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DNA Degradation in Teeth and Bones

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Contents

Summary

A solid understanding of DNA degradation in teeth and skeletal bones is extremely important for forensic (modern) and ancient (degraded over time) DNA analysis of human skeletal remains. In order to maximize the chance for successful DNA analysis, researchers need to know what samples to collect and process. However, earlier studies based on empirical data have revealed inconsistent conclusions, indicating that too many variables affect DNA preservation in these two different types of tissues. For example, analysis of DNA degradation in teeth from two mummies most likely is going to vary depending on the soil activity in the two different areas where the mummies where buried. Through controlled experiments, this study aimed to employ ancient DNA techniques for the recovery and analysis of DNA from heat-treated skeletal bones and teeth originating from sheep (*Ovis aries*). This included to completely separating the extraction and preparation of ancient DNA (degraded DNA) from work with modern DNA, bleach treatment of the laboratory, UV irradiation, fully protective clothing and disposable masks and gloves. DNA degradation in this study was estimated as reduced PCR-amplification, and the goal of the study was to come to an understanding of any possible differences in degradation between the two different tissues.

Soft tissue was removed from the samples to the greatest extent possible prior to heat treatments, to ensure that extracted DNA originated from hard tissues rather than soft tissue. The teeth and bones were subjected to heat at 85^{o}C for 5 – 15 h, and ground into fine powder. To illustrate potential DNA degradation in teeth and skeletal bone samples after heat treatment, amplifications of 125 – 556 base pair sequences from mitochondrial DNA as well as nuclear DNA (sexing) were attempted. This study revealed that the heated skeletal bones contained more DNA than teeth that had been treated in the same way. Teeth samples showed evidence of DNA degradation in templates for two of the three amplicons. Because of the higher DNA content, degradation of skeletal bone DNA was evident only after diluting the original bone samples.

The study demonstrated the feasibility and benefits of using faunal remains to study DNA degradation since contamination with modern sources can be more readily prevented, in particular when experiments are conducted in a dedicated DNA lab space. It has laid a solid foundation for future studies to include more species with more heat treatments and with different temperatures, and has shown that the experimental approach of using faunal materials can act as a highly promising approach for the study of DNA degradation processes in bones and teeth. It can be expected such experimentation will provide important insights for DNA analysis in forensic investigations and archaeological research.

Introduction

Because of their resistance to environmental impacts such as incineration and decomposition, teeth and skeletal bones are the most abundant remains that are preserved in archaeological and forensic settings. When a body has been subjected to fire, one of the most destructive forces of nature, conventional methods for identification can be completely inapplicable because of severe soft tissue damage. Major inorganic components slow down the decomposition and degradation processes significantly, resulting in well preserved dental and skeletal remains (Dr. Dongya Yang, personal communication). Teeth and skeletal bones are often the sole evidential DNA material for identification of individuals who have been deceased for a longer period of time, or have been the victims of fire related deaths, when conventional dental identification is not possible (Sweet & Hildebrand 1998). The improvement of methods allowing for accurate and precise identification from skeletal material from forensic and archaeological sites has been an intense field of study for more than a century and still is (White 2000).

Traditional forensic anthropology aims to study human dental and skeletal remains for identification of sex, age, ancestry (race) and stature estimation, which, once obtained, can provide extremely important information to facilitate positive identification of remains (White. 2000). As demonstrated in many cases, individual identifications of remains need to be confirmed by DNA analysis of the deceased and their relatives (Sweet & DiZinno 1996).

DNA degradation

All cells except red blood cells in an animal body contain nuclei with genomic DNA (nDNA) and mitochondria with mitochondrial DNA (mtDNA). When an organism dies and starts to decompose, body tissues are gradually broken down into their components by the activity of bacteria. The DNA is degraded by the activity of endogenous nucleases and microorganisms, and further destabilized and broken down by processes like deamination and depurination. (Hofreiter *et al.* 2001). To what extent the degradation process affects the DNA relies on two factors: environmental conditions and the time of exposure (Burger *et al*. 1999). The interaction between time and environmental conditions including temperature, humidity, pH and soil chemistry is very complex and makes it extremely difficult to establish any kind of set pattern or correlation between time and stage of decomposition.

Ancient DNA - extremely degraded DNA

Mitochondrial DNA contains only a small number of genes, but since each cell contains thousands of copies, the mitochondrial DNA is often preserved well and available for forensic analysis (Butler & Levin 1998).

The discovery of the polymerase chain reaction (PCR) allowed for amplification of small amounts of DNA, which gave ancient DNA research a real boost, although working with such degraded samples involves problems; one little sequence of contamination can disturb the whole amplification of the extremely damaged ancient DNA (Handt *et al*. 1994).

Failure of PCR amplification is often the consequence of the presence of inhibitors such as humic acid, fulvic acid, biologically degraded products and collagen (Keyser-Tracqui & Ludes 2005, Kalmar *et al*. 2000). Many DNA extraction methods therefore focus on the removal of potential PCR inhibitors, and two of the more frequently used methods include

phenol-chloroform extraction (Faermann *et al*. 1995, Hanni *et al.* 1995) and chelex-based extraction (Walsh *et al.*1991 , Faerman *et al.*1995).

DNA analysis

Analysis of DNA from skeletal bones and teeth holds great potential for accurate and precise identification of remains. It is essential to retrieve adequate amounts of DNA from skeletal remains in the first place, and a solid understanding of DNA preservation in skeletal bones and teeth is critical for successful recovery and analysis of degraded DNA samples. Since teeth and skeletal bones are the most abundant remains, efforts have been made in the field of forensics to enhance the ability to extract enough DNA from skeletal remains (Dr. Dongya Yang, personal communication). PCR to yield amplicons of different sizes from both mitochondrial and nuclear DNA is used to examine the severity of DNA degradation (Handt *et al.* 1994), and for the PCR to reflect DNA degradation it needs to be quantitative and exclude PCR inhibitors since inhibitors can alter the results. It has proven to be a challenging job to recover good quality DNA from skeletal remains since the DNA in bones and teeth has undergone severe degradation, and is often mixed with chemical impurities that can inhibit subsequent PCR analysis, as mentioned above.

While new approaches have been taken to remove PCR inhibition (Yang *et al*. 1998), it is also important to recover more DNA templates in the first place. The bones and soft tissue of the head protects teeth from harsh environmental conditions, including incineration, to a greater extent than bone (Schwarz *et al*. 1991, Spitz 1993, Fisher 2004). However, earlier studies have shown inconclusive results regarding what type of remains hold a better chance of yielding adequate amounts of good quality DNA. Some studies suggest that DNA is better preserved in bones (Kim *et al*. 2008, Hagelberg *et al*.1991), others suggest the opposite (Ginther *et al*. 1992, Rees and Cox. 2010, Alonso *et al*. 2001). A study conducted by Faerman *et al*. in 1995 failed to show any differences at all.

The inconclusive results are mostly based on empirical data from DNA analysis of archaeological remains or some recent skeletal remains. Conflicting empirical studies may in fact reflect the complexity of DNA degradation. There are too many variables involved in DNA degradation and preservation in ancient remains. For example, each burial is unique in terms of pH, moisture and the amount of biological agents such as insects and bacteria, amongst others. Also, each collection of skeletal remains has a different antiquity, and if empirical data are from human teeth and skeletal bones, contamination can be a huge potential problem (Dr. Dongya Yang, personal communication).

When conducting a comparative study, keeping to similar sizes is important to be able to compare the severity of degradation, since small pieces might not give the same amount of protection as larger pieces. When performing DNA extraction from hard tissues, a liquid nitrogen grinding mill is useful when turning the samples into fine powder. A magnetic plunger is thrown back and forth from an electric current in a vial surrounded by liquid nitrogen, to make the sample brittle and also to protect the DNA from degradation by heat. By turning the samples into powder, the surfaced area increases and cells are more easily subjected to the biochemical reagents that break down cells for the release of DNA into the solution (Pretty and Sweet 2001).

Aims

To avoid complex impacts of too many degradation factors, this study was designed to focus solely on the comparison of DNA recovery from skeletal bones and teeth in a controlled lab setting with a single destructive factor - constant heat. Teeth and skeletal bones were exposed to the same heat treatments and ground to powder. DNA extractions were performed, and PCR amplifications were visualized on agarose gels. To avoid potential effects of contamination between lab worker and examined material, faunal species (sheep) were used. Samples were examined in a dedicated forensic DNA lab at the Centre for Forensic Research at Simon Fraser University.

Results

DNA degradation

Two sheep skulls and two femur bones were bought from a local butcher. Pulp and bone marrow were removed at the lab. PCR amplifications from teeth originating from one skull were visualized in the same gels as PCR amplification from the skeletal bone pieces from one femur bone, this referred to as "Sheep set 1", and the remaining set with the second sheep skull and skeletal bone pieces from the second femur was referred to as "Sheep set 2". Approximately the same sizes of four molar teeth fragments per sheep skull and four femur bone pieces per femur bone were subjected to 0 hours, 5 hours, 10 hours or 15 hours of heat treatment in an oven set to 85^{o}C, with one tooth fragment and skeletal bone piece per sheep set per heat treatment. The teeth and skeletal bone pieces were ground in a liquid nitrogen grinding mill (also called cryogenic grinder). Approx 0.11g of powder from each sample was incubated over night and subjected to DNA extraction the following day, with the same volume recovered for each skeletal bone or tooth sample. Three regions of mitochondrial DNA and one region in the sex chromosomes were amplified.

Table 1 shows the effects of heat treatment of the samples. A slightly greater weight loss could be observed for bones (with an average of 7.69 % weight loss) compared to teeth (an average of 1.65 % weight loss), an indication that skeletal bones originally consist of more organic material than teeth.

Table 1. Effects of heat treatment at 85^{o}C for 0h, 5h, 10h and 15h on teeth fragments and skeletal bone pieces.

Hours at 85°C	Weight prior to heating (g)				Weight change after heating at 85^{o}C (g (%))			
	Sheep set [a] 1		Sheep set 2		Sheep set 1		Sheep set 2	
	Teeth	Bones	Teeth	Bones	Teeth	Bones	Teeth	Bones
0[b]	1.00	1.14	0.91	1.16	- 0.02 (2.0)	- 0.04 (3.5)	- 0.01 (1.1)	- 0.01 (0.87)
5	0.93	1.18	0.88	0.96	- 0.03 (3.23)	- 0.07 (5.93)	- 0.02 (2.27)	- 0.10 (10.4)
10	1.09	1.15	1.02	1.12	- 0.03 (2.75)	- 0.09 (7.82)	- 0.04 (3.92)	- 0.14 (12.5)
15	0.99	1.17	0.99	1.08	- 0.04 (4.0)	- 0.11 (9.4)	- 0.03 (3.0)	- 0.12 (11.1)

[a] One set contains of teeth fragments from one skull together with the bone pieces from one femur.
[b] Each heat treatment included four samples; one tooth fragment from each skull and one skeletal bone piece per femur bone.

Amplification of undiluted DNA extracts

The first PCR amplification was performed without dilution of the DNA extracts. No PCR amplification was observed for bone mitochondrial DNA after 0 hours or 5 hours of heat treatment (figure 1). Since it was unlikely that the DNA had been destroyed after 0 hours of treatment, this indicated the presence of PCR inhibitors in the bone samples. Blanks and negative controls showed no PCR amplification.

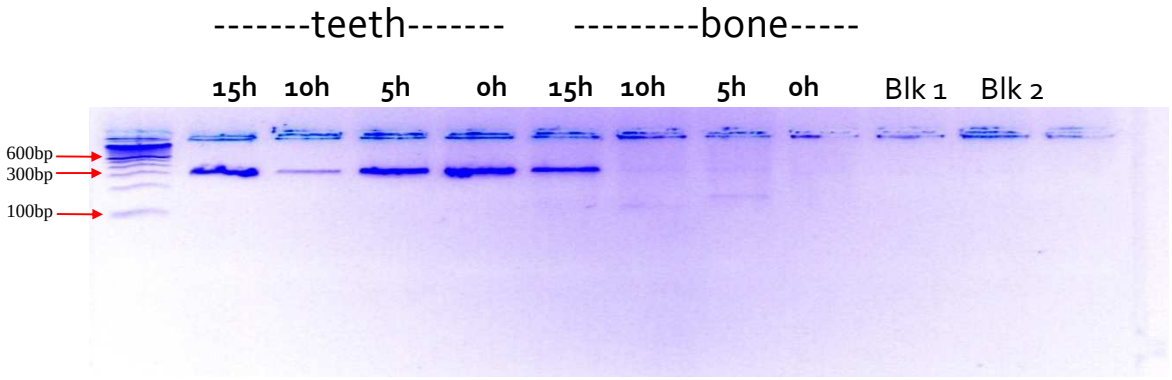

Figure 1. Analysis of 315 base pair amplicons from mitochondrial DNA from undiluted teeth and skeletal bone DNA extracts from sheep set 1. Teeth and skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified at 40 cycles, using primers F624 and R938, and visualized in a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

Amplification of diluted DNA extracts

Extracts were diluted 5-fold and the number of PCR cycles decreased from 40 to 30, to be able to approximate visually the differences in the amount of obtained amplicons on the gel, since the PCR was not quantitative. Any positive controls used in the following amplifications derive from the 15h bone sample from sheep set 1. Sex identification of samples was made from amplifying 220 and 260 base pair regions from the sex chromosomes. Figure 2 shows that the teeth of sheep set 1 were female and the skeletal femur bones in sheep set 1 were male. The results indicated that nuclear DNA was preserved even after 15 hours of heat treatment. Assuming that weaker amplicon bands reflect lower amounts of template DNA, the results suggest that less tooth DNA was present after heat treatment than before, in turn implying DNA degradation due to heat treatment. There was no indication of similar DNA degradation in bones. Blanks and negative controls showed no PCR amplification.

Figure 2. Sex identification of teeth from Sheep set 1 and for the femur bones used for experiments with Sheep set 1. The expected amplicon size was 260 base pairs from the X chromosome and 220 base pairs from the Y chromosome. Teeth and skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified, using primers F90 and R214, and visualized in a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

The sex identification of Sheep set 2 showed that both teeth and bones were male (figure 3). As with Sheep set 1, the nuclear DNA was preserved even after 15 hours, teeth DNA degradation was suggested by comparing the 15 hour sample to the 0 hour sample, while the skeletal bone samples yielded approximately the same amount of amplicons with or without prior heat treatment. The blank and negative controls showed no PCR amplification.

Figure 3. Analysis of sex identification of teeth from sheep set 2 and the femur bones used for experiments with sheep set 2. One band indicates that the individual carries two X-chromosomes (260bp), two bands indicate that the individual carries one X-chromosome and one Y-chromosome (220bp). Teeth and skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified, using primers Amel-F-Bovi and Amel-R-Bovi, and visualized on a 2% agarose gel. Well number 1 shows the 100 base pair ladder. A positive control from the previously amplified (fig 2) 15h bone sample from sheep set 1 was included to permit comparisons of results from these two tests.

When a 125 base pair region of mitochondrial DNA was amplified from teeth and bone samples from Sheep set 1, less amplicons were obtained from all heated samples, suggesting some DNA degradation due to the heating (figure 4). The blanks and negatives did not yield PCR amplifications.

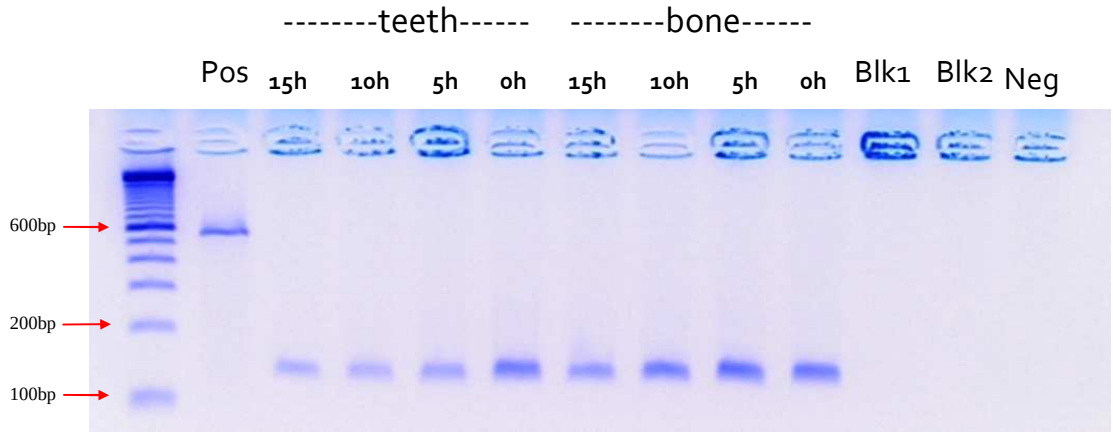

Figure 4. Analysis of 125 base pair amplicons from mitochondrial DNA from diluted teeth and skeletal bone DNA extracts from Sheep set 1. Teeth and skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified, using primers F90 and R214, and visualized in a 2% agarose gel. Well number 1 shows the 100 base pair ladder. The positive control was from the same 15h bone sample from sheep set 1 as for figure 3, with amplification of a 556 base pair region.

Lower amounts of 315 base pair and 556 base pair mitochondrial amplicons upon heating of the template suggested some heat-induced DNA degradation in teeth for set 1 (figure 5). The blanks and negative controls were without PCR amplification. The skeletal bone samples yielded approximately the same amount of amplicons with or without prior heat treatment. Thus, either this DNA was not degraded, the amplification was limited by other factors than the amount of DNA or too much DNA was loaded on the gel to permit discrimination.

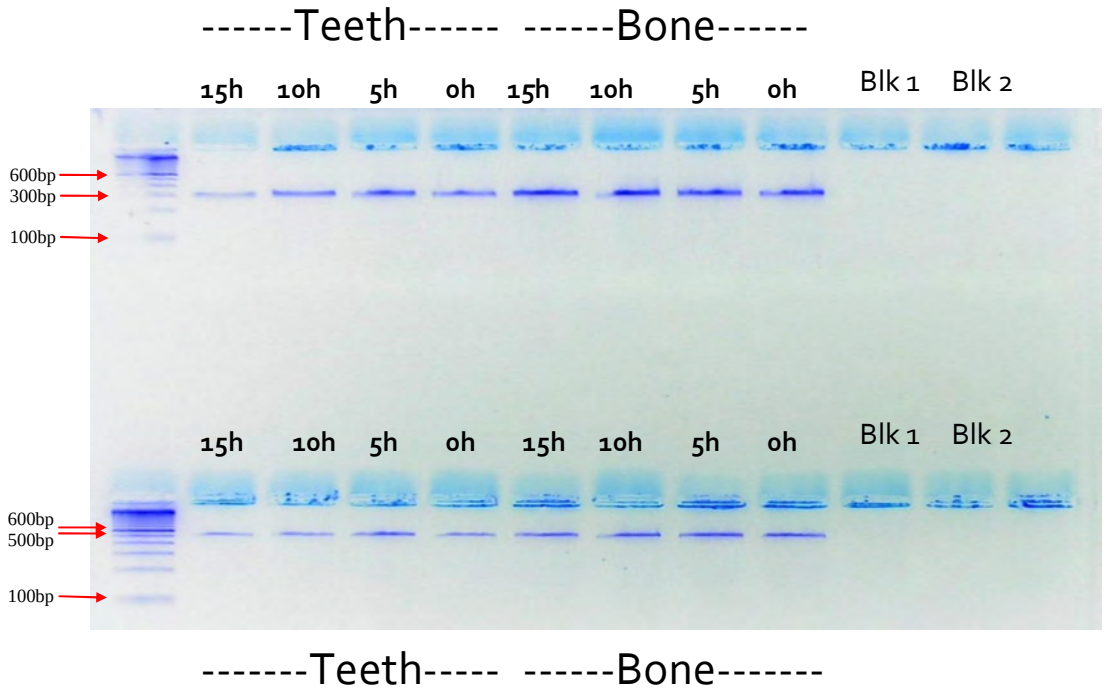

Figure 5. Analysis of 315 (top) and 556 (bottom) base pair amplicons from diluted teeth and skeletal bone DNA extracts from sheep set 1. Teeth and skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified, using primers F624 - R938 and F624 – R1180, and visualized on a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

Also, for Sheep set 2, lower amounts of 315 base pair and 556 base pair amplicons were obtained from the heated teeth samples, suggesting some DNA degradation due to the heating (figure 6). The blanks and negative controls were without PCR amplification. Again, the skeletal bone samples yielded approximately the same amount of amplicons with or without prior heat treatment.

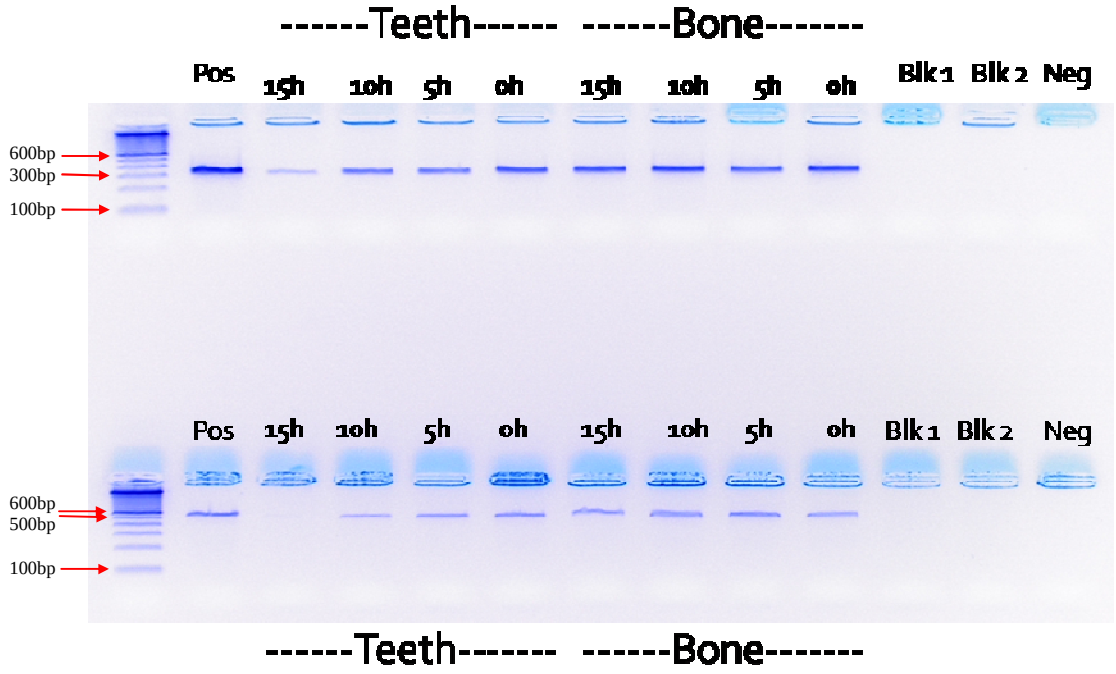

Figure 6. Analysis of 315 (top) and 556 (bottom) base pair amplicons from diluted teeth and skeletal bone DNA extracts from sheep set 2. Teeth and skeletal bone samples were exposed to heat at 85^{o}C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified, using primers F624 - R938 and F624 - R1180, and visualized on a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

These results suggest a correlation between lower amounts of obtained amplicons and increasing time of heat exposure, and also a correlation between lower amounts of amplicons and increasing length of amplicon product. Assuming the differences in band intensity correspond to differences in amounts of template DNA, the bone samples in both sheep set 1 and sheep set 2 yielded greater amounts of amplicons than teeth samples after longer heat treatments.

Comparison between AmpliTaq Regular and AmpliTaq Gold

To test the importance of the DNA polymerase used for identification, AmpliTaq Gold, the standard enzyme used so far, was compared to AmpliTaq Regular. In addition, the bone samples were now diluted 10-fold to see if this would result in noticeably different amounts of amplicons on the gel, and instead of the previously used 2.5 µl of DNA added at the PCR set-up, 1.5 µl DNA was now used for the 315 base pair amplicons (figure 7) and 5 µl DNA for the 556 base pair amplicons (figure 8). Results indicated a lower amount of amplicons from skeletal bone DNA with longer heat treatment. AmpliTaq Gold yielded more DNA for larger amplicons compared to AmpliTaq Regular. The relationship between the apparent amount of amplicon and the severity of heat treatment was more obvious using AmpliTaq Gold (figs 2-6, upper lanes of fig 7 and 8) than using AmpliTaq Regular (lower lanes of figs 7 and 8). In addition, using AmpliTaq Regular resulted in "primer dimers" (figs 7 and 8, lower lanes). Blank and negative controls showed no PCR amplification.

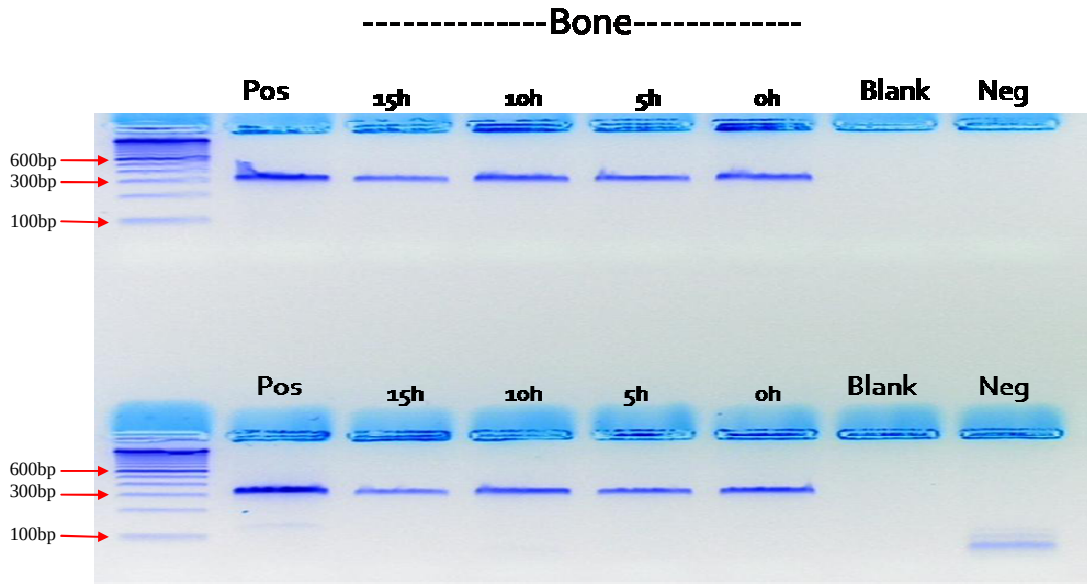

Figure 7. Analysis of 315 base pair amplicons from skeletal bone samples diluted 10-fold from sheep set 2, using AmpliTaq Gold (top) and AmpliTaq Regular (bottom). Skeletal bone samples were exposed to heat at 85°C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified (1.5 µl of DNA), using primers F624 and R938, and visualized on a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

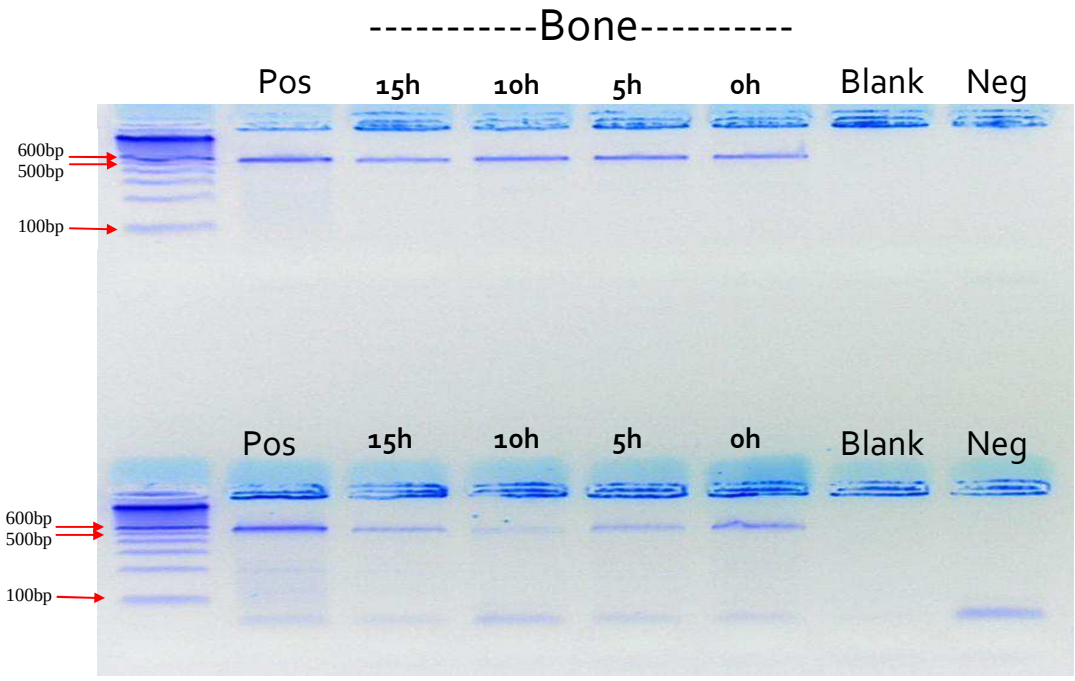

Figure 8. Analysis of 556 base pair amplicons from skeletal bone samples diluted 10-fold from sheep set 2, using AmpliTaq Gold (top) and AmpliTaq Regular (bottom). Skeletal bone samples were exposed to heat at 85^{o}C for 15h, 10h, 5h, or 0h respectively. Heat treated samples were ground to powder. The DNA was extracted and amplified (5 µl of DNA), using primers F624 and R1180, and visualized on a 2% agarose gel. Well number 1 shows the 100 base pair ladder.

Discussion

Results obtained by Handt *et al*. (1994) suggested an inverse relationship between amount of amplicons and length of the amplicons. Handt *et al.* also suggested that if this inverse relationship is not observed, this should be taken as a strong indication of contamination (such as PCR products from former amplifications). The fact that my study demonstrated such an inverse relationship supports the notion that the "ancient DNA techniques" (which are developed to take to extreme measures to minimize contamination risks), as practised at the forensic DNA lab at the Centre for Forensic Research at Simon Fraser University, were adequate for the recovery and analysis of DNA from heat-treated samples.

DNA degradation differences in bones and teeth

Although the method used was not quantitative, the intensity of the amplicon bands could be assumed to reflect differences in amounts of amplicons. Reduced amplification of 315 and 556 base pair sequences in heat-treated DNA from the teeth and skeletal bones of sheep, suggested that the template DNA had been degraded during heating. A larger amount of amplicons was obtained from skeletal bone than teeth through the whole study, indicating that good quality DNA was obtained more easily from skeletal bone than from teeth. This would be applicable both in forensic cases and in cases concerning ancient DNA. Teeth samples showed consistently less amount of amplicons compared to skeletal bone samples with amplicon sizes of 315 and 555 base pairs. Any differences in amount of amplicons in heat treated skeletal bone samples were seen only after diluting the bone DNA extracts, indicating bone samples still contained high amounts of DNA after heat treatments. This observation was expected since bone tissue contains a larger amount of cells compared to teeth.

There was an indication of a small variation between sheep individuals in DNA preservation. When comparing the amount of 556 base pairs amplicons from the 15 hour sample for Sheep set 1 with the same amplicon size from Sheep set 2, there was still a visible band from Sheep set one whereas almost no DNA could be amplified from the 15 hour sample from Sheep set 2. The samples in all were less intense for Sheep set 2 compared to Sheep set 1. It was expected to see differences in DNA degradation since the study was based on two different individuals with different morphology from one another.

Forensic and archaeological importance

At present time there is a disagreement amongst researchers regarding whether teeth should be left embedded in the skeletons of forensic remains for dental identification, or be removed for DNA identification (Dr. Dongya Yang, personal communication). DNA identification of human remains is expensive in terms of both time and money, since it needs to be confirmed that the obtained DNA is not the result of contamination. Similar discussions are applicable in the findings of archaeological remains. Using teeth for DNA information automatically destroys physical pieces of archaeological and anthropological importance. No studies have been performed previously comparing the outcome of DNA analysis between teeth and skeletal bone after exposing the two different tissues to exactly the same treatments. Even though there was not time to perform quantitative PCR in this study, when looking at gained results, it might be favourable to primarily focus on skeletal bones for DNA identification since these yielded better quality DNA compared to teeth. In other words, teeth should be left in their original position for dental identification or archaeological importance. A way to

further support this suggestion, and to make the PCR more quantitative, would have been to test different concentrations of DNA for the amplification. Unfortunately, this was not done.

Use of real time PCR

Real-time PCR was not used in this study due to lack of time, but is necessary for the quantification of the DNA. The technique used in this study can show the differences in degradation patterns between teeth and bone, but cannot give quantitative degradation rate differences. Results given in this study show strong indications that bone yield a greater amount of DNA compared to teeth when being exposed to constant heat at 85^{o}C for the duration of 5 hours, 10 hours or 15 hours of exposure, but without the use of real time PCR it is not possible to tell whether bone or teeth actually *preserves* DNA better than the other, i.e. if bone or teeth have a quicker rate of degradation than the other one. This can be tested by performing real time PCR at different intervals of exposure and then to calculate a function that matches the curve of degradation.

It might be that bones yield a higher amount of usable DNA up to a certain time of exposure due to the higher concentration of cells in bone tissue, but that the DNA after this time of exposure is less preserved than in teeth. Due to the more dense structure and inorganic materials, dental tissue is believed to offer better DNA preservation and hence a slower degradation rate. This would show as a crossover in a diagram over DNA degradation as can be seen in figure 9. Marie Allen, senior lecturer at Uppsala University, supports the suggestion that teeth might preserve the DNA better than skeletal bones in very old samples (personal communication).

If this hypothesis is supported, it would be of great importance for cases concerning ancient DNA where the least amount of physical contact between ancient material and modern sources are sought for, due to the extremely small quantities of DNA present in the ancient material. The amount of physical contact would hopefully decrease if it was shown that teeth preserve DNA better than bone when time of exposure to degradation factors is advanced, since scientists then would know from start what sample to collect for DNA extraction.

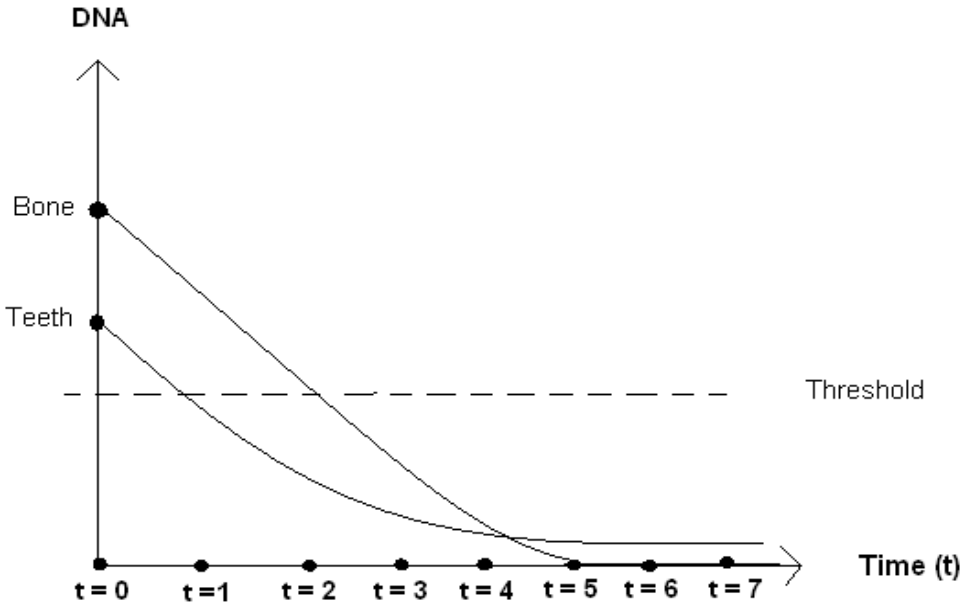

Figure 9. Example of the possible degradation curves for skeletal bones and teeth. (t) indicates a non-determined value for time, starting from the original amount of DNA present in the samples at time zero (t = 0).

Future research

The bone pieces cut from the long bones were mixed during preparation. All bone samples were found to be male but the technique being used can not reveal if the bones used for each experiment originated from the same individual. In future studies, more care should be taken to avoid mixture of samples.

Even though the same conditions and procedure are applied to all samples in an experiment, the extraction efficiency might differ between the samples, resulting in a variation in the amount of extracted DNA. Measuring the amount of DNA after extraction can determine if such is the case.

Further research also needs to include a larger sample size, to examine the effects of species, age and sex on DNA degradation (internal structures might differ depending on age and therefore affect the DNA degradation), to expose the samples to different kinds of heat (e.g. vary temperature and duration, use real fire for the natural fluctuation of temperature), to examine the effects of bone size on DNA degradation (hypothetically, larger teeth would offer better protection of the pulp tissue from heat than small teeth, and larger pieces of bone would offer better protection than small pieces) and to study potential differences in DNA degradation between teeth directly exposed to heat and teeth protected by the skull and tissues, as well as between bone exposed directly to heat and bone naturally covered by tissue. Also, different kinds of environments, e.g. clay, water, moisture and different kinds of biological agents, most certainly would provide a variation of degradation rates.

Finally, studies need to be performed on human tissues. There is a difference in how the teeth grow in sheep compared to humans. The teeth from sheep grow upwards compared to from the centre and out in human teeth, and this variable might have some effect on how the DNA is affected by heat. To be able to trust experimental data in application to forensic cases, experiments need to be performed on human tissues.

Materials and methods

General lab procedures

The pre-PCR laboratory and the post-PCR laboratory were in separate buildings with separate ventilation systems. Bone preparation, DNA extraction and PCR set-up were in different rooms. Procedures were carried out under rigorous contamination controls using disposable gloves and fully covering lab suits. Tubes were UV-irradiated for 10 minutes at 254 nm. Throughout the complete study, sterile and filtered pipette tips were used; bone preparation, heat-treatments, DNA extraction, PCR set-up, PCR amplification and electrophoresis were carried out in separate lab spaces to minimize contamination.

Sample selection and preparation

The teeth and bones were collected from a local butcher and included two sheep skulls and two femurs. The femurs lacked information on whether they originated from the same individual or not.

All the molars and premolars from the skulls were removed with the help of pliers, and greatest precaution was taken to keep the teeth intact with roots. Teeth from skull 1 and skull 2 were kept apart at all times and under strict contamination controls (changing of gloves and frequent cleaning of tools). Teeth were cleaned with toothbrushes and needles in detergent (similar to Yes) and water (about 37^{o}C). As much of the pulp as possible was removed from the teeth by using needles. The molars were then cut in four with the help of a dremel tool, and cut a the way so that each piece contained one root canal. The skeletal bones were cut into small pieces with the help of the dremel tool used previously, and as much of the bone marrow as possible was removed.

Charring

The fragments of molars and femur bones were weighed, wrapped individually in aluminum foil, and placed in trays covered with aluminium foil, one tooth fragment and one femur fragment per tray. The trays were heated to 85^{o}C for 0 hours, 5 hours, 10 hours or 15 hours, after which the bone fragments were weighed again. The samples were crushed into powder using a cryogenic grinder (liquid nitrogen grinding mill).

DNA extraction

DNA extraction was performed using a modified silica-spin column method (Yang *et al*., 1998, 2004, 2005). Approximately 0.110 grams of powder (tooth or skeletal bone powder) was measured for each sample and mixed together with a 2.0 mL lysis buffer consisting of 0.5M ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% sodium dodecyl sulfate (SDS) and 0.5 mg/mL proteinase K. Samples were then incubated overnight at 50^{o}C in a rotating oven. To further digest the proteins in the sample another 50 µL of 20 mg/mL proteinase K was added to each sample after 4 hours. Samples were centrifuged at 2000 *g* for 30 minutes, after which approximately 1.7 mL of the supernatant was transferred to an Amicon centrifugal filter, Ultra-4 (Millipore, Billerica, MA), using a 1 mL pipette set at 850 mL (x2). Amicon tubes were labelled with sample name, date and initials. The Amicon tubes were then centrifuged for 60 minutes at 2000 *g* until the liquid in the column was below 100 µL. 500 µL of PB buffer (Qiagen) was added to the columns, mixed within the membrane of the columns

using the pipette, and then transferred with the original 100 µL of liquid to a QIAquick column (QIAGEN, Hilden, Germany) that was labelled with sample name, date and initials.

To bind the DNA, the columns were centrifuged for 60 seconds at 12800 *g*. The collection tubes were then discarded and the columns were placed in new 2 mL collection tubes. To wash, 400 µL of PE buffer (Qiagen) was added to the columns and these where then centrifuged at 12800 *g* for 60 seconds. The DNA was then washed two additional times with normal PE buffer, with discarding of the old collection tubes and centrifugation for 60 seconds at 12800 *g* between each washing. After the last washing, the collection tubes were discarded and columns were placed in 1.5 mL tubes. 100 µL of EB buffer (Qiagen) was added to the columns which were then incubated at 70^{o}C for 5 minutes, to ensure that the EB solution saturated the membrane. Samples were then centrifuged at 12800 *g* for 60 seconds. 100 µL of DNA was then transferred from the collection tubes to new eppendorf tubes with colored lids (labelled with sample name, date and initials). Columns were put back into the old collection tubes and another 100 µL of EB buffer was added. The columns were incubated at 70 ^{o}C for 5 minutes and then centrifuged at 12800 *g* for 60 seconds. 100 µL of DNA was transferred from the collection tubes to new eppendorf tubes with white lids (to indicate the second pass through of the EB buffer).

Polymerase chain reaction

All the samples were diluted 5-fold (80 µL of ultrapure water to 20 µL of DNA) prior to being amplified.

PCR amplifications were conducted in a Mastercycler Personal (Eppendorf, Hamburg, Germany) in a 25 µL reaction volume (called master mix) containing 50 mM KCL, 10 mM TrisHCl (pH 8), 2.5 mM $MgCl_2$, 0.2 mM dNTP (Fermentas), 1.0 mg/ mL bovine serum albumin (BSA), 0.3 µM each primer (see table 2. Primers were designed beforehand, but has not yet been published), 2.5 µL DNA sample (undiluted or diluted 5 times) and 1.25 U AmpliTaq Gold^{TM} (Applied Biosystems). The tube was vortexed and centrifuged in a micro-centrifuge for 3-4 seconds to collect all liquid. 27 µL of the master mix was then transferred to new tubes and another 3 µL of DNA was added. A positive control (skeletal bone sample from sheep set 1 heated for 15 hours) was included in the analyses of set 2 to permit direct comparison between the two sets of results as well as a negative control along with each PCR set-up.

When comparing AmpliTaq Gold with AmpliTaq Regular (Applied Biosystems), samples were diluted 10-fold. The amount of DNA used for 315 base pair primer was set to 1.5 µl and for the 556 base pair primers the amount of DNA was set to 5 µL. Other than that, it followed the same PCR protocol as for the 5-fold-diluted samples. The concentration of the DNA was not known.

Table 2. Primers

Primer[a]	Sequence 5´-3´	Length of amplicon (bp)	Region
F90	CAC AGA CTT CCC ACT CCA CAA	125	D-loop
R214	ACT CGT TTG CAT GTT TAA GAC AG		
F624	CAC GAG CTT GTT CAC CAT GC	315	D-loop
R938	CAG CTA CAA TTC ATG CTC CG		
F62	CAC GAG CTT GTT CAC CAT GC	556	D-loop
R1180	TAT GCG TTA TGT ATG TGA CCC AG		
Amel-F-Bovi	CAG CCA AAC CTC CCT CTG C	X: 262bp Y: 220bp	nDNA
Amel-R-Bovi	CCG CTT GGC TTG TCT GTT GC		

[a] F, forward, R, reverse

PCR amplification cycles using undiluted DNA consisted of an initial 12 min denaturing step at 95^{o}C, 40 cycles of 30 seconds at 95^{o}C, 30 seconds at 55^{o}C and 40 seconds at 72^{o}C, followed by a final extension step (for more information about PCR, see Appendix paragraph 1.6).

For the 5-fold-diluted samples the PCR amplification cycle was changed to a 60 second extension time at 72^{o}C and 30 cycles instead of 40.

Sex identification for sheep individual 2 was performed using 40 cycles, after failure in detecting any amplification after 30 cycles.

Gel electrophoresis

Five microliters of PCR product were mixed with 5 microliters of SYBR Green^{TM} for visualizing via gel electrophoresis on a 2% agarose gel (TBE buffer from 5x stock solution: 53g of Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA. Dilute to 1x). 100 V for 30 min, and for photographing under a Dark Reader box^{TM} (MoBiTec).

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Appendix

1.1 Teeth and bone

Fire is a major cause of accidental deaths in the United States (Deehan 2008), and according to the Council of Canadian Fire Marshals and Fire Commissioners, about 350 deaths every year are caused by fire. The number one cause of fire related deaths in Canada, and the cause of one out of five fire deaths in Canada and worldwide, is cigarettes (Young 2000).

Environmental conditions also include time as a factor, which leads to the application of teeth and bone to ancient DNA studies.

Through the studies of the teeth and bones of a deceased, the determination of age, sex, ancestry and skeletal idiosyncrasies is possible. Age is easier to determine when the deceased was relatively young at time of death, for example information about tooth formation, tooth eruption and at what age the end of limb bones form and fuse is well documented (White 2000). Sex identification is on the other hand easier to determine in adult skeletons, since sexual characteristics often develop at the age of sexual maturity. Archaeological settings sometimes offer the possibility of comparison between several individuals in a population of skeletons, which makes it easier to gain information about sex, age and race, compared to forensic settings that most frequently involve isolated individuals.

1.2 Morphology of teeth

Teeth consist of dentin, enamel and pulp. The complete tooth consists of a crown covered by enamel and an underlying root covered by the cementum (Gaunt *et al*. 1971). The cementum is a mineralized tissue serving as an attachment for fibres of the periodontal ligament and is thought to yield the most amount of DNA amongst the calcified tissues. Studies on root filled teeth have shown that the pulp is not necessary for the tooth to be consisted as an excellent source of DNA (Sweet & Hildebrand 1998).

When talking about anatomical and clinical crowns, the clinical crown is what is visible in the mouth at any given age. In small children, the clinical crown is smaller than the anatomical crown due to the base of the anatomical crown being hidden by the gingival margin. In elderly people the entire anatomical crown is exposed, often together with a bit of the anatomical root leading to the clinical crown being larger than the anatomical crown. The anatomical root is the part of the tooth typically contained within the bony socket, lying deep to the level if the cervical margin of the enamel.

Teeth consists mainly of dentine which is a mineralized tissue permeated by dentine tubules, each containing a fine protoplastic process of an odontoblast cell which is situated on the surface of the pulp. The pulp chamber is situated in the centre of the coronal dentine. It consists of connective tissue covered by odontoblasts, which are specialized cells concerned with the creation of dentine. Ameloblasts are the cells concerned with enamel matrix formation. Enamel is the highly mineralized layer situated on the outer surface of the anatomical crown.

1.3 Morphology of bone

All bones consist at the gross level of compact and spongy bone. Compact bone is the kind of solid and dense bone found in the walls of bone shafts and surfaces, and it is prohibited from being nourished by diffusion from surface blood vessels. It is instead built up by so called haversian systems, which consists of haversian lamellae. Each lamella contains several osteocytes- living bone cells. Blood, lymph and nerve fibres pass through the haversian canal, running through the core of each haversian system. The spongy bone is mostly found at places where tendons attach or in the ends of long bones and in short bones. The cellular composition in spongy bone is the same as in compact bone, only the porosity differs.

There are three types off cells that are involved in the formation of bone and for maintaining the bone tissue- *osteoblasts*, *osteocytes* and *osteoclasts*. The osteoblasts are bone forming cells that synthesize and deposit bone material. They produce the collagen rich uncalcified organic pre-bone tissue that later is transformed into calcified tissue when crystals of inorganic components are deposited into the matrix. When completely surrounded by the bone matrix, the osteoblasts are called osteocytes and are responsible for maintaining the bone tissue. The osteoclasts are responsible for the removal of bone tissue. This system of constant forming and removal is continuous throughout life.

1.4 Morphological changes in teeth when exposed to temperature and duration

Calcination is the process in which the colour of the teeth (exposed to high temperature and/or duration) turn to blue-gray, then stark and chalky white. At this stage, all that remains are inorganic materials since proteins and water have burned and evaporated. The teeth do not change uniformly due to the content of several different minerals within the structure.

Roots often fracture transversely and crowns along thin cusp margins, and if teeth (or bones) have been heated to high temperatures, sudden cooling by hose streams causes severe fractures. The enamel often falls off the tooth as an intact structure as the underlying dentin shrinks in the heat. Due to the greater content of organic material, dentin shows greater amount of shrinkage than enamel. When all the organic material is burned away, the inorganic crystals tend to collapse into the spaces left by the organic material, although when exposing the heat to temperatures over 800 degrees these crystals fuse to each other inhibiting further shrinkage and fracturing. On average, a tooth exposed to heat shows shrinkage of approximately 10-15% from its original size (Shipman *et al.* 1984, Buikstra & Swegle 1989). According to previous studies, teeth show morphological changes when exposed to heat at different temperatures and duration (Beach *et al*. 2008), as can be seen in table 3.

Table 3. Differences in tooth appearance when subjected to various temperature and duration.

Temperature (^{o}C)	30 min	60 min
204 ^{o}C	No significant morphological changes.	Slight color change in the dentin. The root structure changed from a pale yellow to a yellow color. No color change in the enamel.
260 ^{o}C	The enamel turns very pale brown and is severely starting to detach from the root structure. The dentin turns dark reddish brown.	enamel appears slightly darker.
371 ^{o}C	Enamel is now dark greyish brown with a metallic looking surface. Root dentin has turned from very pale brown to black.	Weight loss.
427 ^{o}C	Crowns separate entirely from roots.	Roots olive-brown color, although mantle dentin light gray.
482 ^{o}C	-	Crown light gray, glossiness gone.
538 ^{o}C	Enamel is now in many small fragments. Color of root structure changes from olive brown to light gray. Root apices brake off from the rest of the root.	-
538-593 ^{o}C	Crown is highly fragmented. Root starting to turn white	-

1.5 Morphological changes in bone when exposed to heat

Cremated bones have been documented to show a variety of colours, all ranging from black to red depending on the temperature and duration of exposure (Shipman *et al.* 1984, Dunlop 1978). Organic and inorganic materials associated with the body, as well as chemical components of the surrounding environment (e.g copper, bronze or iron) further attributes to the different colours. The most frequently noted colours in the heating process ranges from gray-blue, black, gray, gray-white and chalk white (Shipman *et al*. 1984).

A noticed shrinkage of about 10 to 15% after heating bone at temperatures up to 1200^{o}C is said to be dependent upon criteria such as bone type, temperature of exposure and mineral content of bone (Herrman 1977, Strzalko *et al.* 1974). Herrmann (1977) further states that males shrink proportionally more than females due to the higher percentage of bone mineral.

1.6 PCR and real-time PCR

[One single 100µl PCR reaction produces enough template molecules that, if diluted in an Olympic size swimming pool, each 100µl from the pool would still contain 400 intact copies] (Wayne *et al.* 1999. *Annu. Rev. Ecol. System.* 30: page 460)

Polymerase Chain Reaction, or PCR, was formally introduced for the first time in 1986 (Mullis *et al.* 1986), and has become an essential part of molecular biology. The method is very quick and effective where the quantity of DNA sequences is doubled with every cycle. The method relies on repeated alternations between heating and cooling. Two single-stranded oligonucleotides, or primers, are required for the double-stranded DNA, one forward primer and one reverse primer, which need to be sequence specific for the gene of interest. The DNA polymerase most commonly used is the Taq DNA polymerase, originally isolated from the bacteria *Thermus aquaticus.* The PCR consists of 3 phases within one cycle- the denaturing, the annealing and the extension (see figure 10). The denaturation process uses high temperature to break the hydrogen bonds in the double stranded DNA to create two single strands. The annealing process allows for the two primers to attach to the single stranded DNA, at the specific sequence of interest. With the presence of dNTPs and with the help of Taq polymerase, a complementary strand of DNA is synthesized to the original single-stranded DNA, and the final product consist of, yet again, a double stranded DNA (Sequera & Rocas 2003).

When raw estimation of DNA quantity is not sufficient enough, such as the results from a gel electrophoresis, precise DNA quantity can be obtained through the use of real-time PCR. Real-time PCR is an important tool for quantification of the number of ancient DNA molecules from which the PCR starts, and as an indication of good quality. If the PCR starts working on a minimal number of molecules or single stranded DNA, errors in the final PCR products might be colossal since the first cycle give base to all following cycles of amplification, causing errors such as falsely typing an individual as a homozygote (Hofreiter *et al*. 2001)

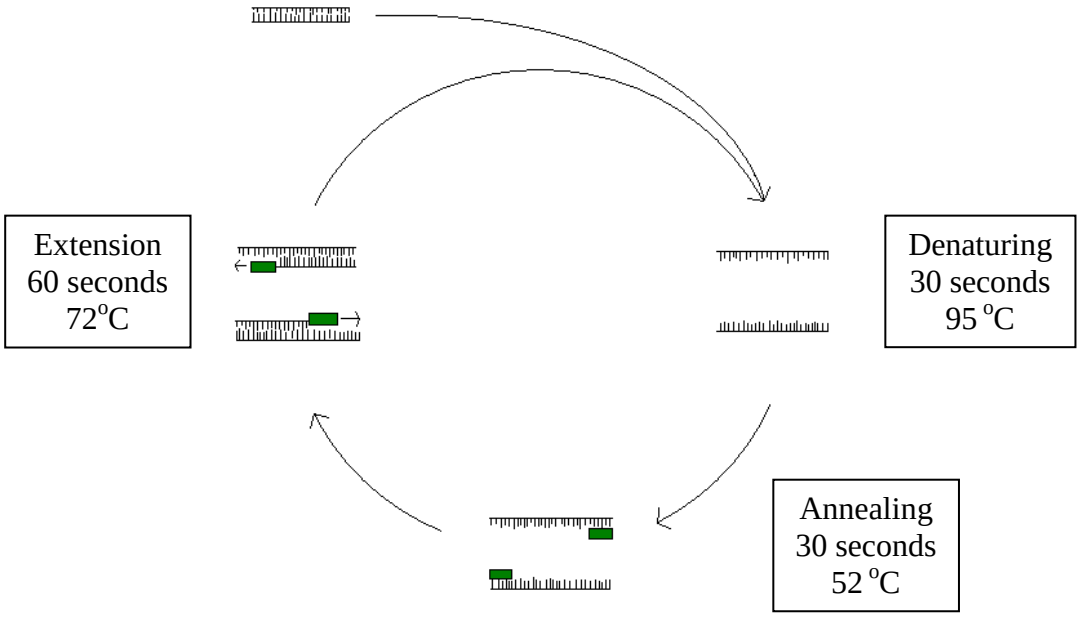

Figure 10. PCR reaction. Picture created by author in paint.

1.7 DNA degradation

All cells except red blood cells contain nuclei with genomic DNA (nDNA) and mitochondria with mitochondrial DNA (mtDNA). When the nDNA is degraded to such a degree that it is no longer usable, focus is turned towards the mtDNA which is present in a much higher number of copies than the nDNA, due to the great number of mitochondria in each cell. Mitochondrial DNA is maternally inherited, which means that the DNA with its unique sequences is exactly the same amongst siblings and maternal relatives (Hutchison *et al.* 1980). Hence it is not as informative as nDNA and cannot provide a positive identification of an individual, but still of great importance during investigations where there is no antemortem comparison sample.

When an organism dies, the DNA starts to gradually degrade by the activity of endogenous nucleases. If the nucleases themselves are inactivated or destroyed, which might be the case in low temperature conditions, high salt conditions or rapid desiccation, some of the nucleic acids might have their transformation to mononucleotides slowed down (Paabo *et al.* 2004, Hofreiter *et al.* 2001). Gradually the DNA molecule is further destabilized and broken down by oxidation and hydrolic processes like deamination and depurination. (Hofreiter *et al.* 2001). To what extent the degradation process has affected the DNA is relying on two factors: environmental conditions to what the sample is being exposed to, and time of exposure (Burger *et al*. 1999). The interaction between time and environmental conditions including temperature, humidity, pH and soil chemistry is very complex and makes it extremely difficult to establish any kind of set pattern or correlation between time and stage of decomposition.

The DNA molecules will eventually suffer from degradation processes enough to be completely destroyed. Approximate estimations indicate that hydrolic damage at neutral pH, physiological salt conditions and a temperature of 15^{o}C would after a period of 100,000 years have completely destroyed all DNA (Paabo & Wilson 1991, Lindahl 1993). Conditions like lower temperature would slow down the degradation processes, and other conditions might speed it up (Collins 2001). Studies show that the DNA is best preserved when under 100, 000 years old and present in environments where UV light is absent and the air is dry and cold (Lindahl 1997, Poinar *et al.* 1996). *Antediluvian DNA* is the term for DNA sequences that are older than one million years (Lindahl 1993), and even though there have been publications about the findings of such (Cano & Borucki 1995, Golenberg 1990), the acceptance of its authenticity are widely challenged (Lindahl 1993**,** Paabo & Wilson 1991).

1.8 Ancient DNA

Ancient DNA touches history in areas of systematic, changes in genetic diversity, migration, ecology amongst others. Origin and spread of diseases can be traced, as well as mutations, and the effects of time and environmental change on genetics (Wayne *et al.* 1999). The first successful aDNA studies where ancient DNA sequences were cloned were performed on tissues from a quagga (Higuchi *et al.* 1984) and from an Egyptian mummy (Paabo 1985). Studies of populations over time and phylogenic relationships of extinct species have been possible due to the big variety of museum collections, where the study of kangaroo rats collected in the early century being the first example (Thomas *et al.* 1990, Groombridge *et al.* 2000, Cooper *et al.* 1996).

Ancient DNA sequences are often mitochondrial since mitochondria contain multi-copy DNA sequences in contrast to the nucleus and its low-copy DNA. The invention of PCR allowed for amplification of only a small amount of DNA sequences and gave aDNA research a real

boost. Animal genes of interest included *cytochrome b* and for plants the chloroplast gene *1,5 biphosphate carboxylase oxygenase* (*RbcL*). Results from aDNA studies are often targets of authenticity claims, as with the DNA sequences from the 17-20-million-year-old magnolia leaf compression fossils from clay sediments of a Miocene freshwater lake (Golenberg 1990). This was the first example of DNA sequences derived from specimens older than 1 million years, but the study was performed without PCR controls, and further attempts of regaining DNA sequences from the same samples have failed. Focus was after this study turned to other extremely old samples like dinosaur bones (Woodward *et al.* 1994) or insects preserved in amber (Cano *et al.* 1995). Amber was thought of as a medium good for the preservation due to its ability to rapidly desiccate whatever was trapped, and also efficiently inhibit bacterial activity (Wayne *et al.* 1999).

Because of the minimal amount of DNA available in ancient tissues, extreme precautions must be undertaken not to contaminate the PCR with extraneous DNA. This includes to completely separate the extraction and preparation of ancient DNA from work with modern DNA, bleach treatment of the laboratory, UV irradiation, fully protective clothing and disposable masks and gloves, by others (Handt *et al.* 1994, Hoss *et al.* 1994). Contamination of ancient DNA is extremely hard to avoid considering that it is enough with only one molecule for it to be contaminated. Contamination might not be solely because of scientists in the lab, but the specimen itself that is contaminated with modern DNA, either human or animal. Hofreiter *et al.* (2001) mentions the problem with human DNA sequences being present in ancient animal remains, and therefore questioning the reliability of several published data concerning ancient human DNA. Hofreiter *et al.* (2001) also mention several authenticity criteria for the determination of ancient DNA, which are seen in table 4.

Table 4. Authenticity criteria for the determination of ancient DNA, and the meaning of the criteria.

Authenticity criteria	Meaning of criteria
Biochemical assay for macromolecular preservation	State of preservation of the specimen should be compatible with DNA preservation, and needs to be examined through amino acid analyses.
Extraction controls and PCR controls	Blank extractions and negative PCR controls are crucial.
Quantification of numbers of template molecules	The quantity of DNA molecules at the beginning of PCR need to be less than 1000. If not, three independent amplifications need to be analysed, with the products cloned and sequenced.
Exclusions of nuclear insertions of mitochondrial DNA	False results might be the consequence from fragments of mDNA present in the nuclear genome.
Amplifications from a second extract	To strengthen results from a study, a second extraction should be carried out to show the same results.
Reproduction in a second laboratory	To confirm results from a study, a sample should be sent from the museum to another laboratory for another extraction, to eliminate laboratory-specific contamination.

1.9 Problems concerning degraded DNA

PCR is an issue with ancient DNA. One little unwanted sequence of contamination can disturb the whole amplification of the highly degraded and damaged ancient DNA. Jumping PCR can be one of the reasons for the formation of chimeric molecules; the incompletion of the copying of damaged DNA templates creates false primers for the continuing cycles of PCR (Paabo *et al.* 1989). Damaged DNA templates are also the reason for errors like the insertion of adenosine residues (Paabo *et al.* 1990).

Successful PCR amplification from degraded DNA samples is complicated due to the many factors interfering with the DNA extraction. Usable results in the PCR amplification is heavily depending on what degradation processes the sample has been exposed to. The DNA might be affected directly through the action of enzymes or oxidation, but the failure of PCR amplification might also be the consequence following the presence of inhibitors such as humic acid, fulvic acid, biologically degraded products and collagen (Keyser-Tracqui & Ludes, 2005, Kalmar *et al.* 2000). Cytosine and thymine residues in ancient DNA extracts might in high concentrations modify through oxidative processes into Hydantoins, heterocyclic organic compounds that prevent the DNA polymerase to function properly and consequently hinder the PCR amplification (Hoss *et al.* 1996). Defaults occurring during the processes of PCR amplification include the insertion of incorrect bases due to deamination products of cytosine, common in ancient DNA samples (Paabo 1989).

One way to work around the problem with inhibitors is to dilute the DNA extract, so that although the amount of DNA is decreased so is the quantity of inhibitors (Fondevila *et al.* 2008). The nDNA is only present in low copy number (Yang *et al*. 1998, Kumar *et al.* 2000) and is constantly getting more and more fragmented as degradation prolongs. DNA markers with long amplicon sizes are therefore ineffective, and short amplicon PCR products are necessary such as mini-STRs and autosomal SNPs.

The challenging research for an effective DNA extraction method for degraded DNA samples has included a variety of different methods and also combinations between a few. Many methods focus on the ability to remove potential PCR inhibitors, for the development of an as efficient method as possible for PCR amplification.

The phenol-chloroform™ method is frequently used in previous research (Faermann *et al*. 1995, Hanni *et al.* 1995) due to the high yield of DNA and the broad range of DNA fragments in various sizes. The DNA dissolves in the aqueous layer while proteins and other non-DNA substances end up in the interface and lower organic phase. Although there is a high yield of DNA, the DNA obtained through this extraction method is not completely pure and hence not optimal for PCR amplification. The use of toxic substances and the many steps of handling also add up to the limitations.

The Chelex™ method uses the chelex beads ability to attract non-DNA-substances, leaving the DNA in the solution (Walsh *et al.*1991 , Faerman *et al.*1995), although the method is not as effective with separating the DNA from unwanted particles as the Silica™ method (Yang *et al.* 1998, Kemp *et al.* 2006, Rohland & Hofreiter 2007). Silica beads attracts DNA molecules when under certain high salt conditions, and the DNA obtained is, because of its purity, highly suitable for PCR amplification. Kijeong *et al.* (2008) documented that using ion-exchange columns after silica bead DNA extraction further purifies ancient DNA samples, and enhances the chances for successful PCR amplification. Ion-exchange columns have been used as a successful DNA extraction method for soil organisms where the DNA samples are highly contaminated with PCR

inhibitors originating from soil (Zaporozhenko *et al.* 2006). Contamination is the huge issue with ancient DNA.

1.10 Amplitaq Gold DNA polymerase in comparison to regular Amplitaq DNA polymerase

Amplitaq Gold™ (Applied Biosystems) DNA polymerase is often preferred over Amplitaq™ Regular (Applied Biosystems) DNA polymerase when performing PCR due to the vast amount of low molecular-weight products that follow using the latter one, also called primer dimers. AmpliTaq Gold is more sensitive than the AmpliTaq Regular and also offers a greater yield of the specific product being amplified. For example, in a study conducted in 2002 by Castle *et al.*, AmpliTaq Gold significantly showed a greater efficiency compared to AmpliTaq Regular by increasing the detection of high risk human papillomavirus (HPV) types with 45%.