A study of post-mortem degradation of teeth to advance forensic DNA analysis as a tool for human identification

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Abstract

The post-mortem decomposition of human teeth is an area of forensic taphonomy that has received little attention. As they are a skeletal element, the diagenesis of teeth is often considered to occur in the same manner as bone. However, there are a number of morphological and chemical differences between these two mineralized tissues, making extrapolation of findings from bones to teeth difficult. With the advent of increasingly sensitive DNA recovery and analysis techniques, successful forensic identification using low levels of DNA present in highly degraded skeletal remains is now possible. As teeth are often the most reliable source of DNA in skeletal remains, an in depth understanding of their decomposition in the post-mortem environment should facilitate more successful identification outcomes.

This research examined the individual tissues of human teeth to address two questions. Firstly, what are the ante-mortem factors that impact on intra- and inter-individual variation in the DNA content of teeth; and secondly, how does post-mortem degradation affect the availability and distribution of DNA in the various tissues of teeth. The overall aim was to provide information to inform sample selection and targeted sampling of teeth for genetic identification of human remains. The low levels of DNA preserved in skeletal remains are associated with compounds that, if co-extracted, complicate the DNA extraction process and can potentially inhibit down-stream analysis. The most problematic of these compounds, which occur naturally in teeth, are calcium and collagen. Targeted sub-sampling of teeth avoids the unnecessary addition of excess amounts of inhibitory compounds and enables extraction of the low levels of endogenous DNA, increasing the likelihood of successful identification of human remains.

This research has confirmed that the roots of the teeth are a better source of DNA than the crown and has shown for the first time that the cementum, which is located on the external surfaces of the roots, is more valuable for nuclear DNA analysis than dentine. In both fresh and decomposed teeth cementum provides a rich source of nuclear DNA, which is easy to access and sample without the need for specialised equipment. Histologically the structure of cementum is maintained during decomposition, whereas pulp is degraded rapidly and dentine loses structural integrity starting at the pre-dentine layer.

Declaration

I certify that this work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously written or published by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Denice Higgins

Signature: Date

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Chapter 1

Introduction

The goal of forensic human identification is the individualisation of human remains by reassignment of a name that was bestowed either at birth or by legal process. Forensic identification may be required for verification of identity of missing person, victims of crime, disaster victims and victims of armed conflicts. Identification of human remains is important for both legal and humanitarian reasons (Black, 2007). From a legal perspective a death certificate cannot be issued until identity is established and many proceedings, such as probate of an individual's Last Will, cannot occur without a death certificate. In the case of criminal misadventure, identification of the deceased can assist in progressing the course of police investigations. In a personal sense, identification of human remains enables family and friends of the deceased person to grieve and allows for the remains to be handled in a culturally and personally appropriate manner.

Traditional methods of identification include visual identification by a family member or friend and comparison of dental or fingerprint characteristics. However, not all remains (e.g. those that are highly degraded or have no ante-mortem records available) are suitable for identification by these traditional means. In these cases DNA analysis may be of assistance. A DNA sample extracted from human remains can be compared to an ante-mortem sample from the presumed individual. An ante-mortem sample may be collected from, for example, a personal item (such as a toothbrush), a pathology specimen, or a DNA database. If such sources of DNA are not available, samples from one or more close relatives can be used for comparison. In the complete absence of a comparative ante-mortem or family reference sample, DNA analysis can be used to provide phenotypic (e.g. eye colour, hair colour, height, gender) (Draus-Barini et al., 2013, Walsh et al., 2013) and biogeographic ancestry information (Zaumsegel et al., 2013, Phillips, 2012), which can help narrow the search for identity. After death post-mortem degradation occurs leading to destruction of the tissues and the DNA contained within them. The hard tissues of the body

are more resistant to degradation due to their mineralized nature so are often all that remains when a body is discovered some time after death. Teeth are unique in that their crowns are covered with enamel, the hardest substance in the body and their roots are incased in bone offering the best chance for survival of genetic material. The over-riding aim of the research presented in this thesis is to provide knowledge to assist the identification of missing persons through the retrieval of genetic information from the teeth in human remains.

An individual's genetic information is contained in the nuclei of their cells (nuclear DNA), and in the mitochondria (mitochondrial DNA [mtDNA]), and is passed on from generation to generation. Nuclear and mtDNA are two completely different genomes with nuclear DNA being passed on from both parents and mtDNA being maternally inherited. In humans, nuclear DNA is bundled up into 46 chromosomes of which there are 22 paired autosomes and two sex chromosomes. A specific region on a chromosome is called a locus, which can occur with different presentations of sequence, referred to as alleles. Each locus consists of two alleles, one on each chromosome. These two alleles may be the same (homozygous) or different (heterozygous) (Kobilinsky et al., 2005). There are three billion base pairs in one copy of the human nuclear genome with approximately 99.7% of the sequence of the bases being identical in all individuals. It is the 0.3% of the base pairs that vary enabling distinction between individuals that are of interest for forensic human identification (Butler, 2011b).

Variant sequences of nuclear DNA in the human genome can occur as repeat sequences, which are made up of a core unit of bases that is repeated multiple times. These core units can occur in many sizes. Medium length core repeats are called minisatellites where as shorter core repeats are called microsatellites. The first form of DNA fingerprinting used

forensically examined minisatellites in the form of variable number tandem repeats (VNTR) (Jeffreys et al., 1985). VNTRs are made up of repeating core units that are 10-100 base pairs long that may be repeated many times (Tautz, 1993). Microsatellites, including short tandem repeats (STR), are now the most commonly used markers in forensic DNA typing. STRs have core repeating units of 2 to 6 base pairs in length with tetranucleotides (having a core repeat unit of 4 base pairs) being the most popular for human identification (Butler, 2005). The smaller size of STRs is advantageous when examining degraded DNA, as they are more likely to be retrieved from degraded tissues than larger fragments.

In forensic DNA analysis, STR markers will undoubtedly be the preferred markers for some time to come due to their widespread use in databases worldwide. Only a limited number of the more than several thousand STRs present in the human genome meet the ideal criteria for forensic use (Butler, 2011a). STRs used in forensic identification need to be efficient, reliable, highly discriminatory, and suitable for the analysis of degraded samples (e.g. short sequence length). They should also have high heterozygosity and a high level of polymorphism, i.e. they should occur with multiple alleles occurring at reasonable frequencies in the population. It is also important that the sequences in the regions flanking the loci are stable and not prone to mutation (Kobilinsky et al., 2005). In addition, to enable use of the product rule in the statistical analysis of profiling results, the loci selected cannot be linked. Multiplexing of STRs (combining a number of fragments in the one DNA test) increases the efficiency of DNA examination and reduces the amount of template DNA required to gain a result; therefore the STRs chosen for use must be able to perform reliably together in one reaction. It is also desirable to have a narrow allele size range to reduce dropout due to preferential amplification of smaller alleles (Butler, 2005). The loci used in human identification are located on non-coding segments of DNA and may be either within or between genes. When naming these loci, those within genes are

labelled using the gene name, e.g. TH01 occurs in the first intron of the tyrosine hydroxylate gene and loci between genes are labelled with their chromosome location, e.g. D5S818 where D =DNA, 5 = chromosome 5, S= single copy and 818 indicates 818th marker described on chromosome 5.

Whilst STR typing of nuclear DNA is currently the most popular technique for human identification (Alonso et al., 2005, Zietkiewicz et al., 2012), single nucleotide polymorphism (SNP) typing is becoming increasingly popular to augment STR typing in cases involving highly degraded DNA (Butler, 2007). A SNP is a variation in DNA sequence at a single base. Less information is gained from a bi-allelic SNP than from a multi-allelic STR so a higher number need to be analysed to gain the same level of discrimination. Approximately 50 SNPs are required to achieve the same level of resolution in human identification provided by 10-16 STR loci (Gill, 2001). Like STRs ideal SNPs need to show high heterozygosity, perform efficiently in a multiplex and be on small amplicons (60-120 bp). There are only a limited number of SNPs that fulfill these criteria.

In highly degraded samples with limited or no nuclear DNA, analysis of mtDNA may provide information despite its lower discriminatory power. As mtDNA is maternally inherited it is the same in all members of the same maternal lineage, giving it much lower discriminatory power than nuclear DNA. MtDNA is more likely to be preserved in degraded tissues due to its presence in higher copy number and its more robust structure (Budowle et al., 2003, Melton and Nelson, 2001). Cells, depending on their type, contain hundreds to thousands of mitochondria, with each mitochondrion containing multiple copies of its own DNA. MtDNA contains two hypervariable regions that are conventionally used for human identification purposes: HVI region (342 bp) and HVII

region (268 bp). HVI and HVII polymorphisms arise through random mutation and are inherited through the maternal lineage. During analysis these regions of the mtDNA are sequenced and compared to a reference sequence. Any nucleotides that differ from this standard are noted. Analysis of mtDNA is complicated by the fact that in some instances not all mitochondria within an organism or even a single cell have exactly the same mtDNA sequence (Zietkiewicz et al., 2012). This is known as heteroplasmy and may be present as single nucleotide substitutions or variations in the length of the hypervariable region. Although heteroplasmy can complicate mtDNA analysis the presence of heteroplasmy detected at identical sites can improve the probability of a match (Ivanov et al., 1996). Due to the higher discriminatory power of nuclear DNA over mtDNA to forensic analysis, the research presented in this thesis primarily focused on nuclear DNA whilst recognising that both forms of DNA can provide information to aid identification.

In degraded remains DNA may be in limited quantity with the areas of interest constituting only a small portion of the total. Selective amplification of these DNA regions can be performed using polymerase chain reaction (PCR) using specially designed multiplexes. The use of a multiplex is less expensive, conservative of sample extract, and quicker to perform than serial singleplex reactions. There are a number of multiplex systems available commercially, including AmpFLSTR Profiler Plus (Applied Biosystems), which is widely used in Australia. This kit co-amplifies the repeat regions of nine STR loci and a segment of the amelogenin gene. The amelogenin gene is amplified using a single primer pair, which generates different length products from the X and Y chromosome allowing gender identification. The other nine loci amplified using this kit are; D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820 (Applied Biosystems, 2006a). The AmpFLSTR Profiler Plus kit (Applied Biosystems) is used for all STR analysis performed throughout this research. Various alleles are determined by the use of capillary

electrophoresis, which separates the DNA fragments according to length. Injection of the sample through a capillary allows the four fluorescently labelled dyes used in Profiler Plus to be separated into distinct spectral components. Each of the four dyes fluoresces at different wavelengths that are detected by a fragment separation instrument. These data are converted into tabular form and then into allele designations using specialised computer software. Once an STR profile has been generated it can be compared to a reference STR profile to determine the likelihood of identity.

Commercially produced DNA profiling kits are optimised to produce profiles from a narrow range of template DNA concentrations. It is therefore necessary to quantify the total amount of amplifiable DNA present in a sample to determine if there is sufficient DNA present to proceed with STR analysis and also to determine how much sample to utilize in the STR reaction. Most STR kits are validated for the addition of approximately 0.5ng of DNA. The recommended quantity of DNA specified by the manufacturer for use with Profiler Plus is 0.5-1.25ng. There are approximately 6.1 picograms of DNA in the nucleus of one cell; hence a minimum of 164 cells is required to yield 1ng of DNA. There are a number of methods available for quantification of DNA but real-time PCR (qPCR) has distinct advantages over other methods. Real-time PCR has a wide dynamic range, it is relatively quick and can be automated, it is extremely sensitive (can detect less than five copies of the target sequence), and it has minimal risks of cross contamination (Valasek and Repa, 2005). In the research presented in this thesis, all DNA quantification was performed using qPCR. The initial quantitative reports examining undecomposed samples utilised a commercial kit, Quantifiler^{TM} (Applied Biosystems). This kit was designed to quantify the total amount of amplifiable human or higher primate DNA in a sample and has been optimized for use with a number of Applied Biosystems instruments and software

(Applied Biosystems, 2006b). These kits are widely used in the forensic community in Australia.

Quantifiler™ kits (Applied Biosystems) contain two 5' nuclease assays: a target specific human DNA assay and an internal PCR control assay (IPC). The target specific assay consists of two primers for amplifying a 62 base pair segment of the telomerase reverse transcriptase gene (hTert), which is located on chromosome 5, and one TaqMan® MGB probe labelled with FAM™ dye for detecting the amplified sequence. The IPC assay consists of a synthetic DNA template that does not occur naturally, two primers to amplify this template and one TaqMan® MGB probe labelled with VIC dye for detecting the amplified sequence (Applied Biosystems, 2006b). The disadvantages of this kit for sole use in this research project were the relatively high cost and the small size of the target fragment. The 62 bp length of the target fragment is not reflective of the quality of the DNA being quantified and is much shorter than the loci targeted by Profiler Plus. To be able to assess the effects of decomposition on DNA quality, quantification of decomposed samples was performed using qPCR with SYBR®green chemistry using two fragment sizes (67 bp and 156 bp) for nuclear DNA and one for mtDNA (77 bp). The fragment details are discussed in the relevant research reports in Chapters 4, 5, and 6. SYBR Green binds to double-stranded DNA (dsDNA), so an increase in DNA product during PCR leads to an increase in fluorescence intensity, which can be measured at the end of each cycle. As SYBR Green binding is non-specific, the inclusion of a heat dissociation experiment at the end of each PCR reaction is required to determine the presence of a single product via visualisation of the heat dissociation kinetics. Each primer pair will amplify a different target, which will melt at a specific temperature.

While major advances have been made in post-extraction DNA technologies, enabling much more information to be gained from very small quantities of DNA, successful genetic analysis is still dependent on the ability to retrieve endogenous DNA from the tissue samples available. DNA extraction involves three basic principles; cell lysis, removal of lipids by addition of a detergent and removal of proteins by addition of a protease. There are a large number of extraction techniques available, including phenol/chloroform (Kalmar et al., 2000), Chelex (Walsh et al., 1991), and silica binding (Hoss and Paabo, 1993). There have been numerous studies undertaken to compare the efficiency of various methods but it is the ability to obtain authentic amplification products from the extracted material that is important. The use of silica extraction methods has been shown to work well for calcified tissues (Rohland and Hofreiter, 2007). The aim of this study was not to repeat studies examining the efficiency of various extraction methods but to examine the teeth themselves and the intrinsic variables within them affecting successful genetic analysis. Hence a protocol that had been tested and shown to be reliable for extracting DNA from teeth was required and a single extraction method was used for all tissues so as not to introduce more variables. I chose to use QIAamp DNA Investigator Kits (Qiagen) following the manufacturer's instructions as this particular kit is designed for purification of DNA from small sample sizes and to ensure no sample-to-sample cross contamination (Qiagen, 2007). The procedure consists of four steps; lysis of the sample with Proteinase K under denaturing conditions, binding of DNA to a silica membrane, removal of residual contaminants by washing and elution of DNA into solution (Qiagen, 2007). In Chapter 2 of this thesis the requirements for an ideal extraction technique for use in a forensic laboratory are examined. This study highlighted that the most important requirements are speed, efficiency, economy, and low risk of contamination. Important considerations for reducing the risk of contamination are limiting handling steps and using automated systems.

Frequently when DNA identification of human remains is required, the remains are severely decomposed or skeletonised. Relatively little advancement has been seen over the last decade in sampling and DNA extraction protocols for bones and teeth. In skeletonised remains, teeth are often the preferred source of DNA as the DNA within them is usually less degraded than that in bones (Alonso et al., 2001, Ricaut et al., 2005). This thesis focused on teeth to gain a better understanding of the dental tissues to inform sample and tissue selection and to inform appropriate and improved sampling and extraction techniques. In Chapter 3 I examined teeth to gain an understanding of the distribution of nuclear DNA within the mineralised tissues of the teeth prior to the influences of post-mortem decomposition. This study also explored the variability between samples and the influence of a number of ante-mortem factors on this variability. As the results of this first study did not entirely reflect the yield from cementum that was expected given what is understood about the histology of teeth, cementum was examined in more depth in Chapter 4. In Chapter 4 the yield of nuclear DNA from cementum was examined in more detail and the potential negative effects of using sodium hypochlorite (bleach) as a means of decontamination were examined. This form of decontamination was used in Chapter 3 and it was considered that this might have affected the nuclear DNA yields generated from cementum. The DNA analyses undertaken in Chapters 4 and 5 were supported by concurrent histological examination of comparable samples.

In a decomposing body, DNA degradation initially is largely biological, being caused by enzymatic autolysis and microbial putrefaction (Alaeddini et al., 2010). If DNA survives these initial attacks it can last for a considerable amount of time, but over extended periods the environment is believed to more directly affect decomposition (Lindahl, 1993). Tissues containing collagen (e.g. teeth and bones) are the last to decompose because collagen has a unique triple helical structure with strong inter- and intra-molecular bonds that are difficult

to hydrolyse (Gill-King, 1997). Once the decomposition of collagen has occurred, hydroxyapatite mineral loss can occur by inorganic chemical weathering (Gill-King, 1997). DNA has been demonstrated to have a strong affinity with hydroxyapatitie and it is believed that DNA can adsorb to the hydroxyapatitie in bones and teeth, indicating a direct relationship between DNA preservation and hydroxyapatite crystallinity (Gotherstrom et al., 2002). Theories relating to the DNA/mineral relationship in decomposing teeth are discussed in Chapter 2 of this thesis.

Post-mortem degradation leads to fragmentation of DNA molecules (Paabo, 1989), hence longer alleles are lost before shorter alleles (Alaeddini et al., 2010). This can result in partial DNA profiles with absence of alleles (allelic dropout) due to unsuccessful/incomplete amplification of longer degraded fragments. These partial profiles are more difficult to interpret and have less evidentiary value than full profiles (Butler, 2005). To assess DNA degradation, this research uses real-time qualitative PCR of both nuclear and mitochondrial DNA, as well as nuclear STR typing results. Whilst the primary focus of this research was nuclear DNA, it is recognised that the two forms of DNA may be preserved differentially across the dental tissues. The impact of DNA degradation on the quality and reliability of STR typing is recognized by forensic scientists but the post-mortem kinetics of DNA degradation in teeth is not well understood. Chapter 5 of this thesis examines the kinetics of both nuclear and mtDNA degradation in the different tissues of the tooth, to highlight the areas of the tooth that would be the best targets for selective tissue sampling, to improve the results of genetic analysis of teeth.

Although high rates of success have been reported for forensic DNA analysis of teeth (Alonso et al., 2001, Milos et al., 2007), this is not always the case. Variable rates of success from tooth samples retrieved from comparable situations have been frequently

noted. The factors affecting success of these analyses are not clearly understood but could be attributed to either ante-mortem or post-mortem influences. In this thesis, these influences are examined to gain a better understanding of inter-sample variability and to allow the formulation of predictive decision models for sample selection.

Ante-mortem factors that may influence DNA recovery from teeth include chronological age and sex of the donor, tooth type (e.g. molars, incisors), and the presence/extent of dental disease (Dobberstein et al., 2008, Gaytmenn and Sweet, 2003). Studies examining these factors and their outcomes are discussed in the review article that comprises Chapter 2 of this thesis, so will not be discussed here. There are other possible variables that have not been considered, such as natural biological variation, systemic diseases, and the functional stresses (e.g. mechanical forces of mastication) to which a particular tooth is exposed. The extent of variation in DNA yields that has been observed from teeth (Gaytmenn and Sweet, 2003, Higgins et al., 2011) makes it difficult to extrapolate meaningful results without very large sample sizes with suitable control on variables (such as age, gender, presence/absence of disease), and this has not always been possible. The study presented in Chapter 3 investigates the extent of variability in DNA yield from teeth, initially focusing on the effects of dental disease, chronological age and sex, to determine the impact of these factors. The extent of variation in DNA yield from teeth was found to be quite large and the effects of dental disease and chronological age were difficult to separate as these two factors were confounded in the samples examined. As a result of these findings, the subsequent studies presented in Chapters 4 and 5 were performed using larger sample sizes and only third molar teeth free from disease. This limitation allowed examination of the effect of chronological age and other potential influences to formulate a better understanding of the effect of these factors. In numerous studies “disease free” is used to mean teeth that are unaffected by dental caries and that are free from restorations but frequently the teeth are not free from periodontal disease. As the effects of periodontal

disease on the DNA content of teeth have not been determined, the teeth used in this research were free from any type of dental disease or dental restorative work.

Post-mortem factors that can influence DNA yield include the time since death and the physical and biological environment both at a microscopic and macroscopic level. Previous research examining these factors has focused primarily on pulp tissue or entire teeth, rather than the individual hard tissues (Alvarez Garcia et al., 1996, Burger et al., 1999, Duffy et al., 1991,Rubio et al., 2012). This makes it difficult to gain an understanding of the effects of post-mortem decomposition on the distribution of DNA within teeth or the relationship between the DNA and tooth mineral. Post-mortem alteration of tooth structure in the individual tissues and the effect this has on DNA in these tissues has largely been overlooked. Chapter 5 examined post-mortem decomposition of individual dental tissues and the effect this had on DNA yield at both microscopic and macroscopic levels. The burial environment used in this study was deliberately fixed to one soil type but natural decomposition in the open environment was permitted. Recordings of soil temperature and moisture were made to enable assessment of the effects of micro-changes in environment on teeth buried close to one another. Decomposition leads to physical and biochemical changes within teeth as well as to the quantity and distribution of DNA in them. These changes make it more complicated to retrieve sufficient DNA from these tissues, potentially requiring changes to the extraction protocols used to maximise success. The research presented in Chapter 6 of this thesis examined the benefit of amending extraction protocols for decomposed samples. A major focus of this research was determination of the factors that affect successful analysis of these teeth and exploration of more conservative and effective targeted sampling techniques.

1.1 Scope of thesis

This thesis aims to synthesise existing information and generate new data on the distribution and post-mortem degradation of DNA in human teeth. From this knowledge, recommendations for nuclear DNA retrieval from teeth for the purpose of human identification are established. The outcomes of this research are aimed at providing practical suggestions for forensic scientists, which are described in five manuscripts, presented in the following chapters.

The first study (Chapter 2) is a review, which draws together pre-existing knowledge of tooth structure, and degradation of both tooth and DNA. This study examined the variables that potentially affect the yield of DNA from teeth, highlighting the current limitations in the field to inform forensic practitioners of criteria important for tooth selection. The individual tooth tissues are discussed, highlighting the potential for decontamination, targeted sampling and DNA extraction protocols to influences DNA recovery.

The second study (Chapter 3) explored the effects of donor age and sex, and the presence of dental disease, on nuclear DNA yield from dentine (from traces of pulp) and cementum. This study revealed a large variation in yield between teeth and demonstrated the difficulties in extrapolating results from confounded variables (donor age and presence of disease), highlighting the need for controlled experiments using large sample sizes and limited variables.

As the first two studies indicated that cementum is likely to be a good source of nuclear DNA, Chapter 4 took a closer look at this tissue. This study demonstrated the value of cementum and explored the effects of indiscriminate sampling and decontamination methods on successful nuclear DNA recovery from this tissue.

Armed with the insights gained from these first three studies, Chapter 5 examined the affects of decomposition of the various tooth tissues. This study revealed the distribution of both nuclear and mitochondrial DNA within decomposing teeth and how this is affected by time. The factors that should be considered for successful sub-sampling of teeth are also highlighted.

The final study reported in Chapter 6 explores the potential for simple variations in extraction protocols to influence DNA yield from decomposed teeth. In this study, the advantages and disadvantages of the use of decalcification and carrier RNA in the extraction process were examined in relation to successful analysis of nuclear DNA from decomposed teeth.

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Chapter 2

Teeth as a source of DNA for forensic identification of human remains: A review

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Chapter 3

Dentine and cementum as sources of nuclear DNA for use in human identification

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Chapter 4

Targeted sampling of cementum for recovery of nuclear DNA from human teeth and the impact of common decontamination measures

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Targeted sampling of cementum for recovery of nuclear DNA from human teeth and the impact of common decontamination measures

Denice Higgins[1*], John Kaidonis[2], Grant Townsend[2], Toby Hughes[2] and Jeremy J Austin[1]

Abstract

Background: Teeth are a valuable source of DNA for identification of fragmented and degraded human remains. While the value of dental pulp as a source of DNA is well established, the quantity and presentation of DNA in the hard dental tissues has not been extensively studied. Without this knowledge common decontamination, sampling and DNA extraction techniques may be suboptimal. Targeted sampling of specific dental tissues could maximise DNA profiling success, while minimising the need for laborious sampling protocols and DNA extraction techniques, thus improving workflows and efficiencies. We aimed to determine the location of cellular DNA in non-degraded human teeth to quantify the yield of nuclear DNA from cementum, the most accessible and easily sampled dental tissue, and to investigate the effect of a common decontamination method, treatment with sodium hypochlorite (bleach).

We examined teeth histologically and subsequently quantified the yield of nuclear DNA from the cementum of 66 human third molar teeth. We also explored the effects of bleach (at varying concentrations and exposure times) on nuclear DNA within teeth, using histological and quantitative PCR methods.

Results: Histology confirmed the presence of nucleated cells within pulp and cementum, but not in dentine. Nuclear DNA yields from cementum varied substantially between individuals but all samples gave sufficient DNA (from as little as 20 mg of tissue) to produce full short tandem repeat (STR) profiles. Variation in yield between individuals was not influenced by chronological age or sex of the donor. Bleach treatment with solutions as dilute as 2.5% for as little as 1 min damaged the visible nuclear material and reduced DNA yields from cementum by an order of magnitude.

Conclusions: Cementum is a valuable, and easily accessible, source of nuclear DNA from teeth, and may be a preferred source where large numbers of individuals need to be sampled quickly (for example, mass disaster victim identification) without the need for specialist equipment or from diseased and degraded teeth, where pulp is absent. Indiscriminant sampling and decontamination protocols applied to the outer surface of teeth can destroy this DNA, reducing the likelihood of successful STR typing results.

Keywords: Cementum, Sodium hypochlorite decontamination, Forensic DNA typing, Skeletal remains, Teeth

Background

In forensic cases involving unidentified bodies often the only sources of DNA for identification are the calcified tissues - bones and teeth. Teeth are a valuable source of DNA [1,2] due to their unique composition and location in the jawbone both of which provide protection from microorganisms and environmental factors responsible for postmortem decay. Surprisingly, little is known about the location of, nor antemortem and postmortem changes in, DNA in teeth. While pulp is recognised as the richest source of DNA in healthy fresh teeth [3] its value is decreased in life by age [4] and dental disease and in death by postmortem degradation (Figure 1). In an ideal dry postmortem environment pulp may mummify [5] and persist for extended periods but in a moist environment putrefaction rapidly leads to complete

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destruction [6]. The hard tissues of the tooth - cementum, dentine and enamel (Figure 1) - are more resistant to postmortem decay but targeted sampling of these tissues for nuclear DNA has not been examined in any depth.

MtDNA and nuclear DNA have been obtained from dentine [3,7-10] but the success rate for short tandem repeat (STR) typing of nuclear DNA is variable [8] and the quantity of nuclear DNA available from dentine is negatively affected by age of the individual and dental disease [9], suggesting a strong relationship between the presence/absence of pulp and recovery of DNA from dentine. In contrast, cementum has been shown to be in some instances a better source of mtDNA than dentine (at least in degraded and ancient teeth) [3,7] and yields of nuclear DNA from cementum are not negatively affected by dental disease or chronological age [9,11].

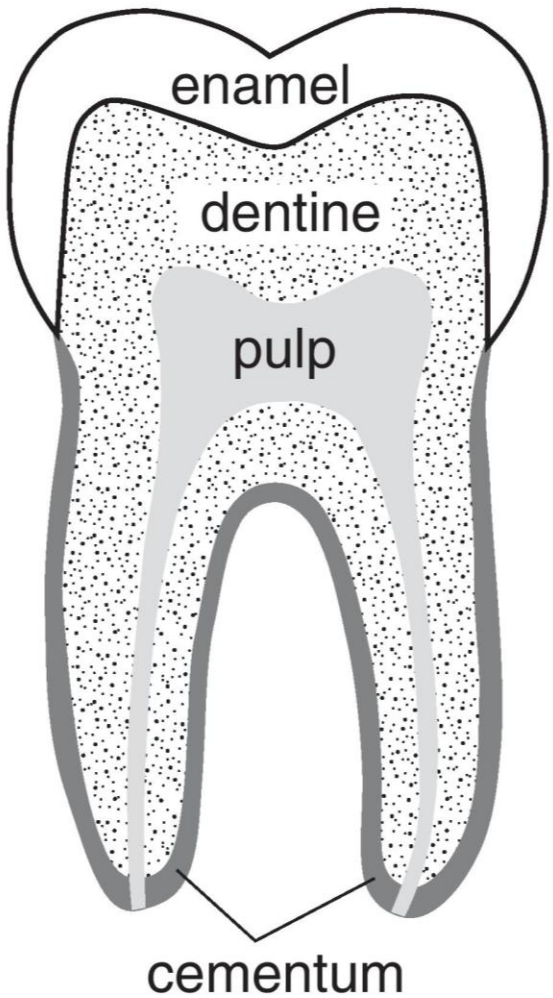

Figure 1 Diagrammatic representation of a human molar identifying the different regions and tissues.

Recovery of DNA from teeth is complicated by mineralisation of the tissues requiring specialised sampling equipment, additional dedicated laboratory space and modified DNA extraction protocols. The major mineral and organic components of teeth - hydroxyapatite (predominantly calcium) and collagen - vary across different dental tissues, with enamel being 96% mineral, dentine 70% mineral and 20% collagen, and cementum 45% mineral and 30% collagen. Pulp is largely cellular and has no mineral content. Both calcium and collagen have been shown to be inhibitors of polymerase chain reaction (PCR) amplification [12] and as such their co-extraction needs to be minimised. A further complication for sampling of mineralised tissues is the fact that an intimate relationship between DNA and hydroxyapatite has been identified in post-mortem samples [13,14] necessitating demineralisation of these tissues for maximum recovery of DNA [15]. Complete demineralisation of bones and teeth using ethylenediaminetetraacetic acid (EDTA) has been shown to improve DNA recovery [15], but requires larger extraction volumes increasing costs and reducing possibilities for automation. EDTA is also a PCR inhibitor so needs to be removed along with calcium and collagen prior to downstream processes.

Improved knowledge of the location of DNA in teeth would facilitate targeted sampling of tissues known to contain nuclear DNA over a range of postmortem intervals and environmental conditions. This could reduce the need for complex and laborious sampling and grinding protocols (including cutting and grinding equipment and dust extraction), allow for smaller sample volume, less calcium and collagen, and reduced dependence on EDTA. Targeted sampling of pulp tissue, which would negate these issues, has been reported via drilling through the crown or by tooth sectioning [16]. However, this does not always have a positive outcome as determining the presence/condition of any pulp tissue prior to sampling is not possible. Cellular cementum may be an important source of nuclear DNA particularly in diseased and degraded teeth where pulp tissue is reduced or absent and therefore the likelihood of retrieving DNA from pulp and dentine is reduced [9]. However, cementum is rarely targeted for DNA analysis and potentially may be removed or damaged during decontamination and sampling.

Prior to sampling, teeth are frequently subjected to decontamination processes aimed at removing exogenous DNA, environmental contaminants and micro-organisms [17]. Decontamination methods vary and include the following, either individually or in combination: removal of the outside layer of the tooth by grinding or sanding [17]; washing/soaking in bleach [18]; washing/soaking in ethanol or in hydrogen peroxide [19]; and exposure to ultra violet (UV) light [20]. Decontamination techniques,

mostly designed to destroy exogenous DNA, have an unknown effect on endogenous DNA, and this may be particularly acute for cementum, which forms the outer surface of the root.

The most commonly reported decontamination methods are removal of the outer surface and washing/soaking in bleach of various concentrations for varying time periods [17]. Studies examining the impact of bleach on bone suggest that endogenous DNA is relatively well protected, possibly due to adsorption to hydroxyapatite [17] or entrapment within mineral aggregates [14,21]. However, it is unknown whether DNA binds to tooth mineral and, if so, at what point during postmortem decay this occurs. Studies examining the relationship between DNA and mineral have been performed on bone [14,21], which is structurally and biochemically distinct from tooth negating the reliability of extrapolation of this data to teeth. No studies have explicitly examined teeth.

The aim of this study is to examine how the efficiency of tooth sampling protocols and the success of DNA profiling might be improved through specific targeting of tissues containing nucleated cells. We confirm the location of nucleated cells in fresh teeth, quantify the yield of nuclear DNA from tooth cementum, and examine the effects of bleach on the nucleated cells/nuclear DNA content of cementum. We show that cementum is a valuable and easily accessible source of DNA in teeth that by virtue of its location is at risk of damage from common decontamination methods.

Methods

One molar tooth was collected from each of 106 volunteer donors along with a blood sample for reference DNA. All work was undertaken under the ethical guidelines and approval from the Research Ethics and Compliance Committee of The University of Adelaide (H-134-2009). Teeth were removed under sterile conditions by registered specialist dental surgeons and placed directly into individually labelled sterile containers.

Initial histological examination

A randomly selected subset of teeth ($n = 4$) was formalin fixed (neutral buffered 10% formaldehyde) for 72 h and demineralised in 10% EDTA at a pH of 7.4. Total demineralisation was confirmed by radiographic analysis. Teeth were embedded in paraffin wax and sliced in 7 μm sections, slide mounted and stained with haematoxylin and eosin. Haematoxylin binds to chromatin in the DNA/histone complex, staining nuclear material a dark violet colour. The location of nuclei in teeth sections was determined by examination under 100×, 200× and 400× magnification using a compound light microscope (Leica Microsystems, Germany).

DNA yield from cementum

A further randomly selected subset of teeth ($n = 66$) was cleaned by gentle curettage with a dental scaler to remove soft tissue remnants and blood, and then wiped with DNA-free saline. Cementum samples, in the form of a coarse powder, were scraped from each tooth using a new disposable scalpel blade for each sample. Care was taken to avoid sampling deep concavities or very tight spaces between roots as these sites can retain soft tissue remnants. All samples collected weighed between 15 and 50 mg, dictated by the availability of tissue and the conservative nature of sampling. All equipment and workbenches were cleaned with 4% sodium hypochlorite before and after sampling each tooth.

All pre-PCR work was undertaken in a dedicated pre-PCR laboratory housed in a separate building to the post-PCR laboratory. DNA extractions were performed using QIAmp DNA Investigator kits (Qiagen, Ilden, Germany), following the manufacturer's instructions for bones and teeth, including the use of carrier RNA. As per this protocol the cementum powder was initially lysed overnight with buffer ATL and Proteinase K at 56°C without prior decalcification. Reference samples were extracted in the same fashion but on a separate day. The final elution volume for each sample was 60 μL. Extraction blanks were included with every set of extractions, one for every three teeth. Extracts were stored at 4°C until quantification and STR profiling.

DNA extracts were quantified using Quantifiler™ Human DNA Quantification Kit, (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM® 7000 Sequence Detection System for real-time PCR (Applied Biosystems, Foster City, CA, USA). Negative and positive controls and seven standards were included in duplicate on each run as directed by the manufacturer. All extraction blanks were quantified. Nuclear DNA concentration was determined using the comparative CT method with unknown samples compared to a standard curve with a range of 0.05 ng/μL to 200 ng/μL. DNA yields were converted to nanograms of DNA per milligram of cementum to allow direct comparison between all samples.

STR profiling of samples and references was performed using Amplf*l*str ProfilerPlus™ (Applied Biosystems, USA). Reactions were performed in 25 μL reaction volumes, consisting of 9.6 μL reaction mix, 5 μL primer mix, 0.4 μL AmpliTaq Gold™ and 10 μL of DNA extract. Cycling was performed on a 9700 GeneAmp Cycler. Amplification parameters consisted of an initial denaturation step at 95°C for 10 min., followed by 28 cycles of 94°C for 60 s, 59°C for 60 s, 72°C for 60 s and a final extension step at 60°C for 45 min.

Capillary electrophoresis was performed on a 3130xl Genetic Analyzer and genotypes analysed using Genemapper ID v3.2.1. A minimum threshold of 50 relative fluorescence

units (RFU) was used for calling alleles and the profiles generated were compared to their respective reference.

Compilation of quantification data and descriptive statistics were undertaken in Excel (Microsoft, USA). Inferential statistical tests were performed using SAS STAT software. Statistical significance was set at $P < 0.05$ for all tests unless otherwise indicated. The distribution of DNA yield from cementum was examined for normality and significant outliers, and was found to be substantially positively skewed. The data were subsequently log-transformed for analysis. A random effects mixed linear model of DNA yield was fitted to the log data using the 'Mixed' procedure in SAS STAT software. The model included the fixed effect of sex and the covariate age, as well as the interaction between sex and age. Tooth identification (ID) was fitted as a random effect.

Effects of sodium hypochlorite

Remaining teeth ($n = 28$) were randomly divided into four treatment groups ($n = 7$ per group) subjected to immersion in bleach of varying concentration for differing time intervals as shown in Table 1.

Teeth were cleaned of soft tissue remnants and blood by gentle curettage with a dental scaler then wiped with DNA-free saline. Bleach treatment was applied as per Table 1, followed by rinsing with sterile saline. Sixteen of the teeth (four from each treatment group) were placed into numbered cassettes and prepared for histological examination as described above. Mounted sections were examined at 100×, 200× and 400× magnification using light microscopy, and photographed and qualitatively assessed for the presence or absence of: soft tissue remnants, nuclei in soft tissue remnants, cellular cementum and nuclei in cementum.

Cementum was sampled from the remaining 12 teeth (three from each treatment group) and DNA was extracted as described above.

Quantification was performed using qPCR with SYBR® green chemistry using a previously published 67 bp nuclear target [22]. The qPCR mix consisted of: 5 μL 2× Brilliant II SYBR® green master mix (Agilent Technologies, USA), 0.15 μM forward primer (GGGCAG TGTTCCAACCTGAG), 0.15 μM reverse primer (GAA AACTGAGACACAGGGTGGTTA), 400 ng/μL Rabbit Serum Albumin, 3.3 μL water and 1 μL DNA extract to a total of 10 μL. All samples were run in triplicate including negative (PCR blank) and positive (dilutions of male genomic DNA, Applied Biosystems, USA) controls and extraction blanks. Cycling was performed using a Corbett 6000 Rotogene real-time PCR thermocycler and consisted of an initial 5 min denaturation at 95°C, followed by 45 cycles of 95°C for 10 s, 59°C for 20 s and 72°C for 15 s. Nuclear DNA concentration was determined using the comparative CT method; unknown samples were compared to a standard curve with a range from 0.033 ng/μL to 8.848 ng/μL. This method offers a standard curve with a lower, smaller range for increased sensitivity.

Table 1 Treatment groups for study of the effects of bleach on the histological appearance of cementum

Treatment group	Treatment
1	None - control group
2	Soaked in 4% bleach for 5 min then rinsed
3	Soaked in 4% bleach for 1 min then rinsed
4	Soaked in 2% bleach for 5 min then rinsed

Results

Histology

Nucleated cells were observed in abundance in the pulp tissues and in and on areas of cellular cementum. They were also noted in accessory canals, in soft tissue inclusions, and in bone and soft tissue remnants that were present in teeth with constricted furcation areas. No stainable nuclear material was visible within dentine. Cellular cementum was more prevalent at the apical ends of the roots and in the furcation areas. A layer of cementoblasts was observed overlaying some root surfaces. The thickness of cellular cementum varied between teeth and was not uniform on all sides of the same roots. In three out of four teeth the cellular cementum was seen to begin approximately two-thirds up the root but on the fourth tooth it started quite close to the enamel junction. Inter-radicular surfaces displayed, in general, thicker cellular cementum than the outer surfaces of the roots. One tooth displayed a number of highly cellular inclusions in the cementum at the apical end of one root, possibly representing the contents of accessory canals. In another tooth a large number of cells were noted trapped between two closely situated roots. Enamel was not present as it is 96% mineral and was totally removed during the demineralisation process.

DNA yield from cementum

Nuclear DNA yield from cementum varied widely between teeth (0.28-173.57 ng/mg, Table 2). The age distribution of tooth donors was biased towards people under the age of 26 years (Figure 2). Seventy-one per cent of donors were aged between 16 and 26 years with every year represented, 17% of donors were aged 29 to 39 years with ages 32 and 38 not being represented, and only 12% of donors were aged between 39 and 60 years with many ages not being represented. The ratio of female to male donors was 59:41. No statistically significant effect was noted of chronological age on DNA yield, although there was a trend showing a decrease in yield with increasing

age. Donor sex did not have a statistically significant effect on DNA yield from cementum.

All cementum samples produced sufficient DNA to yield a full STR profile and there were no discrepancies observed between profiles generated from cementum samples and the corresponding reference sample. We found no evidence of contamination and no dropout of alleles was noted.

Effects of sodium hypochlorite

The control (untreated) group of teeth showed similar histological features to the initial subset of teeth examined. Bleach treated teeth showed a reduction in the presence of and a loss of tissue differentiation in persisting soft tissue remnants in crevices, a reduction in the presence of cementoblasts on the root surfaces, and a reduction in the presence of intact nuclei in the cementum. No structural changes to the cementum were observed.

As seen in Figure 3 the nuclear DNA yields from cementum samples from bleach treated teeth reduced by an order of magnitude at both concentrations (2.5% and 5%) and exposure times (1 and 5 min) in comparison to untreated samples.

Discussion

In fresh teeth cementum is a reliable source of nuclear DNA, and may be an important and easily accessible source when pulp is absent or compromised. The yield of nuclear DNA from cementum, even after sodium hypochlorite decontamination, suggests that sampling solely from the outer surface of the roots leads to successful STR profiling. Histological examination confirmed that cementum is the only dental hard tissue containing nucleated cells. An absence of visible nuclei in dentine suggests that nuclear DNA recovery from this tissue [8,9] may derive directly from pulp tissue with which it is intimately associated (both developmentally and functionally) or potentially from postmortem cellular breakdown allowing DNA to permeate the dentine mineral.

DNA extraction from teeth for human identification frequently involves non-specific sampling (drilling or whole tooth grinding), requiring specialised equipment, laboratory set-ups and lengthy extraction protocols with large volumes of reagents. These features add significant additional cost, time and complexity to tooth-based DNA identification - a major drawback for disaster victim identification (DVI) and other high throughput identification situations.

Table 2 Variation in nDNA yield from cementum adjusted for weight of tooth powder sampled ($n = 66$)

Descriptive statistics (ng/mg)	
Mean	21.26
SD	26.73
SEM	3.29
Min	0.28
Median	14.42
Max	173.57
95% CI	14.65-27.77

Extrapolating our results on fresh teeth to more typical forensic cases involving aged and degraded skeletal remains may not be justified without further research. However, in cases of short postmortem intervals, where human remains are well preserved or in diseased teeth or those from elderly individuals (where pulp is absent or reduced), targeted sampling of cementum as an alternative for DNA analysis and identification offers a number of key advantages. Cementum is readily accessible and easily sampled using manual sampling tools, eliminating the need for specialist equipment to cut, drill and/or grind the teeth thus reducing cross-contamination risks and expense. The DNA extraction process is also simplified and is successful from small sample sizes (15–50 mg) using small volume extraction protocols with the potential for much higher throughput. Cementum contains less mineral than enamel, dentine or even bone, decreasing dependence on EDTA demineralisation steps. In contrast to dentine, DNA recovery from cementum is not adversely affected by dental disease nor age of the individual.

Variation in the abundance and distribution of cellular cementum is to be expected as it is laid down continuously throughout life. In general cementum thickness increases with age [23] but deposition is also affected by functional requirements, the presence of periodontal disease [24] and systemic diseases such as diabetes [25].

Nuclear DNA yields from the cementum of healthy third molar teeth varied by three orders or magnitude (0.28 to 173.57 ng/mg). Previous studies have also shown a large variation in DNA yield from teeth [26,27] but cementum has not previously been examined independently. The reasons for this wide range in DNA yield are unclear. It does not appear to be related to chronological age or sex of the donor, but may be due to variation in the amount of cellular cementum collected from each tooth. Cellular and acellular cementum frequently occur as alternating bands on the tooth and are difficult to distinguish. Despite this, all samples yielded sufficient DNA to produce full STR profiles, confirming the value of targeted sampling of cementum.

External decontamination is often seen as a necessary prerequisite to DNA analysis of postmortem teeth and bones. If teeth are extracted from the jaw under ideal conditions, the value of external decontamination via physical removal or treatment with harsh chemicals needs to be balanced against the negative impact on endogenous DNA recovery. The resistance of teeth to contamination

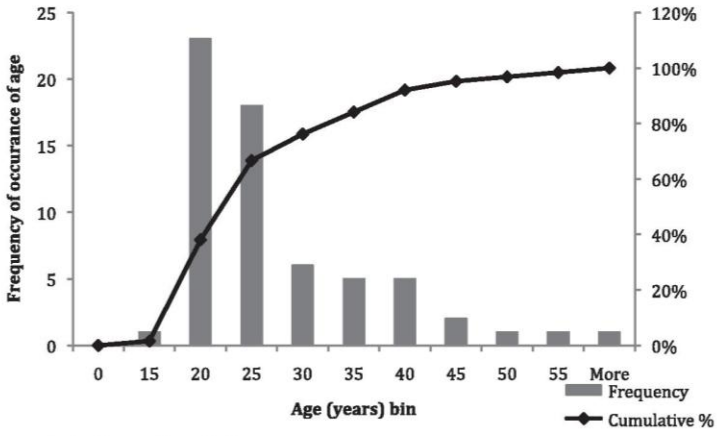

Figure 2 Distribution of chronological age of tooth donors.

even when post recovery handling is not performed in an ideal manner has been demonstrated [28,29] suggesting that severe decontamination measures may not always be warranted.

Bleach, which dissolves soft tissues and destroys DNA, has been used widely as a DNA decontamination measure in ancient DNA research and forensic DNA practice. Despite this, very little is known about the qualitative or quantitative effects on endogenous DNA in human skeletal remains, including teeth. As an important source of nuclear DNA in the hard tissues of teeth, it is important to understand the effects of bleach on cementum. Histological examination of teeth treated with bleach revealed a reduction in the amount of cellular material visible on the outer surface of the root and a loss of tissue definition in remaining soft tissue remnants. This is consistent with previous observations showing that bleach dissolves soft tissue, with effects related directly to concentration, volume and exposure time [30]. Loss of visible nuclei on the root surface, and in the outer layers of the cementum was also observed suggesting an overall loss of nuclear DNA from cementum and associated sources.

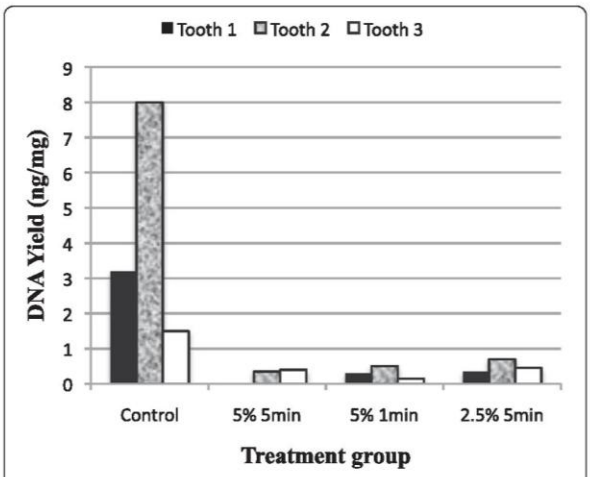

Figure 3 Effects of bleach at varying concentration and exposure time on nuclear DNA yield from cementum.

Quantification of DNA yield from bleach treated teeth showed an order of magnitude decrease in comparison to non-bleach-treated teeth. Other studies examining the effects of bleach have studied bone and did not quantify the effects on the endogenous DNA yield [14,17]. These studies also primarily focused on ancient samples, which potentially differ from samples of a forensically significant time span. In younger samples it would be expected that not all the available endogenous DNA would be bound up in protective mineral aggregates. Salamon et al. [14] included several 'modern' bones in their study and noted DNA outside of the crystal aggregates that was potentially affected by bleach treatment but did not quantify the DNA loss or explore this in any detail. The structural and chemical difference between bones and teeth prevent extrapolation of observations of the behavior of one of these tissues to the other. In our study DNA from cementum treated with bleach was sufficient, in nearly all instances, to produce full STR profiles despite the 10-fold reduction in DNA yield. However, it should be noted that these teeth were healthy fresh samples. In degraded samples it would be expected that the starting amount of DNA would be considerably lower but also that the DNA might be bound to the tooth mineral. Further investigation using degraded teeth of varying postmortem intervals would help understand the true impact of various bleaching regimes on cementum. Potentially teeth at different stages of postmortem decay will display not only differences in their DNA/mineral relationship but also in their porosity influencing the depth of penetration and subsequent effects of bleach.

Dissing et. al. [31] demonstrated in fossilised teeth that bleach penetrated through to the pulp chamber. No studies on the porosity of teeth or depth of penetration of bleach have been conducted on more modern samples.

Grinding the tooth surface has also been reported as an alternative or additional method of decontamination. This method can potentially remove all the available cellular cementum which has been reported to have a maximum thickness in upper molars of 25–1140 μm and 20–700 μm in lower molars [32]. Cellular cementum is generally thickest on molar teeth and is predominantly found at the root tips and between the roots [32]. The histological data from this study supported this distribution pattern and demonstrated an increase in cellularity in areas where the cementum was thickest.

Conclusions

We confirmed that pulp and cellular cementum provide the primary sources of nucleated cells in teeth and demonstrated that cementum is an excellent and easily accessible source of nuclear DNA. Targeted sampling of cementum may be useful in DVI situations where large numbers of individuals need to be sampled quickly, in recently deceased individuals or well preserved remains where specialist laboratory set-up and equipment for sampling and grinding whole teeth are not available, or from diseased teeth or those from elderly individuals where pulp is absent or reduced. Cementum is easily removed from teeth using a scalpel, no special equipment is required and the majority of the tooth is left intact. Cementum samples alone provided sufficient DNA to obtain full STR profiles from all of the teeth examined without a prior decalcification step in the extraction process. Decontamination with bleach reduces the yield of DNA recovered from cementum, which may have a significant effect on STR profiling success of degraded teeth. Tooth extraction from the jaw under controlled conditions may reduce the need for root surface removal or treatment with bleach. However, situations may arise when this is not possible or the teeth available for sampling are no longer in the jawbone. In these cases, the need for more stringent decontamination may be required but should be carried out mindful of possible impacts on DNA in the cementum.

Abbreviations

DVI: Disaster victim identification; EDTA: Ethylenediaminetetraacetic acid; ID: Identification; PCR: Polymerase chain reaction; qPCR: Real time polymerase chain reaction; RFU: Relative fluorescence unit; STR: Short tandem repeat; UV: Ultra violet.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

DH with assistance from JA, JK and GT conceived and designed the study. DH performed all laboratory work, compiled the data, and performed initial interpretation of results. TH performed formal statistics. DH, with assistance from JA, wrote the manuscript. All authors critically revised the manuscript for intellectual content and approved the final manuscript.

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Chapter 5

Differential DNA preservation in post-mortem teeth and the implications for forensic and ancient DNA studies

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Differential DNA preservation in post-mortem teeth and the implications for forensic and ancient DNA studies.

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Abstract

Major advances in genetic analysis of human skeletal remains have been seen over the last decade, primarily due to improvements in post-DNA-extraction techniques. Despite these improvements, all DNA analyses are ultimately limited by the yield of target molecules recovered from the sample. A key challenge for DNA analysis of skeletal elements is that they generally contain only trace amounts of DNA. Enhanced recovery of DNA from bones and teeth by improved sampling and extraction techniques would allow further advancements. However, little is known about the post-mortem kinetics of DNA degradation and whether the rate of degradation varies between nuclear and mitochondrial DNA or across different skeletal elements. This knowledge, along with information regarding ante-mortem DNA distribution within skeletal elements, would inform sampling protocols and facilitate development of improved extraction processes. Here we present a combined genetic and histological examination of the DNA content and rates of DNA

degradation in the different tooth tissues of 150 human molars over short-medium term post-mortem intervals (one to 16 months). Coronal dentine, root dentine, cementum and pulp of 114 of these teeth were subjected to DNA extraction via a silica column method and the remaining 36 teeth were examined histologically. Real time quantification assays based on amplification of two nuclear DNA fragments (67 bp and 156 bp) and one mitochondrial DNA fragment (77 bp) showed that nuclear and mtDNA degraded exponentially, but at different rates, dependent on post-mortem interval and soil temperature. In contrast to previous studies, we identified differential survival of nuclear and mtDNA in different tooth tissues. Histological examination showed that pulp and dentine were rapidly affected by loss of structural integrity, with pulp being completely destroyed in a relatively short time period. Conversely, cementum showed little structural change over the same time period. Finally, we confirm that targeted sampling of cementum can provide a reliable source of nuclear DNA for STR-based individualisation using standard extraction methods, without the need for specialised equipment or large-volume demineralisation steps, even from teeth, which have been buried for 16 months.

1. **Introduction**

Advances in DNA analysis of human skeletal remains are providing high-resolution insights into the origin [1], migrations [2], health [3], biogeographic ancestry [4, 5], phenotype [6, 7] and identification [8-10] of deceased individuals and populations for evolutionary, archaeological, medical and forensic studies. Much of this progress has resulted from post-DNA-extraction advances in polymerase chain reaction (PCR) sensitivity [11], the design and optimization of short-amplicon DNA typing technologies [4], and next generation sequencing [1, 2] that focus on the small amounts of highly degraded DNA recovered from skeletal remains. In contrast, sampling and DNA extraction techniques from bones and teeth have remained largely unchanged since the earliest publications in ancient DNA and forensic biology [12, 13] over a decade ago. These first steps in DNA analysis of skeletal remains are critical and can have a major impact on the amount and integrity of recovered endogenous DNA [14-17], contamination[18], and co-extraction of PCR inhibitors [13, 19, 20], thereby dramatically affecting the success of downstream analysis.

While the differential preservation of DNA in various skeletal elements has been considered [21, 22], relatively little attention has been paid to identifying those skeletal tissues with high ante-mortem DNA content or the relative rates of post-mortem DNA degradation within and between different skeletal tissues. Resolving these issues is critical to future improvements in DNA analysis of skeletal remains and could clarify intra- and inter-individual variation in DNA content of skeletal tissues leading to predictive decision models for sample selection. Also, specific tissues may be identified for targeted post-mortem sampling to maximise recovery of endogenous DNA whilst minimising destructive sampling, the potential for contamination, the co-extraction of inhibitors and the need to remove large amounts of inorganic (hydroxyapatite) and organic (collagen)

fractions. These non-DNA components of bone and teeth are primarily responsible for the large volume/low throughput/high cost nature of DNA extractions from skeletal remains [16, 23], and represent potent PCR inhibitors, if not removed during the extraction process [24]. Targeted sampling, relying on knowledge of ante-mortem DNA content and post-mortem DNA degradation, could allow smaller sample sizes to be processed facilitating the use of medium- to high-throughput techniques and standard laboratory equipment, leading to considerable cost and time saving.

Post-mortem DNA damage has been well characterised, based on theoretical and *in-vitro* studies, and empirical observations of DNA recovered from ancient and degraded samples [25-27]. However, little is known about the kinetics of post-mortem DNA degradation in a real world situation, nor how this varies across different tissues and skeletal elements. Environmental conditions (e.g. temperature, moisture and pH) in combination with time since death (post-mortem interval -PMI) are thought to be the primary factors influencing DNA degradation but the relative effects of environment and time appear to be strongly situation dependent, leading to claims that the rate of DNA degradation cannot be predicted. In contrast, recent work suggests that under a range of conditions, DNA degradation follows a random fragmentation model [28, 29] and, at least in bone, that the rate of mtDNA degradation can be predicted based solely on PMI and ambient temperature [28]. Whilst environmental conditions are believed to have a strong effect on DNA preservation over long PMIs, it is uncertain whether these factors are important over shorter time spans. It also appears that, over long PMIs (hundreds to thousands of years), mitochondrial DNA (mtDNA) degrades at a slower rate than nuclear DNA [28, 30]. It is not known however if the rate of DNA degradation varies across different skeletal elements (bone types and teeth), nor whether the long-term degradation rate and differential degradation of mtDNA vs. nuclear DNA applies at forensically relevant

timescales (days-years). Resolution of these issues is important as sample selection is frequently based on subjective visual interpretation of morphological preservation. In addition, the primary focus, at least for forensic identification, is on individualisation via nuclear short tandem repeats (STRs), which requires relatively large amounts of intact DNA.

Teeth, which are commonly used for ancient DNA studies and forensic identification, offer great potential for targeted sampling at various PMIs, and to examine short-term DNA degradation. The anatomical location (within bony sockets) and morphological structure (particularly covering of impenetrable enamel over the crown) of teeth offer unique protection to endogenous DNA from post-mortem degradation [18, 31-33] and the individual tissues of the tooth show a range of ante-mortem cellularity, organic and inorganic content [34]. This contrasting cellularity and mineral content of the four tooth tissues creates a unique biochemical and anatomical setting to examine the content and post-mortem degradation of DNA. Conventional sampling of teeth for DNA analysis generally follows one of two pathways. Targeted sampling of the pulp, the DNA-rich soft tissue component of the tooth, by splitting open the tooth or drilling in through the crown [35, 36], or non-targeted sampling of the entire tooth (or root). Grinding of the entire tooth provides access to the largest amount of DNA but also includes a large amount of mineral (cementum is 45%, dentine 70% and enamel 97% composed of mineral) that must be removed prior to downstream analysis.

The objective of this study was to investigate the DNA content and rates of DNA degradation in the different dental tissues over short to medium post-mortem intervals. Quantitative real time PCR was used to measure the relative degradation rates across tissues and between nuclear and mitochondrial DNA. A high level of sample homogeneity

and minimal variation in environment was aimed for to gain a better understanding of the effects of ante-mortem factors, temperature and PMI on DNA content and degradation. A concurrent histological examination was also undertaken to improve understanding of the effects of post-mortem decomposition on the tooth tissues and correlate these changes with the DNA results.

2. Materials and Methods

2.1. Sample collection and post-mortem decomposition

One hundred and fifty third molar teeth, free from dental disease, were collected from a total of 85 donors, along with a blood sample for reference profiles. Third molar teeth only were used to reduce variables and because these teeth were available in large quantities from a range of age groups. Donor age varied from 16 to 60 years, with a male to female ratio of 38:47. Teeth were randomly allocated to one of six groups (25 teeth per group: 19 for DNA analysis and six for histological examination). No individual contributed more than one tooth to each group. The six groups represented six PMIs (zero months, one month, two months, four months, eight months and 16 months). To visualise post-mortem structural change over an extended time period two teeth (one incisor and one molar) known to be over 500 years PMI were also studied histologically but were not included in data analysis.

All teeth, other than those in the zero month PMI group, were buried approximately 20 cm deep, randomly spread across two galvanized steel raised beds containing sandy loam. The beds measured 1m high x 1m wide x 3m long and were situated outside without protection from the weather. To facilitate sample retrieval a wire grid was placed on the surface of each bed allowing a grid reference to be recorded against each tooth. The climate in the local area is Mediterranean (i.e. dry summer, sub tropical). Temperature readings were taken at regular intervals (several times a month for short burials and once a month for longer term burials) from six sites (one at each end and one in the middle of each bed, numbered 1 – 3 in box 1 and 4 - 6 in box 2). A burial temperature for each tooth was determined by calculating the average of the temperature recordings from the site closest to the tooth over the course of its PMI.

At the end of each PMI the teeth were retrieved, using stringent collection protocols to minimise risk of contamination, including the use of fresh gloves for each tooth and wiping of all excavation equipment with 3% sodium hypochlorite between retrieval of each tooth. Once a tooth was retrieved it was freed from the bulk of soil by gentle rubbing between gloved fingers and placed in an individually numbered sterile container.

2.2. Histology

Teeth for histological examination were briefly rinsed under running water to remove blood or dirt then immersed in 10% neutral buffered formaldehyde for 72 hours. Subsequently the teeth were rinsed overnight under running tap water then incubated at room temperature in 10% Ethylenediaminetetraacetic acid (EDTA), pH 7.4, with constant stirring until totally demineralised (confirmed by radiographic analysis). After demineralisation, teeth were sectioned into 1/3 and 2/3 sections in a vertical plane, embedded in paraffin wax and sliced in 7 μm sections (starting from the cut sides), slide mounted and stained with Mayer Lillie haematoxylin and counter stained with 1% eosin with phloxine. Haematoxylin binds to chromatin in the DNA/histone complex, staining nuclear material a dark violet colour to allow visualisation of nuclear material.

2.3. DNA sampling and analysis

Teeth for DNA analysis were carefully cleaned of blood/soft tissues or soil with DNA free water and allowed to dry prior to sampling. Cementum samples, in the form of a coarse powder, were scraped from each tooth using a new disposable scalpel blade for each sample. Cementum was identified visually, with sampling restricted to prevent accidental inclusion of dentine. Subsequently the crown was removed from each tooth by cutting a notch, with a diamond disc, at the cementum-enamel junction to a depth of 1mm before striking with a hammer and chisel. Any residual pulp tissue was collected, and then

samples were taken of coronal dentine and then root dentine. Dentine samples were generated using hand turned wire drills and a triangular shaped hobby tool blade, with fresh tools for each sample. Not all dentine was collected to avoid inclusion of cementum or enamel in the sample and to be of similar mass to cementum samples. All equipment and workbenches were cleaned with 4% sodium hypochlorite before and after sampling each tooth. Samples were weighed after collection to allow results to be directly compared.

2.3.1. DNA extraction

All pre-PCR work was performed in a dedicated laboratory located in a separate building to the post-PCR laboratory, following strict protocols including the use of appropriate personal protective equipment. DNA extractions were performed using 14.8-81.5 mg of powdered dentine/cementum using the QIAmp® DNA Investigator kit (QIAGEN, Ilden, Germany), following the manufacturer's instructions for bones and teeth, including the use of poly-A carrier RNA. Reference samples (blood on sterile gauze) were extracted in the same fashion but on a separate day. Samples were eluted in a final volume of 60 μL. One extraction blank for every three teeth (nine samples) was included in each set of extractions. Extracts were stored at -20°C until quantification and STR profiling.

Total demineralisation of bone and tooth samples has been shown to improve DNA yields from ancient and degraded samples[16]. Hence, for teeth in the 16-month decomposition period, tissues available in sufficient quantity were divided into two samples to allow examination of the benefits of decalcification prior to extraction. Where two paired samples were collected one was subjected to the standard extraction process (above). The second sample was demineralised and digested overnight in 1 mL of 0.5 M EDTA, 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/mL Proteinase K at 56°C on a rotary mixer. On the following day these samples were centrifuged to pellet undigested material then the

supernatant was transferred to an Amicon Ultra-4 centrifugal filter (Millipore) and centrifuged at 4000xg for 10 min. Subsequently 1mL DNA free water was added and the sample was again centrifuged for 5 min repeatedly until the residual volume was equal to or less than 200 μL. Once the desired volume was reached 280 μL of ATL was added and the resultant supernatant was transferred to a 2 mL tube and treated in the same manner as the undemineralised samples for DNA extraction and downstream analysis. A total of 12 out of the 19 16-month teeth had sufficient tissue mass to allow dual sampling and extraction. This provided 35 paired samples, 12 from cementum, 11 from coronal dentine and 12 from root dentine. The divided samples weighed 21.3 – 70.2 mg.

2.3.2. DNA Quantification

Quantification of DNA was performed using real time quantitative PCR (qPCR) with SYBR® green chemistry. DNA was quantified using three, previously published, primer sets. The specificity of primers to a single binding site was assessed using a post qPCR melt curve to visualize the dissociation kinetics. Primer details, references and cycling conditions are shown in Table 1.

Table 1. Details of primers and qPCR cycling conditions used for quantification of DNA.

Primer name	Fragment length and reference	Primer Sequence 5'-3'	Cycling conditions
L13258	MtDNA 77bp[14]	ATCGTAGCCTTCTCCACTTCAA	5-minute denaturation step at 95° C, 45 cycles of 95° C for 10 seconds, 58° C for 20 seconds, and 72° C for 15 seconds.
H13295		AGGAATGCTAGGTGTGGTTGGT	
HomoSap_CSF_STR_F	Nuclear DNA 67bp[37]	GGGCAGTGTTCCAACCTGAG	5-minute denaturation step at 95° C, 45 cycles of 95° C for 10 seconds, 59° C for 20 seconds, and 72° C for 15 seconds.
HomoSap_CSF_STR_F		GAAAACTGAGACACAGGGTGGTTA	
HomoSap DQARB1_105F	Nuclear DNA 156bp[38]	AGGTTGCTAACTATGAAACACTGGC	
HomoSap_DQARB1_214R		TGGTTTAGGAGGGTTGCTTCC	

The qPCR mix consisted of 5 μL 2x Brilliant II SYBR®green master mix (Agilent Technologies, USA), 0.15 μM forward primer, 0.15 μM reverse primer, 400 ng/μL Rabbit Serum Albumin, 3.3 μL water and 1 μL DNA extract to a total of 10 μL. All samples were run in triplicate and negative (PCR blank) and positive controls (dilutions of male genomic control DNA, Applied Biosystems, USA) were included on all runs. Extraction blanks were also quantified. Real time PCR was performed on a Corbett 6000 Rotorgene thermocycler.

DNA concentration was determined using the comparative C_T method; unknown samples were compared to a standard curve using the Rotor-Gene 600 Series Software 1.7. The arithmetic mean value of the triplicate qPCR results was calculated for each sample for inclusion in the final analysis.

2.3.3. STR typing

Amplification was carried out using Profiler Plus® (Applied Biosystems, USA). Cycling was performed on a 9700 GeneAmp cycler and consisted of an initial denaturation at 95°Cfor 10 min followed by 28 cycles of 94°C for one min, 59°C for one min and 72°C for one min, followed by a final extension at 60°C for 45 min. All reactions were initially performed in 25 μLvolumes, with 10 μL of DNA (diluted when appropriate to allow a final concentration of ideally between .5 and 1 ng). Capillary electrophoresis was performed using an Applied Biosystems 3130xl genetic analyser and profiling analysis was undertaken using GeneMapper® ID v3.2.1. For samples containing low levels of DNA if peak heights were lower than 50 Relative Fluroscence Units (RFU) the sample was re-analysed a second time and sometimes a third time in 12.5 μL reaction volumes including 5 μL of DNA with a consensus approach being used to identify peaks in these multiple reactions.

2.3.4. Statistical analysis

DNA yields from cementum and coronal and root dentine were converted to ng DNA per mg tooth tissue (ng/mg) for nuclear DNA and fragment copies per mg of tooth tissue (copies/mg) for mtDNA to standardise for differences in sample weights. As pulp was sampled as a whole it was not weighed and is thus reported in ng DNA per uL of extract (ng/uL). A probability level of 0.05 was considered significant for all statistical tests.

A regression analysis was conducted for each of the three quantified fragments (n67, n156 and mt77) using chronological age of the donor (in years), sex of the donor, the plot the tooth was buried in, PMI time (in months), the (arithmetic) average soil temperature of the burial plot over the period of interment, and tissue type as regression predictor variables. A complete model with interaction terms up to order five was fitted. Examination of the

residuals indicated non-linearity. The Box-Cox transformation of the response variable was applied using the *boxcox* function from the MASS library in R. This indicated a log transform of the response variable was appropriate.

A linear mixed effects model was fitted using the R-package 'lme4' [39], and for each fragment type, a backwards step procedure using the significance of predictors was applied to the full interaction model. For simplified models, an analysis of variance test was used to determine if the model had been significantly modified [40]. Both the sex of the donor and burial plot were insignificant factors.

To more closely examine the effects of chronological age on DNA yield for each type of DNA fragment, the data were further subset into two groups of teeth those, which were buried, and those, which were not. As before, a linear mixed effects model was fitted to each subset of the data. For the unburied teeth, the age of the subject and the tissue sampled were treated as fixed effects, and the individual was treated as a random effect. For the teeth, which had been buried, the age of the subject, the decomposition time, the average temperature and the tissue sampled were treated as fixed effects, and the individual and plot were treated as random effects.

To compare the rate of decay of the three different fragment types, a comparison of half-lives was made from the fitted transformed linear models, for various average temperatures. For the nuclear fragments, since there was significant interaction between chronological age and PMI, the half-life for a subject of median age was produced.

3. Results

3.1. Histology

Non-decomposed teeth showed pulp tissue rich in nucleated cells, nucleated cells in cementum and a layer of cementoblasts on the external root surfaces (Figure 1A and 1B). Nuclei were also noted in blood vessels and soft tissue inclusions within the cementum (Figure 1C). In decomposed teeth, pulp tissue showed loss of structural integrity and cellular detail by one-month post-mortem, with a large decrease in presence of nuclei in comparison to fresh teeth (Figure 2A). Teeth subjected to longer PMIs showed a further decrease in the presence of nuclei and an increasing absence of pulp tissue remnants. Dentine showed structural changes in the predentine layer, with the pulpal surface becoming increasingly irregular until at 16 months PMI the predentine layer was completely lost in most cases (Figure 2B). However at the same time period minimal structural change was seen in the cementum layer although a decrease in cementoblasts on the root surfaces and nuclei in soft tissue inclusions was noted (Figure 2C). In the ancient teeth (PMI > 500yrs) no nuclear material was visible, structural breakdown was seen extended through much of the dentine, and some focal destruction was visible in the cementum (Figure 3A and 3B). However, as in the teeth buried for a short period the cementum was far less affected structurally than the dentine.

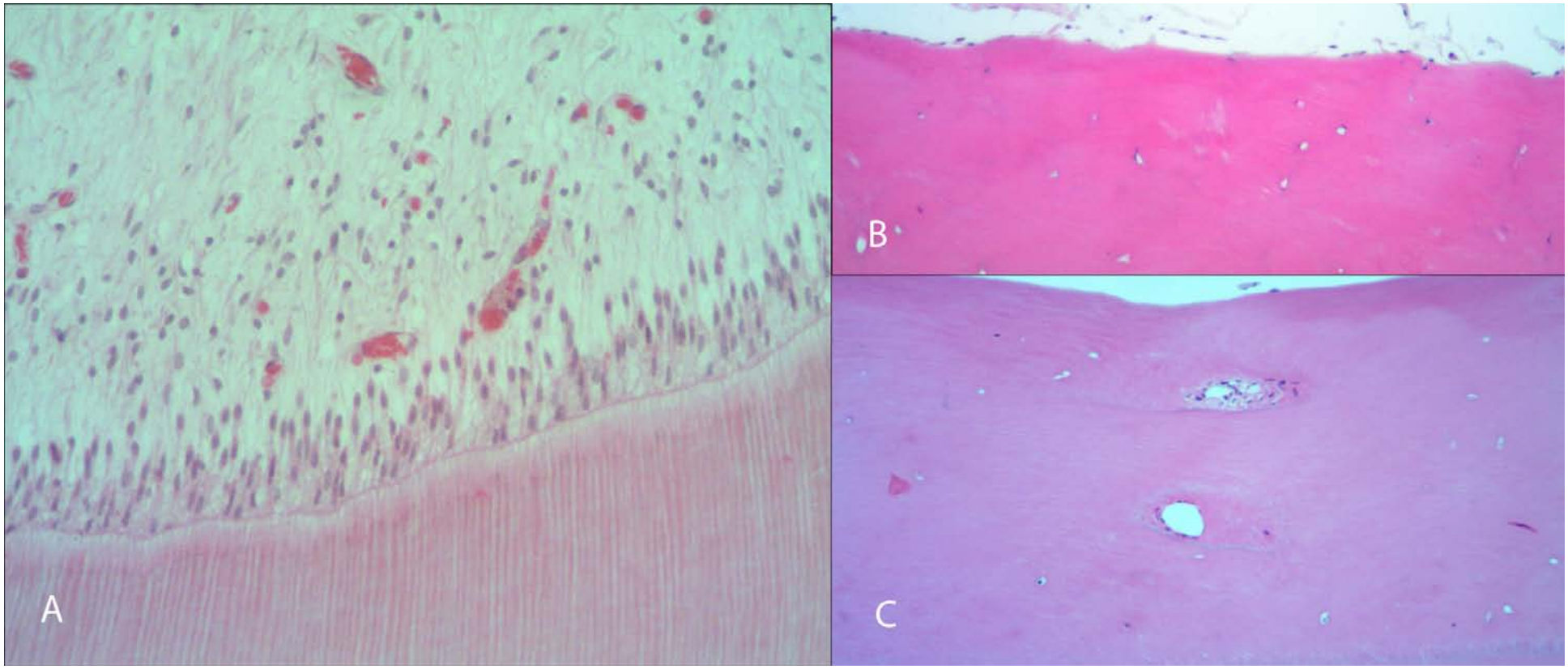

Figure 1.Histological appearance of fresh teeth, (200x) H&E staining. A – Pulp tissue is rich in odontoblasts (cells that form dentine), fibroblasts, defence cells (e.g. histocytes and macrophages), plasma cells, nerve cells and undifferentiated mesenchymal cells. B - Cellular cementum containing cementocytes, in spaces comparable to osteocytic lacunae, with cementoblasts visible on the surface. C – Cementum also had soft tissue inclusions and blood vessels present.

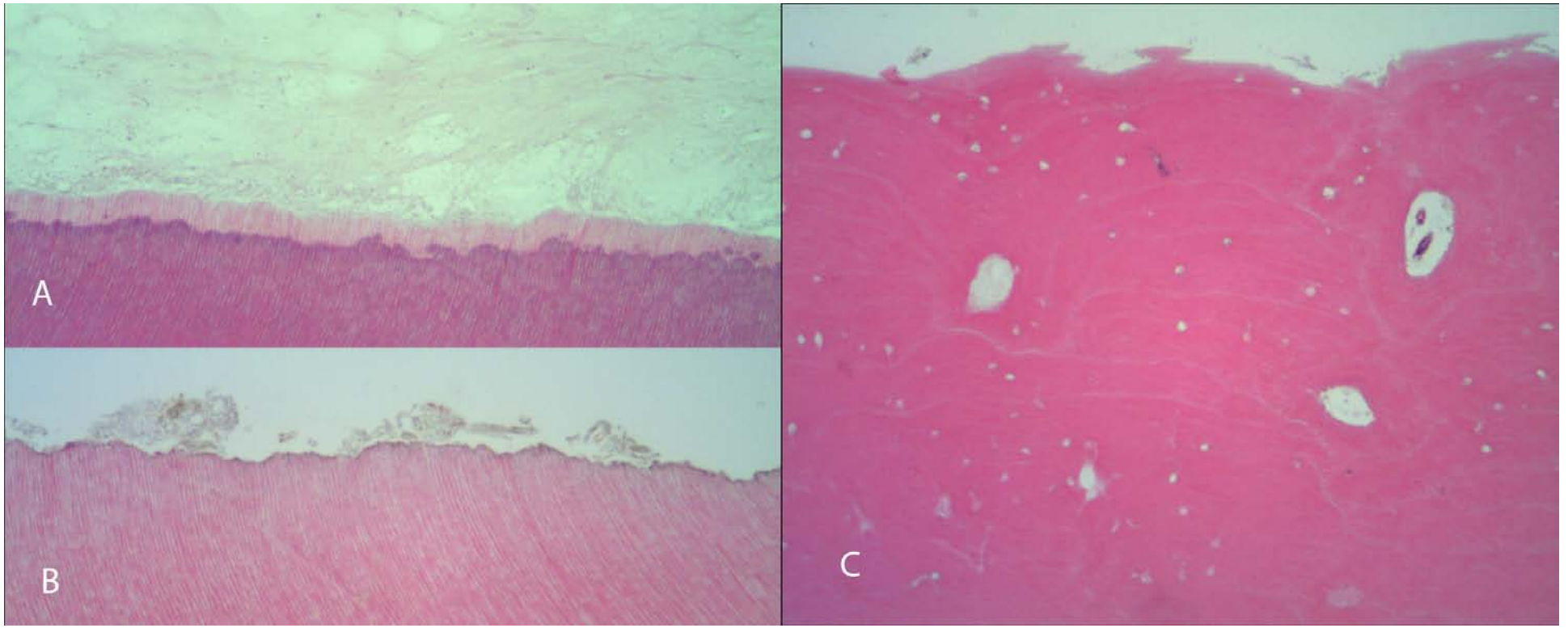

Figure 2. Effects of decomposition on pulp tissue, (40x and 200x) H&E staining. A –Pulp tissue at one month PMI showing loss of structure and nulear material. B – By 16 months very little pulp tissue remained with much of the pulp chamber being empty. Dentine displayes almost complete loss of the predentine layer. C – Cementum at 16 months shows little structural change but loss of cells in soft tissue inclusions and on the external surfaces.

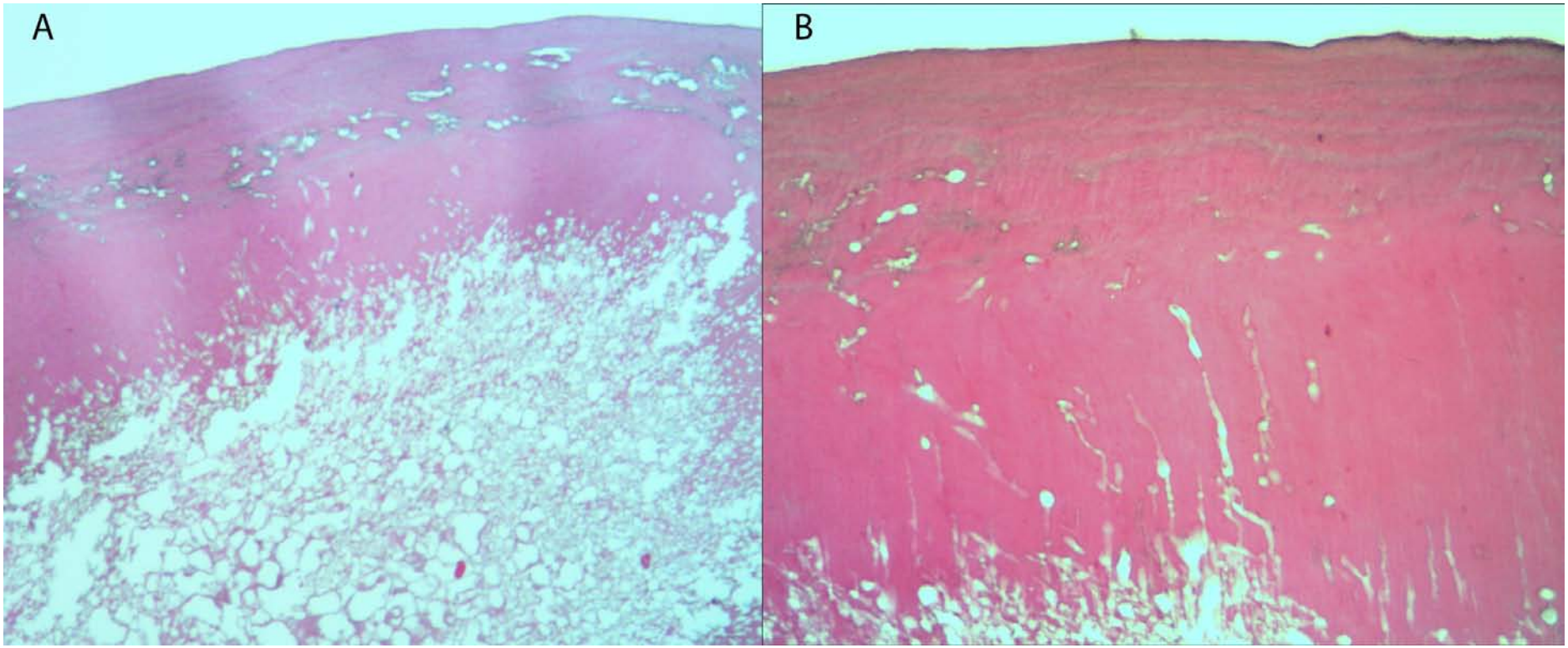

Figure 3. Effect of extended decomposition, (40x and 100x) H&E staining. Histological examination of teeth with a PMI >500 years showed A – Marked structural breakdown of the dentine and no visible cellular material. Areas of focal destruction in the cementum. B – At higher magniification destrction is efficdent traveling outwards along the dentinal tubules with almost the full thickness of the dentine being affected.

3.2. DNA yield

3.2.1. Pulp Nuclear DNA

The percentage of teeth with pulp present declined rapidly as did the yield attained from the pulps that were present (Figure 4). By eight months only 10% of teeth had any remaining visible pulp tissue.

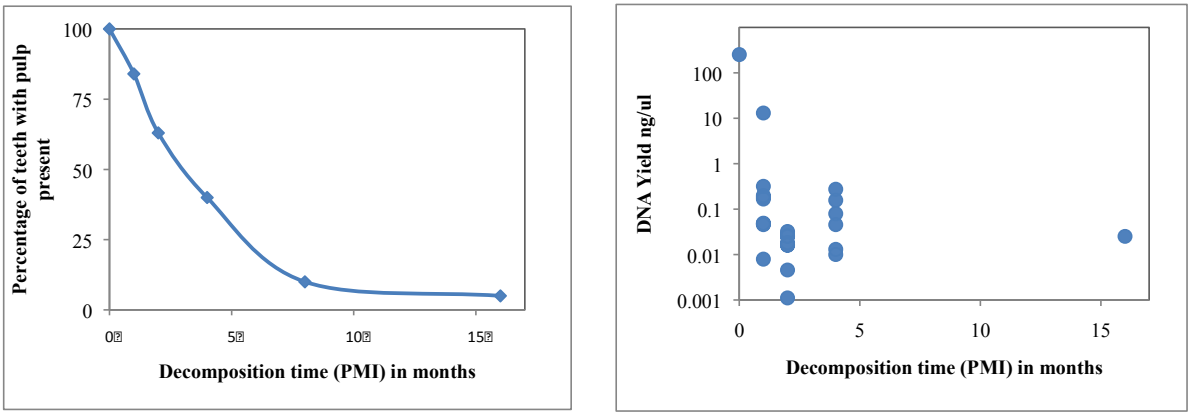

Figure 4.Degradation of pulp tissue. The graph on the left displays the percentage of teeth at each time period that contained any visible pulp and the graph on the right shows the yield of nuclear DNA from pulp tissue (quantified using the 67 bp fragment). Although two teeth in the 8-month group had pulp residue present no nuclear DNA was detected in these samples.

3.2.2. Dentine and Cementum

The yield (taken as the average of the triplicates that were run for each sample) of each DNA fragment per mg of tooth powder from cementum, coronal dentine and root dentine at each PMI examined is displayed in Figure 5. Nuclear DNA is reported in nanograms whereas mtDNA is reported in copies

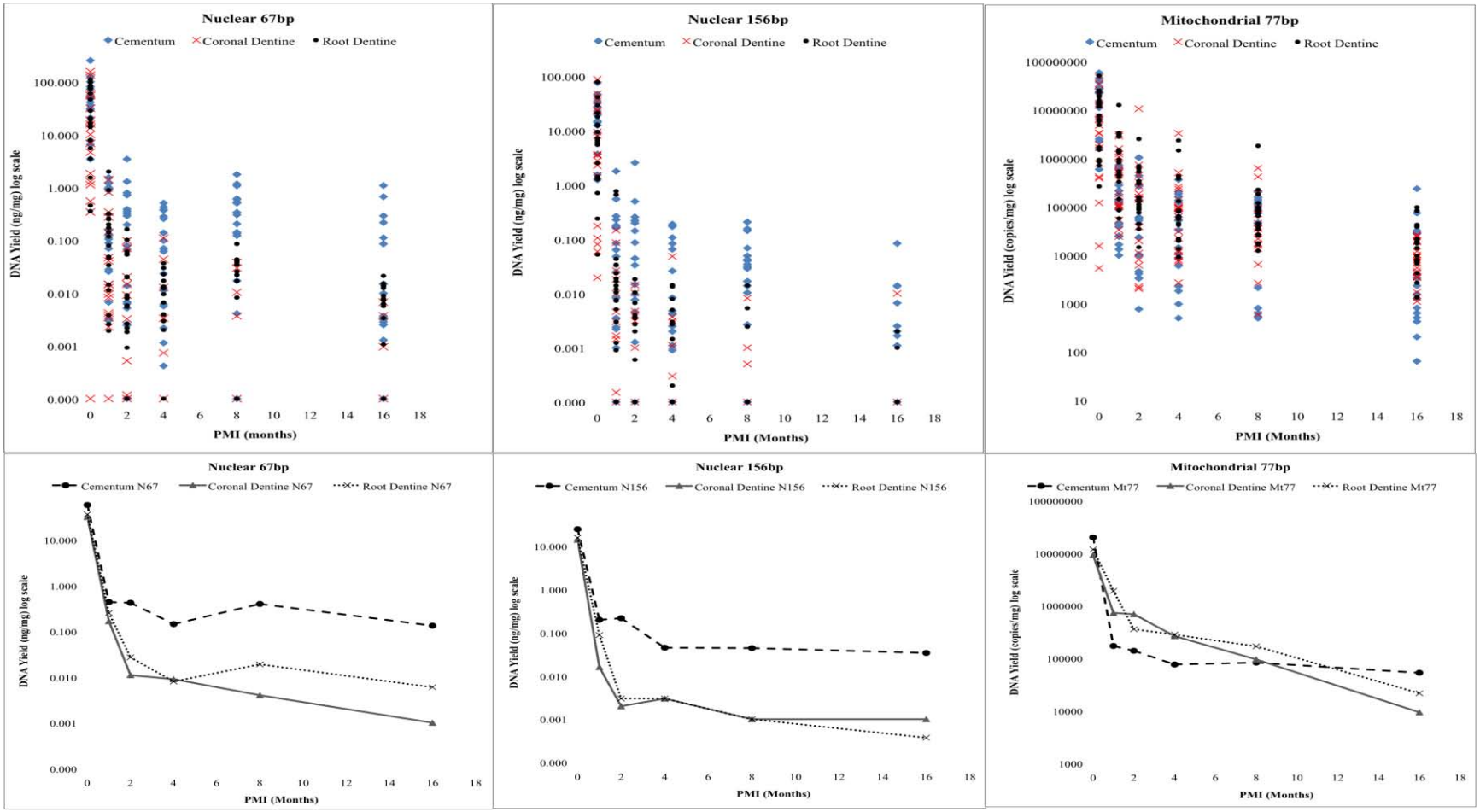

Figure 5: Yield of DNA and mean values from each tissue type at each PMI on a log scale. Nuclear DNA yields are displayed in ng/mg and mtDNA is displayed in copies/mg.All yield are displayed on a log scale. The top row of graphs show the raw data from each of the teeth sampled and the lower graphs show the arithmetic mean value for each tissue at each PMI. The nuclear 67 bp fragment results are displayed in the left hand graphs, the nuclear 156 bp fragment in the centre graphs and the mitochondrial 77 bp fragment in the right hand graph.

Statistical analysis shows:

For the n67 and n156 fragments, tissue type, PMI, average temperature and subject age were significant (Figure 6). For n67, interaction terms between tissue and average temperature; PMI and average temperature, and PMI and subject age were also significant. For n156, interaction terms between tissue and average temperature, tissue and subject age, PMI and average temperature and, average temperature and subject age were also significant.

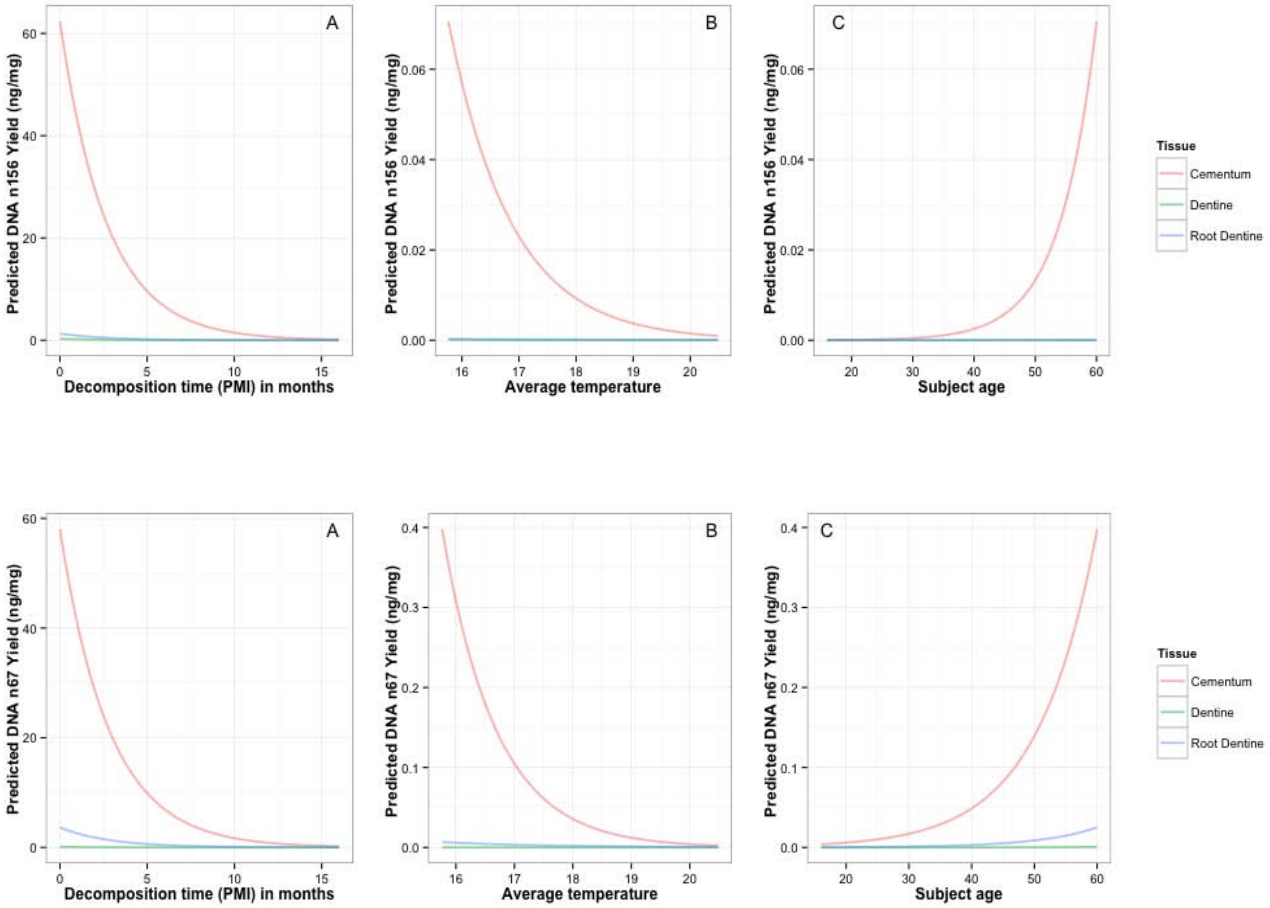

Figure 6: Predicted fragment yield for n67 and n156 at varying PMI, soil temperature and subject age.PMI, temperature and subject age are held constant at sample median values where applicable. (Note the effect of PMI was much greater than that of temperature and age and this is reflected by the Y-axis scale.)

For all observed values of the predictor variables, predicted nuclear DNA yield from cementum was found to be significantly greater than that from dentine. Increase in PMI time and average temperature were found to have a negative effect on nuclear DNA yield. Conversely, an increase in subject age had a positive effect on nuclear DNA yield.

For the mt77 fragment, tissue, average temperature and PMI time were significant (Figure 7). Age of the donor was insignificant. The interaction between PMI time and average temperature was the only significant interaction term found.

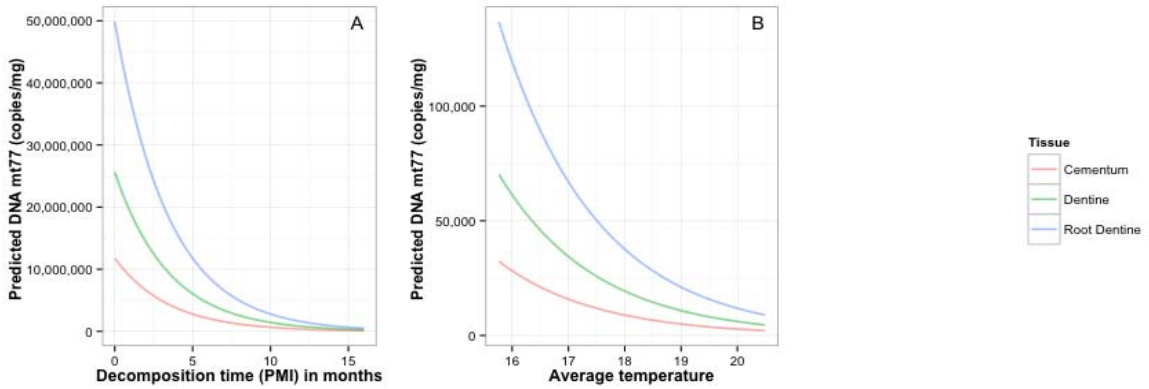

Figure 7: Predicted fragment yield for m77 at varying PMI, and soil temperature.PMI, and temperature are held constant at sample median values where applicable

For all observed values of the predictor variables, predicted mtDNA yield from cementum was found to be significantly lower than that from dentine, with root dentine performing best. An increase in PMI time and average temperature were found to have a negative effect on mtDNA yield.

The average soil temperature calculated for each burial ranged between 15.8°C and 20.5°C with a maximum difference between site never exceeding 2°C (Figure 8).

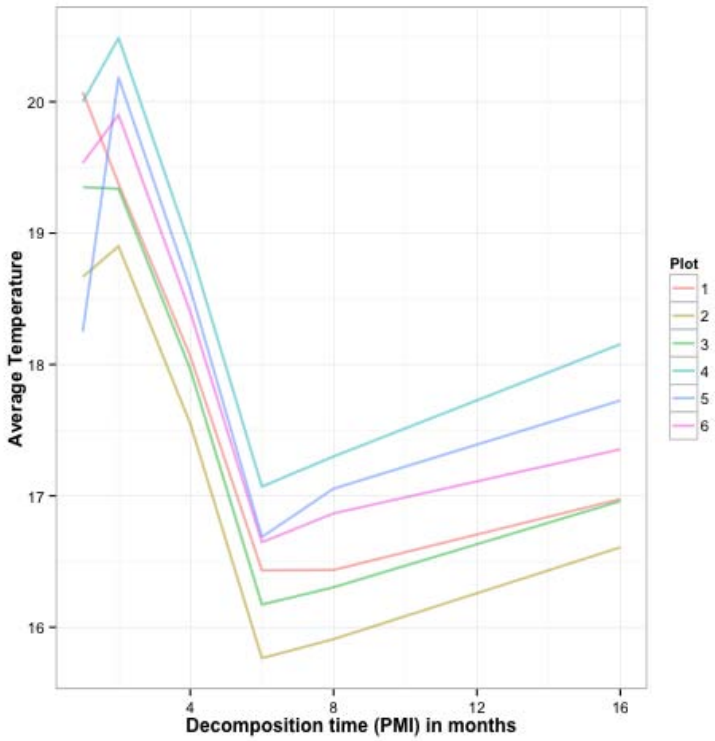

Figure 8.Average burial temperature. The average temperature was calculated for each tooth using soil temperatures for the relevant plot taken over the period of burial. The average temperature for teeth in each PMI group is displayed.

The DNA yield data best fitted an exponential relationship, so regression equations were fitted to indicate the rate of DNA degradation. The predicted half-life for each DNA fragment is shown in Figure 9, which reveals a molecular half-life for the mt77 fragment that is greater than that seen for both the nuclear fragments. In addition the 156 bp nuclear fragment half-life is shorter than that for the nuclear 67 bp fragment.

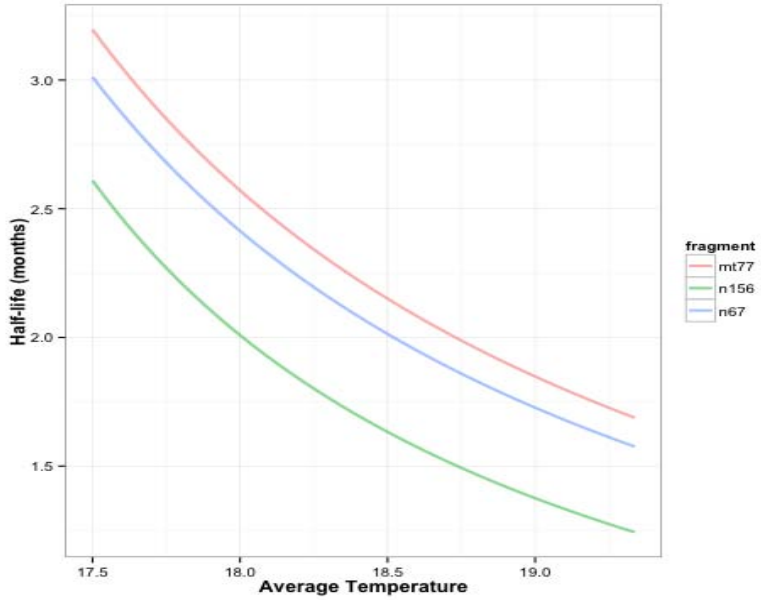

Figure 9.Predicted DNA half-life for varying average soil temperature for each fragment type. For nuclear DNA as the half-life is affected by donor age the average donor age has been selected for the calculation.

3.3. Profiling outcomes

STR typing outcomes achieved from cementum, coronal dentine and root dentine of the decomposed teeth are displayed in the form of a heat map in Figure 10. Cementum gave the highest rate of profiling success, with greater than 50% of samples giving full profiles at each PMI, while coronal dentine showed less than 10% of full profiles after 1 month and root dentine less than 10% success after 2 months. The occurrence of dropout was seen to increase in frequency with increasing PMI. Predominantly dropout affected the longest alleles more frequently than shorter alleles but not exclusively.

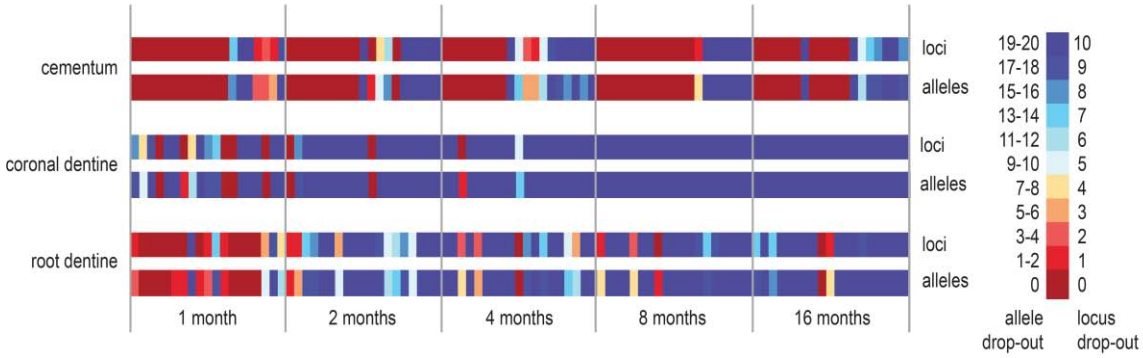

Figure 10.Genotyping success for each of 3 dental tissues from teeth from 5 post-mortem burial intervals. Nineteen teeth were sampled for each time period (1, 2, 4, 8 and 16 months). Allele and complete locus dropout are expressed with colder colours representing lower success and warmer colours representing higher success.

3.4. Effects of demineralisation

Demineralisation of tooth powder prior to DNA extraction led to an increase in DNA yield for all samples that had DNA at detectable levels in their non-demineralised extract except in one instance where the yields were equal. In five instances, DNA was detected in demineralised samples when the non-demineralised portion had no detectable DNA. Nine of the 12 cementum samples yielded DNA in both fractions with the other three yielding DNA from the demineralised portion only. All coronal dentine samples (12) had no detectable DNA in the non-demineralised portions and very low levels of DNA in two demineralised portions, with the rest having no detectable DNA. Half of the root dentine samples (6) had detectable, although low, levels of DNA in the non-demineralised portions and only four samples had low levels of DNA detected in the demineralised portions.

4. Discussion

The present study demonstrates differential distribution of DNA content and preservation across the tissues of human teeth. Cellular cementum is identified as the most reliable source of nuclear DNA once pulp tissue has been lost, whilst root dentine is confirmed as a rich source of mitochondrial DNA. These results suggest that targeted sampling of the most DNA-rich tissues of the tooth will improve the outcome of forensic genetic testing.

This study found a wide person-to-person variation in DNA yield suggesting that the number of cells in teeth differs appreciably between individuals. This variability has also been seen in other studies of teeth [41] and is postulated to be due to various ante-mortem factors. In the non-decomposed teeth sampled all of the tissues yielded nuclear and mitochondrial DNA in sufficient quantity to allow genetic analysis. During decomposition pulp tissue was seen, both histologically and during DNA sampling, to break down rapidly. As pulp tissue is non-mineralised this was not altogether unexpected. However pulp has been demonstrated in previous studies to persist for longer periods than were seen here [35]. In this current study teeth were buried loose in the soil, which likely caused a more rapid rate of decomposition in comparison to teeth that remain in the jawbones during decomposition. Other experimental studies that have compared test samples to actual casework samples report less effects of decomposition in the casework samples [31, 42]. Potentially the decomposition scenario depicted in this current study represents extreme degradation. A large decline in DNA yield was observed in the first month post-mortem likely due to the loss of the soft tissue components e.g. the cementoblast layer on the surface of the roots, pulp tissue and blood vessels. This hypothesis is supported by the appearance of the histology slides, which revealed rapid breakdown of these soft tissue components. Nuclear DNA yields from dentine were reduced to very low levels in most instances by the 8-month interval, whereas nuclear DNA yields were maintained at a

higher level in cementum. This was not unexpected given that the source of nuclear DNA in dentine is the soft tissue of the pulp whereas, in the cementum, the cementocytes, encapsulated in the mineralized tissue, are a major component of the DNA source and are more protected from degradation.

The histological study revealed structural breakdown of the dentine occurring from the pulp interface outwards. The predentine layer, which is the unmineralised layer of dentine adjacent to the odontoblast cell bodies was the worst affected. By 16 months the predentine layer was almost completely lost with the surface of the dentine becoming irregular in appearance. In contrast, the cementum did not show obvious signs of structural change but did show loss of the external cementoblast layer and a reduction in visible nuclei in areas of soft tissue inclusions. At all PMIs examined cementum yielded the highest levels of nuclear DNA. Overall, root dentine provided the highest levels of mtDNA but it was also possible to retrieve reasonable to high levels of mtDNA from cementum. In contrast to these findings a previous study found that cementum was a better source of mtDNA than dentine [14] but this study examined ancient tooth samples. The continued structural breakdown of dentine seen histologically in the ancient samples examined in this current study suggests that as dentine suffers further structural breakdown the mtDNA is subsequently lost, whereas cementum shows more resilience. Cementum also gave the highest rate of success with STR typing at all stages of degradation, frequently being the only tissue to yield complete STR profiles.

Interestingly, the chronological age of the donor had a positive effect on nuclear DNA yield from decomposed teeth. This observation may reflect an increased resistance of older teeth to decomposition. More mature teeth have narrower apices and are more heavily mineralized and less porous than younger teeth [43]. These features potentially restrict the

ability of moisture and microbes to penetrate the outer layers of the tooth, reducing the effects of post-mortem decomposition and DNA degradation. The effect of age was more pronounced in cementum likely due to cellular cementum increasing in thickness with age. Most probably the cementum sampled from younger individuals was primarily acellular cementum.

The DNA yield data generated in this study revealed a different rate of degradation for the different DNA fragments. While very little research has been directed at DNA degradation in the short to medium term, it has been suggested that in the initial post-mortem phase the rate of DNA degradation will be more rapid, due to the activity of nucleases rather than primarily depurinationas is seen in ancient samples [29, 44]. Sawyer et al [44] determined that, for samples with a PMI less than 100 years, the total yield of DNA decreased over time but fragmentation to a small size happened rapidly after death rather than progressively through time. The rate of degradation of mtDNA calculated in this current study was seen to be faster than that seen in a previous study examining mtDNA in ancient moa bone [28]. This is not surprising as the previous study was performed using ancient samples (minimum 1600 years old) and, as the authors noted, their results did not take into account the initial stages of post-mortem degradation, which likely occur at a more rapid rate.

Calculation of a predicted half-life for the 67 bp nuclear DNA fragment and the 77 bp mtDNA fragment revealed very similar results, with a slightly longer half-life recorded for the mitochondrial fragment. A longer half-life for mitochondrial DNA has been supported by the findings of a previous study [45]. In this study by Foran, a number of factors were postulated to affect the rate of DNA fragmentation including the base composition and function of the DNA fragment examined as well as the tissue from which it was extracted

[45]. The similar degradation rates of the n67 and mt77 fragments seen in this study are potentially a reflection of the base composition and length of the two fragments and the short post-mortem period examined. In addition, the rate calculated here for nuclear DNA, but not for mtDNA, was affected by chronological age and an interaction between age and PMI, which complicates comparisons between the two fragments.

The other important finding of this study was that even a relatively small variation in temperature had a significant effect on the yield of DNA from all of the dental tissues. Studies examining ancient teeth have indicated that environmental conditions greatly affect the rate of post-mortem DNA degradation [46], with temperature having the greatest impact [28, 46]. In contrast, experimental studies examining shorter post-mortem time spans often do not note a significant effect of temperature [31, 33]. In this current study we found that temperature significantly affected the degradation rate of both nuclear and mtDNA, with even as little as a 2˚C rise having a negative impact on DNA preservation. Whether the teeth in this study were more significantly affected by temperature due to separation from the jawbone is an important consideration requiring further investigation.

5. Conclusion

The current study has shown clearly that DNA is not distributed evenly throughout teeth but is concentrated in defined areas and that the DNA yield from human teeth is influenced by both ante-mortem and post-mortem factors. The rate of post-mortem degradation of DNA in teeth is dependent on post-mortem interval and burial temperature. This study has shown that in short to medium decomposition time periods even small changes in temperature are important to DNA preservation. Also in the case of nuclear DNA it has been demonstrated that the age of the donor is significant for DNA preservation. This research indicates that the roots of the teeth are far more useful to genetic analysis than the crown with the cementum being particularly important for nuclear DNA analysis. Small samples of dental hard tissues that are amenable to inclusion in standard laboratory workflows can produce reliable and successful STR profiling results if teeth are carefully subsampled.

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Chapter 6

Evaluation of carrier RNA and low volume demineralization for recovery of nuclear DNA from human teeth

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Chapter 7
General discussion and conclusion

7.1 Introduction

This thesis comprises an introduction followed by a series of five chapters in the form of manuscripts, either published, or in preparation for submission, each of which contains discussions and conclusions related to specific aspects of the research carried out during my PhD candidature. In this concluding chapter, the research findings are drawn together to show how they have addressed the overall aims of the study. The significance of the outcomes of this research project is also discussed, together with its limitations, and then suggestions are provided about possible directions for future investigations in this area of research.

Identification of human remains is a complex issue that impacts not only on individuals but also on societies as a whole. Traditional methods of identification are not always successful so that worldwide there are many individuals who remain unidentified indefinitely. Frequently the impediment to successful identification is a lack of post-mortem information that can be derived from the remains. The final avenue for identification is often genetic analysis, which, despite incredible advances in technology, is still reliant on an ability to retrieve sufficient DNA from the available remains. In an attempt to address this key issue, the research reported in this thesis aims to increase our understanding of DNA availability in teeth and of the effects of post-mortem degradation on dental tissues and the DNA in them. This knowledge will allow optimal recovery of DNA from teeth, thereby increasing the possibility of identification of human remains. Teeth were selected for use in this research as they are extremely resistant to destruction (Alvarez Garcia et al., 1996, Schwartz and Schwartz, 1991, Sweet and Sweet, 1995) and have been shown to be a reliable source of DNA for genetic analysis (Alonso et al., 2001, Baker et al., 2001, Milos et al., 2007, Sosa et al., 2012). Additionally teeth have been shown to often yield DNA of better quality than does bone and to be less subjected to

contamination with exogenous human DNA (Alonso et al., 2001, Pilli et al., 2013, Ricaut et al., 2005)

This research has investigated the DNA content of human teeth, with respect to importance to the identification of human remains, ascertaining the tissues of teeth that provide the best source of nuclear and mitochondrial DNA. It has also identified some of the ante-mortem and post-mortem factors that impact on DNA availability. The research presented in this thesis also increases our understanding of the kinetics of post-mortem degradation of the tooth tissues and the DNA in them. The existing literature on this subject, specifically in relation to practical applications for forensic scientists, is inconclusive in a number of important areas. This study sought to gain insight into three key issues:

1. How is DNA distributed through teeth, how variable is this between individuals, and what ante-mortem factors impact on the amount of DNA available in individual teeth?
2. What are the effects of post-mortem degradation on the tooth tissues in relation to the yield and distribution of DNA within them?
3. Are current practices used to isolate DNA from these tissues allowing the full potential of teeth to be realised?

The most salient purpose of this research is to facilitate meaningful assessment of available teeth in forensic identification casework scenarios, to inform forensic scientists about tooth and tissue selection, and to predict likely outcomes under different conditions. The prediction of outcomes should allow forensic practitioners to select appropriate sample handling protocols, extraction techniques and analysis modalities to maximise the chances of positive results and to minimise waste of valuable resources.

It has been argued throughout this research that current methods of sampling teeth are overly destructive and inefficient and do not enable the full potential of teeth to be realised for genetic analysis. This shortfall reflects a lack of understanding of tooth morphology and of knowledge of post-mortem degradation of these tissues. The yield of DNA recovered from any tissue is not only dependent on the amount of DNA actually present but also on other factors, such as the efficiency of the sampling and extraction processes. Therefore, histological studies have formed an integral part of the research performed here to corroborate the DNA yield data. Histological examination of the changes that can occur in teeth, both pre- and post-mortem, has allowed an assessment of whether the DNA yields from the tissues are a valid reflection of the DNA actually present. Histological assessment has also provided useful insights into the potential effects of sampling processes on DNA retrieval, such as highlighting the potential negative impact of common decontamination measures on the DNA yield from cementum.

7.2 Empirical findings and theoretical and policy implications

This work adds to existing knowledge by providing new insights into why DNA is or is not present in specific teeth and in particular tissues, allowing selection of those teeth and dental tissues which will provide the best sources of DNA. The findings of this research enhance our understanding of inter- and intra-individual variation in DNA content of teeth and also provide insights into the factors that need to be considered when selecting the best teeth to sample. Currently, the sampling protocol of choice is to select the largest healthy tooth available and to grind together all of the tissues, with healthy teeth being considered as those free from caries and dental restorations. This research has clearly shown that not all teeth are equal and, indeed, not all tooth parts are equal in value for extracting DNA - in fact, each identification case needs to be considered individually. Tooth selection for DNA extraction should take into consideration several factors, including: availability; health

status of available teeth (including the presence of both caries and periodontal disease); age of the individual (and therefore stage of development of the teeth); and the extent of decomposition.

While dental pulp will always remain the best source of DNA in teeth, this tissue is not always present, being affected by age, disease, and post-mortem decay. Furthermore, sampling of pulp requires specialised skills and equipment that may not always be available. However, it has been shown in this thesis that cementum is frequently a good source of DNA, in particular, nuclear DNA. Indeed, it is sometimes the only source of nuclear DNA of sufficient quantity to allow STR analysis of decomposed teeth. Unlike pulp tissue, sampling of cementum is simple and does not require specialised equipment. This research has also demonstrated that common decontamination methods may have a negative impact on the DNA in cementum and, hence, need to be considered carefully.

The findings of this research add substantially to our understanding of the processes of decomposition that can occur within teeth and of the effects of post-mortem degradation on DNA in teeth. The differential degradation of nuclear and mtDNA, and the vulnerability of pulp and dentine to structural degradation, have been highlighted. Insight into post-mortem DNA degradation in the short to medium term is also provided, contrasting with information provided by previous research examining degradation over more extended periods of decomposition.

The findings of this study have a number of important practical implications for future practice. This research has shown that it is advisable when selecting teeth that the presence of cellular cementum should be a consideration. It has also been demonstrated that the age of the individual (reflecting the stage of development and potentially permeability of the

tissues) is also an important consideration. As tooth development occurs from before birth until approximately the second decade of life, the teeth available in the jaws of one individual represent different stages of development and thus different potential for genetic analysis. Taken together, these findings suggest that careful sample selection and appropriate decontamination and sampling techniques will lead to enhanced outcomes of genetic analysis of teeth in forensic situations. The present study adds to previous findings, confirming that teeth are a valuable resource for genetic analysis of human remains.

On several occasions during the course of this project the opportunity arose to sample teeth from actual forensic cases. Unfortunately, due to ethical restraints, these cases are not reported in detail in this thesis. However the work undertaken on actual cases has highlighted the applicability of the knowledge gained from this research with successful outcomes being achieved from careful sub-sampling of human teeth.

7.3 Limitations of the study and recommendations for future research

The current investigation was limited by a number of factors, including the difficulties in collecting large samples of human teeth. Ideally, decomposition of teeth from the intact body to complete skeletonisation would have been preferred for this research but, given the large sample size required to examine the variables in question, obtaining this number of human remains was impractical. Also issues with burying and subsequently retrieving human remains were a consideration. Consequently, to examine post-mortem decomposition in this study, teeth donated by living individuals were used rather than teeth in situ in the jawbones of deceased individuals. Further studies comparing how ‘loose’ or ‘separate’ teeth decompose compared with those that have been retained in the body would be extremely beneficial to address this short fall. Potentially teeth retained within the jawbone would be less affected by environmental contaminants and the DNA within them

is less likely affected by UV light, moisture and soil microbes as the roots are protected by bone. The pig could be a useful experimental model for research into the variation in decomposition between separate teeth and those retained in the bone. Pigs are physiologically and anatomically closer to humans than other laboratory animals (Fadista et al., 2008) and pigs are frequently used as models for humans in dental research (Popowics et al., 2001). The form of the molars of domestic pigs and humans are grossly similar, although the crown height of human molars is lower than those of pigs and the shape of the molars is simpler. Pigs teeth have also been shown to have a similar dentine and enamel structure to human teeth (Lopes et al., 2006) although the shape is more complex and the enamel is thinner (Popowics et al., 2001). In addition, pigs and humans last shared a common ancestor 83 million years ago (Fadista et al., 2008) and the porcine genome is comparable to the human genome, as it consists of 18 paired autosomes and two sex chromosomes (X, Y) and is approximately 2.7 Gb in size (Fadista et al., 2008). Previous comparison of human and pig genomes has shown extensive conservation, validating the use of pigs in the study of human disease (Hart et al., 2007).

The other major restriction on sample availability is, of course, that teeth cannot be extracted from individuals for the sole purpose of research. They are generally extracted from the jaws for other reasons, e.g. dental disease or orthodontic reasons. Teeth extracted for reasons of dental disease usually come from older individuals whilst healthy teeth extracted for orthodontic reasons usually come from younger individuals. This can lead to sample bias. As has been shown in this research, there are many factors which impact on the DNA content of teeth and the ability to examine these factors in any depth requires samples that are homogeneous with regards to at least some of these factors, e.g. type of tooth and presence of dental disease. Healthy teeth were used in the main part of this study to rule out the effects of disease and dental treatment. However, extracted healthy teeth

tend to be either premolars removed for orthodontic reasons or third molars removed due to lack of space in the jaws. Third molars were chosen for this research because they were available in larger numbers and from individuals of a wider age range than were premolar teeth.

Some variables that could potentially affect the amount and composition of pulp, dentine and cementum, and hence DNA content in third molar teeth, were not included in the study design. These included whether or not the teeth had emerged into the mouth and whether or not the teeth were in occlusal function. Future studies incorporating these variables would be valuable. Also, comparisons of other tooth types, i.e. incisors, canines, premolars and other molars, would also be of value but it would be very difficult to source sufficient healthy examples of these teeth.

This research revealed that decontamination of teeth with sodium hypochlorite potentially has a negative impact on the amount of endogenous DNA in teeth, particularly in cementum. However it did not explore the potential for contamination of tooth tissues with, in particular, human DNA from other individuals or the effects of other methods of decontamination, such as exposure to UV light. No contaminating DNA was detected during STR analysis of samples in this study but all teeth were extracted from the jaws under sterile conditions and were treated in an ideal manner to reduce the risks of contamination. Research has shown that the most likely periods in which contamination of samples occurs is in the initial excavation and collection stage so if ideal sterile practices are followed then contamination can be avoided (Pilli et al., 2013). Unfortunately ideal collection conditions may not always be possible so in reality decontamination of samples may in many cases be a necessity. Contamination of samples with exogenous DNA becomes increasingly of concern as the amount and quality of endogenous DNA declines.

In highly degraded samples endogenous DNA is only present in minute quantities and is likely highly fragmented. Studies have shown that teeth are more resistant to contamination than bone most likely due to the impenetrable nature of enamel and the protection of the roots provided by the overlying bone (Pilli et al., 2013) however they are certainly not immune so further studies exploring the need for decontamination and the impacts of various methods of decontamination are required. Protocols for decontamination should reflect the samples' post-mortem history, the prevailing environmental conditions and the manner of collection, as these factors reflect the likelihood of contamination. Other factors to consider include whether the tooth is retained in the jawbone and whether it is intact or is cracked or fragmented.

Having determined that cementum is an important tissue for nuclear DNA analysis, it remains to be determined what the most appropriate extraction protocols might be that are specific to this tissue. Cementum is less mineralised than dentine or bone so the need for demineralisation steps is likely less than for these other tissues. This present study revealed successful results with as little as 20 mg of cementum powder but the ideal tissue volume to utilise was not examined. Furthermore, only one method of extraction with minor variations was used in this study whereas different methods or modifications to this extraction method may produce even better results.

This research primarily focused on STR analysis of nuclear DNA and while quantification of available mtDNA was performed on samples examined in the main study no mtDNA analysis was undertaken. In the decomposition study reported in Chapter 5 it was demonstrated that all the tissues examined contained mtDNA at all stages of decomposition even when no nuclear DNA was detected. In fact dentine was shown to be a much better source of mtDNA than of nuclear DNA. It would have been possible to

perform mtDNA analysis of these samples although traditional analysis of two hypervariable regions (HVI and HVII) in the control region would have provided limited discriminatory power in a forensic context. A review by Coble et al. of HVI/HVII distribution in a 2002 database of 1655 Caucasians revealed that the most common type (which matches the revised Cambridge Reference Sequence) occurred at a frequency of 7.1% (Coble et al., 2004). While sequencing of whole mitochondrial genomes can help to resolve these common types a less costly and time consuming alternative is to examine selected SNPs in the coding region. Concurrent examination of SNPs and the control region can greatly increase the discriminatory power of mtDNA.

Finally, whilst this research gives some insights into post-mortem degradation of teeth and the rates of DNA breakdown, quantification of DNA fragments of specific size was used to examine the rate of degradation of DNA in the dental tissues. This approach provides a rather restricted view of events because only one mitochondrial fragment and two nuclear fragments were examined. Therefore, the results generated do not provide a clear description of the rate of fragmentation. Next generation sequencing of DNA would allow a more realistic view of the effects of degradation as fragments of all lengths could be examined giving a clearer picture of the breakdown of DNA in these tissues.

7.4 Concluding remarks

The main driving force behind this research was to increase the likelihood of successful identification of human skeletal remains. This has been achieved, with new findings about the distribution of DNA in the tooth tissues and the effects of post-mortem decomposition on DNA yields. This information can now be used to predict outcomes and select the most likely samples and tissues to achieve the maximum chances of correct identification. This research has shown for the first time that the cementum of human teeth is a good source of

DNA and, in teeth without pulp tissue present it is likely to be the best source of nuclear DNA for STR analysis to aid in the identification of human remains. The knowledge gained through this research has already been applied in actual forensic casework and has enabled successful identification of human remains that would otherwise have remained without identity.

7.5 References

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Appendix: Achievements

Grants applied for:

- American Society for Forensic Odontology (ASFO) 2009 – these grants are announced annually and in limited number. I was awarded the maximum amount for my application.
- ARC Linkage Grant – For this grant I approached the Australian Federal Police (AFP), South Australian Police (SAPOL) and Forensic Science South Australia (FSSA). FSSA and SAPOL agreed to be Industry partners and the AFP pledged support in the way of materials and technical advise. Unfortunately this grant was not awarded but FSSA and the AFP continued to support my project.
- Australian Dental Research Foundation (ADRF) 2010. My application for this grant was ranked as the top application nationally making me eligible to apply for the ADIA grant. I was awarded the full amount requested in my application for this grant.
- Australian Dental Industry Association (ADIA) 2010. I was awarded the full amount requested for this grant.

Collaborations established:

- Forensic Science South Australia (FSSA). FSSA has supported my research by provision of materials and technical assistance throughout my PhD and in return I have attended FSSA to assist with casework involving DNA extraction not only from teeth but also from other difficult tissues.
- Australian Federal Police (AFP). The AFP have also provided me with materials and technical support throughout my thesis.

Presentations:

- 2010 ***"Forensic Odontology Research"*** University of Tennessee, Tennessee, USA. Oral presentation.
- 2011 ***"Extraction of nuclear DNA of reliable forensic evidentiary value from human teeth."*** International Society for Forensic Genetics (ISFG) Congress. Vienna, Austria. Poster presentation.

- 2011 ***"Improving the evidentiary value of nuclear DNA from decomposed teeth for use in human identification."*** Australian Dental Industry Association (ADIA) SA Branch meeting. Adelaide, Australia. Oral presentation.
- 2011 ***"Dental tissues as a source of nuclear DNA"*** Australian Federal Police (AFP) Workshop. Canberra, Australia. Oral presentation.
- 2010***"Teeth as a source of DNA for Human Identification"*** National Institute of Forensic Science (NIFS) Biology Scientific Advisory Group (SAG) Meeting, Adelaide, Australia. Oral presentation.
- 2011 ***"Nuclear DNA from teeth for use in human identification"*** Australian Society of Forensic Odontology (AuSFO) International Symposium. Darwin Australia. Oral presentation.
- 2011 ***"Sampling human teeth for nuclear DNA"*** Forensic Science South Australia Biology Group Meeting. Adelaide Australia
- 2012 ***"DNA from Teeth"*** Australian Federal Police (AFP) Workshop. Canberra, Australia. Oral presentation.
- 2012 ***"The Truth in the Tooth"*** Australian and New Zealand Forensic Science Society (ANZFSS) 21st International Symposium on the Forensic Sciences. Hobart, Australia. Poster presentation.
- 2013 ***"Sub-sampling of human teeth for nuclear DNA analysis"*** International Society for Forensic Genetics (ISFG) Congress. Melbourne Australia. Poster presentation.