



## Survival and recovery of DNA from ancient teeth and bones

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### ABSTRACT

The recovery of genetic material from preserved hard skeletal remains is an essential part of ancient DNA, archaeological and forensic research. However, there is little understanding about the relative concentrations of DNA within different tissues, the impact of sampling methods on extracted DNA, or the role of environmentally-determined degradation rates on DNA survival in specimens. We examine these issues by characterizing the mitochondrial DNA (mtDNA) content of different hard and soft tissues in 42 ancient human and bovid specimens at a range of fragment lengths (77–235 bp) using real-time PCR. Remarkably, the standard drill speeds used to sample skeletal material (c. 1000 RPM) were found to decrease mtDNA yields up to 30 times (by  $3.1 \times 10^3$  mtDNA copies on average) compared to pulverization in a bone mill. This dramatic negative impact appears to relate to heat damage, and disappeared at very low drill speeds (e.g. 100 RPM). Consequently, many ancient DNA and forensic studies may have obtained false negative results, especially from important specimens which are commonly sampled with drills to minimize signs of damage. The mtDNA content of tooth cementum was found to be five times higher than the commonly used dentine (141 bp,  $p = 0.01$ ), making the cementum-rich root tip the best sample for ancient human material. Lastly, mtDNA was found to display a consistent pattern of exponential fragmentation across many depositional environments, with different rates for geographic areas and tissue types, improving the ability to predict and understand DNA survival in preserved specimens.

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### 1. Introduction

The recovery and analysis of DNA from human skeletal tissues, teeth and bone, has become a central research tool for scientific fields ranging from ancient DNA to forensics and medical science. Due to the unique value of ancient skeletons for physical anthropology and archaeology (Hanihara, 2008; Hillson, 1979), isotopic studies (Bentley, 2006) and exhibition purposes it is critical that the smallest possible amount of destructive sampling is involved in the recovery of genetic material. Whole teeth or tooth roots are commonly used as samples for DNA studies as the tough enamel and cementum provides improved protection for DNA in many environments, and appears less prone to contamination from modern DNA under certain circumstances (Haak et al., 2008, 2005; Kurosaki et al., 1993; Melchior et al., 2008; Meyer et al., 2000; Rudbeck et al., 2005; Sampietro et al., 2006). Therefore, it is surprising that little is known about the relative amounts of DNA

within the different tissues or regions of teeth, or the potential impact of sampling methods on the resulting quality and quantity of DNA extracted from preserved teeth or bone. Similarly, the interplay between age and depositional environment in the degradation of preserved DNA also remains unclear, due to the lack of detailed empirical studies of the degradation process itself. Knowledge of the degradation process is important not just as a guide for identifying specimens likely to contain DNA but also because an expected negative correlation between DNA template fragment length and copy number is used to support the authenticity of ancient DNA results (Malmström et al., 2007; Pääbo, 1989; Poinar et al., 2006).

To examine these issues and improve the recovery and reliability of endogenous genetic information from ancient and preserved specimens we examined the mitochondrial DNA (mtDNA) content of tooth, bone and hair samples from 40 human specimens covering a range of ages and preservation states (Table S1). We examined the relative quantity and quality (distribution of template fragment lengths) of mtDNA within different tissues, and the impacts of methods used to prepare skeletal samples for DNA extraction. While the analysis of ancient human material brings a much-heightened risk of contamination with

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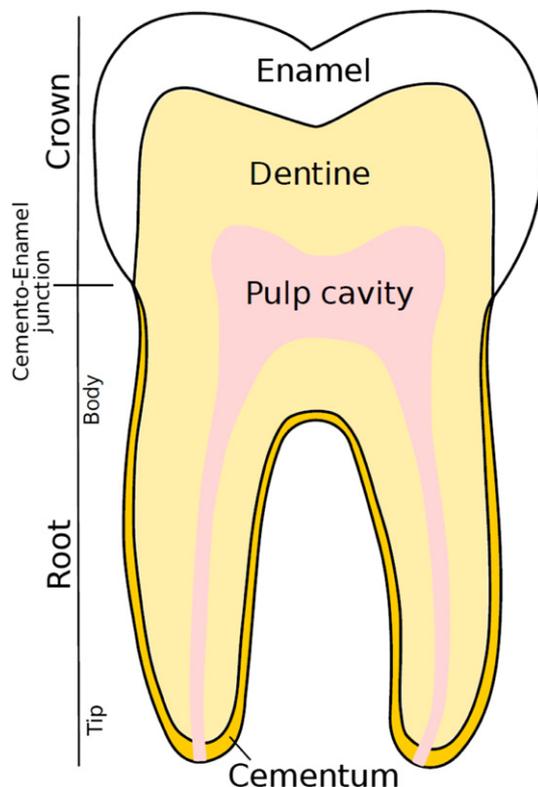
modern human DNA (e.g. Malmström et al., 2005), there is a pressing need within archaeology and forensic sciences to measure these parameters directly, and not with animal models. To minimize the risk of contamination with modern human DNA we focused on skeletal material that has been excavated under carefully controlled circumstances in collaboration with archaeologists. Different samples of the specimens have previously been typed using a highly-sensitive, single-base extension (SBE) multiplex PCR for 22 mtDNA coding region SNPs (Haak et al., 2010) and directly sequenced for the mitochondrial hypervariable segment I (HVS I). To determine whether similar results are obtained from non-human skeletons, we also examined the mtDNA preservation in tooth samples from two permafrost-preserved bison.

The reduced porosity of teeth compared to bone has led to suggestions that the risk of contamination with modern human or environmental DNA is lower in teeth (Rudbeck et al., 2005). The majority of ancient and forensic research using human teeth has focused on different ways to access the internal tooth tissue dentine (Alakoc and Aka, 2009; Cobb, 2002; Gilbert et al., 2003; Merriwether et al., 1994; Shiroma et al., 2004), and has not considered the potential variation in mtDNA content between the different tissues and regions of the tooth (Fig. 1). Dentine lines the pulp cavity and is rich in highly mineralized odontoblastic cells, which consist of a cell body located in the pulp cavity from which long tubules or odontoblastic processes extend into the mineralized dentine (Berkovitz et al., 1992; Carda and Peydro, 2006). Dentine has been a preferred target as it is protected beneath a covering of enamel and cementum, a specialized calcified tissue with high cell density covering the tooth root. It is also possible to sample dentine with minimal alteration to the sample by either drilling into the pulp cavity via the root tip (Cobb, 2002), or by first removing the crown (Drancourt et al., 1998; Merriwether et al., 1994; Rudbeck et al., 2005; Shiroma et al., 2004) which can be subsequently

reattached with minimal sign of alteration. In contrast, cementum has largely been ignored as a source of DNA although it also contains mineralized cells, cementoblasts and cementocytes (Bosshardt and Schroeder, 1991), located in small channels near the dentine boundary (De Leo et al., 2000). Additionally, DNA content may not only vary between tissues, but also with the region of the tooth sampled. For example, within dentine there are 3–4-fold more odontoblastic tubules in the body of the root (65,000–45,000/mm<sup>2</sup>) compared to the apical tip (15,000–20,000/mm<sup>2</sup>) (Franquin et al., 1998), while cementum displays the opposite pattern of cell density (Schroeder, 1986). In contrast, the structure of bison molars is quite different; dentine and cementum are arranged in a parallel columnar structure (Budras et al., 2003), which in this case has the added benefit of providing a simple means of obtaining relatively pure sources of cementum and dentine.

Skeletal samples are generally powdered and decalcified to allow efficient proteolytic digestion of cellular components, irrespective of the method subsequently used to isolate the DNA. However, the physical methods used to powder skeletal remains, has the potential to influence the quantity and quality of recoverable DNA, although this has not been properly investigated. The two main powdering techniques involve directly drilling the sample with a dental burr or drill bit (Alakoc and Aka, 2009; Cobb, 2002; Gilbert et al., 2003; Merriwether et al., 1994; Shiroma et al., 2004), or pulverization with a shaking bead in a bone mill (Meyer et al., 2000; Ramos et al., 1995; Sampietro et al., 2006). Drilling is often preferred where there is a requirement for the specimen's morphological integrity to be maintained (e.g. for physical anthropological research, exhibition purposes (O'Rourke et al., 2000), or forensic identification (Brkic et al., 2000)). Indeed, there is an increasing requirement by museums for key specimens to be sampled via drilling only, despite it being difficult to perform this process in a clean manner outside of an ancient DNA laboratory. In comparison, pulverization in a bone mill is commonly used when a complete or partial tooth or bone sample is available for destructive analysis within an ancient DNA laboratory (Haak et al., 2005; Kolman and Tuross, 2000; Sampietro et al., 2006).

While it is well known that increased temperature rapidly degrades and denatures DNA (Lindahl, 1993), the amount of heat generated during physical powdering methods, especially drilling, has gained little attention. This is potentially a serious issue for bone and teeth, where the crystalline structure is a poor conductor of heat, resulting in rapidly increased temperature from friction or other energy input. It is well known in dental and surgical literature that drill speed is an important determinate of heat generation and the severity of associated harmful physiological effects on living or viable tooth and bone tissue, resulting in necrosis of teeth (Langeland and Langeland, 1968; Schuchard and Watkins, 1965) and bone (Abouzgia and Symington, 1996; Eriksson et al., 1984; Lundskog, 1972; Matthews and Hirsch, 1972). Despite this, drill speed does not seem to have been considered when sampling non-modern teeth and bones, and this parameter is almost never reported in ancient (e.g. Gilbert et al., 2003; Melchior et al., 2008; Yang et al., 1998) or forensic (e.g. Alakoc and Aka, 2009; Malaver and Yunis, 2003) human genetic studies. Our empirical observations suggest that drilling can produce very large heat loadings at the cutting surface, in comparison to shaking-bead pulverizing methods where energy is distributed across a wide area of the sample in momentary bursts, and the large mass of cylinder and mill provide a significant heat sink. It is currently unknown how these common sample preparation methods are influencing the quantity and quality of DNA recovered from skeletal samples. The few previous studies of these issues have been inconclusive, and were both semi-quantitative and reported few details about the drilling process (Cobb, 2002; Smith et al., 1993).



**Fig. 1.** Diagrammatic representation of a human molar, showing the cellular tooth tissues (dentine and cementum) and tooth regions (root body and tip).

Overall, the amount and integrity of recovered DNA is a crucial limiting factor in ancient and forensic research (Handt et al., 1996; Krings et al., 1997). Due to the lack of DNA repair mechanisms after death, DNA is rapidly degraded into short fragments through hydrolysis, oxidative nucleotide modification, the action of cellular nucleases and other hydrolytic enzymes, and bacterial attack (Hoss et al., 1996; Lindahl, 1993; Pääbo, 1989). As a result, the differences in DNA degradation between samples are commonly attributed to environmental factors (Alvarez Garcia et al., 1996; Collins et al., 2002; Dobberstein et al., 2008), especially temperature (Smith et al., 2003) in addition to the time since death. The level of DNA degradation is mainly attributed to post-mortem environmental conditions rather than time since death, since temperature is thought to relate to DNA degradation rates (Smith et al., 2003) and because well-preserved ancient specimens may retain endogenous DNA despite being very old (Shapiro and Cooper, 2003).

The rapid degradation of DNA templates in ancient specimens (Pääbo, 1989) means that any estimates of DNA quantity are directly related to the length of the fragment targeted (Handt et al., 1996). This relationship has been described as an inverse or negative correlation between PCR amplification success and fragment size, and recommended as a test of authenticity for ancient DNA (Cooper and Poinar, 2000; Malmström et al., 2007). Recent methodological advances such as single primer extension (SPEX) and genomic approaches have confirmed the relative abundance of short template fragments in ancient DNA extracts (Brotherton et al., 2007; Poinar et al., 2006). In depth characterisation of DNA quantity in ancient extracts for multiple fragment sizes has been performed using Real Time (q) PCR, primarily on samples from a single post-mortem environment such as Poinar et al. (2006) study, which used six different sized fragments. However, there has been no direct evidence of the degradation process from a range of samples with different post-mortem conditions and tissue types. Additionally, the studies addressing DNA degradation have commonly used at most two different fragment lengths to assess the preservation state of endogenous templates (Colotte et al., 2009; Malmström et al., 2007).

To generate a detailed pattern of DNA template fragmentation, we set out to describe the variation in quantity of ancient mtDNA for a diverse group of samples over a range of fragment lengths. By contrasting tissue types and depositional environments, we aim to reveal general patterns in the underlying fragmentation process that will inform future methodological development in forensics, ancient DNA and archaeological research.

## 2. Materials and methods

All ancient DNA work was conducted at the Australian Centre for Ancient DNA in a dedicated, physically-isolated laboratory some 15 min walk from the laboratory used for post-PCR analyses. Strict protocols were followed to minimize the amount of human DNA in the ancient DNA laboratory, including the wearing of freshly laundered clothes, a full body suit, shoe covers, boots, facemask, face shield and triple gloving, and no personnel movement from the post-PCR environment to the ancient DNA laboratory within a single day. All surfaces in the lab are routinely triple-wiped with bleach, decon and isopropanol. The laboratory is irradiated with ultraviolet (UV) light (30–50 nm) overnight for ca. 4 h. All consumables, disposables, tools and instruments are externally bleached and UV irradiated before entering the lab and then subjected to routine cleaning before, during and after use.

### 2.1. Sample preparation

Descriptions of the 42 bone, tooth and hair samples are presented in Table S1. Rigorous sample decontamination procedures

were followed, with the exterior surface exposed to high-intensity UV irradiation for 20 min, wiped briefly with a bleach (2% (w/v) sodium hypochlorite)-soaked Kimwipe, followed by mechanical removal. The outer tooth surface (ca. 0.5 mm) was removed by a dental burr (1000 RPM). The surface layer of bone samples was removed using a Dremel carborundum cutting disc. Hair samples were immersed in commercial bleach (2% (w/v) sodium hypochlorite) for 5 min and rinsed with 100% ethanol and subsequently dried.

The crown was removed by horizontally sectioning the tooth at the cemento-enamel junction using a diamond-cutting disc (35,000 RPM) to allow access to the internal portions of the tooth.

#### 2.1.1. DNA yields from different tissues and areas within human teeth

The investigation of mtDNA variation between different tissues and regions of the teeth was restricted to the German Neolithic samples (Table S1 and S2) to minimize variation in mtDNA content between samples caused by the post-mortem environment or time since deposition. In this way, the difference in mtDNA quantity between dentine and cementum, and the root body and tip, was investigated using 11 molars and 10 single-rooted teeth (incisors, canines, and premolars). Cementum and dentine were isolated using a dental burr (1000 RPM). To isolate cementum from the external root surface and distinguish it from the inner dentine we used both its macroscopic properties, yellow colour compared to the white dentine, and histology, its bone like, striated appearance compared to the homogenous form of dentine, as visible under a light microscope (Nikon, SMZ-745T, e.g. Lieberman and Meadow, 1992). Dentine was removed by powdering the internal surface of the pulp cavity and in order to avoid mixing the tooth tissues, the dentine was not drilled as far as the cementum border. Single-rooted teeth were chosen for the analysis of the relative amount of mtDNA in the root due to the ease of sectioning compared to multi-rooted teeth (the roots of which are often angular and entwined). The root length was measured with a ruler, and a scalpel was used to mark the halfway point (the border between root body and tip) which was horizontally sectioned using a diamond-cutting disc (35,000 RPM). The root body and tip were then pulverized separately using a bone mill (Sartorius Microdismembrator) at 3300 RPM for 30 s. The bone mill powdering conditions were kept constant for all samples in this study.

Bison teeth were chosen to contrast the relative amounts of mtDNA within tooth tissues as they contain the same hard tooth tissues as humans. Additionally the radiodensity properties of human dentine have been found to be similar to those of bovines (Tanaka et al., 2008), and more so than suid teeth (Fonseca et al., 2004). The dentine and cementum in bison molars are arranged in a columnar structure (Budras et al., 2003). The dentine, attached to the inner and outer enamel surfaces, was separated using a diamond-cutting disc (35,000 RPM) in a vertical sectioning manner (De Leo et al., 2000). The cementum, located in the center of the tooth, is not attached to the surrounding inner layer of enamel. The dentine and cementum samples were subsequently powdered using a bone mill.

#### 2.1.2. The impact of powdering hard tissues on DNA yields

The impact of powdering human skeletal hard tissues was measured on the German Neolithic samples (Table S1 and S2), again to reduce extraneous variables. The impact of standard drill speed (1000 RPM) and pulverization on the retrieval of mtDNA from teeth was assessed for individuals that had two teeth available, one for each powdering method so that enough material was available for the DNA extraction. The impact of drilling and pulverizing on the recovery of mtDNA was assessed by comparing

the average yield from the dentine and cementum studies (which were drilled at 1000 RPM), and the results from the root body versus tip analyses (where pulverization was used) (Table S2). A number of individuals used in the dentine and cementum analyses had not been included in the root body/tip study, so to generate pulverization data from these individuals an entire second tooth root was pulverized (Table S2). It was not possible to investigate the impact of low drill speeds in teeth as a third tooth sample was generally not available per individual. The relative impacts of pulverizing and drilling on bison teeth were also investigated by contrasting the results from the bison dentine and cementum pulverization analyses (above) with separate drilling experiments (at standard speed – 1000 RPM) performed on both tissues.

Bone samples were also used to investigate the impact of pulverization, and high- and low-speed drilling approaches to powdering. Due to the larger size of the bone samples it was possible to perform the analyses on a single bone for each individual ( $n = 6$ ). The bone samples were of varied anatomy, but were predominantly shafts of long bone although a single skull bone (petrous temporal, *pars petrosa ossis temporalis*) was also used (Table S1). Dense cortical bone was examined in each case to minimize between-sample variation. However, variation between bones of varied anatomy may have had an impact on DNA quantification results and it would have been preferable to perform our analyses on a single bone type. Bone samples were cut into small pieces ( $\sim 0.5 \times 0.5$  cm) using a diamond-cutting disc (35,000 RPM) prior to being pulverized in a bone mill. The bone samples were drilled at 1000 and 100 RPM with a dental burr, with each drilling site separated by a minimum distance of 0.5 cm to avoid any potential overlap of temperature effects even allowing for the poor heat conduction of bone (Lundskog, 1972).

## 2.2. DNA extraction

Between 0.1 and 0.2 g of bone or tooth powder was used for DNA extraction experiments, and the mtDNA results were standardized for a 0.1 g starting amount. An extraction blank was included every 3–4 extractions. DNA was extracted using a phenol/chloroform/isoamyl alcohol, pH 8.0 (25:24:1) method after Haak et al., 2008. Hair samples (0.01–0.05 g) were lysed overnight by adding 3 ml of lysis buffer, consisting of Tris, NaCl, Proteinase K, DTT and SDS, and incubating for 24 h at 37 °C under constant rotation (Gilbert et al., 2004). After incubation, DNA was isolated with two extractions of equivolume phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) and one extraction with equivolume chloroform. The DNA was desalted and concentrated using Amicon Ultra-4 filter units (50 kDa, Millipore) with 7.5 ml sterile, filtered water. Final extraction volume was 60–100  $\mu$ l, and extracts were stored at 4 °C before analysis.

## 2.3. qPCR and PCR

Real time PCR (qPCR) was used to determine the amount of DNA in the samples prior to amplification, using a maximum of four fragment lengths, ranging in size from 77 bp to 235 bp. For the bison samples, one HVSI mtDNA fragment (177 bp) was quantified. The primers and standards used are presented in Table S3 and described in the supplementary materials and methods text (S4). All qPCRs were carried out in a total volume of 10  $\mu$ l containing Express SYBR<sup>®</sup> Green ER Supermix Universal (Invitrogen), rabbit serum albumin (0.4 mg/ml), forward and reverse primers (1  $\mu$ M), distilled water and 1  $\mu$ l of DNA extract, with the following reaction conditions: 95 °C/5 min and 50 cycles of 95 °C/10 s, 58 °C/20 s and 72 °C/15 s. Amplifications were performed on the Rotor-Gene 6000 and analysed with the Rotor-Gene 6000 Series Software 1.7.

The haplogroup of the ancient human individuals was determined by amplification of the complete mtDNA control region (Haak et al., 2008; Haak et al., 2005) and parts of the coding region using a highly-sensitive, single-base extension (SBE) multiplex PCR for 22 mtDNA coding region SNPs (Haak et al., 2010).

## 2.4. Sequencing

All successful qPCR and PCR products (i.e. those which are visible on a ethidium bromide stained, agarose (3.5%) gel) were purified using 5  $\mu$ l of PCR product, exonuclease I (0.8 Units/ $\mu$ l) and shrimp alkaline phosphatase (1.0 Units/ $\mu$ l) at 37 °C for 40 min, followed by heat inactivation at 80 °C for 10 min. The purified amplicons were directly sequenced using the BigDye Terminator 3.1 Kit (Applied Biosystems) as per the manufacturer's instructions. Sequencing products were purified using a Multiscreen<sub>HTS</sub> Vacuum Manifold (Millipore), according to the manufacturer's protocol. Sequencing products were separated on the 3130xl Genetic Analyzer (Applied Biosystems) and the resulting sequences were edited and aligned to the revised Cambridge Reference Sequence (GenBank Accession number AC\_000021.2) using the software Sequencher (version 4.7).

## 2.5. Authentication criteria

Due to the high risk of contamination when dealing with ancient human material, a number of precautions were taken to prevent modern contamination. (i) The majority of samples were collected under DNA free conditions after excavation, which included no washing, treating or examining before taking samples. (ii) All preparation and analytical steps prior to DNA amplification were conducted in a clean room area solely dedicated to ancient DNA work located in a physically separated building without any modern DNA work (pre-PCR area). Amplification, cloning and sequencing were carried out in the post-PCR lab. (iii) All steps were monitored by non-template controls and by using bovid samples in parallel. (iv) All individuals were sampled twice from anatomically independent regions and treated independently (extractions performed on separate days). At least eight independent PCR reactions were carried out (four overlapping fragments times two extractions) per individual. A number of samples (those beginning with DEB) were extracted in an independent ancient DNA laboratory to the Australian Centre for Ancient DNA, at the Johannes Gutenberg University of Mainz. These samples were cloned (in accordance with Haak et al., 2010) and an average of eight clones per amplicons was sequenced to detect heterogeneous sequences due to DNA degradation or contamination. All replicable polymorphic sites in combination were consistent with known mtDNA haplogroups, ruling out the post-mortem damage as a potential source for erroneous sequences. (v) The SNP multiplexes (Haak et al., 2010) both confirm haplogroup assignment and provide an ideal monitoring system for ancient human DNA samples, as they directly target SNPs defining all potential contaminating lineages. (vi) Quantitative real-time PCR was carried out on samples to ensure appropriate levels of DNA quantity and to assess DNA quality.

## 2.6. Statistical analysis

A Shapiro–Wilk W test was used to assess the normality of the mtDNA quantity for the tooth tissues and region, powdering technique and fragment lengths. All data were non-normally distributed ( $p < 0.05$ ) and due to this, and the small sample sizes, non-parametric statistics were used for analysis. The differences in DNA quantity between tooth tissues and regions, powdering methods and fragment lengths were assessed using a Wilcoxon Signed-Ranks test.

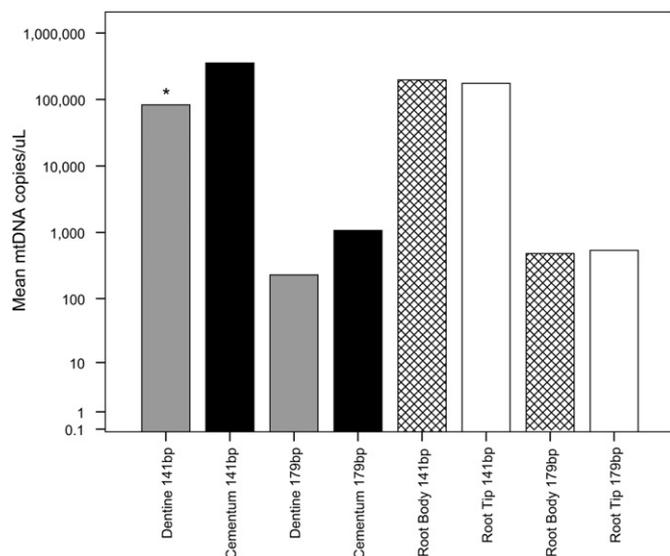
An in-depth description of the statistical analyses used to empirically describe the behaviour of ancient human mtDNA fragmentation, variation in behaviour due to preservation state and the rate of DNA damage ( $\lambda$ ), as estimated from the method described by Deagle et al., 2006, can be found in the [supplementary methods and materials section \(S4\)](#). All statistical analyses were performed using SPSS version 17.0 for Windows.

### 3. Results and discussion

MtDNA was extracted from 40 human remains and two ancient bison teeth (Table S1), using rigorous ancient DNA procedures and decontamination measures (Section 2.1). The number of amplifiable molecules at four different fragment lengths (77, 141, 179 and 235 bp) was quantified using real-time PCR (Table S3) and showed a marked, and predictable, decrease in the number of molecules at longer fragment lengths, demonstrating extensive fragmentation consistent with ancient DNA (Table S2). A minimum of eight identical sequences per individual were obtained from independent sample extractions (Table S2). Sequence damage consistent with known patterns (e.g. Briggs et al., 2007; Brotherton et al., 2007; Pääbo, 1989) was detected very occasionally, and could easily be distinguished via the repeated re-amplification and sequencing of fragments.

#### 3.1. DNA yield from different tissues and areas within human teeth

The recovery of mtDNA was four to five times higher by weight for cementum compared to dentine in ancient human molars (Fig. 2), a statistically significant difference for the 141 bp fragment ( $p = 0.01$ , Table S5). This finding is consistent with histological evidence that cementum has a high cell density, and a similar level of mineralization to bone (Bosshardt and Schroeder, 1991; De Leo et al., 2000). Despite this, cementum is seldom targeted in ancient DNA or archaeological studies although potentially offering a significantly better DNA source. There would also appear to be an increased probability of obtaining nuclear DNA from cementum over dentine in degraded human teeth. Cementum contains nucleated cells within its mineralized tissue whereas in dentine the odontoblast cell



**Fig. 2.** The amount of mtDNA within the different tissues ( $n = 11$ ) and regions ( $n = 10$ ) of ancient human teeth. Due to the extremely large differences in mtDNA quantity between the 141 and 179 bp fragments, results are displayed on a log-linear scale. Cementum contained significantly more mtDNA than dentine (141 bp,  $p = 0.010^*$ ; 179 bp,  $p = 0.477$ ). The root body and root tip were not significantly different (141 bp,  $p = 0.6$ ; 179 bp,  $p = 0.2$ ).

body (with nuclear DNA) is located in the dental pulp, which decays far more rapidly than the mineralized tubules. Therefore, in degraded teeth with no pulp, all that remains in dentine is the mtDNA-rich odontoblastic tubules (Mornstad et al., 1999). Unfortunately, we were not able to test this hypothesis in the current work because we were unable to retrieve nuclear DNA from the samples we tested, presumably due to the extent of DNA degradation in the German Neolithic samples. Although a previous study of modern human teeth has reported an equivalent quantity of mtDNA in cementum and dentine (Malaver and Yunis, 2003), the yield was only approximately quantified following PCR amplification.

Interestingly, bison teeth yielded approximately equal quantities of mtDNA in cementum and dentine (Table S4, Fig. S6). However bovine dentine has been found to contain a greater density of odontoblastic tubules compared to humans (Schilke et al., 2000), which may account for this difference.

Although cementum appears to be the best tooth tissue from which to extract DNA, the position on the surface of the tooth root leads to a potentially increased risk of exposure to contamination. However, this situation is not unique to cementum; dentine is also exposed to the exterior environment through the open structure of the pulp cavity via the apical root tip, and via any cracks or cavities (Gilbert et al., 2005). Cementum can be treated with decontamination methods similarly to bone, including UV irradiation (Lindahl, 1993), mechanical removal of the superficial surface layer and bleach treatment (Malmström et al., 2007). Lastly, roots from *in situ* teeth also benefit from the environmental protection provided by the alveolar sockets.

The similar DNA yields obtained from the root tip and root body (Fig. 2, Table S5) were surprising, given that although both areas contain dentine and cementum, the cementum is both thickest and has the highest cell density at the root tip (Schroeder, 1986). This similarity in results is thought to relate to limitations in the sectioning process where it was necessary to include portions of the lower part of the root body in the root tip sample to gain enough powder for DNA extraction. Previous, semi-quantitative studies of modern human tooth roots have been equivocal about whether the DNA yield is higher from root tip or root body (Franquin et al., 1998; Gaytmenn and Sweet, 2003). Overall, our results suggest that the root tip should be preferred to dentine for genetic analyses as it contains large amounts of cementum, which is expected to significantly increase the chance of obtaining mtDNA (Table 1). Furthermore, the root tip has little morphological research value, and for *in situ* teeth in the maxilla or mandible the sample site is invisible and potentially has less contamination risk. For isolated tooth specimens on display, a cast can be made from an impression taken prior to removal of the tip, although this process may increase contamination risk.

#### 3.2. The impact of powdering hard tissues on DNA yield

We observed a remarkable decrease in the quality and quantity of recovered mtDNA when bone and tooth samples were drilled at

**Table 1**

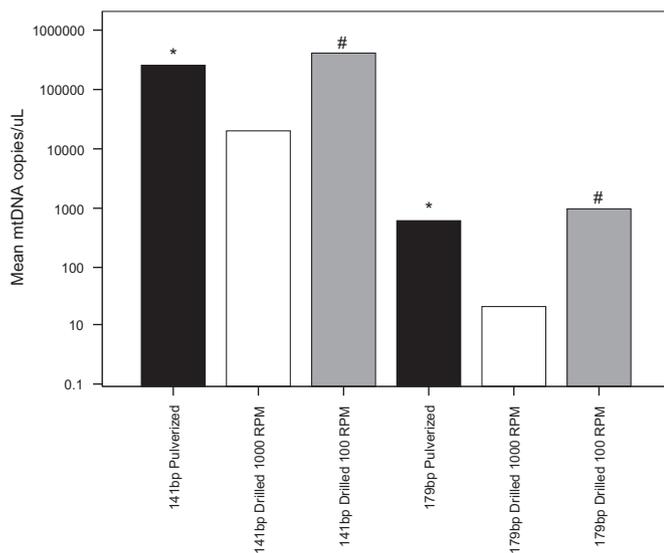
Fragmentation of ancient human mtDNA described using linear, inverse, S-function and exponential models. The models were used to investigate the relationship between DNA quantity and fragment length in degraded samples. The models were fitted to the whole data set and subsets defined by preservation state.

Model	$R^2$ (%)			
	Linear	Inverse	S-function	Exponential
All samples ( $n = 37$ )	18.9	21.3	55.4	63.5
Well preserved ( $n = 20$ )	33.5	36.4	56.7	62.3
Poorly preserved ( $n = 17$ )	10.2	14.8	72.2	78.6

standard speeds (e.g. 1000 RPM) compared to pulverization. This finding is consistent with the observation that heat build up at the tip of the drill bit can be extreme, compared to that generated in a bone mill/shaking-bead system. MtDNA yields from pulverized samples were 5–30-fold higher than drilled samples (Table S7), and significantly more mtDNA copies were found at multiple fragment lengths for both bone (Fig. 3; 141 bp,  $p = 0.028$ , 179 bp,  $p = 0.046$ ) and teeth (Fig. S6; 179 bp  $p = 0.05$ ). The same pattern was found in bison teeth, where drilling reduced the yield of the 177 bp fragments 22-fold (Fig. S6, Table S7). The decrease in yields from drilled samples was particularly apparent at longer fragment lengths, suggesting that heat had a more potent effect on intact, longer molecules.

To investigate the effect of the drill speed on DNA degradation we repeated the experiments using a very slow drill speed of just 100 RPM, via step-down gearing available with specialized dental equipment. While this meant that sampling a bone could take up to 30 min, heat buildup was negligible and mtDNA yields were significantly improved, and similar to the pulverizing method (Fig. 3, Table S6).

The discovery that drill speed is a key determining factor in the ability to extract and amplify mtDNA has major implications for the ancient DNA and archaeological fields, where the most valuable specimens are generally sampled by drilling to minimize damage to the skeleton. Examples include *Homo floresiensis* from which two teams (including the authors) have sampled dentine (not cementum) with drills. Currently, drill speeds are considered largely insignificant and while some protocols recommend the use of 'low-speeds', there are no specifications (Rohland and Hofreiter, 2007). Although the use of high drill speeds on modern bone and tooth tissues have been associated with increased temperature and physiological damage (Eriksson et al., 1984; Langeland and Langeland, 1968; Lundskog, 1972; Schuchard and Watkins, 1965), the situation is somewhat confusing because low drill speeds have also been implicated in temperature rises (Hillery and Shuaib, 1999). The increase in temperature with low drill speeds has been

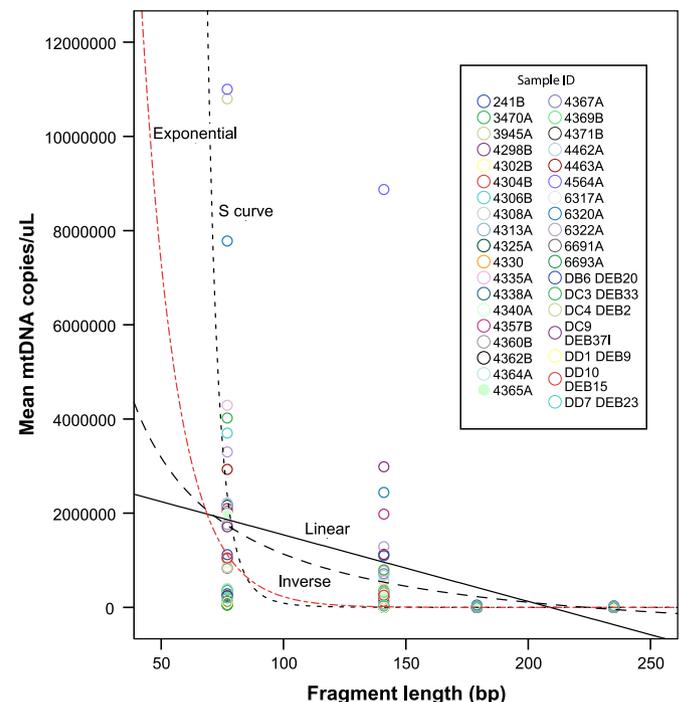


**Fig. 3.** The impact of pulverization and drilling at high- (1000 RPM) and low-speeds (100 RPM) on the recovery of mtDNA from bone. The mean amount of mtDNA is displayed on a log-linear scale. Pulverized bone yielded significantly higher amounts of mtDNA compared to powder produced from drilling at standard speed, 1000 RPM (141 bp,  $p = 0.028^*$ ; 179 bp,  $p = 0.046^*$ ). Drilling at very low speed, 100 RPM, significantly increased mtDNA recovery compared to drilling at 1000 RPM (141 bp,  $p = 0.028^*$ ; 179 bp,  $p = 0.043^*$ ). There were non-significant differences between pulverizing and drilling at low speed (141 bp,  $p = 0.463$ ; 179 bp,  $p = 0.753$ ).

attributed to the considerably greater force (60–120 N) associated with the increased torque of lower speed surgical drills (Karmani, 2006) compared to the modern dental drills (6–24 N) used in the current study. Thus it is likely that both drill speed and pressure need to be carefully monitored to avoid excess DNA damage. Other key measures to reduce the generation of heat include the use of sharp drill bits or burrs that are in good condition (Matthews and Hirsch, 1972). The use of water coolants (Kondo et al., 2000) would appear inadvisable due to the high risk of introducing contaminants.

### 3.3. The degradation of ancient human mtDNA

There is a striking consistency in the fragmentation pattern of human mtDNA across a broad variety of depositional environments, tissue types and time frames (Fig. 4). There is a significant decrease ( $p < 0.01$ ) in quantity of mtDNA templates with each increase of ~50 bp length across the 4 fragment lengths surveyed (Table S8). This pattern, where the vast bulk of ancient DNA is of very short length (e.g. ~80 bp) is in agreement with the results produced using SPEX (Brotherton et al., 2007) and many high throughput studies (e.g. Briggs et al., 2007; Poinar et al., 2006). This highlights that analyses requiring very small fragment sizes are most likely to have sufficient amounts of DNA for reliable analyses. A negative or inverse correlation between fragment length and quantity has been suggested as one of the criteria for authentic ancient DNA results (Cooper and Poinar, 2000; Malmström et al., 2007), and while the data support this general trend, the increased resolution allows a more precise description of the relationship. A variety of models were fitted to the data, and an exponential curve was strongly favored for all specimens, including different tissue types, regions and ages (Table 1, Fig. 4). The exponential model accounts for 63.5% of variation in the data compared to the inverse model, which explains 21.3%. The S-shaped function fitted the data less well than an exponential model, (55.4%), and



**Fig. 4.** Empirically described fragmentation of ancient human mtDNA. A circle denotes an individual ( $n = 37$ ) for which mtDNA was quantified over four fragment lengths (77, 141, 179 and 235 bp). Data were fitted to four models and the amount of variation described by each model for the whole data set is as follows; linear 18.9%; inverse 21.3%; S-function 55.4% and exponential 63.5%.

was investigated to determine whether a step-wise pattern, or threshold length, was important in degradation (for example, greater than 141 bp in Fig. 4). A step-wise pattern has been suggested for the degradation of nuclear DNA, due to histone binding in the nucleosome protecting fragments less than 147 bp in length (Binladen et al., 2006). However, the apparent drop off in quantity above 141 bp appears to be a product of the exponential curve and initial starting quantities. A more accurate description of DNA fragmentation will soon be available using data from a variety of second generation sequencing (shotgun approach) studies of degraded samples, which would provide a highly resolved distribution of fragment lengths.

The observation that template copy number decreases exponentially with increased fragment length across the breadth of samples suggests a common mechanism of fragmentation, with the rate (represented by the slope of the curve in Fig. 4) presumably controlled by depositional environmental conditions and tissue type (Burger et al., 1999; Hoss et al., 1996; Poinar et al., 1996). The steepness of the exponential curve varied considerably with the quality of the genetic material, and the fragment lengths investigated. We examined this issue using a re-scaled (0–1) Euclidean dissimilarity matrix (Table S9) which revealed that well-preserved samples (high amounts of mtDNA as described in S4,  $n = 20$ ) showed the greatest difference in mtDNA copy number between the two longest fragments examined (179 vs. 235 bp = 1.0). In contrast, poorly-preserved specimens (low amounts of mtDNA as detailed in S4,  $n = 17$ ) had few molecules of this length and hence negligible dissimilarity, and instead showed the greatest difference between the shortest fragments (77 vs. 141 bp = 1.0). This pattern illustrates a key problem with quantization approaches often used in ancient DNA and forensics, namely that the number of surviving DNA templates is entirely dependent on the fragment length, while the rate of decrease in template number with increasing length is a curve whose slope is dependent on the preservation state of the DNA.

A major problem with the use of DNA degradation as a guide to authenticity (Malmström et al., 2007) is that old contaminant molecules are also likely to show large amounts of degradation. It has recently been suggested that 'old' contaminants in samples can be distinguished from endogenous ancient DNA through a reduced level of fragmentation (Malmström et al., 2007). However as noted above, the well-preserved samples displayed a reduced negative relationship between fragment length and quantity, dependent on the fragment lengths investigated, making it difficult to resolve whether well-preserved authentic ancient human mtDNA could be distinguished from an 'old' contaminant based on the level of degradation. Furthermore, any contaminants are likely to be physically located in different tissues (e.g. extracellular spaces) and exposed to different degradation rates during preservation and sampling than endogenous DNA, suggesting that fragmentation patterns are likely to be a difficult means to distinguish contaminants.

The distribution of DNA fragment lengths has previously been used to calculate the frequency of DNA damage ( $\lambda$ ) in samples such as marine mammal feces (Deagle et al., 2006). According to a model of random degradation, the amount of amplifiable template will decline exponentially with increasing fragment size in degraded samples (Deagle et al., 2006). The rate of decline is described by a single parameter,  $\lambda$ , the probability of a nucleotide being damaged. This is calculated independent of time and may be considered as an index of the level of DNA degradation, with higher values corresponding to greater levels of damage (Deagle et al., 2006). As our samples were of varied age, the relationship between  $\lambda$  and time was investigated and found to be statistically correlated ( $p = 0.01$ ), accounting for 15% of variation, hence the

**Table 2**

Rate of ancient human mtDNA damage ( $\lambda$ /yr) estimated from the exponential decline in the number of amplifiable mtDNA copies with increasing fragment size for 37 individuals at four fragment lengths (77–235 bp) in accordance with the method proposed by Deagle et al. (2006). The rate of DNA damage was standardized by the age of the sample ( $\lambda$ /yr).

	n	$\lambda$ /yr	Standard deviation	$R^2$ (%)
All samples	37	0.000012	0.000013	82.7
Europe	30	0.0000069	0.0000049	84.0
Europe tooth	26	0.000007	0.000005	84.3
Europe bone	4	0.0000081	0.0000024	79.4
Asia/Tropics	3	0.000035	0.000017	84.2
Asia/Tropics tooth	1	0.000044	0.0000062	77.6
Asia/Tropics bone	2	0.0000156	NA	90.7
South America	3	0.0000364	0.0000051	80.0
Hair	4	0.0000305	0.0000125	81.7

impact of 'time since death' on the rate of DNA damage was removed by standardizing  $\lambda$ /year. The random degradation model fitted the data well and explained between 77.6 and 90.7% of variation in the data (Table 2) independent of how the samples were segregated into post-mortem environment or tissue type. As expected, there were higher rates of DNA damage in samples from warm, humid environments, such as in Asia (China, Silk Road) and the tropics (Bahamas), than in samples from Europe ( $p = 0.01$ ). This finding is in agreement previous studies (Collins et al., 2002; Lindahl, 1993). Although the South American mummy samples were from a high altitude environment, they had significantly higher degradation rates (standardized per year) of DNA damage than European skeletal samples ( $p = 0.00$ ). This may relate to tissue type, as we found that hair (from both South American and African samples) was characterized by higher degradation rates ( $p = 0.00$ ). We recognise that the DNA degradation process in hair may be different to that in bone and teeth, due to the potential for DNA in hair to have already experienced limited degradation during the growth of the hair strand. Furthermore, for a given post-mortem environment DNA from teeth showed less damage than that from bone, although this result was non-significant for European samples ( $p = 0.082$ ) (Table 2). Overall, our findings confirm that although chronological age is a factor, it is not the major determinant of DNA degradation (Smith et al., 2003), and instead environment and tissue type are more important.

#### 4. Conclusions

Overall, our results show that current standard approaches to the genetic analysis of skeletal remains can be extremely detrimental to the recovery of mtDNA, and probably also nuclear DNA. In many cases, this is likely to have degraded endogenous templates to the extent that PCR amplification would be unsuccessful, potentially thwarting the genetic analysis of a number of key specimens. Furthermore, most ancient DNA, archaeological and forensic studies to date have not utilized the most suitable skeletal material, namely cementum-rich material at the tip of the tooth root, and have instead focused on dentine. Finally, the discovery of a consistent pattern of exponential template degradation across multiple environmental and tissue types provides a new means to characterize and analyze site- or region-specific degradation rates, and improve the ability to predict the nature of samples likely to be suitable for ancient and forensic DNA analysis.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jas.2010.11.010

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