroded with a high-powered engraving cutter until the bottom of the cochlea is exposed. Then a small hole is milled into the cochlea, through which a small round drill bit (3 mm – 5 mm) is entered into the bony labyrinth. The bone powder is generated by circular movements and pressure. The powder can be collected through a bleached collection boat that is placed under the skull (Sirak et al., 2017). In the case of an isolated petrous bone that cannot or should not be opened, cochlear bone powder can also be generated by drilling through the inner acoustic meatus. This method was used in the pre combustion bone powder sampling of this experiment to keep the bone as intact as possible for the combustion process. The team at the *Pinhasi Lab* is currently also researching a non-invasive method in which the extraction buffer is injected directly into the auditory canal. Although Evelyn Zelger is involved in this research, it was decided to use an already published and reliable method for this experiment. Post combustion sampling was carried out through the horizontal opening of the petrous bone. This rather radical procedure was chosen to determine the colour of the inside of the petrous bone and the cochlea using the *Munsell Soil Colour Chart*

(Munsell, 1994) to estimate the temperature that had spread inside the petrous bone during the heating process, using the Shipman scale (Shipman et al., 1984). Indeed, the temperature inside does not necessarily correspond to the temperature of the outer surface of the bone or the ambient temperature of the muffle-furnace.

In any case, strict attention must be paid to contamination, which is why it is highly recommended to: clean all materials used, as well as the work surface, before and after each sample with a bleach solution followed by ethanol, to use several layers of gloves and, if possible, to work in a clean room.

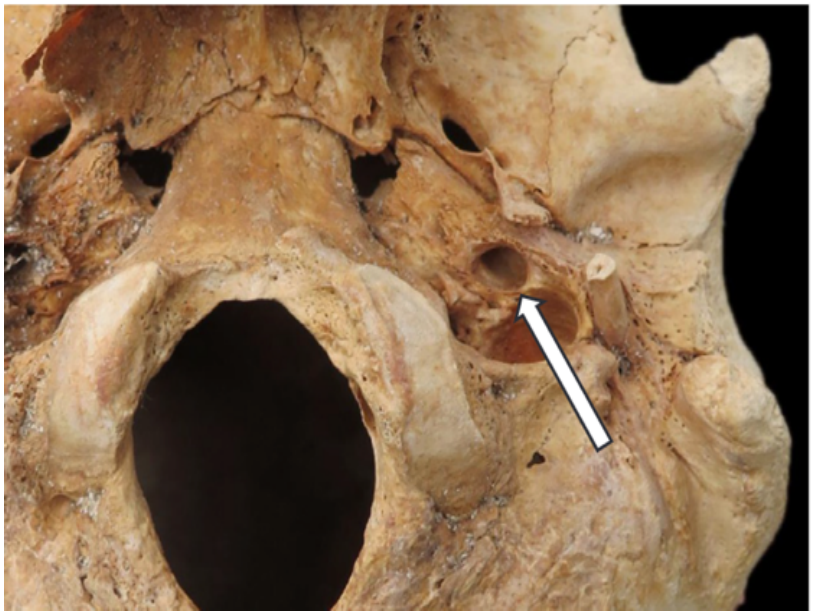


*Figure 14 The bony ridge that separates the jugular foramen from the carotid canal at the base of the skull. The picture was originally published in Sirak et al., 2017.*

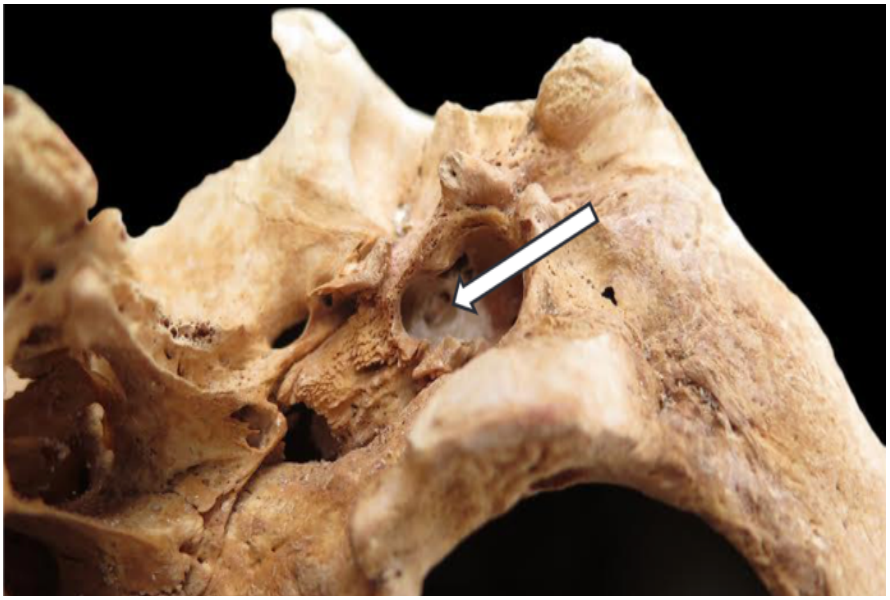


*Figure 15 The opening into the bony labyrinth resulting from the cranial base drilling method. The picture was originally published in Sirak et al., 2017.*

*Pre combustion sampling*

Before the heating process, approximately 50 mg of cochlear bone powder was sampled. To do this, a *Dremel 9100 Fortiflex rotary tool* fitted with a 3 mm grinding bit was used to drill through the inner acoustic meatus. Before the drilling process, all bits were treated in a bleach bath and rinsed with an ethanol solution. Two layers of gloves were worn throughout the process, the top layer was changed regularly, new drill bits were used for each bone, and the entire work area was cleaned with bleach to avoid contamination. The bone powder was stored in 1.5 ml *eppendorf* DNA low bind tubes. The pre-burned cochlea powder samples are used as comparative values for the degradation levels of DNA under the respective temperatures.

*Post combustion sampling*

The burnt petrous bones were exposed to a short-wave UV light for 30 minutes in a cross-linker before the bone powder sampling starts. The burned petrous bones were cut horizontally with a *Dremel 9100 Fortiflex rotary tool* fitted with a small-sized cutting bit. The cochleas of the samples up to a combustion temperature of 300 °C ware carefully cut out. The samples above 300 °C combustion temperature had such a brittle trabecular and dens bone around the cochlea that it could be broken out with bare fingers. To obtain bone powder, the cochleas were placed individually in a grinding jar with a grinding ball, both of which were previously cleaned with bleach and ethanol and irradiated with short-wave UV light for at least 30 minutes. The grinding jar was then clamped in a mixer mill and shaken at maximum frequency for two 30-second sessions. A pause of ten seconds was taken between sessions to allow the generated heat to dissipate and not affect the final result. The bone powder of the individual samples was then stored in single 1.5 ml *eppendorf* DNA low bind tubes. The cutting was carried out in Vienna in the *Ron Pinhasi* ancient DNA grinding lab. A protective suit with a hood, a face mask, two layers of gloves and plastic socks were worn to avoid contamination. Also, before the drilling process, all bits were treated in a bleach bath and rinsed with an ethanol solution, the workstation was cleaned with bleach after each sampling process, and the top layer of gloves is changed regularly

*Figure 16 The orientation mark along which the burnt petrous bones were cut horizontally. The picture shows burnt PB 4.*

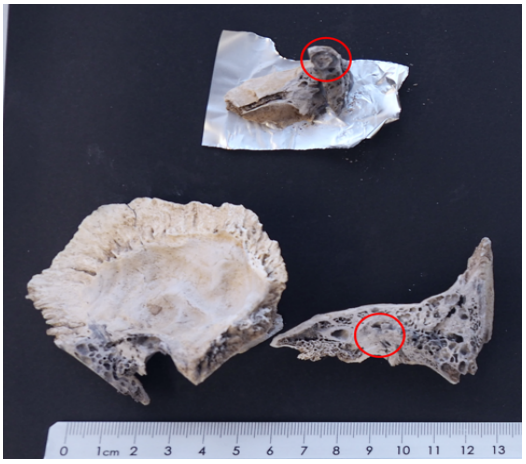


*Figure 17 Horizontally cut petrous bone with severed squama. The cochlea has been cut in the middle and the bony labyrinth is clearly visible. The colour of the cochlea is visibly darker than the rest of the bone. The picture shows burnt PB 4.*

### *5.2.3 Solutions and Buffers*

*Table 4 Ingredients of the extraction buffer*

**Extraction Buffer**

| | **final concentration** | **20mL** |
|---|---|---|
| **EDTA (0.5 M)** | 0.45 M | 18 mL |
| **Proteinase K (¬15 mg/mL)** | 0.25 mg/mL | 333.33 µL |
| **H20** | up to X mL | 1.7 mL |

*Table 5 Ingredients of the binding buffer*

**Binding Buffer**

| | **final concentration** | **260 mL** |
|---|---|---|
| **Guanidine hydrochloride (MW 95.53)** | 5 M | 124.3 g |
| **Isopropanol** | 40 % | 104 mL |
| **Sodium Acetate pH 5.2 (3 M)** | 90 mM | 7.8 mL |
| **Tween-20 (10 %)** | 0.05 % | 1.3 mL |
| **Up to 260 mL with H20** | - | - |

*Table 6 Ingredients of the TET buffer*

**TET Buffer**

| | **final concentration** | **10 mL** |
|---|---|---|
| **1M Tris-HCl (pH 8.0)** | 10 mM | 100 µL |
| **EDTA (0.5 M)** | 1 mM | 20 µL |
| **10% Tween-20** | 0.05 % | 50 µL |
| **dH20** | - | 9 mL 830 µL |

*Table 7 Ingredients of the EBT buffer*

**EBT Buffer**

| | **1 mL** | **10 mL** |
|---|---|---|
| **Qiagen EB buffer** | 59.97 µL | 9 mL 995 µL |
| **Tween-20** | 0.03 µL | 5 µL |

*Table 8 Ingredients of the Blunt-End Repair Master Mix*

**Blunt-End Repair Master Mix (M1)**

| | **1 x** | **20 x** |
|---|---|---|
| **End Repair Enzyme Mix** | 3.5 µl | 70 µl |
| **End Repair Buffer** | 7 µl | 140 µl |
| **ddH20** | 34.5 µl | 690 µl |
| **Total** | 45 µl | 900 µl |

*Table 9 Ingredients of the Adapter Ligation Master Mix*

**Adapter Ligation Master Mix (M2)**

| | **1 x** | **22 x** |
|---|---|---|
| **ddH20** | 10 µl | 220 µl |
| **T4 DNA ligase buffer (10x)** | 4 µl | 88 µl |
| **PEG-4000 (50 %)** | 4 µl | 88 µl |
| **Adapter mix (100 µM each)** | 1 µl | 22 µl |
| **T4 DNA ligase (5 U/µL)** | 1 µl | 22 µl |
| **Total** | 20 µl | 440 µl |

*Table 20 Ingredients of the Adapter Fill-In Master Mix*

**Adapter Fill-In Master Mix (M3)**

| | **1 x** | **22 x** |
|---|---|---|
| **ddH20** | 13.5 µl | 297 µl |
| **Thermopol reaction buffer (10x)** | 4 µl | 88 µl |
| **dNTPs (10 mM each)** | 1 µl | 22 µl |
| **Bst polymerase, large fragment (8 U/µL)** | 1.5 µl | 33 µl |
| **Total** | 20 µl | 440 µl |

*Table 11 Ingredients of the qPCR Master Mix*

**qPCR Master Mix (M4)**

| | **1 x** | **40 x** |
|---|---|---|
| **Blue S'Green qPCR 2x Mix (*Biozym*)** | 10 µl | 400 µl |
| **IS7 (5 µM)** | 1.6 µl | 64 µl |
| **IS8 (5 µM )** | 1.6 µl | 64 µl |
| **ddH20** | 5.8 µl | 232 µl |
| **Sample** | 1 µl | |

*Table 12 Ingredients of the Indexing PCR Master Mix*

| | **Indexing PCR Master Mix (M5a)** | | | **Indexing PCR Master Mix (M5b)** | |
|---|---|---|---|---|---|
| | **1 x** | **20 x** | | **1 x** | **20 x** |
| **Pfu Turbo Cx Poly** | 1 µl | 20 µl | | 1 µl | 20 µl |
| **10 Rxn buffer** | 5 µl | 100 µl | | 5 µl | 100 µl |
| **P5 (5uM)** | 2 µl | | | 2 µl | |
| **P7 (5uM)** | 2 µl | | | 2 µl | |
| **dNTPs (10mM)** | 1 µl | 20 µl | | 1 µl | 20 µl |
| **Template** | 10 µl | | | 3 µl | |
| **ddH20** | 29 µl | 580 µl | | 36 µl | 720 µl |
| **Master Mix per rxn** | 36 µl | | | 43 µl | |

*5.2.4 DNA Extraction*

The DNA extractions were performed according to the standard extraction protocols for bone powder of the *Pinhasi Lab*, in this case the protocol according to Dabney (Dabney et al., 2013a) with *Roche High Pure Extender Tubes*. The extraction was performed in a clean room in a *Thermo Scientific™ MSC-Advantage™ Class II Biological Safety Cabinet* under the highest possible sterile standards. To minimise contamination, a bodysuit, face mask, plastic socks and two layers of gloves were worn, the top layer was changed several times and sterilised with bleach before each entry into the safety cabinet. All tubes containing the bone powder were also sterilised on the outside with bleach before being taken to the clean room. However, any foreign contamination that may have been introduced into the bone powder through the sampling process or was simply already present in the inner ear cannot be removed. 50 mg bone powder was mixed with 1 mL of an extraction buffer solution containing 0.5 M EDTA and proteinase K. The mixture was vortexed and incubated under rotation for 18 hours at 37 °C in a *ThermoMixer C*. Then 13 mL of binding buffer was added to the Roche Tubes. The 1.5 mL tubes containing the powder solution were centrifuged at 13,000 rpm for 2 minutes to allow the bone pallet to settle to the bottom of the tube. The supernatant was then transferred to the 13 mL binding buffer, and then centrifuged at 1,500 xg for 4 minutes. The spin columns were then broken from the Roche extenders and spun dry for one minute. 650 µL PE wash buffer was added to the spin column and centrifuged again at 6.000 rpm for 1 minute. The flow-through was removed and the washing step repeated. The spin column was then spun dry by centrifuging at maximum speed for 1 minute and placed in a clean 1.5 mL *eppendorf* DNA low bind tube. DNA was eluted from the silica membrane by adding 25 µL TET buffer, then the tube was incubated at 37 °C for ten minutes and centrifuged for 30 seconds at maximum speed. This procedure was repeated to obtain a total of 50 µL elute.

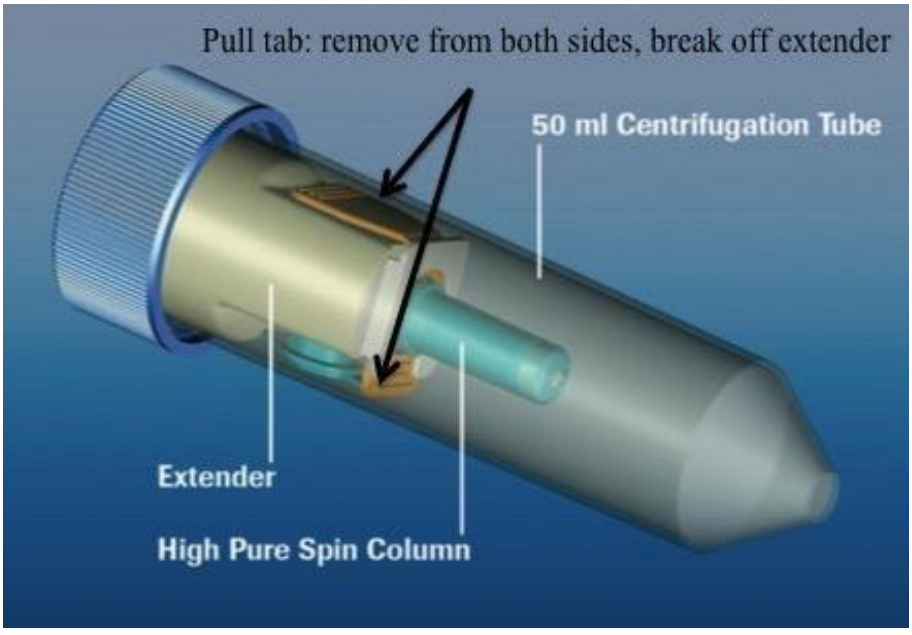



*Figure 18 Roche High Pure Extender Tube. The original image was published on the manufacturer's website. Available online at: https://lifescience.roche.com/en_at/products/high-pure-viral-nucleic-acid-large-volume-kit.html#details [last accessed: 7.7.2022].*

*5.2.5 Library preparation*

Library preparation was performed according to the protocol of Meyer and Kircher (Meyer & Kircher, 2010), which is specific for *Next Generation Sequencing*. First, a blunt-end repair was performed using a Master Mix (M1), which was mixed with the 25 µL DNA extract. The mixture was then incubated at 25 °C for 15 minutes and at 12 °C for five more minutes. For the following clean-up step, a *Qiagen MinElute PCR Purification Kit* was used, which was designed for very short molecules (30 – 80 bp). To do this, five times the sample, in this case 200 µL, of PB binding buffer was added to each *Qiagen* column, then the 40 µL of each sample were added. The column was centrifuged for 1 minute at maximum speed (13,000 rpm) and the flow-through was discarded. Then 650 µL of PE wash buffer was pipetted into the column and again centrifuged for 1 minute at 13.000 rpm. The flow-through got discarded and the column centrifuged again at maximum speed for 1 minute to remove all remaining PE buffer. The spin column was removed from the collection tube and placed in a new 1.5 mL *eppendorf* DNA low bind tube. Then it was eluted with 20 µL EBT elution buffer and centrifuged at maximum speed for 1 minute after waiting for 1 minute. The spin column was discarded, the double-stranded adapters were ligated to the DNA in the next step with a Master Mix (M2) containing T4-DNA ligase and the samples were incubated at 25 °C for 30 minutes. The previously described washing procedure using *Qiagen MinElute PCR Purification Kits* was repeated at this point. The adapter sequences were then loaded with Bst polymerase (M3) during an incubation step of 30 minutes at 37 °C, followed by thermal inactivation of the enzyme at 80 °C for 20 minutes. Finally, a 1:40 delusion of the samples was prepared with 39 µL TET buffer, which is used for the subsequent real time qPCR.

*5.2.6 Indexing*

The real time qPCR determines how many cycles are needed to sufficiently amplify at least 1,000 copies of each original library fragment to make sure no complexity is lost. First, a Master Mix (M4) with a *Biozym* Blue S'Green qPCR 2X Mix containing a hot-start DNA polymerase, blue and green dye, an IS7 primer (5 uM), and an IS8 primer (5 uM) were prepared. 19 µL of each reaction was pipetted into each tube. To this, 1 µL of standard curve was added to the standard curve tubes and 1 µL of sample to each additional tube. PCR amplification was then performed using the following temperature cycling profile: 2 minutes at 95 °C, 40 cycles of 10 seconds at 95 °C, 20 seconds at 60 °C, followed by a final cycle of 20 seconds at 70 °C.

An PfuTurbo Cx HotStart DNA Polymerase Master Mix (M5a) was used to perform indexing PCRs with one unique P5 and one unique P7 indexing primer per sample. For samples that require 8 cycles or fewer according to real time qPCR, the amount of input material must be reduced, and three reactions are performed so that these samples are also amplified for at least 9 cycles to generate approximately 1,000 copies of each original library fragment. 3 µL of library was thus added to the freshly prepared PCR mix (M5b). PCR amplification was performed using the following temperature cycling profile: five minutes at 95 °C, *n* cycles of 15 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 68 °C, followed by a final cycle of five minutes at 68 °C. For the following clean-up, *Qiagen MinElute PCR*

*Purification Kits* were used as previously described, except that 2 µL NaAc (3 M) was added in the first step to prevent the solution from becoming too basic. For samples with three times 3 µL sample input, 750 µL of PB buffer with 6 µL NaAc (3 M) was pipetted into a fresh 1.5 µL tube. The 50 µL of each of the three reactions for this sample were added to the tube. Then 750 µL of the mixture was pipetted into the *MinElute* tube, spun at maximum speed for 1 minute, the flow-through was discarded, then the remainder of the mixture was added to the same column, spun again at maximum speed for 1 minute, and the flow-through discarded again. For washing and eluting, the protocol described earlier was followed.

### *5.2.7 Next Generation Sequencing*

The concentration of the individual PCR reactions was measured with a *Qubit 3*, the length of the DNA fragments was determined with the *Agilent 2100 Bioanalyzer* according to the manufacturer's guidelines. Based on the concentrations indicated by the results from the Qubit and the only if the results of the Bioanalyzer were positive, the samples were pooled in equimolar ratios. All samples did pass this quality control whereby PB 1 und PB 8 showed relatively low values. *Next Generation Sequencing* was performed the *Vienna Bio Center* on an *Illumina NextSeq500* platform.

### *5.2.8 Bioinformatical analysis*

The bioinformatical analysis was carried out with the help of Daniel Fernandes, PhD who is part of the *Pinhasi Lab* team. The applied pipeline is a custom ancient DNA bioinformatics pipeline consisting of the following programs: *FastQC* was used for a first quality control, *cutadapt* to remove the adapters, *Burrows-Wheeler Aligner (BWA)* to align the nucleotide sequences against a reference sequence such as the human genome, and *Integrative Genomics Viewer (IGV)* was used to visualise the genomic datasets.

# 6. Results

The present results have shown that DNA extraction is still possible at a combustion temperature of 500 °C. As expected, all eight samples lost more or less weight in accordance with the temperature at which they were exposed. Surface discolouration also occurred in all samples. For the samples with a burning temperature of 450 °C and 500 °C, the surface discolouration with an analysis according to Shipman et. al. (Shipman et al., 1984) indicates a higher combustion temperature (Shipman stage III – IV, 526 °C – 940 °C). Another observed result is the change towards lower deamination frequencies due to the combustion.

## 6.1 Taphonomic changes and temperature mapping

### *6.1.1 Colouration*

PB 1 was burnt at a temperature of 300 °C and shows an overall reddish brown colour. Secondary colourations are reddish yellow, with a slight pink note. The Shipman stage II (286 °C – 525 °C) classification can be applied to all three areas, outer surface, petrous part and cochlea, with the cochlea showing a slightly greyer colouration compared to the rest of the bone. The combustion temperature therefore matches the Shipman stages. A more detailed description of the staining of all samples can be found in Table 13.


*Figure 19 Burnt PB 1 with broken Squama*

PB 2 has black to very dark brown colouration. Parts of the squama have reddish grey to pinkish white colouration. Black discolouration is seen around the inner meatus. The cochlea itself is mainly brown-black in colour with only a few light spots, which is why it has been classified as Shipman stage II (286 °C – 525 °C). The rest of the bone was classified as Shipman stage II – III (286 °C – 645 °C), all stages are consistent with the actual combustion temperature of 400 °C.

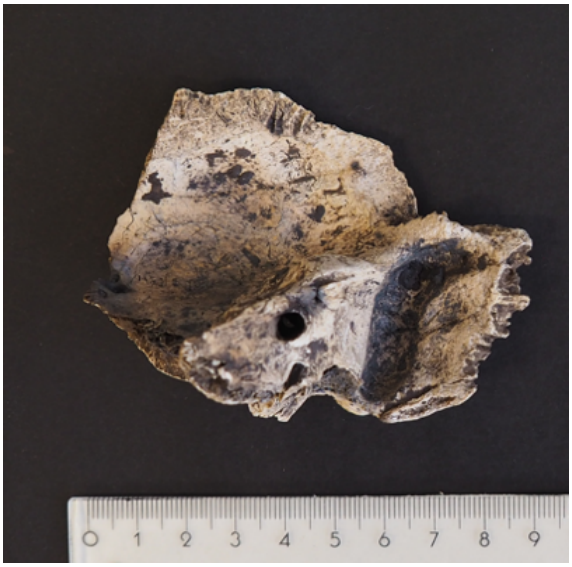


*Figure 20 Burnt PB 2*

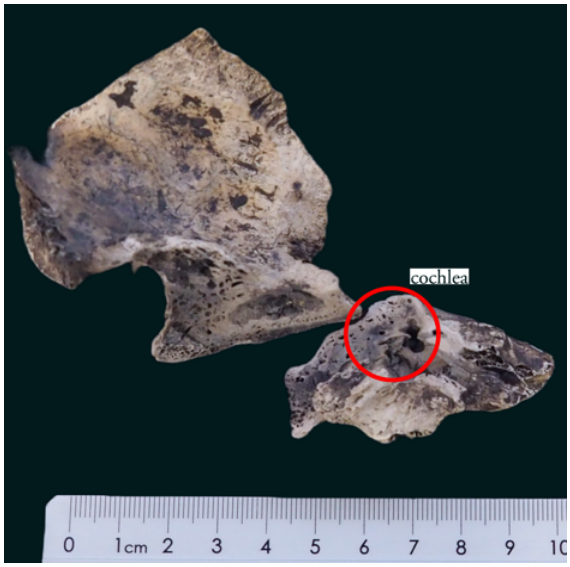



*Figure 21 Horizontally cut PB 2. The colour of the cochlea is visibly darker than the rest of the bone.*

PB 3 was heated to 220 °C and is mostly yellow to very pale brown in colour. Reddish to pinkish white components are also present. The petrous part has more brown components, which is why it was categorised as Shipman stage I – II and the rest as stage I, which can be mapped to the combustion temperature.

*Figure 22 Burnt PB 3*

PB 4 was classified in Shipman stage IV in the outer surface and the petrous part, the cochlea in stage III – IV. This was due to the light grey to white colouration of the bone. The cochlea was slightly thinner grey in colour and had dark brown to black areas. Shipman stage IV corresponds to a temperature range of 626 °C – 940 °C, which is above the actual combustion temperature of 500 °C. Shipman stage III – IV is also just above the actual combustion temperature with a range of 526 °C – 940 °C.

*Figure 23 Burnt PB 4*

*Figure 24 Horizontally cut PB 4 with severed squama. The colour of the cochlea is visibly darker than the rest of the bone.*

PB 5 mainly shows a discolouration from light brown to dark grey, with the area around the inner meatus showing the darkest colouration. There are also a few pale yellow to reddish brown discoloured areas. The entire bone was classified as Shipman stage II (286 °C – 525 °C) which is in accordance with the combustion temperature of 350 °C.

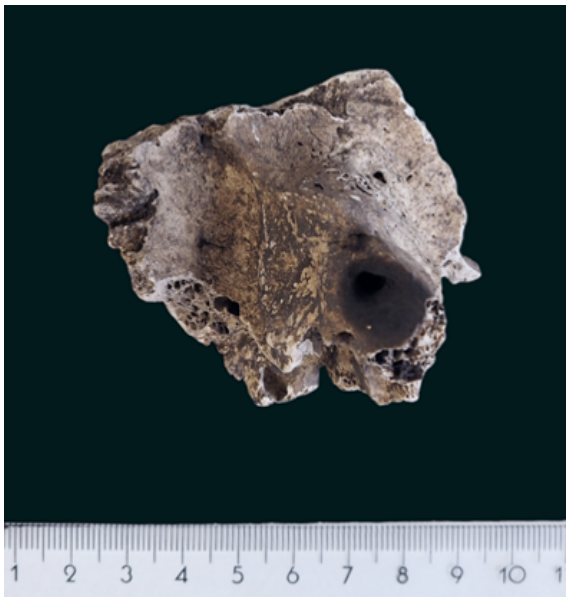


*Figure 25 Burnt PB 5*

PB 6 was heated to a temperature of 145 °C and still shows a typical colouration for a dry unburnt bone. The bone is mainly very pale brown to yellow and shows some pinkish white to reddish-yellow staining. Therefore, the sample was assigned Shipman stage I (20 °C –285 °C).

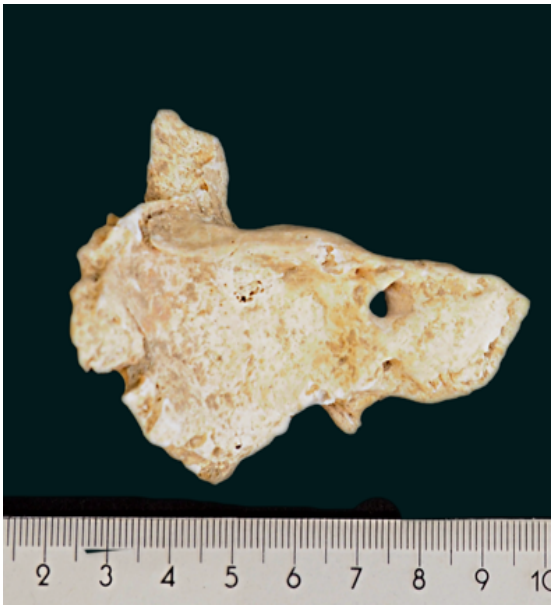


*Figure 26 Burnt PB 6*

PB 7 shows a light brownish grey main colouration. White to pinkish white tones are interspersed. The cochlea is somewhat thinner grey and was therefore assigned to Shipman stage III – IV, the rest of the bone was assigned Shipman stage IV. The estimated temperature range of 626 °C – 940 °C (Shipman stage IV) or 526 °C – 940 °C (Shipman stage III – IV) also exceeds the actual combustion temperature of 450 °C for this sample.

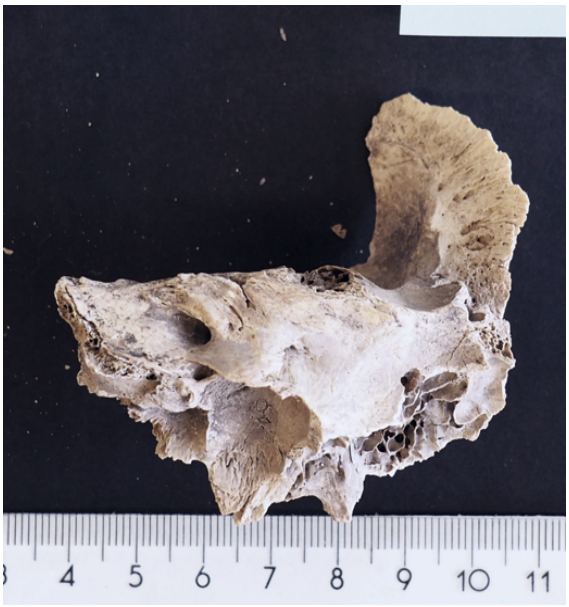


*Figure 27 Burnt PB 7*

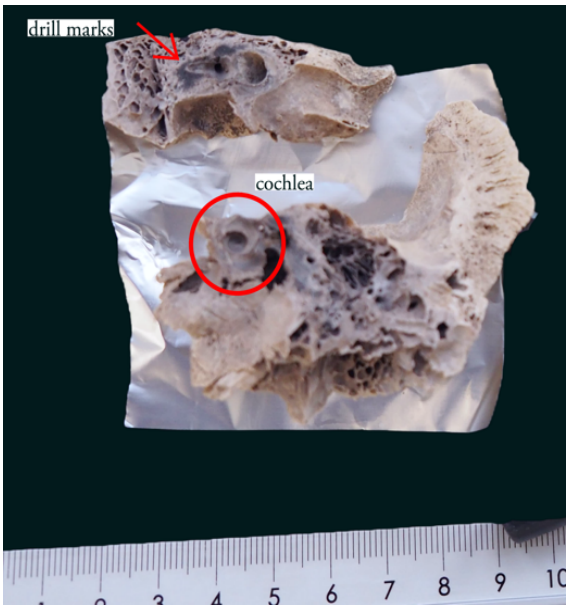



*Figure 28 Horizontally cut PB 7 with visible drill marks from the pre burning bone powder sampling. The colour of the cochlea is visibly darker than the rest of the bone.*

The outer surface and the petrous part of PB 8 were assigned to Shipman stage I – II (20 °C – 525 °C). This is evidenced by the yellowish brown to very brown colouration of the surface. In addition, reddish brown to pinkish areas are visible. The colour of the cochlea is more yellow with some very pale brown to brown discolouration. Therefore, the cochlea was classified as stage I (20 °C – 285 °C). All stages also agree with the combustion temperature of 270 °C for this sample.


*Figure 29 Burnt PB 8*

*Table 13 Colour coding of the samples according to the Munsell's colour code and the Shipman Scala (1984)*

| ***Sample*** | ***Munsell's colours code*** | ***Munsell's colours*** | ***Shipman (1984) stage*** | ***Shipman (1984) temperature estimation*** |
|---|---|---|---|---|
| **PB 1** | | | | |
| Outer surface | 5YR 2.5/2; 7.5YR 6/6, 8/4, 8/3, 7/2; 10YR 3/1, 2/0, 4/6, 4/3; 10YR 3/1, 3/3, 4/2 | Dark reddish brown, reddish yellow, pink, pinkish grey, very dark grey, dark brown, dark greyish brown | II | 286 °C – 525 °C |
| Petrous part | 5YR 2.5/2; 7.5YR 6/6, 8/4, 8/3, 7/2; 10YR 3/1, 3/3, 4/2; 10R 2.5/2 | Dark reddish brown, reddish yellow, pink, pinkish grey, very dark grey, dark brown, dark greyish brown, very dusky black | II | 286 °C – 525 °C |
| Cochlea | 10YR 2/1, 5YR 5/2, 3/1 | Black, reddish grey, very dark grey | II | 286 °C – 525 °C |
| **PB 2** | | | | |
| Outer surface | 10YR 2/1; N9-5/0; 7.5YR 8/2, 7/4; 5YR 7/2, 3/2, 7/1, 6/2 | Black, blue grey, pinkish white, pink, pinkish grey, dark reddish brown, light grey | II – III | 286 °C – 645 °C |
| Petrous part | 10YR 2/1, 2/2; N9-5/0; 7.5YR 8/2; 5YR 7/2, 2.5YR 2.5/1, 7/1, 6/1 | Black, very dark brown, blue grey, pink, dark reddish brown, reddish black, light reddish grey, reddish grey | II – III | 286 °C – 645 ° |
| Cochlea | 7.5YR 8/3, 10YR 7/2, 2/1, 2/2 | Pink, light grey, brown, black, very dark brown | II | 286 °C – 525 °C |
| **PB 3** | | | | |
| Outer surface | 5YR 8/2, 8/3, 8/4, 10YR 8/8, 7/3, 3/2, 6/6, 5/6 | Pinkish white, pink, yellow, very pale brown, very dark greyish brown, brownish yellow, yellowish brown | I | 20 °C – 285 °C |
| Petrous part | 7.5YR 8/3, 7/8, 10YR 8/6, 7/3, 3/2, 6/6, 5/6, 5/8 | Pink, reddish yellow, yellow, very pale brown, very dark greyish brown, brownish yellow, yellowish brown | I – II | 20 °C – 525 °C |
| Cochlea | 10YR 8/8, 7.5YR 8/2, 8/4, 5YR 8/4 | Very pale brown, pinkish white, pink, yellow | I | 20 °C – 285 °C |
| **PB 4** | | | | |
| Outer surface | 7.5YR 8/2, 8/3, 8/4; 10YR 7/1, 7/2, 8/1 | Pinkish white, pink, light grey, white | IV | 626 °C – 940 °C |
| Petrous part | 7.5YR 8/3, 8/2, 8/4; 10YR 7/1, 7/2, 8/1 | Pinkish white, pink, light grey, white | IV | 626 °C – 940 °C |
| Cochlea | 2.5YR 8/1, 7/1; 7.5YR 5/1; 10YR 7/2 | White, light reddish grey, grey, light grey | III – IV | 526 °C – 940 °C |
| **PB 5** | | | | |
| Outer surface | 7.5YR 6/3, 8/1, 5YR 5/3, 5Y 8/2, 8/3, 10YR 3/1, 3/3, 4/2 | Light brown, white, reddish brown, pale yellow, very dark grey, dark brown, dark greyish brown | II | 286 °C – 525 °C |
| Petrous part | 7.5YR 6/3, 8/1, 7/8; 5YR 5/3, 5Y 8/2, 8/3, 10YR 3/1, 3/3, 4/2 | Light brown, white, reddish yellow, reddish brown, pale yellow, very dark grey, dark brown, dark greyish brown | II | 286 °C – 525 °C |
| Cochlea | 10YR 7/1, 2/2; 7.5YR 2.5/1, 5/1 | Light grey, very dark brown, black, grey | II | 286 °C – 525 °C |
| **PB 6** | | | | |
| Outer surface | 10YR 8/3, 8/4; 7.5YR 8/2, 8/4, 8/6 | Very pale brown, pinkish white, pink, reddish yellow | I | 20 °C – 285 °C |
| Petrous part | 10YR 8/3, 8/4, 8/6; 7.5YR 8/2, 8/4, 8/6 | Very pale brown, yellow, pinkish white, pink, reddish yellow | I | 20 °C – 285 °C |
| Cochlea | 7.5YR 8/4, 8/6 | Pink, reddish yellow | I | 20 °C – 285 °C |

| | | | | |
|---|---|---|---|---|
| **PB 7** | | | | |
| Outer surface | 7.5YR 8/3, 8/2, 8/4; 2.5YR 8/1; 10YR 7/2, 8/1, 6/2 | Pink, pinkish white, white, light grey, light brownish grey | IV | 626 °C – 940 °C |
| Petrous part | 7.5YR 8/3, 8/2, 8/4; 2.5YR 8/2; 10YR 7/2, 8/1, 6/2 | Pink, pinkish white, white, light grey, light brownish grey | IV | 626 °C – 940 °C |
| Cochlea | 7.5YR 7/2, 7/3, 7/4, 5/1 | Pinkish grey, pink | III – IV | 526 °C – 940 °C |
| **PB 8** | | | | |
| Outer surface | 10YR 5/8, 6/8, 7.5YR 6/6, 5/6; 5YR 5/3, 8/3, 5/6 | Yellowish brown, reddish yellow, strong brown, reddish brown, pink, yellowish red | I – II | 20 °C – 525 °C |
| Petrous part | 10YR 5/8, 6/8, 7.5YR 8/3, 6/6, 5/6; 5YR 5/3, 8/3, 5/6 | Yellowish brown, pink, reddish yellow, strong brown, reddish brown, pink, yellowish red | I – II | 20 °C – 525 °C |
| Cochlea | 10YR 7/1, 8/6, 8/4; 7.5YR 5/2 | Light grey, yellow, very pale brown, brown | I | 20 °C – 525 °C |

### *6.1.2 Weight loss*

*Table 14 The weight loss of the samples ordered by combustion temperature*

| ***Ref. no.*** | ***Combustion temp. [°C]*** | ***Pre comb. weight [g]*** | ***Post comb. weight [g]*** | ***Weight loss [g]*** | ***Weight loss [%]*** |
|---|---|---|---|---|---|
| PB 6 | 145 | 18.10 | 17.00 | 1.1 | 6.08 |
| PB 3 | 220 | 25.99 | 23.58 | 2.41 | 9.27 |
| PB 8 | 260 | 14.80 | 13.92 | 0.88 | 5.95 |
| PB 1 | 300 | 28.64 | 24.17 | 4.47 | 15.61 |
| PB 5 | 350 | 19.50 | 17.70 | 2.43 | 12.46 |
| PB 2 | 400 | 39.95 | 31.41 | 8.54 | 21.38 |
| PB 7 | 450 | 23.70 | 16.81 | 6.89 | 29.07 |
| PB 4 | 500 | 54.65 | 39.28 | 15.37 | 28.12 |

As expected, all samples have lost a considerable amount of weight, Table 14 shows the exact numbers. It can be seen that the weight loss tends to be proportional to the combustion temperature. Sample PB 6 was burnt at the lowest temperature of 145 °C and has the lowest weight loss of 6.08 %. PB 7, with a combustion temperature of 450 °C, and PB 4 with 500 °C are the samples that were exposed to the most intensive heat and lost the most weight with 28.12 % and 29.07 % respectively. However, sample PB 8 has a weight loss of 5.95 % at a combustion temperature of 260 °C, which means it has lost less weight in percentage terms than its predecessor PB 3, which was burnt at a lower temperature (220 °C). Similarly, PB 5, with a weight loss of 12.46 % at a combustion temperature of 350 °C, shows a lower weight loss than PB 1, which was exposed to a maximum temperature of 300 °C and lost slightly more, namely 15.61 % weight. However, these discrepancies are minor, and the sample set is relatively small so that we can still speak of a trend towards a proportional relationship between weight loss and combustion temperature.

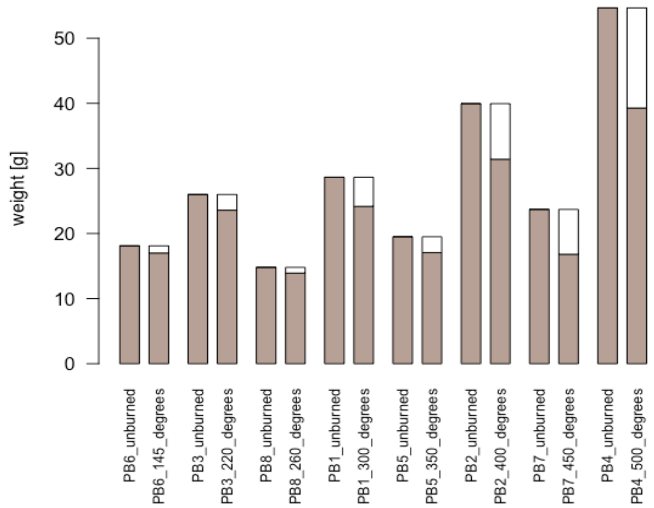



*Figure 30 The weight loss of the samples ordered by combustion temperature in comparison to the unburnt petrous bones.*

## 6.2 DNA analysis

All samples were sequenced with an Illumia (*NovaSeq XP SR100*) instrument at the *Vienna Bio Centre* on behalf of the *Pinhasi Lab*. The sequencing was a first screening run. Deeper sequencing would be possible, but the time available for this experiment was a limitation. However, a deeper sequencing is still possible at a later stage.

All samples were processed as described in the Material and Methods part and a blank was prepared for each extraction and libraries preparation. The results of the blanks show a relatively small number of unique human reads (75 – 25,682), which is within the normal range, and a length of 44 – 46 bp. All blanks show no damage patterns. Table 15 shows the results of the samples in detail.

All sample results show between 2,342,284 and 1,394 unique reads, a length of 70 to 41 bp and the endogenous human DNA content is between 68.47 % and 3.16 %. The damage pattern shows a range of 18 % to 6 % on the 5′ side of the reads and 20 % to 6 % on the 3′ side of the reads.

PB 1, shows relatively low reads with 19,742 unique reads and has no damage pattern. PB 7 and PB 8 also have a low number of reads, with 24,128 and 1,394 unique human reads respectively. However, both samples show a damage pattern of 11 % on the 5′ side of the reads and 10 % on the 3′ side of the reads (PB 7) and 11 % on the 5′ side of the reads and 11 % on the 3′ side of the reads (PB 8). PB 6 already shows relatively few reads (24,838) in the results of the unburnt sample and as expected, shows a further reduction (8,197) in the burnt sample. However, this sample also shows a damage pattern, and the chromosomal sex (M) could be determined. In general, all modern pre-combustion samples (PB 5 – PB 8) have lower unique reads than the ancient samples. The low starting point of the reads naturally also has an effect on the post combustion samples, which are therefore also relatively low, except for PB 5. PB 5 has more unique human reads after the burn (2,342,284) than before the burn (1,398,276). To know the exact reason for this, a deeper sequencing would have to be done.

*Table 15 Results of the DNA analysis sequenced with an Illumia (NovaSeq XP SR100) instrument at the Vienna Bio Centre ordered by combustion temperature.*
*UB = unburned*

| ***Ref. No.*** | ***Combustion temp. [°C]*** | ***total reads*** | ***endogenous [%]*** | ***unique reads*** | ***Mean length [bp]*** | ***Deamination frequencies [5'\|3' - %]*** | ***Chr. Sex*** |
|---|---|---|---|---|---|---|---|
| PB 6 | 145 | 52,113 | 19.06 | 8,197 | 41 | 0.18 \| 0.20 | M |
| | UB | 65,670 | 45.24 | 24,838 | 40 | 0.17 \| 0.16 | M |
| PB 3 | 220 | 5,441,927 | 47.88 | 2,183,177 | 43 | 0.06 \| 0.06 | F |
| | UB | 6,617,603 | 48.26 | 2,616,929 | 65 | 0.22 \| 0.18 | F |
| PB 8 | 260 | 56,506 | 3.16 | 1,394 | 59 | 0.11 \| 0.11 | - |
| | UB | 5,550,166 | 12.43 | 582,994 | 58 | 0.25 \| 0.24 | M |
| PB 1 | 300 | 272,896 | 12.86 | 19,742 | 70 | N/A \| N/A | - |
| | UB | 7,062,960 | 59.37 | 3,510,814 | 58 | 0.24 \| 0.21 | F |
| PB 5 | 350 | 4,194,517 | 68.47 | 2,342,284 | 58 | 0.17 \| 0.15 | M |
| | UB | 6,857,486 | 24.49 | 1,398,276 | 55 | 0.24 \| 0.22 | M |
| PB 2 | 400 | 548,738 | 33.67 | 120,466 | 48 | 0.11 \| 0.11 | F |
| | UB | 4,807,465 | 38.61 | 1,550,878 | 54 | 0.32 \| 0.28 | F |
| PB 7 | 450 | 92,028 | 32.71 | 24,128 | 52 | 0.11 \| 0.10 | - |
| | UB | 7,748,235 | 0.66 | 43,247 | 45 | 0.12 \| 0.12 | M |
| PB 4 | 500 | 1,659,674 | 22.22 | 244,995 | 41 | 0.12 \| 0.12 | F |
| | UB | 4,257,291 | 19.34 | 684,128 | 57 | 0.24 \| 0.22 | F |

# 7. Discussion

## 7.1 Colouration

The experiment clearly shows the gradual discolouration of a bone under the influence of heat. Except for PB 7 (450 °C) and PB 4 (500 °C), the actual staining matches the expected staining in all sample parts. PB 7 and PB 4 show a light greyish, almost white discolouration, indicating greater exposure to heat than they were actually exposed to. A mainly black colouration was expected. This suggests that the time of heat exposure plays a crucial role in the biochemical processes leading to the discolouration. As PB 4 was burnt first, the oven was completely cooled down and the sample was in the closed oven for a total of 90 min. PB 7 was in the closed oven for a total of 60 minutes. Another explanation could be that due to the old age of the bones, which mainly applies to PB 4 (approx. 2,500 – 3,000 years old), the organic components in the bone matrix were already strongly degraded and thus disintegrated more rapidly during the combustion process. The storage of the bones also plays a role and may have led to the fact that PB 7 already had rather strongly degraded organic components before combustion. The modern samples all had relatively low unique human reads (see the DNA analysis part of this chapter) even in their unburnt status but were treated in the same way as the ancient samples. This suggests that there was an extrinsic or intrinsic factor that had a positive effect on the degradation of the DNA present.

## 6.2 Weight loss

As far as weight loss is concerned, the samples behaved as expected and confirm the trend towards a proportional relationship between combustion temperature and weight loss, as already shown by Gonçalves and collaborators (Gonçalves et al., 2013), among others. The existing deviations from proportionality are minor and to be expected and the sample size is relatively small, so it can still be assumed that a larger study under similar conditions would again underline the trend towards a proportional relationship between weight loss and combustion temperature. Nevertheless, the factor ancient vs. modern should also be included here. The fact that sample PB 7 (modern), which was burned at 450 °C, lost slightly more weight (29.07 % in total) than PB 1 (ancient), which was burned at 500 °C (28.12 % in total), can also be explained by the fact that modern samples tend to show more severe heat-induced changes than ancient samples. This is due to the fact that in the ancient samples a large part of the water that was incorporated into the bone matrix has already evaporated over time. Likewise, far more organic components of the bone matrix have already degraded than is the case in modern samples. This affects the reaction of the samples in the combustion process. If there is less material that can undergo heat-induced degradation, then there will be less difference between the pre and post combustion samples. In order to be able to reach concrete conclusions, however, a histological analysis of the samples would be required.

## 7.3 DNA analysis

After this experiment, it can be confirmed that the cochlea is a suitable sample for the recovery of usable DNA fragments in severely degraded bone. All results above 25,682 unique reads (threshold defined by the highest amount of unique human reads detected in the blanks) and with a damage pattern can be considered positive results, all others negative results. In the extraction blank although, a chromosomal sex determination (XX - chrY: 24/1244) was possible. This, in combination with the 25,682 unique reads and the non-existent damage pattern, indicates a small amount of modern contamination. Since none of the samples belong to an identified individual, no conclusions can be drawn about the accuracy of the results.

Except for the results of PB 1, which can be considered a negative result, and PB 8, that yield a low percentage of endogenous DNA, all burned samples show a high to moderate percentage of endogenous DNA (68.47 % – 19.06 %). This is a very promising result and underlines the relevance of research in this area. Indeed, it is expected that larger-scale studies may also reach similar results. However, no more DNA was recovered from the modern burnt samples than from the ancient burnt samples. The lowest value of endogenous DNA was indeed found in the modern PB 6, which was heated at the lowest temperature of 145 °C. This is most likely due to the condition of the sample before combustion. The petrous part of the bone was open laterally and the otic capsule with the cochlea were exposed. The highest value of endogenous DNA was extracted from PB 5. As this value is higher than the value of unburnt PB 5, the result should be further confirmed by a deeper bioinformatical analysis and/or deeper sequencing. It can generally be said that the four modern samples (PB 5 – PB 8) do not show ideal conditions even before combustion (UB). In addition to the untypical behaviour of PB 5 just described, PB 6 already shows only 24,838 unique human reads in the unburned state and thus already starts with a value below the defined threshold. PB 7 contains only 0.66 endogenous (%) reads in the unburned state and PB 8 shows a very high reduction in reads in the post-combustion sample, despite the relatively low combustion temperature of 260 °C, although the results of its unburned sample were better than those of the other modern samples. Overall, one can argue that the ancient samples performed better than the modern samples. All samples were treated exactly the same. However, there is always the possibility that a sample will react differently to the entire extraction and sequencing pipeline due to intrinsic factors or the way and place it was buried or stored. The exact reason for the failure of the post-combustion sample PB 1 and the poor results of PB 6 to PB 8, both unburned (UB) and burnt, could not be determined.

Nevertheless, PB 2 – PB 4 show a pattern in the reduction of reads and the reduction of damage frequency. PB 5 and PB 8 also show a reduction in damage frequency. It is also highly encouraging that between 36.71 % and 22.22 % of endogenous DNA could be extracted from the samples heated to the higher temperatures of 400 °C – 500 °C. This result is consistent with the results of DNA analyses performed with aDNA methodologies on burn victims by the team around Daniel Gaudio (Gaudio et al., 2019).

Since soft tissue is usually still attached to a human body when it is burnt in a real-life-scenario and this experiment was carried out with already degraded samples (ancient samples from circa 900 – 400 AD, modern samples from the 1970s), it can be assumed that even higher DNA yields can be achieved in a real-life low-temperature burning (> 800 °C) or when the body was exposed to the heat source for a limited period of time. However, further studies are needed to confirm this hypothesis.

There can be no doubt that the sample size chosen for this experiment is not sufficient to obtain statistically meaningful results. But especially with very sensitive samples like human skull bones, experiments with a small sample size are of great importance. As pilot studies, they lay the foundation for larger studies that are crucial for gaining knowledge in the respective field.
What was certainly confirmed by the results of this pilot study, however, is that the sampling strategy chosen is crucial to the outcome. Using bone powder from the densest region of the human skeleton as a sample, the Dabney protocol (Dabney et al., 2013a) and purification with *MinElutes*, which is suitable for molecules as short as 30 bp, had a positive effect on the results of the experiment and are therefore ideal strategies for obtaining amplifiable DNA molecules at this stage of research. aDNA methods are more expensive and time-consuming, but the experiment clearly shows that this methodology is more promising for complex and highly degraded samples than conventional forensic extraction methods, where valuable DNA could only be found up to a maximum of 210 °C (Fredericks et al., 2015). The method used for this experiment should therefore definitely be further developed and improved. In particular, there is a need for further studies on the use of aDNA extraction methods in combination with *NGS* in complex forensic contexts, also beyond fire related scenarios. Areas of application include remains that have been in water for a long time or exposed to other extrinsic factors such as severe drought and UV radiation. One concrete area of application that urgently needs more attention and effort is the identification of migrant deaths, of which there are thousands every year as a direct result of brutal and inhumane border regimes, such as the border in the desert between Mexico and the US, or the European Fortress with its border in the East and the Mediterranean. Whereas the most deaths worldwide are recorded in the Mediterranean Sea with more than 21,200 deaths between 2014 and 2020 (*World Migration Report 2022*, 2021). The application of these methods in such delicate forensic context and the relevance of the methods in court require a stable data pool and a meaningful number of peer-reviewed published studies. The potential of aDNA methods and *NGS* for Forensic Anthropology is too great to forego expanding this data pool.
However, for use in real life forensic scenarios, the *Cranial Base Drilling Method (CBDM)* should always be used to obtain the required bone powder. This method is far less invasive than the collection methods used in this experiment. *CBDM* leaves the skull structure largely intact and is therefore the ideal method when it comes to the ethical and moral standards of working with recently deceased human remains. Furthermore, working in a clean room, as was done in the present study, is not essential. Other studies with similar scenarios, such as Daniel Gaudio's in 2019 (Gaudio et al., 2019), have been conducted in modern laboratories. However, in a modern laboratory, extra care is needed in terms of

contamination, especially with highly degraded samples. But here, too, *Next Generation Sequencing* offers an advantage. With this sequencing method, the deamination pattern of a DNA sample can be determined. In the case of nucleotide bases, hydrolytic deamination occurs during the degradation process. These are modifications that cause the affected nucleotide bases to be misread by DNA polymerases. The base most affected by deamination is cytosine (C). During deamination, cytosine becomes uracil (U) and leads to the incorporation of adenine (A) during DNA replication. As a result, visible substitutions of C to T or G to A occur (Dabney et al., 2013b). This makes it possible to distinguish a degenerate sample from a sample from a person who is still alive. However, the risk of cross-contamination remains.

What this experiment has also shown is that it is possible to bridge the gap between Forensic Anthropology and other disciplines, in this case the field of aDNA research or genetics. Evelyn Zelger had no previous experience in the field of laboratory work with genetic material and was only trained by the *Pinhasi Lab* team in the course of this study. She then carried out all the methods herself. So, there is a strong call to approach applied science courageously and to engage with disciplines that are supposedly beyond the boundaries of one's own discipline. Especially since an interdisciplinary approach gives all researchers a broader view, as different disciplines bring different approaches and perspectives to the topic at hand. The work in the *Pinhasi Lab*, where anthropologists, archaeologists, geneticists, (micro- )biologists and bioinformaticians work together, is the best example of a successful interdisciplinary approach. Studies such as Helena Correia Dias' on DNA methylation for age determination (Correia Dias et al., 2020a; Correia Dias et al., 2022; Correia Dias et al., 2020b), Daniel Gaudio's on DNA analysis of identified burn victims using *NGS* (Gaudio et al., 2019), Saskia Ammer's on stable isotopes and geographical origin (Ammer et al., 2020a; Ammer et al., 2020b), among others, are already pointing in a direction where Forensic Anthropologists are increasingly turning to microanthropological methods. Approaches from the fields of microbiology, genetics and chemistry are increasingly being carried out by Forensic Anthropologists themselves and show a trend towards taking responsibility beyond the triage role of Forensic Anthropology in complex identification processes. This offers the advantage that the entire chain of analysis can be carried out by fewer people, so there is less risk of falsifying the results. Also, the interpretation of the results is easier if the interpreters can carry out the whole sampling process by themselves which entails: the recovery of the human remains, the analysis of the bones, and the bone powder sampling for DNA analysis. This is particularly relevant for very critical samples, as special care and accuracy are required.

# 8. Conclusion

This research aimed to contribute to narrowing the knowledge gap on the exact threshold for DNA survival under the influence of heat. The results of the study show that even at a combustion temperature of 500 °C, viable DNA molecules can still be extracted from cochlear bone powder using the Dabney protocol (Dabney et al., 2013a) in combination with *NGS*. While the sample size and the analysis of unidentified individuals limit the significance of the results, the use of the chosen methodology in future studies offers the possibility of establishing a promising DNA analysis practice in sensitive identification cases in Forensic Anthropology. Following this pilot study, there are several possibilities to continue the research. Probably the two most important areas of approach after this experiment are a study with identified individuals and a larger sample size. The identified individuals are necessary to be able to verify the results obtained and a larger sample size for statistically relevant results. Since it will be difficult to make a larger number of human skull bones available, animal bones may have to be used as well. It would also be interesting to compare actual fire incineration with heating in an electric muffle furnace, as was conducted for the present experiment. An American-Portuguese research team recently conducted such a comparison focusing on isotope ratio values in burnt bone material and showed that there are indeed differences between the two combustion methods (Sarancha et al., 2022). Furthermore, an experiment should be carried out at higher temperatures. Although a maximum temperature of 500 °C was deliberately chosen for this study to counteract the absence of soft tissue, the present results cannot give an indication of the temperature up to which successful DNA extraction is possible. It should be pointed out once again that a fire in a forensic context can reach between 600 °C and 1,200 °C. The exact relationship between heat and the deamination patterns offers further starting points for future studies. Indeed, the results of this experiment showed that exposure to heat causes DNA strands with deaminated nucleotide bases to be shortened. This study could clearly contribute to the expansion of knowledge regarding the behaviour of DNA under the influence of heat. The experiment also shows that the field of Forensic Anthropology is not limited to the analysis of whole and fragmented bones but is also capable of conducting microanthropological studies that give the discipline additional prestige and relevance.

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# 9. Index of Tables

Table 3 Shipman, P., Foster, G., & Schoeninger, M. (1984). Burnt bones and teeth: an experimental study of color, morphology, crystal structure and shrinkage. *Journal of archaeological science, 11*(4), 307-325.

Table 13 Shipman, P., Foster, G., & Schoeninger, M. (1984). Burnt bones and teeth: an experimental study of color, morphology, crystal structure and shrinkage. *Journal of archaeological science, 11*(4), 307-325.

All other tables were created by Evelyn Zelger herself and are based on the data collected.

# 10. Index of Figures

Figure 1 The graphic was created by Emilia von Breitenberg. The original is published in Potuguese in: Palhares Machado, C. E., Deitos, A. R., Velho, J. A., & Cunha, E. (Eds.). (2022). Tratado de Antropologia Forense - Fundamentos e Metodologias aplicadas à Prática Pericial.

Figure 2 The graphic was created by Emilia von Breitenberg. The original is published in Potuguese in: Palhares Machado, C. E., Deitos, A. R., Velho, J. A., & Cunha, E. (Eds.). (2022). Tratado de Antropologia Forense - Fundamentos e Metodologias aplicadas à Prática Pericial.

Figure 3 Schmidt, C. W., & Symes, S. A. (2015). The analysis of burned human remains (2 ed.). Academic Press.

Figure 14 Sirak, K. A., Fernandes, D. M., Cheronet, O., Novak, M., Gamarra, B., Balassa, T., Bernert, Z., Cséki, A., Dani, J., Gallina, J. Z., Kocsis-Buruzs, G., Kővári, I., László, O., Pap, I., Patay, R., Petkes, Z., Szenthe, G., Szeniczey, T., Hajdu, T., & Pinhasi, R. (2017). A minimally-invasive method for sampling human petrous bones from the cranial base for ancient DNA analysis. BioTechniques, 62(6), 283-289. https://doi.org/10.2144/000114558

Figure 15 Sirak, K. A., Fernandes, D. M., Cheronet, O., Novak, M., Gamarra, B., Balassa, T., Bernert, Z., Cséki, A., Dani, J., Gallina, J. Z., Kocsis-Buruzs, G., Kővári, I., László, O., Pap, I., Patay, R., Petkes, Z., Szenthe, G., Szeniczey, T., Hajdu, T., & Pinhasi, R. (2017). A minimally-invasive method for sampling human petrous bones from the cranial base for ancient DNA analysis. BioTechniques, 62(6), 283-289. https://doi.org/10.2144/000114558

Figure 18 Roche Life Science. Available online at: https://lifescience.roche.com/en_at/products/high-pure-viral-nucleic-acid-large-volume-kit.html#details [last accessed: 7.7.2022].

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