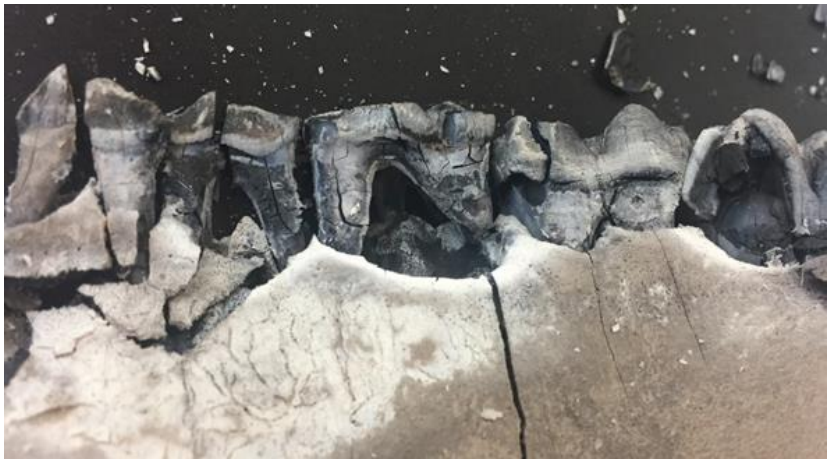
Retrieving DNA from incinerated teeth: Implications for forensic identification



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Bachelor of Dental Surgery

Graduate Diploma in Forensic Odontology

School of Biological Sciences

Faculty of Sciences

The University of Adelaide

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy at The University of Adelaide

May 2020

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

In the name of Allah, Most Gracious, Most Merciful

Dedicated to my amazing husband, my wonderful mother and my late grandparents who would have been so proud of me.

Table of Contents

Thesis Abstract

Loss of lives due to building fires, vehicle explosions, terrorist bombings and bushfires require immediate response and identification of any deceased. The temperatures reached in these catastrophic events can widely vary from approximately 300°C to more than 1000°C. This can be sufficient to leave humans remain in an extremely fragile and fragmented state. In such circumstances, teeth can survive the harsh condition and are therefore one of the most frequently used biological tissues used for DNA profiling. Currently there is very little empirical data to determine the opportunity for successful genetic typing from teeth incinerated at varying temperatures. A process whereby the generation of DNA data from highly burned teeth can be predicted would be highly beneficial. If there is a prediction of DNA typing, then a process to generate these data would further aid in the process of human identification from such sample types.

The research in this thesis highlights the development of three novel pre-screening methods for incinerated teeth:

- *a temperature-prediction tool*
- *a DNA viability triage*
- *a diagnostic workflow*

A holistic approach is utilised in this thesis that integrates multiple analytical methods such as colourimetry, X-ray diffractometry, scanning electron microscope and DNA analysis. The research data in this thesis are generated and analysed in the following forms: photographic images, colourimetric values, hydroxyapatite crystallite size, X-ray diffractogram, microscopic images and quantified DNA.

The research is presented stepwise following the chapters:

Chapter 1 – A comprehensive review of the current literature relating to the study of incinerated teeth including the structure, DNA contents and investigative approaches.

Chapter 2 – The practicality of a fire simulation as an experimental approach to incinerated teeth was tested and evaluated. The decision to incinerate teeth using a furnace was made.

Chapter 3 – The integration of spectrophotometric and x-ray diffraction analyses to investigate incinerated teeth was established.

Chapter 4 – Based on the validation study in Chapter 3, a tool to predict temperature-exposure of incinerated teeth was developed and validated.

Chapter 5 – nDNA and mtDNA analysis from incinerated teeth was discussed. Using the data from Chapter 4 and the data of the quantified DNA, a diagnostic triage for DNA viability in incinerated teeth was established. In addition, an optimised workflow for the investigation of incinerated teeth that will be used for DNA analysis was proposed.

Overall, this thesis presents novel and robust pre-screening methods that can offer important information prior to DNA analysis of incinerated teeth. In addition, this research provides an opportunity to advance the understanding about the forensic value of incinerated teeth in predicting temperature and assessing DNA viability. A major implication of this research is the possibility to implement the developed methods in forensic identification using incinerated teeth.

Thesis Declaration

I, Rabi'ah Al-Adawiyah binti Rahmat certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written 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 in my name, 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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Rabi'ah Al-Adawiyah Binti Rahmat

May 2020

Biography

"I would like to be remembered as someone who was not afraid to do what she wanted to do, and as someone who took risks along the way in order to achieve her goals."–Sally Rise, the first and the youngest American astronaut to have travelled to space.

The author was born on September 21st, 1987 and raised in Kuala Lumpur, Malaysia. She received her high school education from a prestigious boarding school at the Tunku Kurshiah College (2000 – 2004) in Malaysia. In 2011, she was conferred with a Bachelor of Dental Surgery from The Vinayaka Missions University in India, with a first-class degree. The following year, she started her professional career as a general dentist at the Malaysian Ministry of Health (2012-2013) and at the Faculty of Dentistry, The University of Malaya (2014). In 2015, she was awarded a scholarship by The University of Malaya for a postgraduate study. By the end of 2015, she completed a Graduate Diploma in Forensic Odontology at The University of Adelaide, with distinction. In 2016, she commenced her PhD candidature at the University of Adelaide with most of her studies were supported by the Ministry of Education, Malaysia and The University of Malaya, Malaysia.

The author is a forensic-trained dentist, with substantial case work experience. She was involved in the disaster victim identification of the MH-17 plane crash and other cases relating to dental identification. Her field of interests are the identification of burned human remains and disaster victim identification. She plans to continue her passion in forensic science research, specifically in the interdisciplinary approach of human identification.

Acknowledgements

"We must believe in ourselves as no one else will believe in us, we must match our expectations with the competence, courage and determination to succeed."–Rosalyn Yallow, Noble prize winner in Physiology or Medicine

My PhD is a roller coaster of a journey, but I would not have it any other way. This remarkable and humbling experience has made me the person that I am today. Many obstacles became tolerable because of an amazing support system that I am blessed to have. The work of this thesis could not have been possible without the encouragement and guidance of many incredible people. I would like to express my heartfelt gratitude to each one of them.

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in forensic DNA seemed to be impossible at first, but here I am today, presenting a forensic DNA related work. I am forever indebted to his unwavering support.

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My family and friends in Malaysia, for keeping me in their prayers. Especially my doting mother for lending her ears when I wanted to fume out my frustrations, and always motivating me to complete this PhD journey.

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And finally, I want to specifically honour **my dear husband, Shah Faiz Shawaluddin** who has been in this ride together with me since day one. He has been with me through ups and downs. He is my solid rock during my lowest moment. His unconditional love, care and support are my strength to endure this journey. Thank you for dealing with me patiently. Mainly, thank you for being so selfless and for making sure that I completed this journey safe and sound.

Publications, Grants and Presentations

"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained." – Marie Curie, first woman and only woman in history to ever win a Nobel Prize twice, and the only human to ever win a Nobel Prize in two different sciences.

Publications

1. Rahmat RA, Humphries MA, Austin JJ, Linacre AMT, Raven M, Self P. Integrating spectrophotometric and XRD analyses in the investigation of burned dental remains Forensic Sci Int. 2020 [Published].
2. Rahmat RA, Humphries MA, Austin JJ, Linacre AMT, Malik A. Novel diagnostic models to predict nuclear DNA and mitochondrial DNA recovery from incinerated teeth [Prepared manuscript].
3. Rahmat RA, Humphries MA, Austin JJ, Linacre AMT, Self P. The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data [Prepared manuscript].
4. Rahmat RA, Humphries MA, Austin JJ, Linacre AMT, Malik A. Retrieving DNA from incinerated teeth: implications for forensic identification [Prepared manuscript].
5. Rahmat RA, Humphries MA, Austin JJ, Linacre AMT, Malik A. An improved method of preparing teeth to optimise DNA yield. In draft stages [Prepared manuscript].
6. Rahmat RA and Austin JJ. Lessons learned from a fire simulation as an experimental approach to study burned dental remains [Prepared manuscript].

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Presentations

1. Incinerated teeth as a source of DNA for human identification. Poster presentation. The Australian and New Zealand Forensic Science Society, ANZFSS 23rd International Symposium 18-22 September, 2016.
2. The forensic identification of burned remains: Dental evidence recovery for DNA analysis. Poster presentation. The 5th Malaysian Summit of Australia. 20 April, 2016.
3. Identification of burned remains by DNA analysis in teeth. Poster presentation. 10th Annual Florey Postgraduate Research Conference. 29 September, 2016.

Chapter 1
General Introduction

Retrieving DNA from incinerated teeth: Implications for forensic identification

Establishing the identity of a deceased person is important for medical, legal and ethical reasons. From a forensic point of view, human identification must be conducted diligently using validated scientific methods that involve biology, chemistry, physics and statistical analyses. Forensic identification of a deceased person is necessary in criminal investigations (unexplained natural death, homicide or suicide), after natural or man-made accidents and disasters (bushfire, earthquake or motor-vehicle accident), and in war crimes or genocide [1, 2]. Methods of identification that are recognised by the International Police Organization or INTERPOL to establish an identity of a deceased body are fingerprint analysis, dental identification and genetic Deoxyribonucleic Acid (DNA) profiling [3]. These methods are performed using similar approaches whereby post-mortem data obtained from a deceased body are compared to the corresponding ante-mortem evidence.

Teeth and bone samples are the most reliable sources of DNA when the process of sample collection is greatly delayed or the deceased body is subjected to unfavourable environmental condition [4]. DNA-based analysis has been applied to burned fragments and successfully identified severely burned remains in much forensic casework [5, 6], and it is heavily used in many disaster victim identification (DVI) cases. For example, in the Australian bushfires known as the Black Saturday fires on 7 February 2009 across Victoria, 173 human lives were lost [7, 8]. DNA analysis has contributed to 41% of total positive identifications [9]. In another instance, the World Trade Centre attack on September 11, 2001, DNA analysis was used to identify more than 3000 fragmented burned remains [7]. With as many as 15000 body parts recovered in the tragedy, many victims are still left unidentified today. After 16 years, a continuous effort in DNA research has enabled a positive identification of one of the victims through DNA analysis of an archived bony fragment [10]. In the MH17 airplane crash on July 17th 2014 in Eastern Ukraine, despite being crushed, co-mingled and exposed to extreme heat, 98.2% of the collected post-mortem samples were able to provide highly informative DNA genotyping results [11].

A part of this research study focussed on the likelihood of retrieving DNA from incinerated teeth. In a deceased person, DNA samples can be retrieved from a wide-range of biological samples including blood, muscle, hair, bones and teeth [12]. In fire cases such as vehicle explosion and fuel combustion (suicide or homicide), temperature can go up to more than 600°C [13-16], At this temperature, human remains can be severely burned to an extent that the soft tissues such as skin, blood and muscles tissues are completely destroyed. High temperature has a strong influence on the reduction of DNA quality and quantity [17]. As the temperature increases, direct heat-exposure to the soft tissues may disrupt the structural integrity of its DNA content and consequently the DNA becomes compromised for DNA analysis. In contrast, the matrix in teeth and bone have a high mineral content that is intertwined with the organic matter, protecting DNA from direct heat-exposure [18-21]. Due to this, teeth and bone are valued sources of DNA [12, 22]. However, teeth have better DNA preservation and are less likely to get contaminated than bones [11, 23, 24]. Moreover DNA retrieved from teeth has been shown to be higher quality than DNA retrieved from bones in some circumstances [25].

DNA degradation

Following death, the manner by which the body is kept, preserved or disposed (buried, submerged, burned, and frozen) has a profound impact on the rate of tissue decomposition [26]. Tissue decomposition induces DNA fragmentation that subsequently reduces the likelihood of obtaining a complete DNA profile [27]. Biological samples recovered in cases where human remains have been subjected to harsh environmental conditions often contain low quantity and compromised quality of DNA templates to the extent that PCR amplification would be unsuccessful [28]. The samples might also carry substantial PCR inhibitors and contaminants such as collagen type I, humic acids, tannins, incorrect concentrations of Ca^{2+} and Mg^{2+} ions [29-32]. In these circumstances, selecting appropriate samples, sampling techniques and DNA extraction methods would improve the success of DNA amplification [33]. Thus, a comprehensive understanding about DNA structure and the pathway of DNA degradation can assist the effort to optimise the retrieval of genetic information from the degraded samples. This is important to increase the efficiency of the subsequent DNA analyses.

DNA structure

DNA is a double-helical structure that looks like a ladder consisting two strands of deoxyribose sugar-phosphate groups and four different nitrogenous bases (nucleobases) [12, 34, 35] (Figure 1). Both deoxyribose sugar-phosphate backbones are connected through the nucleobases and bonded by hydrogen bonds, and twisted to form the double helix [12, 34, 35]. Each strand is a series of polymerised deoxyribose sugar and phosphate groups that are linked through highly-resistance phosphodiester bonds [36]. The basic structure of DNA is called a nucleotide. A nucleotide consists of a deoxyribose sugar, a phosphate group, and a nitrogenous base (Figure 3b). The deoxyribose sugar contains five carbon atoms (C1 – C5) in which the nitrogenous bases is attached to C1 through N-glycosyl bond and the phosphate group is attached to C5 [12, 37]. The nucleobases are of two groups, purines which consist of adenine (A) and guanine (G), and pyrimidines which consist of cytosine (C) and thymine (T) [38]. Pyrimidine groups are more stable than purines [37]. According to Chargaff's rule [38], bases from each group are paired together in the following pattern; A = T and G = C. This complementary pairing of bases is called a base pair (bp), which forms the basic measurement unit of a DNA fragment [12]. The genetic data are stored in DNA in the order that the paired nucleobases are arranged along the deoxyribose sugar-phosphate backbones [12, 34].

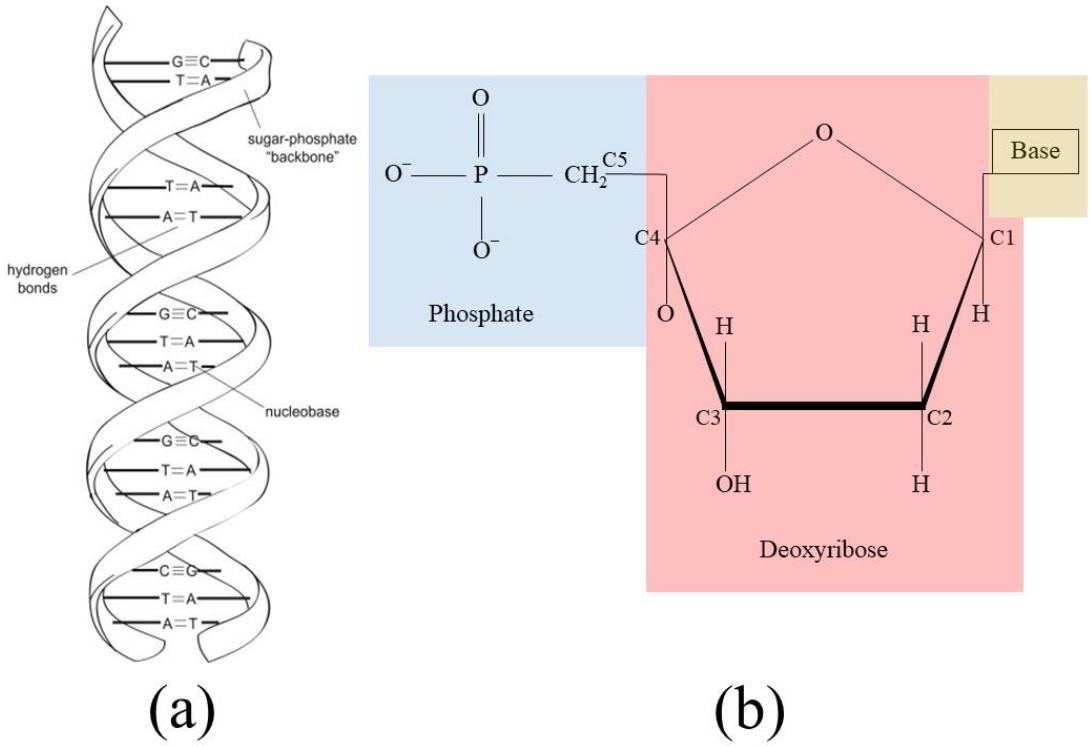

Figure 1— DNA structure. (a) The double-helix DNA is made up from a series of polymerised sugar deoxyribose-phosphate and paired nucleobases that are attached through hydrogen bonds. (b) Nucleotide, the basis unit of DNA. The nucleotide structure comprises of a deoxyribose sugar, a phosphate and a nitrogenous base. Image was modified from [12, 34].

Pathway of DNA degradation

DNA is a highly labile covalent molecule [39]. Being a chemically reactive molecule, DNA responds to the exposure of several physical and chemical agents and is susceptible to damage [33]. The degree of DNA degradation can be influenced by temperature, moisture, oxygen levels, ultraviolet radiation, microbes, soil composition and pH [40-43]. The integrity of the DNA structure is compromised when biological samples are exposed to harsh environmental conditions such as fire, high humidity and prolonged immersion in water [33, 39]. Factors affecting the DNA integrity are microbial activity (secretion of lytic enzymes), reactive compounds (nucleases, oxygen and metabolites), and radiation (cosmic, UV from the sun and geological) [33, 34, 39, 44]. DNA structure may alter or its strands may break depending on the underlying causes and these structural changes can adversely affect DNA amplification during PCR or mistyping of the target loci [33].

In a deceased person, a change of physiological process occurs whereby the blood circulation that transports oxygen and nutrients supplies to the body cells is

ceased [33]. These supplies are vital for a cell to produce energy in the form of adenosine tri-phosphate (ATP) [26]. Oxygen-deprived tissues undergo failure at cellular levels including metabolism and repair mechanism that is evident through the observable stage of decomposition [26]. The depletion of the ATP level in the cells interrupts the body system from functioning normally [33]. The permeability of all membrane bound organelles increases, leading to leakage of hydrolytic enzymes from damaged lysosomes [26]. The cellular volume will increase to a point that the cell membranes rupture [40]. The reduction of the oxygen levels triggers the activation of the anaerobic reaction that leads to the accumulation of acidic by-products. An acidic environment in a cell promotes DNA degradation [26]. This low pH also activates the hydrolytic enzymes which in turns cause loss of cells adhesion or scientifically known as tissue necrosis [26]. Necrosis is characterised by absence of cell nuclei [26].

Intracellular enzymes such as endonucleases and lysosomes are released from the lysed cell and initiate DNA fragmentation [45]. Lysosomes remove histones from the nucleus and aid the endonucleases to break DNA molecules into smaller fragments [26, 33, 46]. These enzymes also catalyse hydrolytic and oxidative reactions of DNA in cells. The hydrolytic reaction involves the detachment of the purine groups from the nucleotide (depurination), whilst the oxidative reaction results in the modification of the nitrogenous bases and ribose-phosphate scaffold [45]. Certain cations or positive-charged ions such as Mg^{2+} and Ca^{2+} have a substantial role in the activation of the lytic/endonucleases enzymes. Figure 2 shows the target sites for oxidation, hydrolytic and enzymatic attacks that disrupt the integrity of DNA structure that causes DNA instability and leads to degradation.

Heat-induced DNA damage

Forensic biological samples that are collected from a crime scene may have been exposed to exogenous environmental insult such as high temperatures, ultraviolet irradiation or humidity [36, 47]. These harsh conditions are damaging to the structural integrity of DNA. The likelihood of retrieving sufficient amounts and quality of DNA from biological samples rapidly decreases with increasing heat intensity and duration [33, 46]. DNA is naturally hydrated [39]. Each nucleotide in a DNA structure has 8-10 water molecules that are bound to it [44]. Temperature can vastly affect the rate of DNA fragmentation by promoting degradative hydrolytic process [33]. Continual

temperature increment and prolonged heat exposure cause loss of water in cells. When temperatures rise above 110 °C, hydrolytic reactions in DNA are initiated in which hydrogen bonds become unstable and gradually break [39].

Due to the susceptibility of the N-glycosyl bond to hydrolytic attack (Figure 2), this bond can easily break [33]. This leads to depurination and the weakening of the DNA chain. The DNA instability is further augmented by the breakage of the DNA strand at the 3'-phosphodiester bond [45]. This is supported by a recent finding in a study using FTI-R analysis, in which the ribose-phosphate scaffold in DNA can be destroyed by heat [48]. Another form of heat-induced DNA damage is hydrolytic deamination [39, 49]. The N-glycosyl bond is intrinsically labile, thus it increases the likelihood of the heat-exposed DNA structure to hydrolytic deamination [39]. The most frequent DNA base that undergoes hydrolytic deamination is cytosine residue which then forms uracil [44].

Following extensive or intense exposure of biological samples to high temperature, the fragmentation of DNA can reduce its size to 200 bp and lower [50]. The small size of DNA fragments makes amplification of longer targets problematic, creating issues for PCR-based analyses of STR loci. High temperature also promotes the activity of DNases which further decreases the half-life of amplifiable DNA [33]. DNA lesions characterised by strand breaks, abasic sites, or modified bases prevent DNA polymerases from incorporating nucleotides and extending primers [33, 51]. Damaged DNA molecule, modified bases and low DNA yield can greatly hinder a successful PCR and subsequent genotyping.

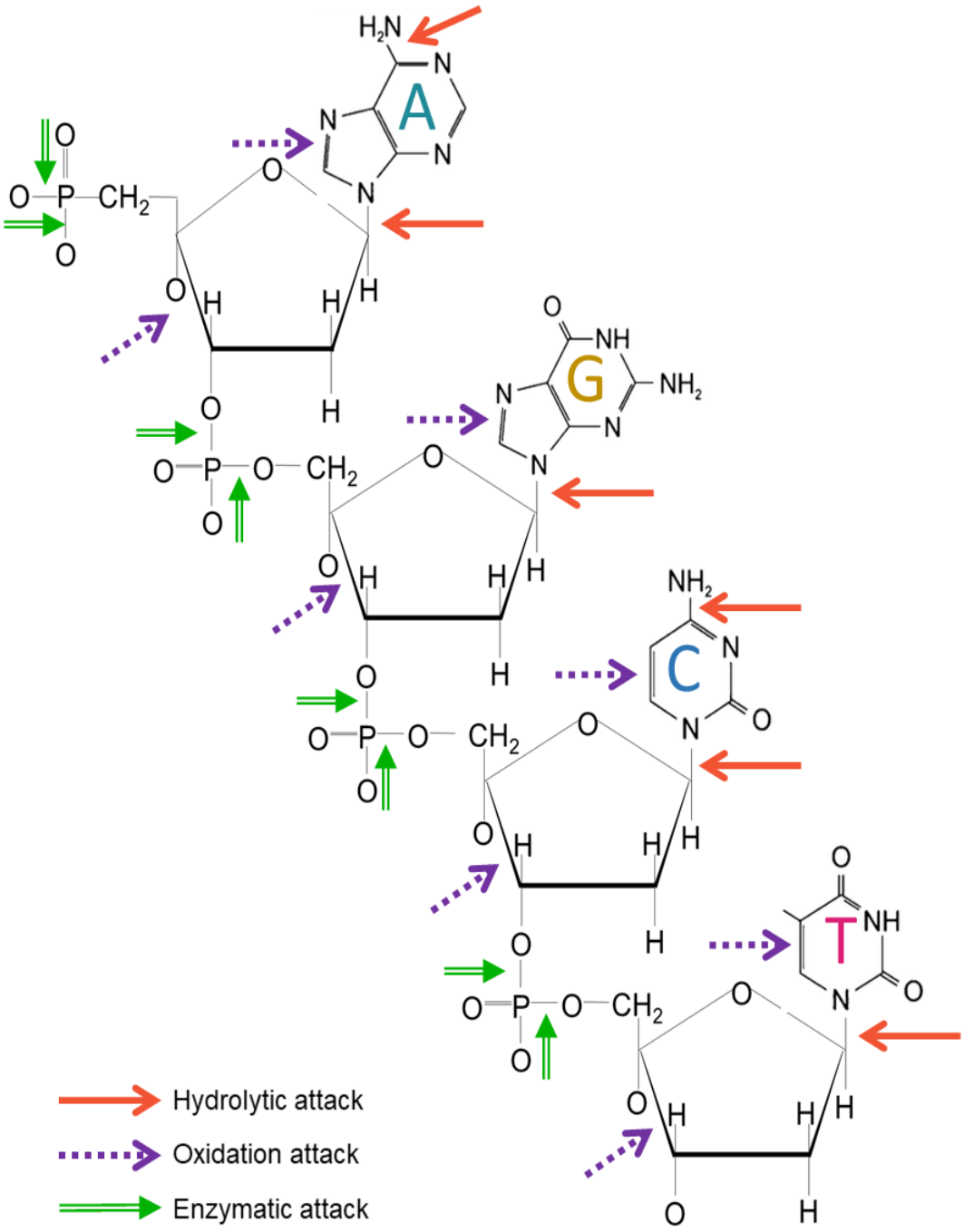

Figure 2— Arrows show target sites for oxidation, hydrolytic and enzymatic attacks that disrupt the integrity of DNA structure and resulting in DNA degradation. Diagram was recreated, modified and adapted from review articles by Alaeddini *et al.* [33] and Lindahl [39]. A: Adenine, G: Guanine, C: Cytosine, T: Thymine.

Assessment of DNA degradation

DNA degradation can be assessed using a real-time quantitative PCR (qPCR), whereby amplification and detection of two target sequences of different lengths [52, 53]. qPCR is a highly sensitive technique and less laborious to quantify levels of gene

expression in biological samples [54]. qPCR has been shown to be beneficial for a highly degraded sample containing low number of templates, detecting PCR inhibitors, Y chromosome (male-specific DNA) and mitochondrial DNA [33, 55, 56].

qPCR allows the generation of PCR products to be monitored in real time [36]. Using a fluorescence dye such a SYBR® Green can aid in the detection of the PCR products generated in each cycle [57]. The dye binds with the double-stranded DNA molecules and fluoresces under UV light [57]. An alternative sensitive assay is TaqMan® system that consists of a fluorescent labelled probe [58]. The assay works by splitting a target-specific probe by the 5' to 3' exonuclease activity of Taq DNA polymerase, producing in an increase production of the reporter [56]. Other than a fluorescence dye, components of qPCR are template DNA, Taq DNA polymerase, as set of primers (forward and reverse), magnesium chloride, nucleotide triphosphates and reaction buffer [36].

The process of qPCR is the same as the conventional PCR. A PCR cycle consists of the following steps: denaturation, annealing and extension [36]. The most critical step is annealing because the primers can only specifically anneal to their target sequences at the optimum melting temperature, usually between 50°C to 65°C [54]. Melting temperature is the temperature at which half of a particular DNA strands will be dissociated [36]. Otherwise, a nonideal temperature can cause a nonspecific annealing of the primers such as a self-complementing primer that forms a loop, primers complementing each other that forms primer-dimers or primer anneal non-specifically elsewhere in the DNA template [36]. Each qPCR run should include a duplicate or triplicate, and no template control (NTC) should be included to check for DNA contamination and primer-dimers formation [54]. Prior to qPCR, it is important to emphasise on designing primers and selecting target sequences that can ensure efficient amplification of DNA products (amplicons) [54] (Table 1).

Table 1—Recommended primer design and selecting a target sequence [54].

Primers	Target sequence
Guanine-Cytosine of 50% and 60%	Guanine-Cytosine of 50% and 60%
A melting temperature of 55°C to 65°C	75 – 150 bp fragment length
G or C at the end of the primers	No secondary structure

The qPCR assay can be validated by analysing the reaction efficiency and target specificity using sample standards [54]. This analysis can be performed once qPCR cycles are completed, through the assessment of a standard curve and a melt curve [57, 59] (Figure 3a and 3b). A standard curve is used to determine the qPCR efficiency (Figure 3a). According to Taylor *et al.* [54], the efficiency of qPCR is a measure of the conversion rate at which the polymerase transform the assay to amplicon. At 100% efficiency, a qPCR could amplify DNA products at the rate two-fold per cycle [54]. A single sharp peak shown in the melt curve confirms specificity of primer annealing [54] (Figure 3b). In addition, a gel electrophoresis method can authenticate the expected size of the qPCR products and a smear on the gel suggests the amplicons are degraded [27, 60].

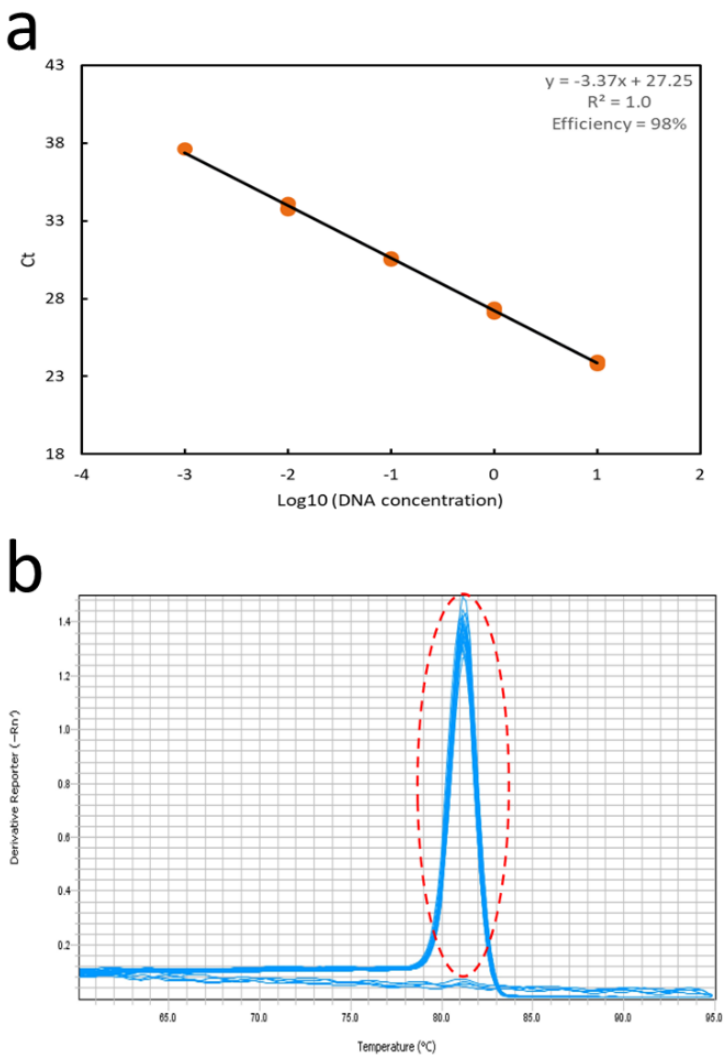

Figure 3— Validation of a qPCR assay: (a) The standard curve shows the efficiency of a qPCR by plotting the log of starting quantity of the template against the C_t values. This standard curve was created from a 10-fold dilution of five points DNA standards. Each dilution was an average C_t value of the standard triplicate. The linear regression line, along with the correlation coefficient (r^2), are indicators of the degree of the qPCR assay optimisation. (b) The melt curve shows a single peak (in the dashed line). This confirms specificity of primer annealing.

Incinerated teeth as a source of DNA

A successful genetic analysis of burned human remains is heavily reliant on the quality of DNA recovered from the sample. DNA in blood and muscle tissue exposed to high temperatures is often severely degraded and susceptible to contamination [11]. These complications may hamper DNA analysis. Teeth are located in the mouth, embedded between the tongue and the facial and musculoskeletal structure [61]. This is a strategic location that offers substantial protection from direct contact with heat or other assaults especially for the posterior teeth. Thus, teeth often

survive fire which make them to be an ideal source of DNA when identifying severely burned human remains [62, 63].

The composition of teeth

A tooth is of two distinct parts, a crown and a root(s) (Figure 4). The foundation of a tooth is made up of three layers of dental tissues namely enamel, dentin, and pulp (mentioned according to the inward arrangement) and a supporting tissue on the root surface that is cementum [4, 64]. The major compositions of these tissues are hydroxyapatite minerals (inorganic material) and collagen type-I (organic matter) [65, 66] (Table 2). Each tissue contains hydroxyapatite (HA) of different ratios that results in varying physicochemical properties, which are discussed in the following subsections. This heterogeneous composition contributes to the robustness of the overall tooth structure. To give an overview, enamel and cementum provide coverings to the elastic dentin and the delicate pulp. Both tissues also create an impermeable layer that prevent the infiltration of external assaults, foreign matters or contaminants such as direct heat, fluid or microbes into the teeth. The tooth roots gain an additional physical protection from the surrounding jaw bone as teeth are firmly embedded in the tooth socket of the jaw bone.

i. Enamel

Enamel is the covering layer of the crown. It is made up of 96%wt minerals embedded in 1%wt of organic matter and 3% of water [67] (Table 2). The high mineral content makes enamel the hardest tissue in vertebrates and that it can withstand the mechanical forces such as mastication, hot beverages and acidic food [68]. On a downside, the high mineral content makes it brittle. Due to its acellular nature, no DNA is present in enamel. Enamel and alveolar bones provide a physical barrier for DNA in dentin, pulp and cementum teeth from direct access of heat, bacteria and other environmental insults.

ii. Dentin

Dentin contributes to the bulkiness of a tooth [68] and is formed by cells named odontoblasts that secrete a predentin matrix that eventually mineralizes into mature dentin [64]. The matured dentin has dentinal tubules that housed the processes of odontoblasts [64]. The tissue extends from the crown to the root forming the most of

the tooth structure and surrounds the pulp chamber [69]. Dentin contains 70%wt mineral and 20%wt organic matter (collagen) (Table 2) that provide substantial compressive and tensile strengths to the teeth, compensating the brittleness of the overlaying enamel [66, 67].

iii. Pulp

Pulp is a soft tissue and forms the core of a tooth (Figure 4). It consists of tooth cells including odontoblasts, fibroblasts and undifferentiated ectomesenchymal cells, and a vascular bundle of nerves and blood capillaries. The space containing the pulp is known as a pulp chamber and it is enclosed by the dentin except at the apical region of the root where the vascular bundle enters [68]. Pulp enables teeth to response to stimuli such as hot, cold, pain and numbness, and provides a pathway for the transportation nutrients and oxygen to maintain the vitality of a tooth. A healthy pulp being highly vascular and 100% cellular can provide substantial amounts of DNA [70]. However because pulp is purely a soft tissue, it will undergo putrefaction and decomposes faster than dentin and cementum and its DNA content is significantly affected by age, dental diseases and post-mortem decomposition [71-73].

iv. Cementum

Cementum covers the surface area of the tooth root and provides an anchorage underpinning the dentin in the root [64, 68, 69]. Although it is a part of the whole tooth, it is a supporting tissue because it was formed from dental follicles during the development of the tooth root, which also explains the location [69]. Based on its structure and function, cementum is generally classified into two types: acellular cementum and cellular cementum [68]. Acellular cementum contains no cementocytes and extends from the cemento-enamel junction (CEJ) to the root apex and cellular cementum contains cementocytes and present on the apical third of the root [69]. Thus, only cellular cementum can provide DNA. Cementum alters in response to the functional requirements of the tooth, thus the thickness of cementum increased as more is deposited on the root surface by the cementoblasts [71, 74]; these are the cells that contain DNA.

Table 2— The approximate proportion of enamel, dentin and cementum presented in percentage by weight (w/w) and percentage by volume (v/v) [4, 64, 67, 69, 75-77].

Constituent	Enamel		Dentin		Cementum	
	%by weight	% by volume	% by weight	% by volume	% by weight	% by volume
Water	3	8	10	20	12	22
Organic matter	1	2	20	30	23	33
Mineral	96	90	70	50	65	45

Hydroxyapatite: the principle mineral of teeth

Teeth are of calcified tissues consisting nano-sized mineral crystals tightly distributed in an aqueous-organic matrix [67]. This mineral component greatly contributes to the durability of teeth. Hydroxyapatite, $Ca_{10}(PO_4)_6OH_2$ (HA) is the principle mineral of teeth. It is present in the form of bioapatite crystal and is non-stoichiometric [66]. The biological HA has substantial carbonate ions in the teeth and other skeletal tissues, making it different from the pure form of HA [66]. The average size of HA in unburnt teeth is a range of 15 – 22 nm in diameter [78]. Enamel has relatively larger HA crystallite and higher crystallinity compared to HA in dentin due to the trivial organic content (<1%) [79, 80]. The degree of crystallinity and size of HA can be influenced by various factors: heat-exposure, diagenesis, humidity, pH, microbial attack and soil environment [80-84].

DNA contents in teeth

Pulp, dentin and cementum are sources of DNA in teeth [63, 65, 71]. However, the DNA yield from these individual tissues was found to vary greatly [71, 74, 85]. On the other hand, enamel being the most mineralised tissues (96%wt of HA) contains no DNA due to being acellular. In addition, in contrast to the crown of the tooth, the root DNA can be obtained from the tooth roots because roots are composed of pulp, cementum and dentin [74, 85]. Although dentin and pulp are present in the crown, the DNA yield from crown is ten times less that retrieved from roots because of its predominant enamel content [85].

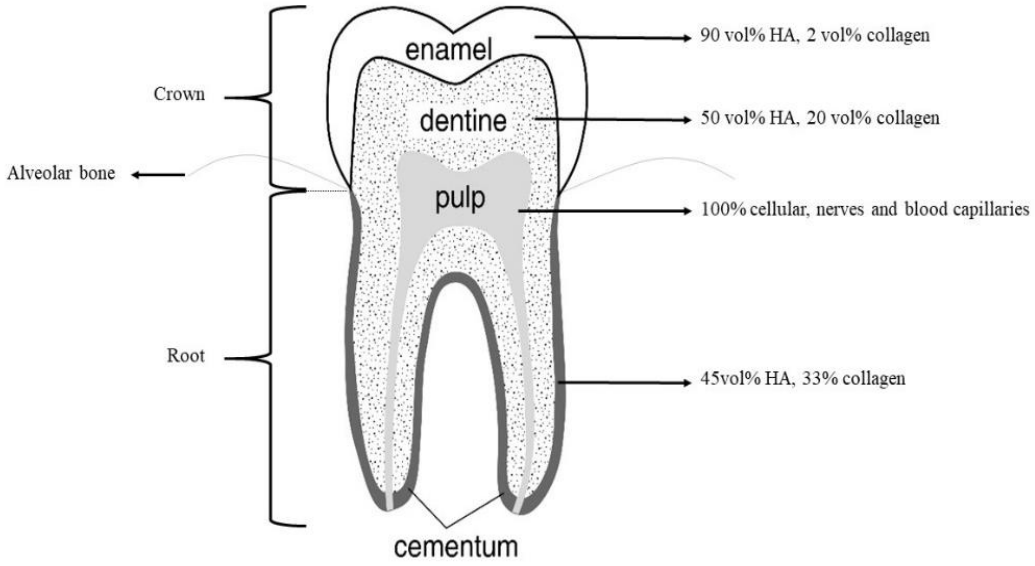

Figure 4— Schematic diagram of a human molar showing tooth parts (the crown and the root), dental tissues (enamel, dentin and pulp), tooth-supporting tissues (cementum) and the relative distribution of hydroxyapatite (HA) mineral and collagen of each tissue presented in percentage in volume, v/v (vol%) and DNA in teeth. Image taken and modified from [65].

DNA in dentin and cementum are found to be preserved longer than DNA in pulp when subjected in unfavourable post-mortem conditions [70]. The DNA preservation is due to the adsorption of DNA to hydroxyapatites [41, 86, 87]. The negatively charged phosphate groups in the backbone of the DNA molecule bind to hydroxyl sites on the hydroxyapatite. This particular arrangement aids in preservation of DNA in teeth by retarding the chemical break down of DNA structure [41].

Survivability of DNA in incinerated teeth

In many forensic identification cases of fire victims using genetic analyses, teeth have been the main source to sample DNA [9, 11, 62, 63, 88]. Amplifiable DNA can still be retrieved from teeth burned at 300 °C [89-91]. Tsuchimochi *et al.* [92] claimed that no PCR product could be obtained after teeth were incinerated at 400 °C. The success of positive DNA amplification is however greatly determined by the fragment size as shorter fragments have greater chance to be amplified [72]. Genetic analysis of skeletal remain is known to be greatly impaired when the temperature reaches over 800 °C because organic matters, including DNA, are completely destroyed [93, 94].

However, analysis using mitochondrial DNA (mtDNA) may be particularly favourable because it can be extracted in greater quantity due to their abundant amount

in cells relative to nuclear DNA (nDNA) and the mtDNA is in a protective membrane, then if any DNA survives this heat insult then mtDNA has more chance of remaining intact [94]. Also, DNA recovery is better in molar and premolar teeth because these teeth afford more heat protection compared to incisors and canines because of the strategic location at the back of the oral cavity between cheeks and tongue [61]. Moreover, molar and premolar teeth have larger pulp volume [95, 96] and number of roots compared to incisor and canine teeth [96], thus, more DNA recovery is expected from them [4].

Investigative approaches to analyse incinerated teeth

Analysis of incinerated teeth can be a challenging process due to their fragile structure. During the incineration process, intense heat-exposure alters tooth structure at the macroscopic and the microscopic levels. Macroscopic alterations are usually visible with the naked eye which include changes in size, colour and shape [97-99]. Microscopic alterations such as crystallinity, chemical composition and histological structure can be assessed using a specific tool or analysis [79, 100-102]. Similar to burned bones, these alterations can be highly informative in forensic and archaeological contexts because such details can be used to infer, biological profiles of the burned remains, the temperature and duration of fire-exposure and pre-burning condition of human remains [98, 103-107]. Due to that, various analytical techniques have been available to examine the structural changes in teeth. Each technique offers different information and it is selected to fit the purpose of the analysis. It is thus important to address the investigative approaches of incinerated teeth bones.

Experimental approaches

It is important to design a solid incineration experiment because burned skeletal remains recovered in forensic fire case or during archaeological field excavations might have been exposed to a broad range of temperature. The temperature of various fire scenarios is different (Table 3): a wooden campfire is in the range of 400°C – 700°C [105, 108]; house fires are in the range of 700°C – 900°C [108]; funerary cremation is in the range of 900°C – 1000°C [109]; fire involving motor vehicles is in a range of 800°C – 1100°C [110, 111]; fire-storm such as bushfire can increase beyond 1000°C [112].

Sandholzer [101] has synthesised various laboratory-based heating methods to incinerate teeth and dental materials from previous studies' regimes on human teeth [97, 113-117]. In the study, he grouped the heating method into two by associating it to a real fire: (1) an incineration with a constant temperature exposure is thought to be comparable with the sudden thermal shock generated by a fire; (2) an incineration with an incremental temperature increase reflects a deliberate temperature increase due to the presence of protective structures [101, 118, 119]. Most of this research did not take the fire behaviour into account.

Faigrieve [108] has described the role of combustible material and oxygen availability in determining the temperatures, its fluctuation and durations of fires. Moreover, factors such as the body location, topography of the surrounding environment and presence of fire accelerants also influence the variability of peak temperatures across different parts of the body [120]. There is still lack of study conducting a heating experiment using a real fire to investigate incinerated teeth. This could be because conducting such experimental approach is inconvenience, time consuming and require researchers to consider many variable factors that influence the heat intensity.

Compositional changes in incinerated teeth

Fundamentally, the gradual changes in colour and crystallinity are attributed to the result of compositional changes in teeth [105, 121, 122]. When the temperature increases, teeth begin to lose water. Complete dehydration and the intense heat induce combustion of organic matters leading to substantial loss of organic content and the expansion of the HA crystals [123]. Reactions and changes in incinerated teeth are best described through various stages (Table 3).

Table 3—Overview of the temperature induced changes in burned teeth summarised from previous studies [89, 91, 97, 99, 101, 105, 111, 116, 122, 124-127].

Stages	Temperature	Reactions	Morphological changes	Colour changes	Crystal size changes	DNA viability	Related burning scenario
Dehydration	100-600 ˚C	Hydroxyl bonds break. The loosely bound water and bonded water are removed from the apatite minerals	Micro-fracture and friable crown	Neutral white, yellowish to brown	Mild increased	Yes	Open camp fires
Decomposition	500-800 ˚C	Combustion of organic materials and carbonisation	Increased chequered patterns in crown and cracking of dentin, separation of crown and root	Dark brown, black to greyish	Mild increased	No	Fire pits, prairie fires
Inversion	700-1100 ˚C	Complete loss of organic matters, expansion of hydroxyapatite crystal and its conversion to β-tricalcium phosphate	Shattering of enamel, root exposed	Greyish white, light blue-grey to chalked white	Significantly increased	No	Cremation furnaces
Fusion	<1600 ˚C	Melting and coalescence of crystals	Enamel and rooted shattered.	Calcined white	No further increased	No	Burning cars and houses, bushfires

Morphological changes of incinerated teeth

Burned teeth shrink in size with an approximate 10–15% reduction [105]. The size reduction occurs when inorganic crystals collapse into the spaces left by the organic material. Merlati *et al.* [115] found that tooth crowns shatter at 800°C, and that the tooth root will shatter at 1100 °C. Uneven crack lines on the crown are due to different proportions of organic contents in enamel and dentin. Dentin seems to exhibit greater shrinkage than enamel, likely due to its greater organic content [98]. Also, dentin contracts and expands slower than enamel, thus inducing stress at the dentino-enamel junction, which producing a partial or even complete shattering of the crown of a tooth. Above 800°C, no further size reduction or damage occurs because the crystals fuse to each other, which inhibits further heat-related shrinkage and fracturing [105].

Analysis of colour alterations

Colour is a visual, perceptual property in human beings. The colour of a tooth is a product of light propagation within its structure before it reaches the observer's eye [128, 129] (Figure 5). Following that, the light sensitive cells in the eye respond to the spectrum of light and the perceived colour is described [130] (Figure 5). The tooth changes colour when heated because of the organic and mineral orders are modified and this affects the light propagation [129, 131]. A visual observation and a subjective interpretation has been the conventional practice to describe the colour changes of burned skeletal remains [98, 105, 132, 133]. Perceptions and interpretations of colour and colour comparisons are highly subjective and greatly varied amongst observers [134]. Thus, the main drawback of such practice is that an objective communication of a particular colour is difficult. Eye fatigue, age, experience, degrees of light exposure and other physiological factors can influence an observer's colour perception [135, 136].

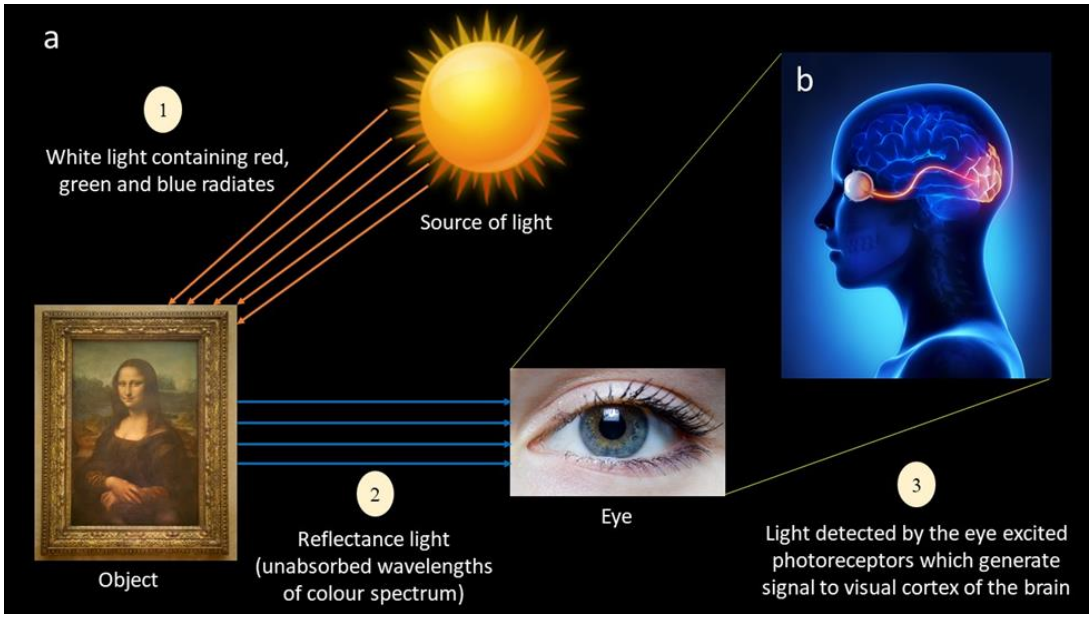

Figure 5— The process of how human's brain perceives the colour of an object. The colour of an object is that part of the energy radiated by a light source that is not absorbed by the object but reflected. The physiological process in the brain creates visual formation.

Based on the summary of temperature induced changes presented Table 3, colour changes correspond to the stages of organic-inorganic material alterations in the teeth as the heating temperatures increased. Previous studies highlighted that the colour changes in teeth and bone are attributed to the loss of organic content [13, 98, 105, 137]. In addition, the changes of colour in teeth exposed to a certain temperature can be varied [101]. For instance, a tooth incinerated at 300°C may look partly unaffected by heat but some parts of the tooth may turn brown. The uniformity of the colour changes seen in a tooth at a particular temperature is due to its different mineral content across dental tissues; enamel is 96% mineral, thus the subtle colour change whilst dentin has a substantial amount of organic material that combust upon heating [98] (Table 3). There is a minimal colour change from neutral (yellowish ivory) to light brown at the early stage of incineration (100°C – 500°C) because teeth are only losing water and no alteration of organic-inorganic content. When the combustion of organic matters occur between 500°C and 800°C, teeth get carbonised and turn darker brown or black. Beyond 800°C when the organic matter is completely burned and no carbon left, the colour become lighter, ranging from bluish grey, chalky white to calcined white.

Colour has been broadly used to be indicator of the range of temperature to which skeletal remains has been exposed to [13, 105, 106, 114, 125, 138-140]. Based

on previous studies by other researchers [105, 137, 141, 142], Becdelievre *et al*., [143] summarised the association of the observed colour changes in bone with corresponding temperature-exposure as follows:

- Up to 200/300°C: orange, yellow, yellowish, tan, ivory and white
- 200/300°C to 550°C: dark brown and black
- 300°C to 700°C: grey, light grey, blue grey
- 600°C to > 1000 °C: white, pale yellow white, white to light grey

Teeth and bone show similar colour changes, but the alterations are not necessarily occurred at the same pace. The colour changes between teeth when exposed to the same temperature. For instance, at 300°C to 500°C, teeth and bone can turn to brown-black to grey [101, 105, 143, 144].

Munsell Soil Colour Chart [145] has been broadly utilised in forensic science and archaeology to interpret the colour changes in heat-treated skeletal remains [133, 146-149]. The Munsell colour system represents the entire colour spectrum and provides a systematic approach of interpreting colour into three components - hue, value and chroma [105, 150]. Shipman *et al.* [105] and Frederick *et al.* [148] described the colour changes in heat-treated teeth and bone using the Munsell chart. Using the Munsell-colour chart to assess colour changes in incinerated teeth, Shipman *et al.* [105] summarised the changes as follows: (1) pale yellow and yellow, (2) reddish brown, very dark grey-brown, neutral dark grey, and reddish-yellow, (3) neutral black, with medium blue, (4) light blue-grey and light grey; (5) neutral white with some medium grey and reddish-yellow. Nevertheless, the reliability is questionable because this method is subjected to individual perception and observer error [151].

It should be noted that temperature-specific colour schemes should be used prudently as this method is subjective and provide a vague estimation of a temperature range [138]. In a survey study by Krap *et al.*, the accuracy and precision of the colour based temperature estimation of thermal-treated bone among forensic and physical anthropologists were low [134]. This demonstrates the need to assess the colour changes using a quantifiable approach such as colourimetry. Colourimetry

is a method to measure colour using the trismulus values (X, Y and Z) devised by the CIE (Commission Internationale de l'Eclairage)[152, 153]. Colourimetric data including lightness (L), chromaticity (a and b), whiteness (WI) and yellowness (YI) can be measured using spectrophotometer [125, 154], or from the digital photographic analysis of the skeletal remains [155]. Spectrophotometers measure the colour using the standard light D65 illumination which resemble the colour observed in a daylight condition [152, 156]. This method reduces the risk of inter- and intra-observer errors. In recent published studies, colourimetry analysis was shown to estimate temperature-exposure of incinerated teeth with high accuracy [125, 154].

Analysis of HA crystal alterations

There are various methods to investigate the crystallite structure in teeth. The most frequently applied methods in previous studies are X-ray diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FT-IR) [78, 79, 157]. The application of small-angle x-ray scattering method to assess incinerated teeth has been reported in some studies [158, 159]. In this study, XRD was the method of choice to assess the heat-induced changes of HA crystallite size in teeth. This is because XRD is capable of showing distinct differences in crystallite size and a sharpening of diffraction peaks on a produced diffractogram at high temperatures [87, 105, 160]. For example, the diffraction pattern of HA mineral in unheated teeth has a notable wider peak than the peak in teeth heated at 600°C which indicates the mineral crystal has increased in size.

X-ray diffraction (XRD) has been a widely used technique to assess the crystallinity changes of thermally-treated teeth and bone [78, 161, 162]. It is a recommended approach to estimate the temperature-exposure of burnt human remains [78]. However, similar changes of the crystallite structure can also occur in diagenetically-altered skeletal remains [81, 163]. Crystallite size increases in both burnt and fossilised skeletal remains due to the decomposition collagen [80, 123]. Archaeologists have been using XRD analysis to characterise and date fossil skeletal remains that had undergone diagenetic evolution [81, 164]. This demonstrates that the crystallinity quantification should not be an exclusive method to indicate the degree of heat-induced changes of HA. Thus, the sole application of XRD to estimate temperature-exposure of burnt skeletal remains is not recommended [154].

Much attention has been given to the study of HA crystal changes in bone [6, 162, 163, 165, 166]. It should be noted that the average crystallite size of HA in unburnt teeth is larger (~22 nm) than in unburnt bone (~17 nm), but when both structures were exposed to 900°C, the crystallite size increase more in bone compared to in teeth [78]. Moreover, crystallites derived from bones and teeth react differently to heat because teeth have layers of dental tissues (enamel, dentine and cementum) containing different proportion of mineral, thus the need to investigate bones and teeth separately [78, 139]. For instance, in this research study, it was evident that hydroxyapatite transformed into whitlockite in teeth but not in bone that were heated more than 800°C [139].

Genetic Analysis

Genetic analysis has been widely applied in forensic and archaeological sciences, from paternity testing, criminal cases to identification of unknown remains [12]. INTERPOL has recognised the application of genetic analysis as a primary identifier in the forensic identification of missing person cases [167]. The application is highly reliable because of its power to associate a sample with an individual with a high degree of certainty [168, 169]. DNA stores genetic information such as physical features and other traits that can be passed down through a family lineage [12]. Various information valuable to resolve identity of can be retrieved from biological samples including of that have been exposed to harsh environments such as an extreme fire condition [5].

Human genome

The entire DNA within a cell that carries genetic information of an organism is called the genome [37]. The human genome is the first vertebrate and largest genome to be sequenced and it has approximately 3.2 billion DNA bases [12, 36, 170] (Figure 6). Genes comprise about 2% of the 3.2 billion bases of the genome [170, 171]. Most of these genes, approximately 75% is extragenic and have similar nucleotide sequence which is not forensically beneficial [36]. Humans share 99% similarity of genome and are differentiated at approximately 1% of the genome [171]. Only certain parts of the genome are analysed in forensic identification, for example 13-15 STR loci and 40-60 SNPs [172-174].

Based on its location in the cell, there are three types of DNA, namely nDNA, mtDNA and chloroplast DNA (only in green plants) [37]. Nuclear DNA accounts for the majority of genomic DNA and it is organised into chromosomes by coiling around positive-charged histones [12]. In the nucleus, human genetic data are distributed into 23 pairs of chromosomes [175]. The total chromosomes are equally inherited from both parents [12]. Mitochondrial DNA is different. It is inherited solely from the mother, circular and the size is 16,570 bp [176]. There are thousands of mitochondria in the cytoplasm of a cell, making it particularly suitable for the identification of degraded human remains [177].

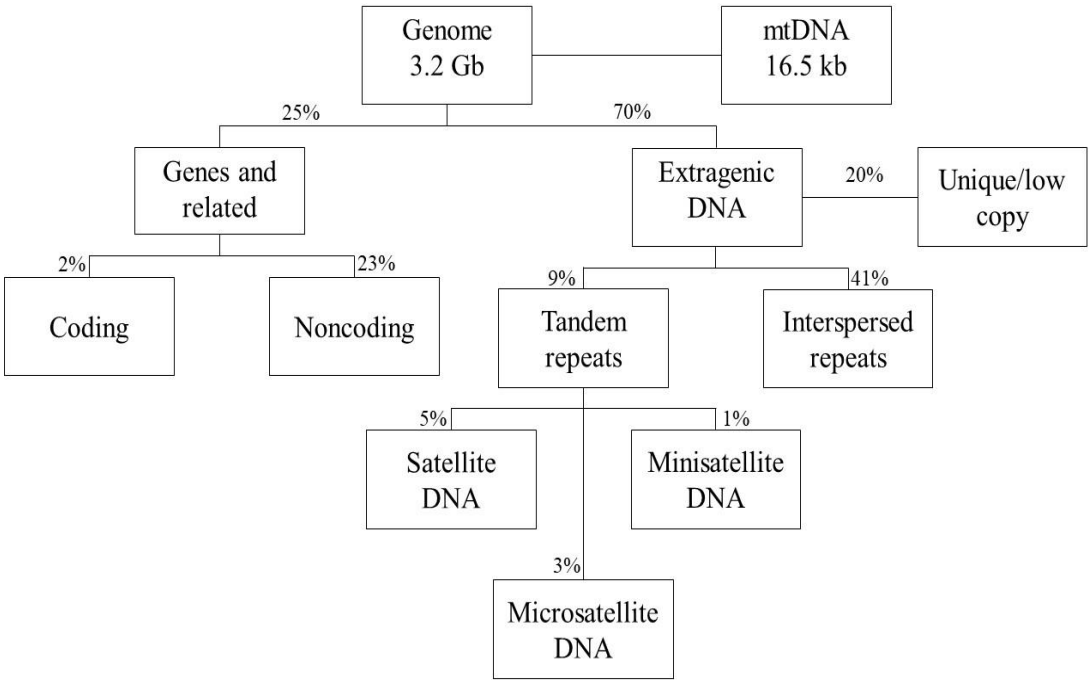

Figure 6— The fractions of a human genome. The stratification of the DNA types is based on its structure and function. Image taken and modified from [12, 178].

DNA profiling

The analysis of a forensic biological sample to generate a DNA profile is called DNA profiling [179, 180]. DNA profiling is broadly used for human identification in many forensic cases such as mass disaster, crime investigation and missing persons [7, 181]. The principle of DNA profiling is a comparison between a DNA sequence of a tissue sample from an unknown deceased body (a post-mortem sample) to a DNA sequence of a reference sample (an ante-mortem sample) of a known missing person through a number of defined process [7] (Figure 7). The ideal application of DNA profiling in forensic context is to match the reported missing

person with the post-mortem samples of biological evidence, with a high degree of certainty [36, 182].

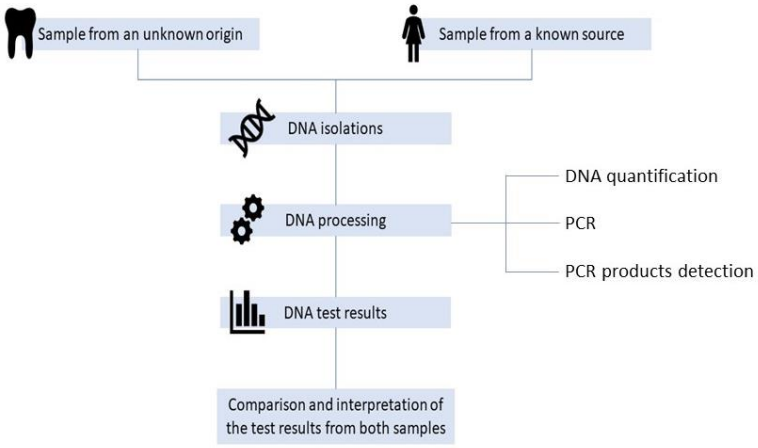

Figure 7—General steps of DNA profiling.

For a DVI operation, the International Society of Forensic Genetics (ISFG) has listed different types of post-mortem samples depending upon the condition of the body [4] (Table 4). Based on ISFG recommendation #3, multiple samples should be taken from every body and body part as soon as possible [4, 167]. This recommendation can certainly be applied for a routine identification as well unless the body has undergone an extensive fragmentation. On the other hand, ante-mortem samples can be sourced from direct references which are deceased's biological samples (e.g. blood stain cards, stored biopsy tissues, semen, extracted tooth) and personal items (e.g. toothbrush, mouth guard or hairbrushes) or from indirect references which are first-degree biological relatives (e.g. blood or buccal swabs from parents, children, siblings or known identical twin) [4, 5, 183]. Similar to post mortem samples, ISFG recommendation #4 has suggested multiple collection of direct and indirect ante-mortem samples should be performed for each missing person [4].

Table 4— Recommended types of post-mortem samples based on the condition of a deceased body. Table was reproduced from [4].

Condition of body	Post-mortem samples
Intact whole body without decomposition	Blood and buccal swabs
Fragmented body without decomposition	Blood (if available) and deep red muscle tissue (~1.0 g)
Intact/fragmented body with decomposition	Long compact bone (~4-6 cm cut) and/or healthy teeth without fillings (molar preferable) and/or any bone (~10 g; preferably dense cortical bone)
Severely burnt bodies	Long compact bone (~4-6 cm cut) and/or healthy teeth without fillings (molar preferable) and/or any bone (~10 g; preferably dense cortical bone) or urinary bladder swab

In an operational laboratory, these samples will be subjected to a sequence of procedures which aim to retrieve DNA contents, target specific DNA segments and ultimately generate a DNA profile for comparative analysis [184, 185]. All procedures are equally important and served specific purposes [36, 184] (Table 5). Thus, these procedures have to be performed meticulously. In addition, when possible it is important to have dedicated laboratories separating pre- and post-PCR procedures and to adhere to a strict contamination protocols to minimise cross-contamination [36, 185].

Table 5—Procedures involved in DNA processing.

Procedure	Description
Extraction	A process to lyse cells to release the DNA molecules, purify them from other cellular materials, and prepare the DNA into a format compatible with the next procedure [184].
Quantitation	A process to measure the DNA quantity recovered from extraction and to determine the appropriate amount of DNA template to include in PCR amplification [186, 187].
Amplification	A process called as Polymerase Chain Reaction (PCR) whereby a specific region on DNA strands (loci) are targeted and replicated (amplicons) to produce high number of the amplicons [187, 188].
Separation	A process in which DNA fragments of different sizes are pulled apart to distinguish one fragment to another [189].

DNA genotyping methods

There are various sources of DNA polymorphisms throughout the genome [170, 171]. Repetitive sequences on polymorphic regions formed the genetic

variability between individuals and it constitutes 1% of the genome [170, 171, 190]. The repetitive sequences that are known as satellites are located near to the midpoint of a chromosome and have a core repeat unit varying from two to thousands of base pairs [12, 170, 191]. While there are specific repeated sequences throughout the genome in all humans, the lengths of the repeated sequences are quite different [192]. This is the area of interest in forensic genetics. Various methods of typing genetic polymorphisms developed, applied and improved in the forensic identification using DNA analysis [193]. The continuous advancements in forensic genetics have hugely contributed to more sensitive, reliable and robust genotyping techniques.

i. VNTR

The first polymorphism used for DNA profiling is variable number tandem repeats (VNTRs) or known as minisatellites [194]. Restriction fragment length polymorphisms typing, or RFLPs was first described in a published paper in 1980 [195]. RFLP typing of VNTR was first applied for a couple of forensic identifications in the United Kingdom [192, 195, 196]. However, the application of RFLP typing of VNTR requires a large amount and a good quality of DNA [197]. RLFPs method applies the advantage of the endonucleases ability to cut DNA strands at specific nucleotide sequence (restriction sites) [195, 198]. This produces DNA fragments of different lengths. Successful RFLPs requires DNA to be intact, thousands of bases (1000 – 20000 bp) in length and at least 50 ng in weight [182, 192]. It is also a nucleotide sequence specific and structure sensitive method, thus any changes or alteration of a nucleotide sequence due to environmental insults can halt DNA fragmentation. Thereby, RFLP method is not suitable for cases with limited or degraded DNA.

ii. STR-based typing

Short tandem repeat (STR) typing has been the most dominant method for forensic DNA profiling [36, 189]. The structure of typical STRs used in forensic genetics is tetranucleotide (4 bp) core repeat units that have between 5 and 20 alleles with length around 100 to 350 bp [199]. STRs are highly polymorphic with a small size of loci [200, 201] which is smaller than amplified fragment length polymorphism (AmpFLP) such as D1S80, the early PCR-based forensic testing

[196]. STR analysis works efficiently with degraded DNA compared to AmpFLPs [196]. STR typing enables DNA profiling of poor-quality samples that is usually the case for forensic samples. Because of the use of the PCR, less than 1 ng of DNA template is required for STR analysis [193]. Smaller-size STR amplicons have been developed by moving the PCR primers closer to the STR repeat region [202]. This initiative was taken to overcome the problem of an incomplete or a unsuccessful DNA profiling using degraded samples [203].

Over the years, STR systems have been rapidly developed and become the standard tool for forensic laboratories worldwide [204, 205]. From a human identification point of view, STR markers should ideally be able to demonstrate the highest variability among individuals [206]. In 1994, the Forensic Science Service (FSS) in the United Kingdom established efforts to create robust and powerful multiplexed sets of 12 STRs including the quadruplex (QUAD) and SGM [207]. Then, a commercial STR kit, The AmpFℓSTR® SGM Plus replaced the SGM and has been used around the world [203]. In the United States of America, a set of 13 STR markers has been selected when comparing DNA profiles using a widely applied software package, the Combined DNA Index System (CODIS) [204]. Some of the loci are CSF1PO, FGA, THO1, TPOX and vWA [208]. In Australia, the use of PowerPlex 21 system combined with Profiler Plus markers has been commercialised and widely used in many forensic identification cases [9, 167, 209].

The length variation between alleles can be identified by performing capillary electrophoresis (CE) on amplicons [210]. This is then followed by a nomenclature convention recommended by the DNA Commission of the International Society for Forensic Haemogenetics (ISFH) [211]. For example, TH01 9.3 is referred to sequence alleles TH01 with nine complete repeat motifs and three incomplete repeat motifs [212]. However, this nomenclature does not include sequence differences between alleles that may be caused by transversions, transitions, insertions, deletions, and inversions of one or more nucleotides. Nevertheless, the high discrimination power of size-based alleles is sufficiently robust that it can provide sufficient forensically relevant information [213]. The random match probability of two individuals sharing the same alleles at each STR loci within their DNA is more than one in a billion [214]. However, DNA should be intact and well preserved to successfully amplify and detect STRs, especially those with loci longer than 200 bp

[215]. To overcome this shortcoming, unlinked mini-STRs (D1S677, D2S441, D4S2364, D10S1248, D14S1434, and D22S1045) were introduced to generate shorter amplicons (< 125 bp) from degraded forensic samples [216, 217]. The exposure of samples to environmental insults such as high temperatures can promote the fragmentation of DNA, thus reducing the DNA template till it no longer retains 5′ and 3′ target regions for STR amplification [218]. This is supported by a finding in a recent study whereby low DNA yields with high degrees of degradation hinder complete DNA profiling from burned skeletal remains using STR genotyping [219].

Nowadays, the sequence data of STR genotyping can be generated with a high throughput and wide coverage using massively parallel sequencing (MPS) technology [212]. It is also known as next generation sequencing (NGS) or the next generation of post-Sanger sequencing [220]. Forensic STR polymorphisms are more informative when typed by MPS because sequencing these STR loci provides extra information in the form of sequence variation, which is not possible when measuring amplicon length using capillary electrophoresis (CE) [221, 222]. Another advantage of using MPS is that allows large sample multiplexing, improved mixture deconvolution, and the simultaneous analysis of different types of markers [223]. For example, STR polymorphisms and SNP panels can be multiplexed to retrieve further information on both ancestry and phenotype [221, 224]. The simultaneous use of multiple markers (STRs and SNPs) can provide higher discrimination power and increase the likelihood of DNA profiling success. In a recent study using the Ion S5™ System, and the ForenSeq DNA Signature Prep Kit on the MiSeq FGx™ system, MPS multiplexes have shown the ability to generate more probative information from challenging samples that have been subjected to fire, cremation and thermal degradation [225].

iii. SNP-based identification

Single nucleotide polymorphism-based typing is a more recent molecular genetic method for samples that contain too degraded DNA or extremely low DNA templates, particularly in cases where STR typing fails to yield sufficient results to obtain complete profiles with high discriminatory power [226, 227]. Highly degraded DNA from these biological samples can produce stochastic effects and the higher molecular weight STR loci can be barely amplified in STR typing [228, 229]. As a

result, short SNP fragments have been widely used in forensic identification of degraded samples [33]. Single nucleotide polymorphisms (SNPs) are useful genetic polymorphic markers for forensic identification [230]. SNPs present in millions of positions along the DNA chromosomes in human genome as a single base pair [226, 231]. SNPs make up approximately 85% of human variation [171, 231]. SNPs are less polymorphic and mostly are biallelic, making them less informative for identity testing than STR typing [182, 199]. The discriminatory power of SNP typing to differentiate between individuals is three to five times lower than STR typing [182, 199]. Between 50 and 80 SNPs are needed to obtain the discrimination similar to the STRs [173].

SNPs have been used extensively as genetic markers in the SNaPshot® assays to analyse identity and forensic DNA phenotyping (FDP) [199, 232]. The forensic applications of SNPs include: (1) human identification using identity-informative SNPs (IISNPs); (2) kinship analysis and paternity testing using lineage-informative SNPs (LISNPs); (3) biogeographic ancestry (BGA) using ancestry-informative SNPs (AISNPs); (4) external visible characteristics (EVCs) such as eye, hair and skin colours using phenotypic-informative SNPs (PISNPs) FDP process includes the potentials of SNPs to infer biogeographical ancestry and externally visible characteristics[224, 227]. In a recently published study, an advanced screening tool that consists of a mini-multiplex SNaPshot® screening tool was developed [233]. The tool enables the comparison of short mitochondrial and nuclear DNA targets, and the assessment of biogeographic ancestry (AISNPs), lineage (LISNPs), and phenotype single nucleotide polymorphisms (PISNPs) [233].

Advantages of SNPs over STRs are the short PCR amplicons (minimum of a single locus) suitable for the analysis of degraded DNA and high multiplexing potential, low mutation rate which is ideal for ancestry affiliation, and enables many forensic applications other than human identification [179, 185, 201]. SNPs are more stable with a mutation rate of 10^{-8} in comparison to STRs that have a mutation rate between 10^{-3} to 10^{-5} [234]. Unlike SNPs, STRs alone cannot be applied for forensic DNA phenotyping (FDP) [224]. FDP is a DNA intelligence tool that is used to use to predict eye, hair and skin colour [233, 235]. SNPs are highly valuable in cases where no or partial DNA profiles are obtained from STR typing [180]. A recent study by Emery *et al.* [219] has suggested the possibility that DNA fragments of

approximately 50 bp surviving in skeletal remains burned at more than 550°C. Therefore a DNA extraction can be performed using ancient DNA protocols and a DNA genotype obtained using SNPs.

A number of techniques can be used to detect SNPs including restriction-enzyme digestion, Sanger sequencing, primer extension and allele-specific hybridisation [36]. The initial two techniques are not feasible for forensic cases that require the analysis of 50 to 80 SNPs dispersed around the genome [36]. Several primer extension methods have been developed including the mini-sequencing reaction, pyrosequencing and allele-specific extension. The SNaPshot® mini-sequencing assay is the most versatile SNP detection method for forensic DNA profiling due to its multiplexing ability and high sensitivity [224]. The multiplexing technique enables integration into operational forensic laboratories without the requirement for any additional equipment. Additionally, a SNP*for*ID 52-plex IISNP assay can be a supplementary assay to STR analysis of samples containing low quantity and quality of DNA [236]. The SNP*for*ID 52-plex profiles have been successfully retrieved from highly degraded teeth and bones, including a burned femur [237, 238]. The modern sequencing technologies (e.g. MPS) have enabled the advancement of a forensic marker that was designated a microhaplotype [239, 240]. Small segments of DNA (<300 bp) with two or more SNPs within a molecular distance defining three or more haplotypes [221]. Current data characterised 129 loci from 55 populations worldwide show a promising use of these microhaplotypes appear to be useful in forensic identification, ancestry inference and determining lineage [221].

iv. Mitochondrial DNA

Mitochondrial DNA (mtDNA) typing has been used in forensic identification when the biological samples are severely compromised for nuclear (nDNA) STR-based typing and to the extent that the STR analysis is no longer successful [241, 242]. mtDNA is characteristically different compared to nuclear DNA, it is a closed circular molecule that codes for 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs and located extranuclear, that is in cytoplasmic mitochondrial organelles [243, 244]. The circular structure of the mtDNA may be the factor that makes it more resistant to nuclease activity than nuclear DNA, making it resilient against challenging

environmental conditions [193]. The human mitochondria contain discrete genome (different to nuclear DNA) and is 16, 569 bp in length [36, 171, 243, 244]. mtDNA PCR primers can produce robust amplification from as little as 0.5 picograms, 200 fold less than required for STR analysis [175].

MtDNA is high in copy number, does not recombine, and in mammals is maternally inherited [245]. These characteristics make mtDNA a valuable genetic marker for forensic identification. The high copy number of mtDNA is particularly useful when cellular material in the biological evidence is limited, for example in hair shafts and faecal samples [50, 246]. Teeth and bone are also an excellent source of mtDNA in post-mortem degraded human remains [247, 248]. In contrast to nDNA, the non-recombining and maternal inheritance of mtDNA allow maternal relatives from several previous generations to be reference samples [245]. This makes mtDNA excellent for the lineage reconstruction of the maternal side of an unidentified remain, and it is particularly useful in mass disaster investigations [249].

Traditionally, forensic identification using mtDNA has relied on HV1 and HV2 sequencing [241, 242]. These two hyper variable regions can be sequenced using the Sanger sequencing method [250]. With the MPS technology, an improved DNA method such as a complete mtDNA genome sequencing approach has been developed [245]. An MPS tiling method for simultaneous mtDNA genome sequencing using 161 short overlapping amplicons (average 200 bp) can be applied for degraded samples [251]. Commercial kits known as the Precision ID mtDNA has been developed using this approach [252]. In fact, mtDNA whole genome sequencing using the Applied BiosystemsTM Precision ID mtDNA Whole Genome Panel (Thermo Fisher Scientific) was successfully applied in the recent identification of the skeletal remains that were recovered from the *La Belle* shipwreck [253].

PCR

RFLPs was quickly superseded by the application of polymerase chain reactions (PCR) method on short tandem repeats (STRs) in 1985 [196, 200, 254, 255]. This was rather quicker, easier to perform. PCR is a powerful tool that can amplify low quantity (less than 1 ng) and quality DNA (less than 500 bp) into millions of copies [188, 254]. PCR works on an enzymatic-catalysed mechanism in which a target region on DNA is copied and replicated exponentially over 30 cycles

[188, 256]. Without PCR that generates abundant copies of DNA, many forensic samples with degraded DNA could not be analysed. The PCR components including *Taq* DNA polymerase, oligonucleotide primers to target a specific region (sequence) of DNA and repeated thermal cycling protocol to replicate the targeted sequence can yield PCR products known as amplicons [257, 258]. Nowadays, PCR is the main method for the identification of human remains in forensic casework and ancient DNA contexts [29, 259]. Various DNA genotyping methods are benefited from PCR products including short tandem repeat (STR) typing assays, single nucleotide polymorphisms (SNP) and mitochondrial DNA analysis [53, 188, 201, 203].

Sampling techniques of teeth

There are a number of techniques for sampling teeth because the quantity and type of DNA yield from different dental tissues is widely-varied [71]. The preferable sampling method is the one with the minimal likelihood of causing tooth destruction. For a vital tooth, pulp is the recommended source of DNA because it contains rich blood capillaries [4]. To recover pulp for DNA analysis, techniques that have been used are sectioning longitudinal [70] or horizontal of the tooth [23, 72], and drilling a small hole through the crown surface to expose the pulp cavity [260]. When a tooth becomes non-vital due to bacterial infection, advanced age and post-mortem cellular degeneration, pulp is no longer an ideal source of DNA [65, 71, 72]. In this case, dentin is the next targeted portion for DNA extraction [261].Pulp may still be recovered in teeth exposed to low-intensity heat or incinerated teeth which structure is still intact.

However, teeth recovered at a fire scene can be fragmented such that only roots or fragments are left and no vital pulp is available due to desiccation, thus pulp sampling is no longer an option. Due to the coverage provide by enamel and cementum, the DNA rich dentin is preserved and ideal for yielding DNA. Also, as mentioned in a previous section 3.1.2, dentin contains only the process parts of odontoblasts, and not the cell bodies containing nuclei. Thus, DNA in dentin will remains available post-mortem. Horizontal splitting has also be applied to recover dentin [262]. Gustafson [263] has described a method to recover dentin from teeth in which two thirds of the root is removed to get access to dentin.

Enamel and cementum also protect DNA within teeth in various environments and from contaminants, therefore, whole teeth or tooth roots are commonly used as samples for DNA studies [264]. In previous studies, complete pulverisation of whole tooth has shown to contribute to high success rates of DNA typing [25, 265] . However, in these studies the technique was applied to unburned teeth. Rubio *et al*. [127] have used pulverisation technique using liquid nitrogen with a freezer mill to reduce incinerated teeth to powder form. However more studies are essential to verify the best method of sampling incinerated teeth.

Pigs as an analogue for humans in forensic research

This thesis serves to seek a better understanding about the forensic value of incinerated teeth, specifically the relationships between the heat-induced structural alterations and the viability of DNA recovery. To achieve the aims of the thesis, *in situ* pig teeth were chosen as a substitute for human samples. This was because of the difficulty to obtain sufficient human jaws with intact teeth of similar age, and where the human bodies had not been preserved in formalin. Although obtaining human cadavers is not impossible, the quantity and age of donors is certainly limited for forensic sciences [266]. In addition, pig carcasses are more readily available and require less vigorous ethical approvals, therefore enabling large numbers of samples to be included in a range of experiments. The use of pigs also minimises the confounding variables such as age, tooth wear and dental disease. Experiments using analogues are easier to control these confounding factors [266]. In this research, intact and sound teeth were selected from adult pigs of roughly the same age (approximately two years of gestational age), so variation including tooth development, dental diseases and tooth wear would be minimal (tooth eruption and root formation were completed).

Pigs (*Sus scrofa*) have been used as human proxies in many forensic studies such as forensic anthropology [106, 162], forensic DNA [267-269] and forensic taphonomy [270, 271]. Pigs share general similarities with humans in various aspects including structure, musculature, and weight [162]. The dentition of pigs and humans share close similarities in terms of size, morphology and composition [272, 273]. Both pigs and human are omnivores, contributing to the structural resemblance of enamel and dentine in both species [274]. In addition, previous studies have shown

that similar thermal alteration patterns were observed between the skeletal remains of humans and pigs [105, 106, 273].

By using pig mandibular segments with *in situ* teeth, and by selecting tooth types (molars and premolars) that resemble human teeth, the structural changes and DNA viability after incineration can be investigated in more realistic forensic settings. The use of isolated human teeth to study the effects of incineration contributes less value to human identification in fire-related forensic casework, or to the estimation of fire temperatures based on the condition of the teeth [269]. The data in the following core chapters were completely derived from pig samples, therefore the results may not be directly applicable to human teeth. Nonetheless, the findings of the thesis established a model for further validation work of incinerated teeth involving human specimens. This is in line with a recent suggestion that human cadavers are required for final validation of forensic methods [266].

Research direction and aims

The investigation of the microstructural alterations of burned skeletal remains has been demonstrated to be reliable for estimating the temperature-exposure (43, 58-62). Although the trends of surface colour and crystallinity changes in teeth are similar to that observed in bones [97, 98, 105, 132], notable distinctions of the changes between teeth and bones have been demonstrated which suggests that teeth and bones are to be analysed separately [78, 158]. Many studies have applied colourimetric and XRD analyses on incinerated teeth [97, 101, 107, 125, 159], the applications of both analyses together in real forensic or archaeological scenarios were rarely reported and not necessarily included in casework protocols for the identification of burned remains.

Several researches have been done to explore the value of colour [127, 148], and HA mineral [41], in predicting DNA viability in teeth and bones. DNA degradation has been associated with the increase of crystallinity in the HA, thus HA can be used to indicate the presence of DNA in the material [41]. A study by Rubio *et al.* [127] on incinerated teeth indicates an inverse correlation of DNA concentration with spectrophotometric values. Similar study has been done on bone by Fredericks *et al.* [148] in which they recommend the use of calorimetry to determine DNA concentration in heated bone. Findings of deleterious effects of heat

on DNA structure and mineral transformation in teeth, suggest that these could be used as a potential tool for the screening of samples for integrity prior to DNA analysis [48, 275]. Though DNA is generally known to be less likely viable in body tissues of burned remain, this research was encouraged by the belief that no biological sample should be disregarded as a potential source for DNA recovery. Moreover, the association of structural alterations of incinerated teeth and DNA contents within them is has not been studies rigorously.

In general, this study focussed on exploring the attributes of morphological changes of incinerated teeth specifically the colour and HA crystal size in providing valued information in forensic context such the estimation of temperature-exposure and the viability of DNA. This thesis presents comprehensive empirical findings of a thorough investigation of teeth incinerated at various incineration temperatures that encompassed from the experimental approach, analytical techniques, structural changes and DNA viability. The aims of this research were of four-fold and these were addressed accordingly in the next chapters:

- To investigate the feasibility of a fire simulation as an experimental approach to investigate incinerated teeth (Chapter 2)

- To explore the reliability of the combined use of spectrophotometric and XRD analyses in the investigation of incinerated teeth (Chapter 3)

- To develop a predictive tool to estimate temperature-exposure of incinerated teeth (Chapter 4)

- To develop a diagnostic model to assess DNA viability in incinerated teeth by quantifying the colour and hydroxyapatite crystal size (Chapter 5)

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Chapter 2

Lessons learned from a fire simulation as an experimental approach to study burned dental remains

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Lessons learned from a fire simulation as an experimental approach to study burned dental remains

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Abstract

Fire simulation has been a less common incineration method compared to a laboratory-based heating experiment using a furnace in the study of burned dental remains. However, unlike fire simulation, furnace incineration does not consider the dynamics of fire behavior. This research aimed to evaluate the practicality of a fire simulation to burn dental remains and to investigate changes in teeth that were burned in their natural state, protected by the oro-facial structure. Fire simulation was performed in a burn chamber using fresh pig heads with intact teeth. Fire behaviour, temperature changes of the chamber and morphological changes of the burned teeth were recorded. Temperatures fluctuated throughout the experiment varying from 90°C – 600°C, and other than charred skin and calcined anterior teeth, blood, muscles and posterior teeth were hardly affected by heat and flames. Although this experiment attempted to simulate a fire scenario, it was evident that variable factors contributing to the fire behavior are difficult to control. Firstly, factors such as the amount of oxygen and airflow influence the intensity of the fire. Secondly, substantial melted fat from pig heads aggravated an anticipated explosion during the burn. This study suggests that a fire simulation is not necessarily a feasible method to incinerate teeth for the study of burned dental remains.

Keywords

Forensic science; Fire simulation; Human identification; Burned dental remains; Experimental approach

Introduction

Burned dental remains are usually found at fire scenes such as residential fires, motor vehicle accidents, explosions and bushfires [1-3]. Teeth are complex structure in which the major components are hydroxyapatite minerals (HA) and collagen [4, 5]. The proportion of these major components change upon heat-exposure [6-8]. The compositional changes can be manifested through the alterations of colour, fracture, size and crystallinity [8, 9]. These thermal alterations are routinely examined to extract information pertaining to fire, such as type of fire and the temperature estimation at which the burned dental remains were exposed to [10, 11]. Burned dental remains have also been heavily used in the identification of severely burned human remains using dental comparisons and DNA analysis [12-16]. Since teeth serve as valued forensic evidence, extensive investigations of burned dental remains changes have been conducted to address issues pertaining to recognizing skeletal remains, recovery, scene reconstruction, methods of analysis, and interpretation of the structural changes [1, 17, 18].

However, fire simulation is not a frequently used experimental approach to study burned dental remains. This could be because the intensity of fire is difficult to control. Multiple factors influence the intensity of fire, these include temperature, duration of heat exposure, airflow of the surroundings and type of fuel [19-21]. Conducting a fire simulation should be considered because temperature of a real-fire scenario greatly fluctuates and burned dental remains retrieved from crime scenes are exposed to the dynamic of fire [9, 22, 23]. The temperatures of house fire ranges between 700 – 900 °C [22, 24], motor-vehicle accident ranges between 800 – 1100 °C [25], and wild fires can reach up to 2000 °C [17].

A laboratory-based experiment using a furnace has been an alternative method to incinerate teeth in the previous studies investigating dental burned remains [26-30]. In contrast to a fire simulation, a furnace incineration generally does not produce flame, unless there is combustible material present. Flame is a hot incandescent zone of fuel gases or vapors and oxygen where combustion occurs [23]. Unlike the controlled manner of furnace incineration, the combustion process in fire is not constrained by any specified variable such as the availability of oxygen and the airflow of the surrounding [31, 32]. The absence of flame distinguished a furnace incineration from a fire simulation.

Pope and Smith [33] investigated burn patterns of skulls that was subjected to a fire simulation. In a recent study by Carroll and Smith [34], the value of fire simulation experiments in the analysis of burned bone from both archaeological and forensic contexts was investigated. However, both studies were limited to burned bone. In the current study, a real-fire scenario was constructed by incinerating pig heads in a compartment fire. The study aimed to assess the practicality of a fire simulation as an experimental approach in the study of burned dental remains and to evaluate the morphological changes observed in teeth burned in a fire simulation.

Materials and method

The samples used in this research were six intact pig heads *(Sus scrofa domesticus)*: four two years old adults and two six months old juveniles. These ages were chosen based on the tooth developmental stages. All permanent teeth have erupted around two years of gestational age, whereas all deciduous teeth have erupted around six months of gestational age.

The fire simulation was performed in collaboration between the author with the University of Adelaide Forensic Odontology Unit, South Australian Police (SAPOL) Forensic Service Branch, Country Fire Service (CFS) and Metropolitan Fire Service (MFS) (Figure 1). It was conducted at the South Australian Country Fire Services State Training Centre, Brukunga, South Australia, Australia.

A compartment fire was constructed and controlled by a group of experts from CFS and MFS. The burning process took place in special burn chambers (refer to supplementary data). Each chamber was a confined space of rectangular shaped that measured 2.5 m × 2.0 m × 4.0 m with doors at the entrance and both sides to allow access for the recovery team. A steady-state fire was constructed for this fire simulation. The fuel was sourced from liquefied petroleum gas and timber. Thermocouples with metal-sheathed fibre-glass extension wires were placed at different heights of 1.2, 1.7 and 2.3 m – one in the bottom left corner of the room was used to detect and document the temperature inside the burn chamber. The temperature changes throughout the burning process were continuously monitored through a screen that was in a control area in an adjacent building. The data of the entire burn process were recorded electronically. The fire was extinguished using high-pressure water spray by the firefighters according to the standard operating procedures of CFS [35].

Figure 1—South Australian Country Fire Service personnel participating in the fire simulation.

Fire simulation 1

In the first simulation, a compartment fire was constructed to simulate a house fire. A mock-up scenario, where there were three adults and two juveniles in a living room, were created inside the chamber. All the pig heads were placed on four solid metal frames at two different heights: 1.6 m and 1.0 m. The heights were based on the estimation of an average standing adult that was 1.6 m, and an average standing juvenile and a sitting adult that was 1.0 m. All three adult pig heads were placed separately: two pig heads at 1.6 m and one pig head at 1.0 m. The two juvenile pig heads were placed together on a solid steel frame of 1.0 m height. All pig heads were exposed to fire at the same time for a duration of 20 min before the fire had to be abruptly extinguished for safety reasons (Figure 2).

Figure 2—The flashover had forced the fire personnel to extinguish the first fire simulation immediately for safety reason.

Fire simulation 2

The method of incineration was revised to obtain complete burning of soft tissues. In the second fire simulation, only one adult pig head was involved. In contrast to the compartment fire in the first fire simulation, the type of fire that was constructed to burn the pig head was a flame immersion (Figure 3). The duration of the burning process for both fire simulations was 20 min.

Figure 3— An adult pig head was incinerated in a flame immersion (second fire simulation).

Recovery of the burned remains

Following both fire simulations, all the pig heads were recovered using the protocol at the scene as suggested in the Disaster Victim Identification Forensic Odontology guide [36]. The recovery team was led by a Forensic Odontologist and a scene coordinator from SAPOL Forensic Response Section of Fire Investigation and was performed by MFS personnel (refer to supplementary data). Removal of the jaw and teeth were performed under laboratory conditions on a sterilized workbench. The surface of the bench was cleaned with 4% sodium hypochlorite prior to the placement of the burned pig heads for contamination control purposes. The jaws were then photographed and digitally stored in the following formatting: Tagged Image File Format (TIFF) and Joint Photographic Experts Group (JPEG). The characteristic changes of the teeth including colour changes, fracture pattern and fragility were interpreted based on visual observation.

Results

Fire simulation 1

In the first simulation, the temperatures of the fire fluctuated throughout the burn process and varied greatly between different areas in the burn chamber (Figure 4). Within a few minutes of the burn, the temperature was 90°C. From then the temperature spiked up to a maximum of 577.6°C. The temperature was stable and then the temperature rapidly decreased to 75°C at the end of the 20-min burn before a flashover occurred. The flashover had forced the fire to be immediately extinguished by the firefighters using high-pressure water spray (Figure 2). It was observed that the features of all pig heads, two adults and two juveniles were visually similar externally as they were completely burned, blackened and carbonised.

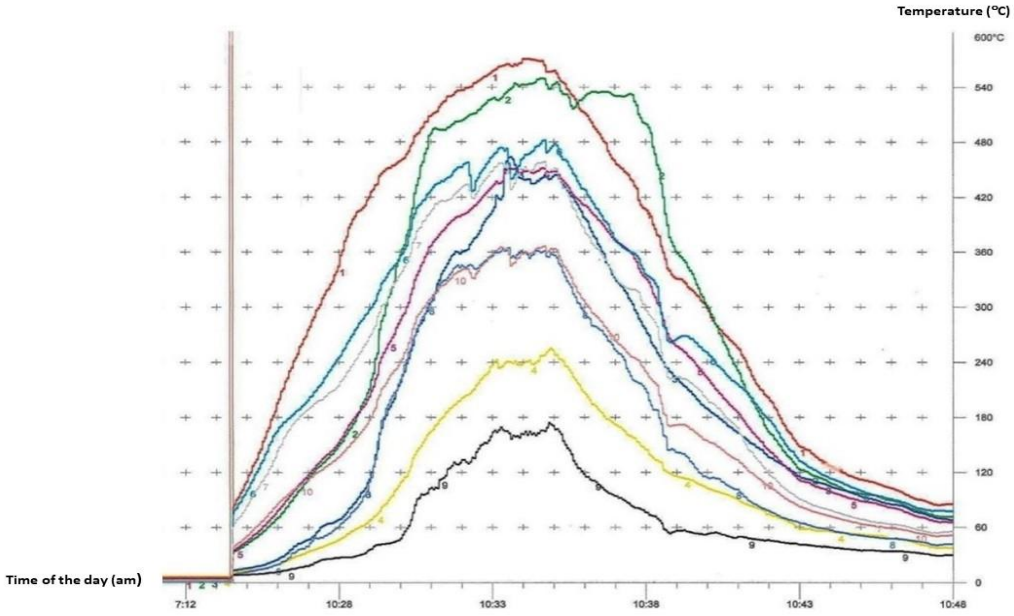

Figure 4—An example of a graph depicting real-time temperatures in the burn chamber that was recorded during the fire simulation. Different coloured lines signify different area in the chamber. The red line represents the front upper area of the chamber where the pig heads were placed. The temp

Fire simulation 2

In the second simulation, wood fuel was placed directly beneath the pig's head supported by a metal framework. After evaluating the outcomes of the first simulation, the combustion technique was modified to attempt for a complete burn of soft tissues of the pig head and the varying levels of incinerated teeth. Following the direct exposure to the fire immersion the visual appearance of the pig head was completely burned, blackened and carbonised (Figure 5). The maximum temperature

of the fire was 120°C and decreased to 45°C after 20 min. The maximum temperature was about five times lower than that in the first fire simulation.

Figure 5—The condition of an adult pig immediately after its recovery from the second fire simulation (fire immersion). The pig head looked completely charred, blackened and carbonised.

Morphological observation of the teeth

Table 1 showed physical changes of the teeth in adult and juvenile pig heads. Anterior teeth were burned and calcined whilst posterior teeth revealed minor changes. The anterior teeth were completely calcined and became ashen white. They were extremely fragile, thus easily crumbled when being handled. No dislodgement of anterior roots was noted. All molar teeth in adults and juvenile pig heads were, however, barely affected by the fire. The premolar teeth adult and juvenile pig heads turned blackish, but were still structurally intact. No dislodgement of tooth or any of its part was noted in both adult and juvenile pig heads. All pig heads, especially from the first simulation, were not completely incinerated. The soft tissues surrounding the jaws of adults and juveniles including gums, tongues, palates, cheeks mostly still present and barely burned by the fire.

Table 1—Post-incineration examination of the teeth revealed calcined anterior teeth and minor changes in the posterior teeth.

Fire simulation	Pig head	Anterior teeth	Posterior teeth
1	Adult		
	Juvenile		
2	Adult		

Discussion

Currently, little is known about the technical details of a fire simulation for the study of burned remains. A similar issue was mentioned in a recent study whereby the current literature lacks critical documentation and evaluation of open air field experimentation [34]. This paper discussed the findings of conducting a fire simulation experiment in investigating burned dental remains and lessons that can be learned from the fire simulation.

Lesson #1

Incineration effects of a fire simulation on teeth were different than the effects of incineration using a furnace. Using a furnace, teeth are subjected to a consistent and controlled incineration. As a result, a series of colour changes is usually observed in burned teeth as temperature or duration of exposure increased. However, in a fire simulation, the physical changes of burned teeth were not uniform across the mandibular arch. The anterior teeth became ashen white and fragile due to the direct

exposure of heat from the fire that led to a calcination process in the tooth structure. This resulted from evaporation of the water content and the complete combustion of the organic materials in the teeth [8]. The posterior teeth of both adults and all molars in juveniles were barely affected by the fire due to the protection afforded by the substantial thickness of jaw bones and soft tissues. The result of this study is supported by a previous study which states that the characteristics of teeth do not change uniformly [6]. The presence of flame and the temperature fluctuation during the burning process justifies the non-uniform incineration of the teeth.

Lesson #2

The fire had to be abruptly extinguished due the untenable spread of a flashover. Flashover is the ignition of an object below a scorching fire by radiation that can occur when the upper layer of the hot gas temperature ranges between 500°C to 600°C [37]. In this simulation, the temperature spiked up to its highest point of 577.6°C during the flashover. Although liquefied petroleum gas and timber were used as fuels, melted pig fats that were dripping down from the head had interfered with the compartmentalised fire that progressively became a flashover. Melted pig fats are ignitable liquids that were overlooked and had amplified the temperature of the fire. A combination of substantial content of fat, presence of char, and a flame caused the subcutaneous fat of the pigs to render [38].

Lesson #3

A fire simulation is not an easily reproducible condition. How much ever meticulous and rigorous an experiment is being constructed, there will be variables that are difficult to control [39]. Simulating a fire scene is considerably difficult because of the uncertain changes such as: fuel supply, flickering of flames, exposure to sources of high temperature, radiant heat, airflow, and availability of oxygen. These changes are underlined as factors influencing the intensity of a fire [21, 31, 32, 40, 41]. In this study, the musculoskeletal structure of pig head was not burned and only the skin on the surface of the heads was charred. These results have also been observed in a recent study whereby deer carcasses retained a lot of soft tissue and the bones displayed a wide range of colour alterations following a fire simulation similar to this study [34]. This occurred because a complete cremation can only be achieved when the temperature exceeds 700°C [42], whilst the maximum burning temperature

attained in the first fire simulation of this study was 577.6°C. In addition, complete thermal decomposition of the whole body can only be obtained when burning conditions are inconsistent in relation to temperature, fuel and oxygen [43].

Lesson #4

Mutual understanding, skills integration and collaborative efforts between forensic scientists and forensic fire experts are important for better communication and to enhance evidence handling skills at the fire scene. In addition, the need of forensic specialists to attend forensic scenes that require their expertise has been an ongoing debatable issue, as in many countries, most of the scene are attended by police personnel only [44]. Macoveciuc et. al [45] emphasised the need for a forensic anthropologist presence in evidence recovery at a fire scene. This suggestion corresponds to the recommendation made by Kelty and Julian [46], in which forensic experts from respective fields should voice their expectations and explicitly define their needs to prevent unnecessary frustration.

Limitations

Firstly, the burn chamber is an existing burn facility that has been a regular site to conduct fire-related trainings, but no fire simulation of burning animal or human body specimen had ever been performed at the facility previously. This fire simulation was the first of its kind that was performed at the facility, thus no standard or cautionary measures customized for the animal burning were available at the time of this fire simulation. Numerous factors affecting an uncontrolled fire such as concentrations of various gases, heat transfers, solid dynamics and combustion should be thoroughly assessed prior to conducting a fire simulation.

Secondly, three specimens consist adult pig heads, have raised the question of similarity with humans' jaws as having thick jaw tissues caused heat protection of teeth that was evidently obvious. In a study related to odontology DVI training, Berketa et al. [47] have found that there are differences compared to humans in anatomy and behaviour of pig heads following exposure to heat and fire. The average thickness of jaw tissues between juvenile pigs and adult humans is found to be similar, with 1.54 cm for pig and 1.38 cm for human [48]. However, in addition to juvenile pig heads, we had also used adult pig heads which were substantially bigger than the average adult human heads.

Thirdly, pig teeth were used as proxies to human teeth. This is because both species share similar tooth size, morphology composition and period of development [49-51]. The enamel and dentine in pig teeth and human teeth are also similar because both are omnivores [52]. In addition, previous studies have shown that similar thermal alteration patterns were observed between the skeletal remains of human and pig [8, 50, 53]. Despite the similarities, it must be noted that pig teeth have a greater number of cusps, more irregularities and less resistant to compressive stress compared to than human teeth [54, 55].

Future work

The observations and the lessons obtained from this study has increased our knowledge of a fire simulation as an experimental approach to study burned dental remains. This study has demonstrated the challenges of conducting a fire simulation to incinerate dental remains including laborious work and environmental variables including wind and melted pig fats are difficult to control. Additional research questions concerning other variable factors that can affect the thermal alterations of the teeth such as the combustion products, smoke and the chemical mixtures generated in a real-life fire, can be addressed in future work involving fire simulations.

Future work could construct a more realistic fire simulation that closely mimics a fire event leading up to death such as an accidental house fire or a fire to conceal a murder such as a deliberate cremation. The structures of the skeletal remains that were burned using different types of fires were shown to change at different rates [34]. The combustion from the simulated house fire was rather intense but rapid (160 min), whilst a pyre fire demonstrated a continuum and it took a longer time (210 min) to reduce the skeletal remains of a deer to calcine [34]. Perhaps, full pig carcasses can be used as human proxies rather than just the pig heads. This is important as it allows the reconstruction of the body position of fire victims and to observe the behavioural alterations of the animal remains following the fire exposure. This may provide insights on how human bodies might behave in a fire catastrophe. A recent study of a 20-minutes fire simulation involving deer carcasses has demonstrated the joint flexure of the limb muscles, which explained the occurrence of a pugilistic position [34].

Furthermore, one may consider sourcing un-embalmed human cadavers or cadavers where the bodies were not treated with preservative substances such as formalin or balsam as specimens in a future research. This would allow comparative analysis of the fire simulation effects between pigs and human, and subsequently it can contribute further knowledge to the current research. In fact, a recent study has suggested that pig cadavers are an ideal proxy in most forensic experiments and human cadavers are only necessary in comparative studies involving other species or to validate forensic methods [56].

Conclusion

This study provides justifications on the practicality of a fire simulation as an experimental approach for the study of burned dental remains. A fire simulation is not always feasible due to the variability of the factors influence burning. Nevertheless, this study was a simple pilot study that offers insight for the future research that intends to utilise fire simulation to investigate burned dental remains.

Conflict of interest

The authors declare no conflict of interest with this research.

Ethics

The handling of the animal remains in this study was done in line with the University of Adelaide Animal Ethics.

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Supplementary data

Lessons learned from a fire simulation as an experimental approach to study burned dental remains

S 1—The special burn chambers at the South Australian Country Fire Services State Training Centre, Brukunga, South Australia, Australia where the fire simulation experiments took place.

S 2—The compartment fire to simulate a housefire was constructed and controlled by the fire personnel from the South Australian Country Fire Service and the South Australian Metropolitan Fire Service.

S 3— The recovery of an adult pig head was performed by CFS and MFS personnel following the second fire simulation.

Chapter 3

Integrating spectrophotometric and XRD analyses in the investigation of burned dental remains

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Contribution to the paper	Research planning, project administration, samples analysis, data curation, data interpretation, visualisation, wrote manuscript and acted as corresponding author.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above).
ii. permission is granted for the candidature in include the publication in the thesis.
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Melissa A. Humphries		
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Signature		Date	21/04/2020
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Contribution to the paper	Conceptualization, resources, supervision, manuscript evaluation and funding acquisition		
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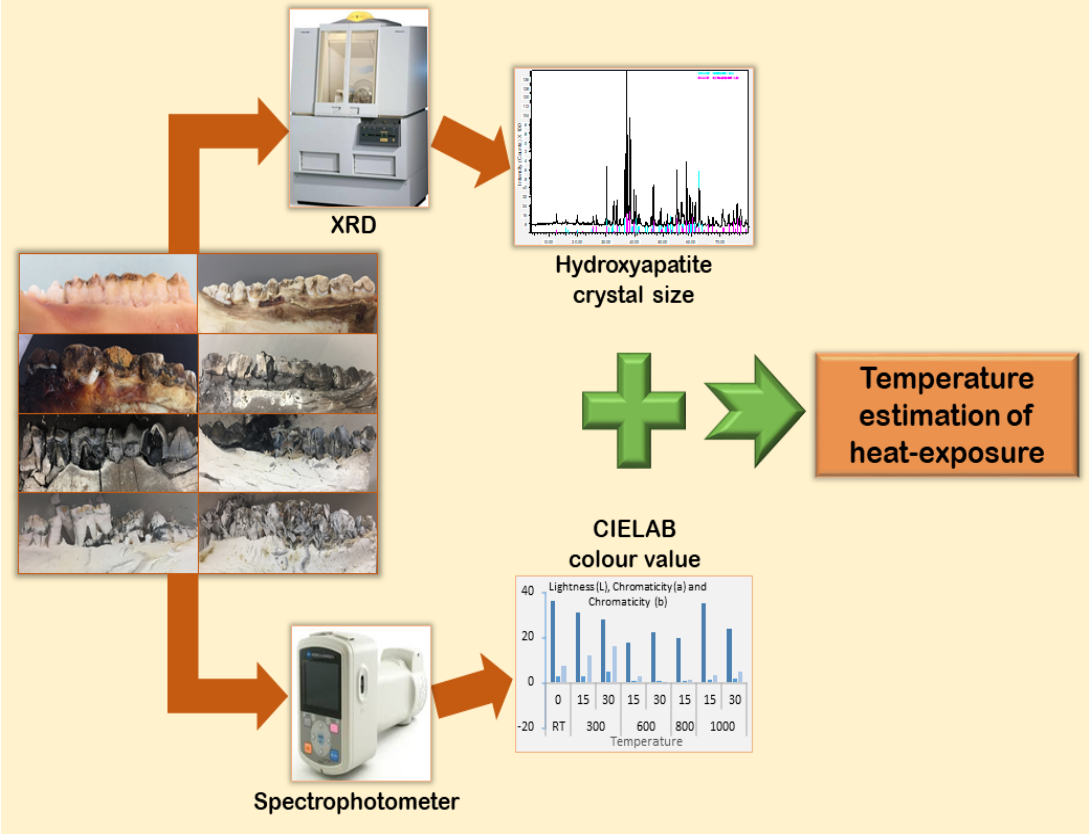

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Integrating spectrophotometric and XRD analyses in the investigation of burned dental remains

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ABSTRACT

Heat alters colour and crystallinity of teeth by destruction of the organic content and inducing hydroxyapatite crystal growth. The colour and crystallite changes can be quantified using spectrophotometric and x-ray diffraction analyses, however these analyses are not commonly used in combination to evaluate burned dental remains. In this study, thirty-nine teeth were incinerated at 300–1000 °C for 15 and 30 min and then measured using a spectrophotometer and an x-ray diffractometer. Response variables used were lightness, L*, and chromaticity a* and b* and luminance (whiteness and yellowness) for colour, and crystal size for crystallinity. Statistical analysis to determine the attribution of these variables revealed yellowness and crystal size were significantly affected by temperature ($p < 0.05$), whilst duration of heat-exposure showed no significant effect. This study suggests the inclusion of both spectrophotometric and x-ray diffraction in investigating thermal-heated teeth is useful to accurately estimate the temperature teeth are exposed to.

1. Introduction

The resilient structure of teeth to withstand the test of time and adverse environmental conditions means that teeth are frequently recovered from archaeological settings. Through burned dental remains, archaeology experts can analyse and interpret the demography, cultural practice and past ritual ceremony. Teeth also can be crucial evidence for human identification after a fire. Analysis of temperature-dependent characteristics of teeth including the alterations of colour and crystal size can provide contextual information about a fire's condition such as fire temperature, and eventually facilitates an investigation of burned dental remains [1–4]. For this reason, the investigation of burned dental remains is of ongoing interest in forensic science and archaeology. Teeth are the hardest structure of the human body and the heat insulation gained from the surrounding musculoskeletal structures, usually survive high temperatures and are the least of all body parts affected by the fire [5,6]. Burned dental remains found in archaeological sites (e.g. cultural cremation practice), and forensic cases (e.g. aircraft accidents, vehicle and house fires, and bushfire) are commonly fragmented [7]. Fragmented tooth crowns are usually recovered at a fire scene because they tend to break and fall apart due to direct heat exposure [8]. These dental fragments are suitable to be used for spectrophotometric and x-ray diffraction (XRD) analyses.

Teeth are made up of inorganic and organic components in various proportions. For example: enamel is composed of 97 wt.% of inorganic matter (hydroxyapatite) and 3 wt.% of organic matter (collagen, proteins and lipids); and dentin is composed of 70 wt.% of hydroxyapatite and 30 % of organic matter [9]. The organic matter in teeth is present in an aqueous-gel environment where collagen, proteins and lipids are kept hydrated [10]. On heating, teeth undergo extensive structural changes and the ratio of mineral-organic contents is gradually altered. From 110 to 260 °C, dehydration occurs in which hydroxyl bonds break and eventually

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teeth lose any water molecules [11]. During this time, collagen gradually degrades [12]. When the temperature is increased up to 500 °C, combustion of the organic matter begins to occur especially in collagen-rich dentin [13,14]. The depletion of organic matter decreases the pH inside the tooth and leaves voids allowing the growth of hydroxyapatite (HA) crystals to fill up the interspaces further reducing the environmental pH to less than pH 4.2 [15]. HA is at its the most stable at neutral pH, therefore in an acidic environment HA crystals become unstable [16]. With an excess of Mg^{2+} ions, the growth of HA is halted and HA transformed into a more stable form of calcium phosphate known as whitlockite [$Ca_9Mg(HPO_4)(PO_4)_6$] [17]. Above 1100 °C, HA crystals melt and coalesce to each other [18]. This process can be reflected through alterations in the outward appearance of teeth including colour and crystallinity [18].

1.1. Colour analysis

The colour of teeth is said to be influenced by factors including lighting conditions, translucency, opacity, light scattering and gloss [19]. As the intensity of heating increases, tooth colour progressively changes. In general, the sequence of colour changes is from its neutral colour (yellowish white), to brown, black, blue-grey, and finally chalky white [8,20,21]. Factors affecting these changes are temperature, duration, oxygen availability and other related influences [10,22]. Traditionally, many studies have analysed colour changes of incinerated teeth visually and compared the observation to a colour guide such as the Munsell colour system [1,2,10,20,21]. Despite the claim made by Shipman et al. [10] that the Munsell colour chart system offers a standardised, reproducible interpretation of quantified colours, the reliability of such colour chart can be argued because the interpretation process is subjected to individual perceptions and variations among observers.

More generally, the interpretation of colour based on visual observation is subjective because this method entirely depends on an individual's perception [19,21]. Due to this subjectivity, the ability of individuals to describe colour is inconsistent from time-to-time [23]. Hence, it is not surprising that colour descriptions of heat-treated teeth vary from one study to another [1,10,20]. The lack of objectivity in visual observation method has led to a suggestion to use a quantitative analysis to measure colour changes [21]. A study by Rubio et al. [24] has evaluated colour of heat-treated teeth using a spectrophotometer. According to their study, the application of spectrophotometry using a Commission Internationale de l'Eclairage lightness (L^*) and chromaticity (a* and b*) (CIELAB) colour system is reliable as it provides objective colour data with high accuracy. CIELAB was developed by the International Commission on Illumination. Colour values revolve within CIELAB space are lightness (L^*) and chromaticity (a* and b*). Spectrophotometry also provides tristimulus values (X, Y and Z) that can be used to calculate whiteness and yellowness (luminance) of teeth.

1.2. Crystallinity analysis

Crystallinity is an attribute of conformational order within a crystal lattice [25]. Teeth exhibit small size of HA crystals around 22 nm with irregularities in the lattice [4]. Crystallinity measurement has been used to indicate thermal modification of HA minerals in teeth and bones [25]. The growth of HA crystals can be measured using an x-ray diffraction (XRD) analysis, where the output is a diffraction pattern consisting of a set of diffraction peaks. Sharpening of the diffraction peaks in XRD patterns is associated with increased temperature of thermal treatment and increased HA crystallite size [10,26]. Piga et al. [4] has indicated the use of XRD to estimate burning temperatures ranging from 200 to 1000 °C.

1.3. Aims of the study

Temperature and duration of heat-exposure are known to influence the degree of structural alterations in teeth including colour, crystallinity and texture [27]. This study aimed to quantitatively evaluate effects of temperature and heat-exposure duration on colours and crystallinity of teeth. CIELAB colour space (L*a*b*), luminance (WI and YI), and HA crystallite size were selected as the response variables against a range of temperatures and durations of exposure. We hypothesised that all response variables should change as both temperature and duration change. We also hypothesise that the profile of the observed changes should be the same across all variables. This study also aimed to evaluate the potential of colour and crystallinity of teeth as indicators to estimate temperature of fire.

2. Methods

2.1. Sample preparation

Mandibular jaws from domestic pigs *(Sus scrofa domesticus)* were obtained from a local abattoir. The pigs were adults of average two years age in which teeth development was completed. Only mandibular bones with fully developed, sound and intact premolars and molars were selected. Mandibles with posterior teeth that had carious lesions were excluded.

Four mandibles were selected. Each mandible was cut into two segments, making a total of eight segments. Each segment had the first premolar as the anterior border and had the second molar as the posterior border. All the attached muscles and fat were completely removed from the bone surfaces using sterile surgical blades. The surgical blades were changed for every mandibular segment. The mandibular segments were cleaned with distilled water. The number of samples are 48 teeth (n = 48). Five teeth from a mandibular segment were removed and kept as control samples at room temperature.

2.2. Furnace incineration

The incineration process of the samples was performed in a controlled condition using an electrical furnace (Ward, Serial No: 12098, South Australia) at a laboratory operated by CSIRO Land and Water Division, Urrbrae, South Australia. Mandibular segments were incinerated at each of the following temperature/time combinations: 300 °C/30 min, 600 °C/30 min, 1000 °C/30 min, 300 °C/15 min, 600 °C/15 min, 800 °C/15 min and 1000 °C/15 min. Each mandibular segment was placed in a crucible with the buccal surface facing upward. It was then positioned in the centre of the pre-heated furnace for the assigned duration.

2.3. Visual examination

Post-incineration, changes in the teeth were examined by visual observation and were recorded as photographic images in the following formatting: Tagged Image File Format (TIFF) and Joint Photographic Experts Group (JPEG). Feature changes including colour, fracture, and any damage seen in teeth were interpreted subjectively.

2.4. Spectrophotometric analysis

Colour was measured using a portable handheld spectrophotometer (CM-700d, Konica Minolta Sensing Americas, Inc., U.S.A).

An 8-mm target mask with plate was attached at the lens to switch the illumination area. Colour data software (SpectraMagic™ NX CM-S100w) was used to operate the instrument from the computer, record measurements, process the data and for file management.

The spectrophotometer was calibrated twice prior to usage. The first calibration was a zero calibration using a zero calibration box. The second calibration was a white calibration using a white calibration cap with calibration data. The parameters of the spectrophotometer were set to a uniform colour space as recommended by CIE [28], the observer angle was 2° and the illuminant condition was D65. The data collected were the CIELAB L*a*b* values and the luminance values that are X, Y and Z. L* value indicates the lightness of an object's colour on a scale from 0 (black) to 1 (white), and a* and b* are the chromaticity of an object's colour. The value of positive a* is for the redness and negative a* is for greenness. The value of positive b* is for the yellowness and negative b* is for blueness. Measured values of a* and b* near to zero indicates an object of neutral colour, either white or grey.

The whiteness index (WI) and yellowness index (YI) were calculated from the luminance values to analyse the whiteness and yellowness of the teeth. The calculations were performed using the formulae proposed by ASTM Method 313 [29]:

$$WI = Y + 800(0.3127 - X) + 1700(0.3290 - Y)$$

$$YI = 100\frac{[100(1.2985X - 1.1335Z)]}{Y}$$

The buccal surface of the tooth crown was the target area to measure the colour. Five measurements were recorded at the same target area and the average of these measurements were calculated to obtain the final measurement.

2.5. XRD analysis

Forty-eight teeth were examined at the X-ray Diffraction Laboratory, CSIRO Land and Water Division, Urrbrae, South Australia, to measure the crystal size of HA. The tooth crowns were removed and manually ground into a powder form in an agate mortar and pestle. Fine powder (~50 mg) was sprinkled onto Si low background holders for XRD analysis. XRD patterns were recorded with a PANalytical X'Pert Pro Multi-purpose Diffractometer using Fe-filtered Co Kα radiation, automatic divergence slit, 2° anti-scatter slit and fast X'Celerator Si strip detector. The diffraction patterns were recorded at a scan rate of 2.43 °C two theta per minute giving an overall counting time of approximately 30 min. Phase identification was performed on the XRD data using in-house software (XPLOT) and HighScore Plus (from PANalytical) search/match software. Calculations of crystallite size were performed using the TOPAS refinement parameter "Cry Size L" that is suggested in the TOPAS-Academic technical reference [30].

2.6. Statistical analysis

All statistical analyses were completed using the statistical software R [31]. Correlations between response variables were analysed using the built-in 'cor' function. This was necessary to ascertain if multivariate analysis was required. Response variables which are theoretically related and moderately correlated should be fit in a multivariate model. If response variables are weakly correlated than separate univariate analyses can be completed. Alternately, extremely high correlations between response variables suggest redundancy and the number of response variables can be reduced to a representative set.

Linear models were fit to the chosen response variables using the 'lm' function. Possible variable transformations were explored using the boxcox function of the MASS package and all post hoc analyses were completed using a Tukey HSD correction to control the family-wise error rate.

3. Results

3.1. Visual inspection

Due to the bony protection afforded to the roots, the colour changes primarily focused on the coronal portions of the teeth (Fig.1). For 15-min heating, teeth colour changed from neutral white-yellowish to opaque white with no signs of crack lines or fracture at 300 °C. At 600 °C, the tooth crown turned light grey in colour and the crowns begun to noticeably crack. Visible fracture lines formed through enamel. Premolars became metallic grey and molars turned into black in colour at 800 °C, with fragmentation dramatically increased and fracture lines intensified all over the crowns. At 1000 °C, the teeth ultimately turned white with complete disintegration of the tooth crowns. There was loss of bone, making extended fracture lines on the roots visible. For 30-min heating, the observation contrasts with the 15-min heating at 600 °C where tooth crowns became greyish black and disintegrated whilst the roots were dislodged as bone cracked. Teeth became chalky white at 1000 °C and horizontal fracture lines were noted on the roots.

3.2. Spectrophotometric data

The spectrophometric data for the average values of L*, a*, b*, WI and YI of tooth groups at every temperature, separated by duration, are shown in Fig. 2. L* values decreased between control group and 800 °C. At 1000 °C, L* value increased, with the L* value of 15-min group similar to the control group. L* of the 15-min group were higher than L* values of 30-min group at 300° and 1000 °C. No apparent changes were observed between chromaticity a* for control teeth with the a* of 15-min group at 300 °C but a twofold-increase of a* for the 30-min group. At 600 °C, a* of the 15- and 30-min group reduced markedly approaching zero for white colour. The 800 °C 15-min group shows similar values to the 600 °C 30-min group. a* slightly increased from 800 °C to 1000 °C with a* value of the 30-min group almost twice the a* value of the 15-min group. Chromaticity b* increased from the control group to 300 °C, with double-hike for 30-min group and half-increment for 15-min group, changing for a more saturated yellow colour. A sudden drop of b* value at 600 °C for both durations with the yellow of 15-min group less saturated and the 30-min group changed towards blue colour. At 800 °C, b* was lessened and becoming more neutral yellow. b* was increased by threefold from 800 °C to 1000 °C, but the yellow saturation for 15- and 30-min groups were lower than the control group.

WI decreased from the control group to 300 °C for both time durations. WI kept on declining till 600 °C for the 15-min group. WI increased for the 600 °C 30-min group and only increased at 800 °C for the 15-min group. However, WI for the 1000 °C 15-min group rised markedly, in stark contrast to the 30-min group where the increase was minor. The highest value of YI is manifested in the 300 °C 30-min group before it significantly decreased at 600 °C with the lowest value seen in the 30-min group. A increase of YI is observed from 800° to 1000 °C

3.3. XRD data

Table 1 lists the average crystallite size of unheated and heated tooth samples. Control samples have an average crystallite size of 63.8 nm. Hydroxyapatite crystals gradually shrank when teeth were heated at 300° and 600 °C for 15 min. However, the 300 °C 30-

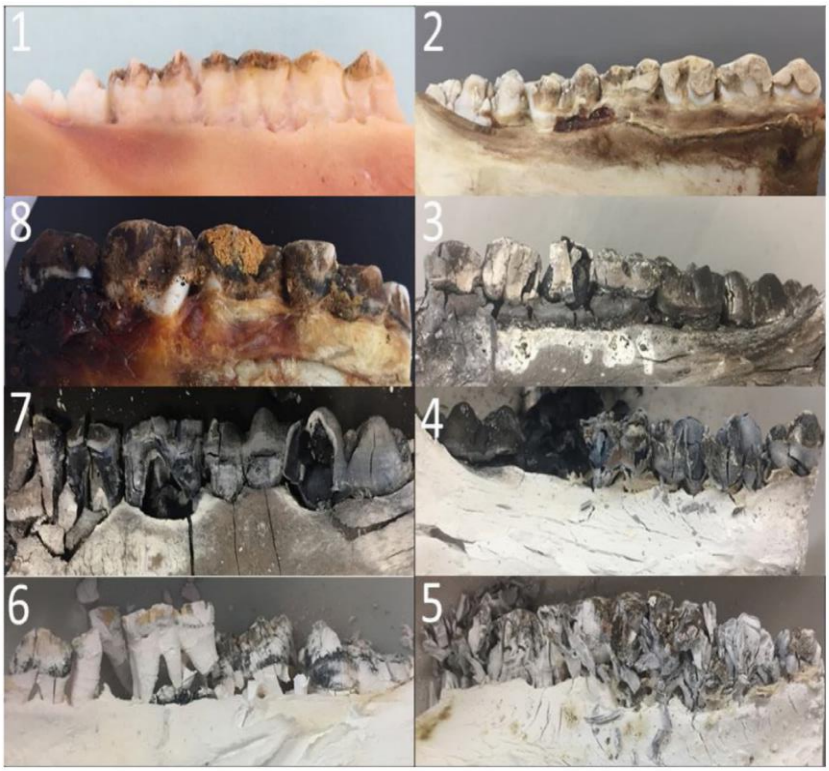

Fig. 1. Clockwise from top left: 1) Unheated teeth 2) Teeth heated at 300 °C for 15-min 3) Teeth heated at 600 °C for 15-min 4) Teeth heated at 800 °C for 15-min 5) Teeth heated at 1000 °C for 15-min 6) Teeth heated at 1000 °C for 30-min 7) Teeth heated at 600 °C for 30-min 8) Teeth heated at 300 °C for 30-min.

min group shows dramatic size reduction but a minor decrease in size at 600 °C. The crystallite size was then doubled after heating at 800 °C for 15 min. A dramatic increase, with more than 25-fold augmentation of crystallite size, was observed in teeth heated at 1000 °C for both durations.

Diffraction patterns of control and heated teeth are presented in Fig. 3. The diffraction (002) peaks corresponding to hydroxyapatite crystallite and used for crystallite size calculation are marked in Fig. 3. In general, the patterns denote a trend concerning the crystallite growth of samples heated for both 15- and 30-min durations: (i) no notable peak narrowing for control teeth and teeth heated up to 800 °C; (ii) Peak narrowed dramatically at 1000 °C. Peak widths of teeth heated for 30 min appearing similar those heated for 15 min. Interestingly, calcite or calcium carbonate ($CaCO_3$) was only detected in samples heated at 300 °C for 30 min. As expected, the formation of whitlockite was identified in tooth samples heated at 800 °C and 1000 °C.

3.4. Statistical analysis

The correlation between response variables was first tested to inform whether multivariate analysis techniques were required. Based on correlation coefficients (for details see Table 2 in supplementary data), strong correlations were found between a* and b* with YI ($p > 0.01$) and between L* and WI ($p > 0.01$), thus a*, b* and L* were omitted from further analysis with YI and WI forming the representative set of response variables. The correlations shown between CS, WI and YI were all weak, justifying the use of separate, univariate analyses rather than a multivariate technique. The predictors for each of these response variables are temperature and duration.

Two-way ANOVAs were then conducted considering both the main effects and interactions between temperature and duration for each of the three response variables, CS, WI and YI. Both CS and WI required log transformations to adhere to the assumptions of homoscedasticity.

3.5. Crystallite size (CS)

Two-way ANOVA revealed a significant effect of temperature on CS ($F(4,31) = 199.45$, $p < .001$) and failed to find a significant main effect of duration ($F(1,31) = 0.17$, $p = .683$) or interaction between duration and temperature ($F(2,31) = 1.25$, $p = .302$). Follow-up post-hoc Tukey HSD pairwise comparisons revealed that the CS of teeth incinerated at 1000 °C was between 15.8 and 48.5 higher than the controls, 25.8 and 64.9 higher than 300 °C, 35.1 and 88.2 higher than 600 °C and 14.1 and 43.2 higher than 800 °C (95 % confidence intervals), regardless of duration. The only other observable differences were higher CS at 800 °C than 600 °C (1.3–3.9) and higher CS at 600 °C than at room temperature (0.3–0.9) but these differences are both relatively small compared to those observed at the highest temperature.

3.6. Whiteness (WI)

There were no significant main effects or interactions observed for the two-way ANOVA of temperature and duration on WI (Temperature: ($F(4,31) = 1.14$, $p = .059$), Duration: ($F(1,31) = 0.60$, $p = .597$), Temperature*Duration: ($F(2,31) = 2.45$, $p = .103$)). However there was a trend toward significance for the duration variable. Further investigation with a larger data set may be able to explore this difference further.

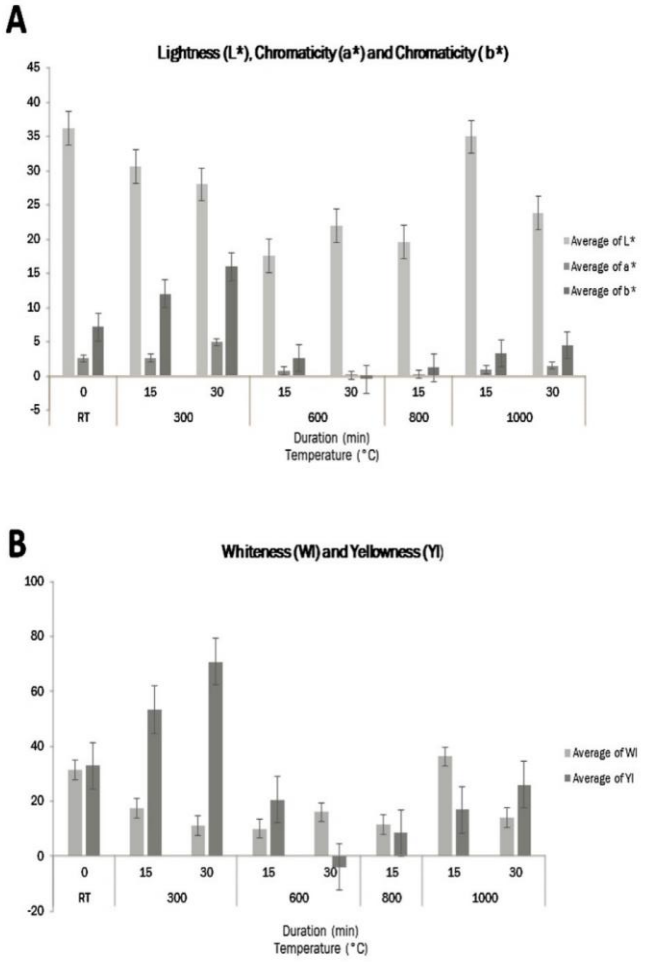

Fig. 2. Mean values for (A) lightness (L*), chromaticity (a*) and chromaticity (b*) and (B)whiteness (WI) and yellowness (YI) in tooth groups exposed to room temperature (RT), 300°, 600°, 800° and 1000 °C for 15- and 30-min. (N = 48).

3.7. Yellowness (YI)

The two-way ANOVA of temperature and duration on YI revealed the most complex results in the analysis with a significant interaction between the two predictor variables ($F(2,31) = 15.50$, $p < .001$). Pairwise comparisons with a Tukey HSD correction (for details please see Table 3 in supplementary data) found the following:

1 YI peaked at 300 °C. YI was significantly higher at 300 °C than any other temperature, regardless of duration.
2 At 600 °C, YI was between 6.2 and 42.6 higher after 15 min, than after 30 min. This was the only significant difference based solely on duration.
3 Teeth exposed to 600 °C for 30 min were between 18.6 and 55.1 higher in YI than the room temperature teeth and between 6.4 and 42.8 higher than teeth exposed to 800 °C for 15 min. However, teeth incinerated at 600 °C for 30 min were significantly lower in YI than teeth incinerated at 1000 °C for 15 min (2.6–39.0) and for 30 min (10.6–49.2).

Table 1
Average crystallite sizes of hydroxyapatites in teeth left at room temperature (RT) and teeth heated from 300 to 1000 °C for 15 and 30 min (min) were calculated from (002) peak diffraction pattern. In this study, crystallite size was measured in nanometer (nm). 1 nm =10 Å (Angstrom).

Temperature (°C)	Duration (min)	Mean Crystallite Size (nm)
RT	0	63.8
300	15	51.1
300	30	39.7
600	15	34.4
600	30	31.4
800	15	73.5
1000	15	1940.0
1000	30	1999.0

4. Discussion

This paper presents findings from spectrophotometric and XRD analyses of heat-treated teeth, and discusses the relationship between colorimetric variables, L*, a*, b*, WI, YI and HA crystal size. Two durations of heat-exposure, 15 and 30 min were analysed to investigate the effect of duration on colour changes and crystallite size. Both durations were specifically chosen because previous research has shown colour does not change in teeth after 30 min of incineration [20].

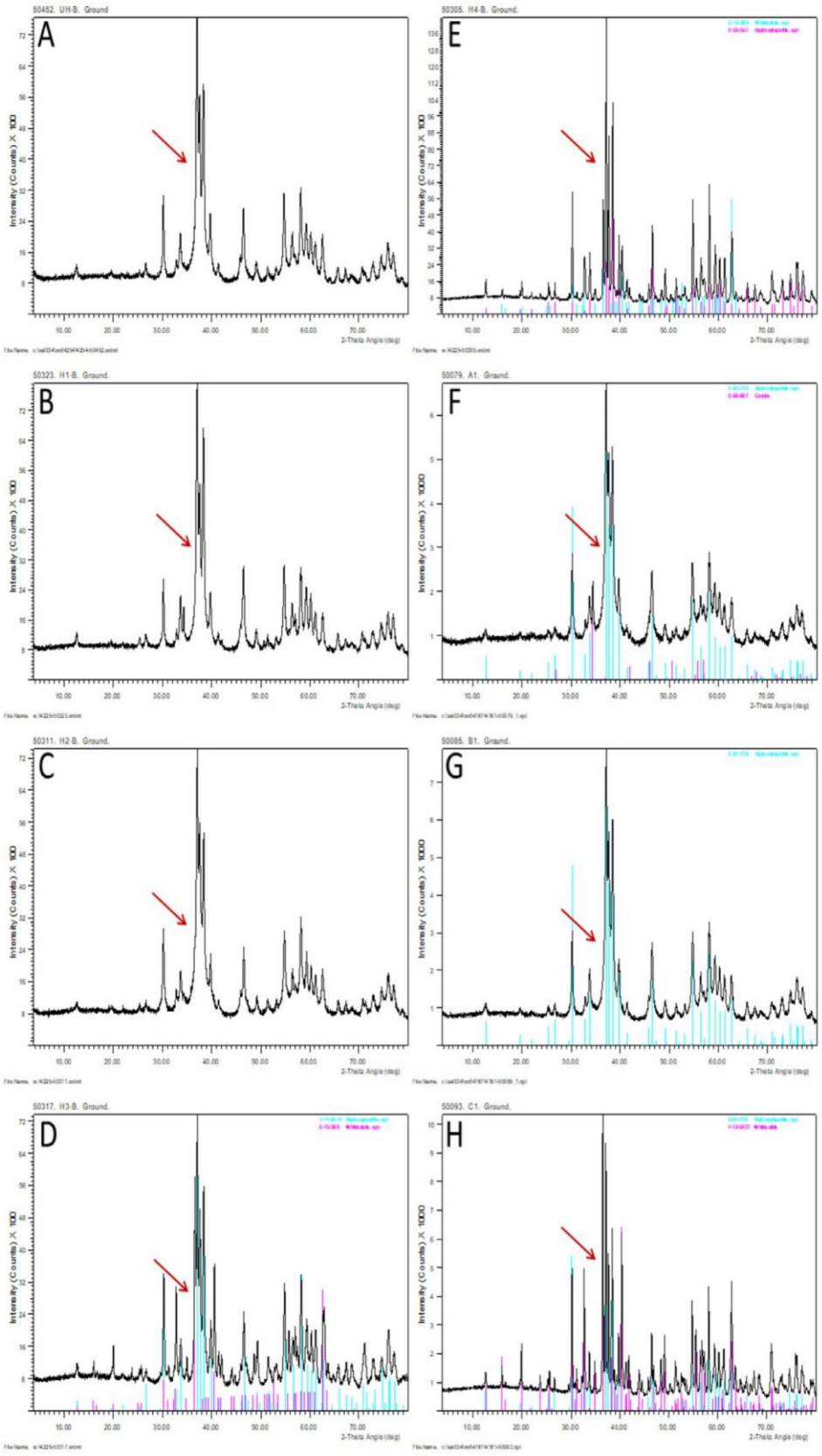

Fig. 3. Diffraction patterns of first premolars tooth samples: (A) Unheated (B) Heated at 300 °C, 15 min. (C) Heated at 600 °C, 15 min. (D) Heated at 800 °C, 15 min. (E) Heated at 1000 °C, 15 min. (F) Heated at 300 °C, 30 min. (G) Heated at 600 °C, 30 min. (H) Heated at 1000 °C, 30 min. The diffraction (002) peak of each sample pattern is pointed with an arrow. Sample heated at 300 °C shows the presence of calcite. Samples heated at 800 °C and 1000 °C for 15- and 30-min (A, D, E, and H) reveal the addition of whitlockite.

4.1. Spectrophotometric analysis

Despite the visual observation of teeth heated for 15-min and 30-min being different, our spectrophotometric results were generally unable to find a significant effect of duration on colour variables or crystal size ($p < 0.05$). This finding is in stark contrast with a previous study in which duration of heat-exposure was shown to affect tooth colour changes [21]. Our results also showed that the lightness, L^* value, is not affected by temperature. This finding contradicts the findings in a study by Rubio et al. [24] that L^* is a valuable determinant to estimate the incineration temperature. Our findings are interesting considering that dehydration was shown to have significant effect on the lightness of teeth [32]. However, the dehydration process is only accountable for the heating up to 500 °C [8,10]. In our study, the measure of lightness decreased from the room temperature to 600 °C, a range where teeth were losing the water component due to dehydration. Enamel was mainly affected as it is entirely made up of inorganic components and therefore, it became progressively porous. This finding is supported by studies in which the lightness of tooth was found to be proportionate porosity of enamel and decreased when they are dehydrated [33,34]. In addition, a major advantage of using the CIELAB colour system is that the difference between a standard and the measured colour can be quantified [35].

4.2. XRD analysis

In general, the most significant transformation of HA crystallite phase occurs after 800 °C. Reduction in crystallite size in 300 °C–600 °C sample groups could be due to the dehydration process that altered the chemical structure of HA. By 800 °C, HA crystallites progressively expanded to fill up the interspaces resulted from the combustion of organic matters. The sudden and striking growth of the crystallite size noted in teeth heated at 1000 °C is consistent to a previous study by Piga et al. [4].

The observed narrowing of the diffraction peaks is related to changes in the crystallite size that increases with heating temperature. Unheated teeth show relatively broader peaks than heated which reflect a poor crystalline apatite. Diffraction peaks became narrower and sharper, corresponding to an increase in the crystal size of hydroxyapatite and towards a more orderly crystalline arrangement in lattice plane, are observed as the temperature increased up to 1000 °C. These results are consistent with the most significant structural changes of the bone mineral occurring between 600 °C and 800 °C, as previously reported [10,25,36].

Interestingly, calcite only appeared in samples heated to 300 °C for 30-min. No calcite was detected in specimens of the 300 °C 15-min group. It was also absent in specimens of the 600 °C group. During heat treatment, calcium carbonate is formed when hydroxyl ions (OH^-) of hydroxyapatite are replaced by carbonate ions CO_3 [37]. This might explain why there is a marked decrease in size for the 300 °C 30-min group. Calcite is the most stable form of calcium carbonate ($CaCO_3$) [38]. It was reported that in bone, calcium carbonate formed after a complete combustion of organic matter around 600 °C and it disappeared after heating at 900 °C [39]. Contrasting to that, other studies have claimed that complete loss organic matters was at 350 °C, with carbonate ions decreasing around 400–700 °C [40,41]. By far, findings on the temperature at which calcite is formed and loss are greatly varied and mostly inconsistent. Being the third most abundant component of skeletal minerals [42], accidental carbonate contamination by adsorption on the surface of apatite crystals cannot be ruled out. The mechanism of calcite formation exclusively in the 300 °C 30-min samples is still obscure. Thus, further studies on the occurrence of calcite in burned dental remains are needed.

We found that at temperatures over 800 °C whitlockite formed. Whitlockite is a product of thermally-heated hydroxyapatite where over 800 °C, HA crystals coalesce to each other [43,44]. This finding corresponds to the whitlockite seen on the XRD pattern in human teeth heated after 750 °C [45,46]. The claim that no definite temperature at which transformation of bioapatite to whitlockite occur is therefore arguable [45]. The evidence of hydroxyapatite becoming whitlockite can aid in the estimation of temperature. The results presented here suggest that identification of whitlockite in burned teeth means the teeth have been heated at no less than 750 °C. This is an important indicator since the temperature of house fires, motor-vehicle accidents and fire disasters are 750 °C and above [7,47]. Also, whitlockite was consistently identified in all teeth heated to more than 800 °C, a finding that supports a study by Piga et al. of which whitlockite formed systematically in teeth heated at 750 °C [45]. It is worth noting that whitlockite can be formed as a result of other conditions in biological human systems such as pathological calcifications which include dental calculus, salivary stones, soft-tissue deposits and arthritic cartilage [48].

There was, however, a dramatic increase in crystallite size at 1000 °C suggesting that the HA crystallites may have recrystallised and expanded drastically to fill the voids created by dehydration and organic matter destruction. Dehydration or loss of water in the enamel and dentin occur in between 100–400 °C where hydroxyl bonds break during the process and water molecules evaporated, followed by the loss of organic matter after 400 °C [9]. Sudden increase of HA crystal size is noticeably consistent for all teeth heated at 1000 °C. This finding could be a useful feature to indicate if a dental remain has been subjected to temperatures higher than 800 °C. Our results correspond to a previous study where a marked increase in crystallinity with more ordered crystal lattice has been found in teeth heated above 700 °C [4].

4.3. Recommendations

We recommend the integration of spectrophotometric and XRD analyses on burned teeth to estimate the maximum heating temperature with greater accuracy. The credibility of spectrophotometric and XRD analyses when used individually to recognise burned skeletal remains and to estimate the maximum heating temperature have been proven in some studies [10,49,50]. Yet, the conclusions from the individual use of spectrophotometric or XRD analysis alone provide weak evidence of incineration for three reasons. Firstly, any change of colour seen in tooth does not necessarily means that it has been burned. The colour change can be influenced by multiple factors such as taphonomic effects (skeletal weathering and decomposition) [51] and lifestyle (diet, nicotine staining or fluorosis) [19]. Secondly, the growth of hydroxyapatite increased in a diagenetically altered teeth as seen in bone [52]. Diagenesis is a process that starts after the vertebrates die in which taphonomic factors such as elements of burial soil and the presence microorganisms influenced the alteration of the chemical structure and induced recrystallization in teeth and bones [53,54]. The increased of crystallinity was found to be inversely correlated with the collagen contents [55]. Thirdly, as an increase of crystal size is notable only from teeth heated more than 700 °C, the sole use of XRD is not very useful for estimating low temperatures.

Since the application of spectrophotometric and XRD analyses on real archaeological or forensic scenarios are still uncommon, we also recommend both analyses to be incorporated in the future research investigating heat-treated teeth. Combining analytical techniques to investigate burned skeletal remains has been suggested in a previous archaeology research to improve the robustness and accuracy of the interpretation [56]. Measuring the colour and crystallinity of

heat-treated teeth using both analyses have enabled us to obtain quantitative data with precision and accuracy. Both analyses are also cost-effective, require small amount of sample (0.5 mg) and the procedure is relatively simple. Indeed, instrument analyses offers objective, quantifiable, repeatable and quick measurement readings [57]. In this study, the spectrophotometer was calibrated to a white reference prior to measuring the teeth. The white calibration step allows the normalisation of the spectrophotometer to correctly obtain correct transmittance or reflectance factors throughout spectrum [58]. The application of spectrophotometer also can minimise interpretation errors [59]. XRD on the other hand has been claimed to be the ideal mean to express the crystallinity of a bioinorganic phase [45].

5. Conclusion

The longevity of burned dental remains has greatly benefited studies of archaeological and forensic sciences. This study recommends the integration of spectrophotometry and XRD analyses to be a routine practice in casework upon the discovery of burned dental remains. Simultaneous application of both analyses has proved to be able to accurately estimate maximum heating temperature of teeth. Also, the inclusion of both analyses in the investigation of burned dental remains could potentially amplify the strength of evidence in forensic identification and archaeological casework.

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Ethics

The handling of the animal remains in this study was done in line with the University of Adelaide Animal Ethics.

CRediT authorship contribution statement

Rabiah A. Rahmat: Project administration, Methodology, Investigation, Data curation, Visualization, Writing - original draft. **Melissa A. Humphries:** Formal analysis, Writing - original draft, Visualization. **Jeremy J. Austin:** Conceptualization, Resources, Supervision, Writing - review & editing, Funding acquisition. **Adrian M.T. Linacre:** Supervision, Writing - review & editing. **Mark Raven:** Resources, Formal analysis. **Peter Self:** Resources, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare no conflict of interest with this research.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.forsciint.2020.110236.

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Supplementary data

Integrating spectrophotometric and XRD analyses in the investigation of burned dental remains

S 1—Correlation coefficients between the values of response variables including CS, L, a, b, WI and YI.

Response variables	CS	L*	a*	b*	WI	YI
CS	1	.227	-.144	-.176	.315	-.159
L*		1	.282	.358*	**.887****	.210
a*			1	**.915****	-.077	**.907****
b*				1	-.017	**.966****
WI					1	-.138
YI			.			1

CS=Crystallite Size; L=Lightness; a=chromaticity of redness-greenness; b=chromaticity of yellowness-blueness; WI=Whiteness index; YI=Yellowness Index; Correlation is highly significant at **p < 0.01; Correlation is moderately significant at * p < 0.05.

S 2—Results of the pairwise comparisons with a Tukey HSD correction for yellowness index (YI)

300°C Duration	Temperature (Duration)	Lower	Upper
15	RT	2.2	38.6
	600 (15)	14.6	51.0
	800 (15)	26.8	63.2
	1000 (15)	18.2	54.7
	600 (30)	39.0	75.5
	1000 (30)	8.0	46.6
30	RT	19.6	56.1
	600 (15)	32.0	68.5
	800 (15)	44.2	80.6
	1000 (15)	35.7	72.1
	600 (30)	56.5	92.9
	1000 (30)	25.7	64.1

Temperature in degree Celcius (°C) and duration in minutes (min).

Chapter 4

The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data

Statement of Authorship

Title of paper	The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data
Publication Status	Unpublished and unsubmitted work written in a manuscript style
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Principal Author

Name of Principal Author (Candidate)	Rabiah Al-Adawiyah Binti Rahmat		
Contribution to the paper	Research planning, project administration, samples analysis, data curation, data analysis and interpretation, visualisation, wrote manuscript and acted as corresponding author.		
Overall percentage (%)	80%		
Signature		Date	04/05/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above).
ii. permission is granted for the candidature in include the publication in the thesis.
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Melissa A. Humphries		
Contribution to the paper	Statistical analysis and interpretation		
Signature		Date	21/04/2020

Name of Co-Author	Jeremy J. Austin		
Contribution to the paper	Resources, supervision, manuscript evaluation and funding acquisition		
Signature		Date	23/04/2020

Name of Co-Author	Adrian M. T. Linacre		
Contribution to the paper	Supervision and manuscript evaluation		
Signature		Date	21/04/2020

Name of Co-Author	Peter Self		
Contribution to the paper	Resources and XRD investigation		
Signature		Date	08/04/2020

Manuscript prepared for submission

The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data

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Abstract

This study presents a novel tool to predict temperature-exposure of incinerated pig teeth as a proxy for understanding impacts of fire on human teeth. Previous studies on the estimation of temperature-exposure of skeletal elements have been limited to that of heat-exposed bone. This tool was constructed based on the colourimetric and hydroxyapatite crystal size variables using data obtained from unheated pig teeth and teeth incinerated at 300°C, 600°C, 800°C and 1000°C. Through an analysis of variance, colourimetric and crystal size variables demonstrated the ability to predict incineration temperature. A predictive tool was developed using a multinomial regression model of colourmetric and crystal size data. A qualitative variable that allows an expert to describe the certainty of temperature of burning based on the observation of the tooth appearance was added to the model. As a result, a model predicting the temperature-exposure of the incinerated teeth into the category from which it was developed has an accuracy of 95%. Cross-validation of the full model using a different data set demonstrated an accuracy of 79%. Qualitative assessments of the heat-induced structural changes including visual observation, SEM, diffraction pattern, and mineral identification

were included as supplementary analyses in the tool to support the resultant predicted temperature. This tool along with the supplementary analyses is a holistic, robust and reliable approach to estimate temperature of heat-exposed pig teeth, with high accuracy, and may act as a valuable proxy to estimate heat exposure for human teeth in forensic casework.

Keywords: Teeth; Temperature estimation; Heat-induced change; Colourimetry; X-ray Diffraction; Multinomial regression model

Introduction

Predicting the maximum temperature of exposure of burned skeletal remains can greatly benefit the forensic investigation of a suspicious fire. It can aid in reconstructing the events leading up to the fire and to provide clues about what type of temperature a deceased body may have been exposed [1]. The pattern of heat alterations on the skeletal remains may indicate the burn is suspicious or accidental [2]. The heat-induced changes in burned teeth and bone when analysed using an appropriate method can help to piece together relevant information pertaining to the burned human remains [3].

Although teeth are resilient to heat, the structures are subjected to biochemical changes including the organic content, crystal size and the structural integrity of DNA molecule. These changes are temperature-dependent [4-7], such that teeth are a potential source of evidence to determine the temperature of the fire. In addition, the condition of the teeth recovered from burned victims is dependent on the maximum temperature the fire has achieved [8]. Thus, a precise and objective interpretation of the heat-induced changes in teeth can be a reliable approach to estimate the temperature of fire.

Colour model

Colour has been a predominant feature used to suggest the temperature to which the skeletal remains have been exposed [7, 9-15]. Visual observation is used frequently to identify these colour changes in heat-induced skeletal remains due to its simplicity. In general, the sequence of the colour changes in teeth and bone heated at an increasing temperature is similar and predictable, starting from the neutral colour of yellowish ivory, dark brown, charred black, light grey to calcined white [7,

16-19]. However, it must be noted within the temperature range the teeth and bone to which they were exposed, the colour change may vary. For example at 300°C to 500°C, teeth and bone can turn to brown-black to grey [7, 17, 20, 21]. Sandholzer [22] and Krap *et al*. [23] advised that temperature-specific colour schemes should be used cautiously and colour changes were assumed to be of minor value for forensic investigations because this method is subjected to individual perception and observer error.

Estimating temperature based on colour changes has progressed into an objective method that uses colourimetric analysis. Colourimetry is an analytical technique to specify, evaluate and predict the colour appearance using the 3-dimensional trismulus values (X, Y and Z) devised by the CIE (Commission Internationale de l'Eclairage)[24, 25]. Colourimetric data of lightness (L*), chromaticity (a* and b*), whiteness (WI) and yellowness (YI) can be obtained directly using a spectrophotometer [4, 11], or obtained indirectly from the digital photographic analysis of the skeletal remains [26]. Spectrophotometers use the standard light D65 illumination which enables the measurement of the colour to be taken under conditions that resemble daylight [24, 27]. Measuring colour using a calibrated instrument such as a spectrophotometer eliminates the risks of observer bias and errors. In recent published studies, colourimetry analysis that utilised a spectrophotometer to measure the colour was shown to be reliable method to estimate the temperature-exposure of incinerated teeth [4, 11].

Crystallinity analysis

Crystallinity analysis has been used in studies of heat-induced skeletal remains [28-30]. Hydroxyapatite (HA), the major mineral in teeth and bone shows the characteristics of nanosize crystallite, low crystallinity and high lattice strain [29]. Burnt teeth and bone show an increase of the crystallite size and crystallinity, and a reduction of the lattice strain [30]. X-ray diffraction (XRD) has been a widely used mineralogical technique to assess the changes in the crystallite structure of thermally-treated teeth and bone [30-32]. The diffracted x-rays produce diffraction patterns that consist of peaks [33]. The sharpening of the peaks indicates that the crystals are expanded in size, high crystallinity and lower lattice strain [32]. In previous studies, the increase of crystallite size and mineral transformation (HA to whitlockite, WHI)

indicated that the teeth and bone were exposed to temperature more than 700°C [4, 30, 34].

Pig teeth as a human proxy

Pigs *(Sus scrofa)* have been used as a proxy for humans in forensic experimental studies [14, 32, 35, 36]. This study was designed such that teeth should remain *in situ* within the jaw bone during incineration to retain the natural heat insulation for the tooth roots. Due to the limited availability and ethical concerns to obtain and incinerate human teeth that are embedded in the mandible or maxilla, pig teeth were chosen to substitute human teeth. Pig and human teeth share close similarities in terms of size, morphology and composition [37, 38]. In addition, previous studies have shown that the thermal alterations of teeth and bone of pigs and human are consistent [7, 14, 32].

Aims

This paper presents the assessment of colourimetric and hydroxyapatite crystal changes in incrementally-heated pig teeth, specifically with aims: (i) to validate the relationship of colourimetric value and hydroxyapatite crystal size of incinerated teeth with the heating temperature; and (ii) to develop a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data.

Materials and methods

Sample preparation

Fifteen mandibular jaw segments of adult domestic pigs (*Sus scrofa domesticus*) were obtained from a local abattoir. Each mandibular segment consisted of six posterior teeth; three premolars and three molars. All the attached muscles and fat were completely removed from the bone surfaces using sterile surgical blades. The surgical blades were changed for every mandibular segment. The mandibular segments were cleaned with distilled water. All dissection work was performed in the School of Animal and Veterinary Sciences, Roseworthy, The University of Adelaide, Australia.

Furnace incineration

Three mandibular segments that held a total of eighteen teeth were kept as control samples at room temperature (27°C). A total of twelve mandibular segments were incinerated for 15 min at the following temperatures: 300°C, 600°C, 800°C and 1000°C; with three mandibular segments in each temperature group. Each mandibular segment was contained in a crucible with its buccal (outer) surface facing upward. It was then positioned in the centre of the pre-heated furnace. The incineration process was performed in a controlled condition using an electrical furnace (Ward, Serial No: 12098, South Australia) at a laboratory operated by CSIRO Land and Water Division, Urrbrae, South Australia.

Unerupted, partially erupted and broken teeth (n = 9) were excluded from this study. In total, 96 skeletal samples comprising 81 teeth and 15 mandibles were included for further analysis post-incineration. Only teeth samples (n = 81) were used for the statistical analysis to develop the predictive tool. Bone samples were only used in the visual, scanning electron microscopy and X-ray diffraction pattern analyses for comparison purposes.

Visual examination

Post-incineration, gross changes in the teeth and bones including colour, fracture and dislodgment were documented digitally. Images of the samples were kept in the following format: Tagged Image File Format (TIFF) and Joint Photographic Experts Group (JPEG).

Scanning Electron Microscopy (SEM)

SEM was used for qualitative analysis of the microstructural changes in incinerated tooth crowns and bones. Incinerated tooth crown and bone samples from each temperature group were selected randomly for SEM analysis. Comparisons of the tooth and bone samples were made across the temperature groups (300°C – 1000°C). All electron microscopic work was performed at Adelaide Microscopy, the University of Adelaide.

Unheated samples and samples heated at 300°C were subjected to the dehydration protocol, which was an ascending ethanol series from 70% (v/v) to absolute ethanol (2x10 min). Samples incinerated at 600°C – 1000°C were already

completely dried. All samples were then chemically dried using hexamethyldisilazane (HMDS). This step was performed with full precautions including wearing the necessary personal protection gear in the fume hood. Samples were mounted on aluminium stubs with the buccal surface facing upward, sputter coated with 5 nm platinum, and examined in a Philips XL30 Field Emission Scanning Electron Microscope (ESEM-FEG, at 10 kV; FEI Company, Hillsboro). The buccal surface of each sample was examined and photographed at increasing magnifications of 1000, 2000, 3500, 5000, 8000, 10000, 15000 and 20,000×. Imaging was performed using the built-in secondary electron (SE) detector. The surface texture of the samples was visualised and recorded in digital formats: Tagged Image File Format (TIFF) and Joint Photographic Experts Group (JPEG).

Colourimetric analysis

Quantitative analysis of colour changes was only performed on teeth (n = 81). The colourimetric measurement could not be performed on nine teeth because these had crumbled completely post incineration. Colour was measured using a portable handheld spectrophotometer (CM-700d, Konica Minolta, Inc., Chiyoda City, Japan). An 8-mm target mask with plate was attached at the lens to switch the illumination area. Colour data software (SpectraMagic™ NX CM-S100w, Konica Minolta, Inc., Chiyoda City, Japan) was used to operate the instrument from the computer, record measurements, process the data and for file management. The colour measurement was performed according to the protocol described previously [4]. The buccal surface of the tooth crown was targeted for measuring the colour. Five measurements were recorded for each sample and the average of these measurements were computed by the spectrophotometer. The data collected were the following; CIELAB lightness (L*), chromaticity a* and b*; whiteness (WI) and yellowness indices (YI).

XRD analysis

Eighty-one tooth crowns were subjected to XRD analysis to collect crystallography information of the samples including the crystal size, diffraction pattern and mineral phase identification. Nine teeth had crumbled completely post incineration, thus had insufficient surface area for XRD analysis. Quantitative analysis of the crystal size was performed only on teeth whilst qualitative analyses including the assessment of diffraction patterns and transformation of the mineral

phases were performed on teeth and bones. All XRD analyses were performed at the X-ray Diffraction Laboratory, CSIRO Land and Water Division, Urrbrae, South Australia, according to the protocol described previously [4]. The tooth crowns and bones were manually ground into a powder in an agate mortar and pestle. Fine powder (~50 mg) was sprinkled onto Si low background holders for XRD analysis. Data of XRD patterns were recorded with a PANalytical X'Pert Pro Multi-purpose Diffractometer (Malvern Panalytical Ltd, Malvern, United Kingdom) using Fe-filtered Co Kα radiation, automatic divergence slit, 2° anti-scatter slit, and fast X'Celerator Si strip detector at a scan rate of 2.43°C two theta per minute giving an overall counting time of approximately 30 minutes.

Rietveld refinement of XRD data

The data of XRD patterns were processed for phase identification and crystal structure characterisation using the Rietveld refinement (RR) method. Complete RR procedures from uploading the XRD data, peak profile fittings, viewing XRD patterns and generation of the graphs were performed using the Profex-BGMN bundles 3.14.3 software [39]. Refinement parameters are summarised in the supplementary data (S1). The crystalline phases of hydroxyapatite (HA) and whitlockite (WHI) were determined from a comparison of the registered patterns in the International Centre for Diffraction Data (ICDD) powder diffraction file (PDF)[40].

Crystal size determination

The HA crystal size (CS) of all samples was obtained from the Profex-BGMN software [39]. Hydroxyapatite (HA) has an anisotropic and hexagonal crystal structure (refer details in the supplementary data, S1). The crystallography of hydroxyapatite suggests that HA crystals have two axes, a- and c- axis that may grow differently in the two unique directions. Thus HA crystals will not have the same dimensions, as the c-dimension (0,0,1) of the crystal may be different to the a-dimension (1,0,0) of the crystal. Based on the output of crystallite size (1,0,0) and crystallite size (0,0,1), Profex-BGMN software computed a value for an average crystallite size in the (1,1,1) direction [41, 42]. The crystallite size was calculated in metric units of nanometre (nm).

Model development

A temperature prediction was developed based on statistical analyses of the collected colourimetric and CS data of unheated teeth and teeth incinerated at a range of increasing temperatures (n = 81). Statistical analyses were performed using the statistical software R [43]. Initially, the relationships between L*, a*, b*, WI, YI, CS with the temperature (27°C, 300°C, 600°C, 800°C and 1000°C) were investigated using correlation coefficient analysis. Correlations between variables were analysed using the built-in 'cor' function and were graphically displayed in a form of a correlation matrix, using the 'corrplot' package. The weakly and moderately correlated response variables were used in separate univariate analyses. Alternately, high correlations between response variables suggest redundancy and the number of response variables can be reduced to a representative set.

Normality tests were conducted to check if the observations in each group were normally distributed. Analysis of variance (ANOVA) was performed for the selected response variables using the 'aov' function to compare means between the temperature groups. Tukey HSD post hoc analysis was completed using a correction to control the family-wise error rate and to compares the difference between each pair of means with an appropriate adjustment. Statistical significance was accepted at $p < 0.05$.

A multinomial regression model was fitted into the selected variables. The function used to generate the multinomial regression was 'multinom' from the 'nnet' package. Temperature groups of 27°C, 300°C, 600°C, 800°C and 1000°C that were used to incinerate the teeth were applied as categorical responses in the model. To ensure the replicability of the results, the seed of the random generator was fixed. To improve the prediction accuracy of the model, a qualitative variable that was a confirmatory observation of the burnt based on the appearance of a tooth was added to the model.

Model validation

This model was subjected to a k-fold cross validation, with k = 5. This means the data was split to five groups. The model was applied to the first four groups and then it was validated against the predictions it generated for the final group. To verify

the stability of the model, the k-fold cross validation was performed five times, using all combinations of the five groups.

To test the reliability of the model, it was further validated using a different set of data from a previous study [4]. Samples were categorised probabilistically into a specific temperature group (27℃, 300℃, 600℃, 800℃ and 1000℃). The overall accuracy of the model demonstrates the percentage of the correct temperature classifications for the test data set. An online tool enabling prediction using this model was built using the 'Shiny app' package [44].

Results

Qualitative assessments of teeth and bone

Gross macrostructural alterations

The gross macrostructural alterations of the external surfaces of incinerated teeth and bone were different (Table 1). In teeth, the general progression of the colour from unheated to 1000°C changed from yellowish cream, opaque white, metallic black, light grey to chalky white. In contrast, the colour changes in bones were from soft beige, polished white, blackish grey, whitewash white and chalky white.

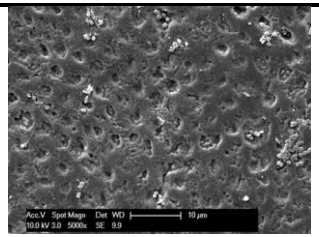
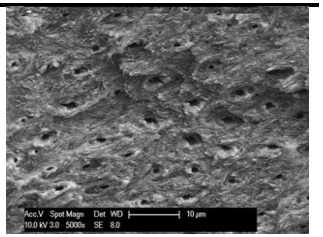
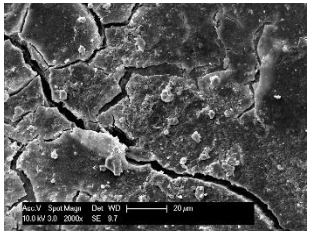
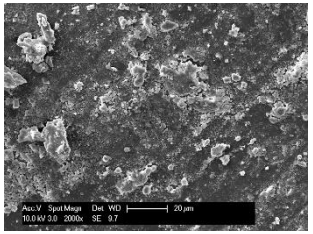
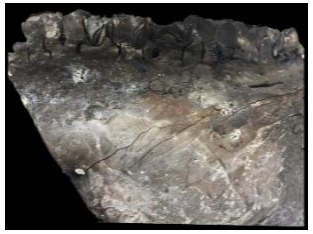
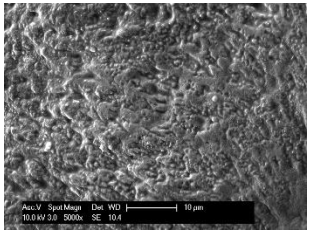
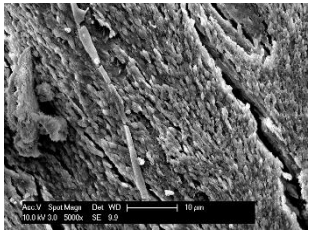
SEM

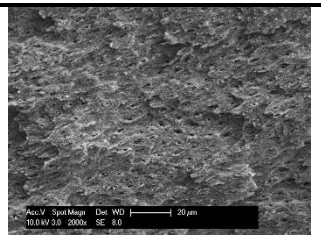
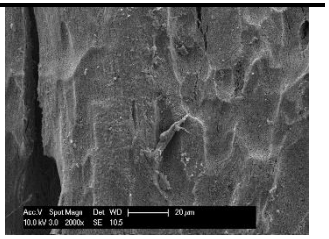
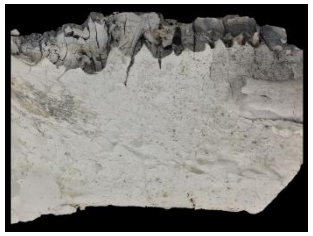
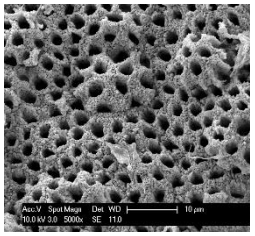
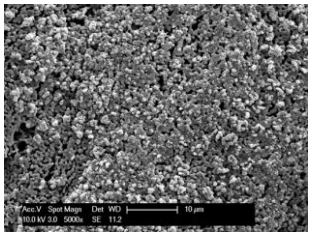
Table 1 shows the comparisons of surface characteristics of unburnt and incinerated tooth crowns and bones under the SEM magnification range of 2000x to 5000x. At 300°C, the enamel of the tooth crown demonstrated crack lines whilst the bone surface appeared intact with irregularities. At 600°C, fracture lines on the tooth crown appeared to extend to the underlying dentine. The extended fracture lines of enamel into the underlying dentine overlapped with the macro-feature observed visually in which enamel cracked and exposed the dentine. Some pores on the surface became noticeable. The bone begun to show striated fissures that were parallel to each other with flaking and lifting. The pitted and gritty surface on the tooth and bone surfaces became extensive.

At 800°C, tooth crowns revealed corrugations on the surface with generalised pores. The surface irregularities on the bone had softened and it revealed a stalactite pattern. The fissures became wider and the bone demonstrated porosity. At 1000°C,

the tooth surface became porous and had a honey-comb appearance. Tooth crown revealed corrugations on the surface with an enhanced size of the pores. The bone surface showed a small nodular and a “melting ice” pattern with no increased in porosity compared to that seen on the tooth surface.

Table 1—The macrostructural and microstructural alterations of the external surfaces of teeth and bones that were unheated and incinerated at 300°C, 600°C, 800°C and 1000°C.

Temperature (°C)	Visual observation	Macroscopic features		SEM observation	
		Teeth	Bone	Teeth	**Bone**
Unheated		Ivory white to pale yellowish cream.	Soft beige to light cream.		
300		Opaque white; intact.	Polished white or light beige; intact.		
600		Charred black with metallic white spots; vertical fracture lines	Blackish grey with brown and white patches; minor cracked lines		

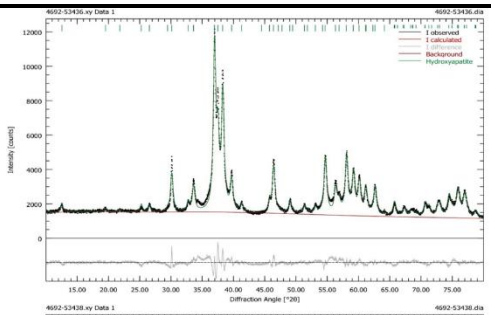
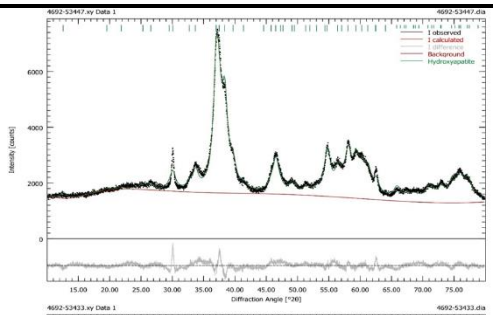
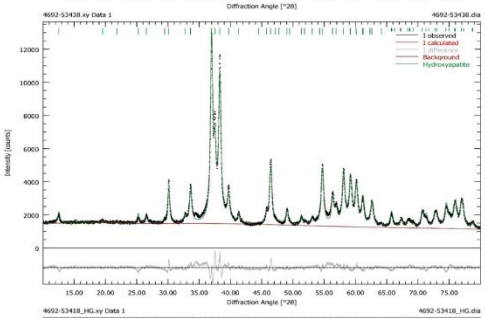
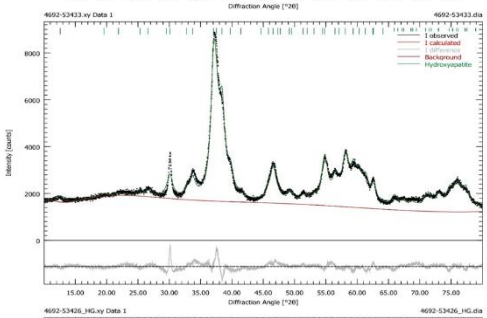
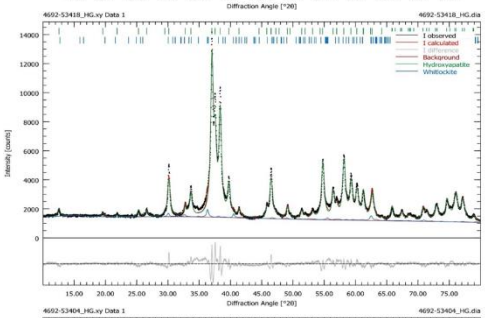
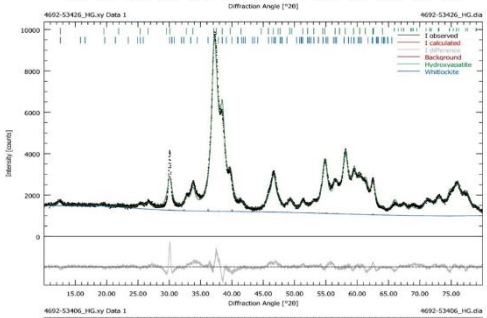
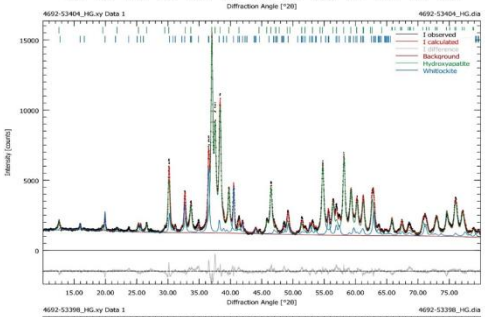
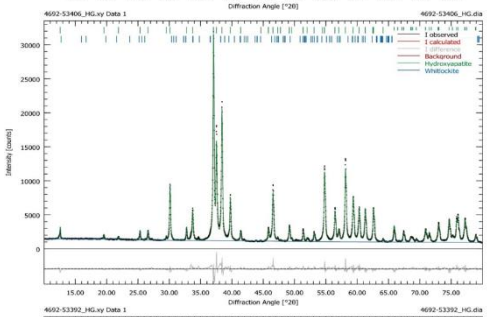
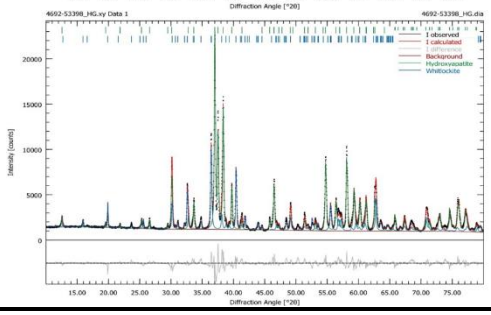
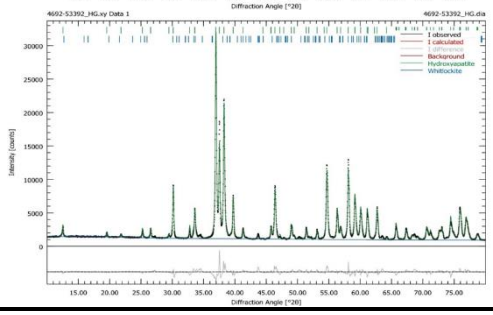
800		Light grey and matte black; fragmentation of crowns; delicate and easily crumbled crowns; Loose roots.	Whitewash white with light greyish spots; flaking of the periosteum.		
1000		Chalky-white with light grey patches; chequered pattern on crowns and extended to roots; extremely fragile and easily dislodged.	Chalky-white with grey spots and pink tints; periosteum detached; visible patina fracture lines underlying periosteum.		

Diffraction Patterns

In general, the Rietveld refinement of all samples demonstrated excellent agreement between observed and calculated diffraction profiles. Table 2 demonstrates the comparisons of the diffraction patterns of all the samples including at 27°C – 1000°C. All the observed peaks of the diffraction patterns matched the peaks of standard HA. Both diffraction patterns of unheated teeth and bone exhibit broad diffraction peaks. However, the diffraction peaks of the unheated bone samples appear notably wider than the unheated teeth. At 300°C, no noticeable changes were observed in the width of the peaks and they appeared to be similar to the peaks observed in unheated samples.

At 600°C, the width of the peaks was narrower and the intensity was increased for both teeth and bone. At this temperature, traces of WHI phase was detected in tooth sample. At 800°C both diffraction patterns demonstrated remarkably sharpened peaks and increased intensity. However, bone especially demonstrated a sudden sharpening in comparison to the bone sample heated at 600°C. Both diffraction patterns show exceptionally sharp peaks at 1000°C.

Table 2—Comparisons of the diffraction patterns of teeth and bone that were unheated and incinerated at 300°C, 600°C, 800°C and 1000°C.

Temperature	Teeth	Bone
Unheated		
300		
600		
800		
1000		

Identification of mineral phase

An overview of crystallite size and mineral phase changes in incinerated teeth is presented in Table 3. A size reduction was noted from temperature 300°C (μ = 21.68, SD = 8.56) to 600°C (μ = 17.09, SD = 6.77), followed by a sudden increase of crystallite size when the heating increased more than 600°C (μ = 41.23, SD = 22.75). The percentage of WHI was inversely proportional to the percentage of HA. WHI was not detected in unheated teeth and teeth heated at 300°C. From 600°C to 800°C, WHI content increased and is strongly correlated to the HA crystallite size. Half of the teeth heated at 600°C showed the presence WHI mineral (N = 56).

Table 3—The mean (μ) and standard deviation (SD) of crystallite size (CS) and mineral phases of hydroxyapatite (HA) and whitlockite (WHI) in unheated teeth and incinerated teeth against temperature from 27°C to 1000°C. (n = 81).

Temperature °C	μ±SD CS (nm)	HA percentage	WHI percentage
27	22.66 (7.88)	100% (0.00)	0% (0.00)
300	21.68 (8.56)	100% (0.00)	0% (0.00)
600	17.09 (6.77)	99% (0.01)	1% (0.01)
800	41.23 (22.75)	69% (0.27)	31% (0.27)
1000	60.48 (22.74)	64% (0.19)	36% (0.19)

Development of a temperature prediction model

Correlations between variables

Fig. 1 is a correlation matrix that displays the overall correlation coefficients between all variables. Circles that are blue indicate a positive correlation and the red, a negative correlation. The size of the circle is proportionally related to the degree of the correlation strength. Positive correlations were noted between temperature with WI and crystallite size. Else, negative correlations were observed between temperature and L*, a*, b* and YI. Both directions of correlations confirm that as the temperature increased, the samples appeared more porous, whiter, less yellow and absorbed more light.

Anything with a correlation coefficient above $r = 0.9$ was considered strong enough that the variables were essentially replicates of the other. Three variables, a*, b* and YI, showed strong correlations to each other with $r = 0.91$ for the YI and b*

correlation. Thus, b* was not used in the subsequent analysis. The table of correlation coefficients is available in the supplementary data (S2).

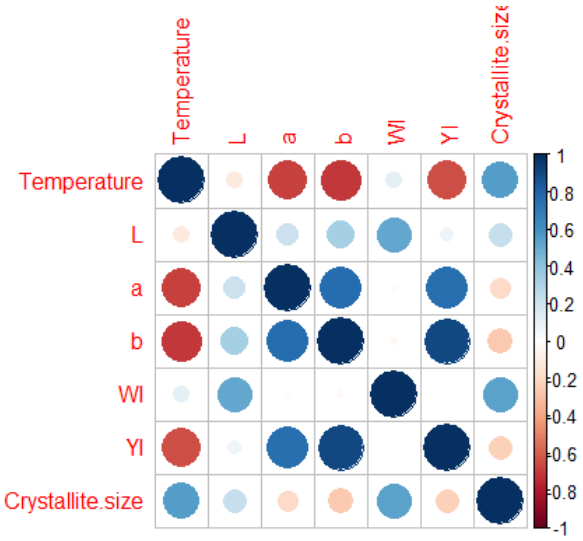


Figure 1—A correlation matrix shows the correlation coefficients of all variables of the total tooth samples (n = 81): temperature, lightness (L*), chromaticity a* and b*, whiteness (WI), yellowness (YI) and crystallite size (CS). Blue colour indicates a positive correlation and red colour indicates a negative correlation. The intensity of colour and the size of the circle follows the strength gradient of the correlation. A darker and larger circle indicate a stronger correlation. A lighter coloured and smaller circle indicates a weaker correlation. Empty box with no circle indicates no correlation.

ANOVA

The differences of each response variable, L*, a*, WI, YI and CS (log transformed to meet test assumptions) split by temperature groups (27°C, 300°C, 600°C, 800°C and 1000°C) were tested using one-way ANOVAs (Fig. 2 and Fig. 3). The results confirmed that there was a significant main effect of temperature for each variable. Normality and homogeneity of variance tests are presented in the supplementary data, S2. Post-hoc Tukey's multiple comparison tests determined where the significant differences of means between temperature groups were for each response (refer the details in the supplementary data, S2).

Following are the summary of the ANOVA results:

- L* can discriminate 600°C and 800°C with other temperature groups (Fig. 3a).
- a* can differentiate 27°C, 300°C with temperature 600°C and above (Fig. 3b).
- WI can discriminate 1000°C to 300°C, 600°C and 800°C (Fig. 3c).

- YI can distinguish 27°C, 300°C and 600°C with each other, and with 800°C and 1000°C (Fig. 3d).
- CS can differentiate 1000C from all other temperature groups, 27°C, 300°C, 600°C and 800°C (Fig. 4).

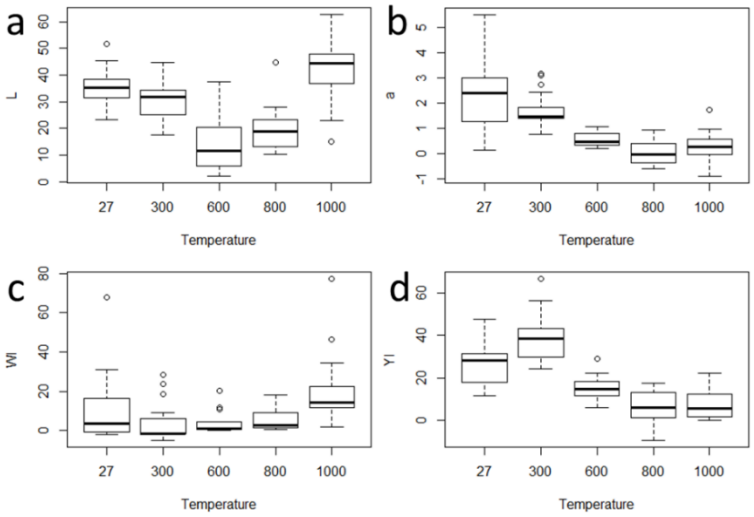


Figure 2—Boxplots show the differences of observed response variables (y-axis) split by temperature groups of the colourimetric variables against the temperature groups, 27°C, 300°C, 600°C, 800°C, 1000°C (x-axis): (a) Lightness, L; (b) Chromaticity a; (c) Whiteness index, WI; (d) Yellowness index, YI (Please refer the details in the supplementary data, S2) (n = 81).

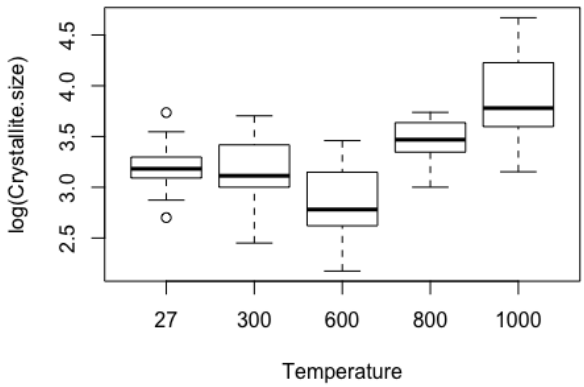


Figure 3—Boxplots show the differences of observed crystallite size (y-axis) split by temperature groups of the colourimetric variables against the temperature groups, 27°C, 300°C, 600°C, 800°C, 1000°C (x-axis) (please refer the details in the supplementary data, S2) (n = 81).

Multinomial regression analysis

Results from the initial ANOVAs indicated that L, a*, WI, YI, and CS are all excellent parameters to identify and discriminate temperature groups (refer details and code in the supplementary data, S2). However a model with these predictors alone did not generalise as well as desired. To improve the accuracy of the model, a qualitative

variable “burned” was added as a predictor. The “burned” variable consists of the following options: uncertain (27°C and 300°C); definitely burned (600°C, 800°C, 1000°C). As such, this incorporates the expert knowledge of the person using the model by requiring them to first classify the tooth into one of these categories. The classification matrix original fitting of the multinomial regression model L*, a*, WI, YI, CS and ‘burned’ as predictors, demonstrates the improvement of the accuracy up to 95% (refer details in the supplementary data, S2).

Validation of the temperature prediction model

The final multinomial regression model that predicts the temperature-exposure of incinerated teeth into a category of incineration temperature category is presented in Table 4. This model was validated using 19 samples from a previous study [4]. Applied to the new data, the model classified 79% of the samples into the correct temperature groups, misclassifying only those from the highest temperature group (1000°C).

Table 4—The multinomial regression model that was developed and validated to predict the temperature-exposure of incinerated teeth (n = 19). A tool enabling prediction using this model was built online and it can be found at **https://melsapps.shinyapps.io/Predicting_temp_of_burned_teeth/**

Coefficients:							
	Intercept	Burned	a	YI	Crystallite.size	WI	L
300	-164.41	34.27	-28.65	4.28	0.32	-1.62	2.20
600	70.27	113.91	13.55	-0.12	-2.54	2.23	-4.23
800	1.47	68.84	-37.69	1.18	0.66	0.21	-1.32
1000	-48.97	104.36	-38.51	1.34	0.82	0.05	-1.03
Std. Errors:							
	Intercept	Burned	a	YI	Crystallite.size	WI	L
300	2.94	0.00	15.56	0.77	0.43	1.40	0.44
600	0.79	0.79	1.73	2.81	1.53	8.78	7.89
800	1.60	0.79	1.45	2.81	1.53	8.77	7.88
1000	1.84	1.84	1.70	2.81	1.53	8.77	7.88
Residual Deviance:	22.77						
AIC:	78.77						

Discussion

This paper presents a newly developed tool to predict temperature-exposure of teeth by assessing the colourimetric and hydroxyapatite crystal changes in incremental-heated pig teeth. Several qualitative analytical approaches including visual observation, SEM, diffraction patterns analysis and mineral identification were added to optimise the findings of the heat-induced changes and to ensure comprehensive measures were taken to achieve the aims.

Temperature prediction model

Variables correlation

This study is an extension of a preliminary study [4], in which the prospect of using both spectrophotometric and XRD analyses to investigate burned dental remains was assessed. That study tested the correlation of colourimetric (L, a*, b*, WI, YI) and crystal size (CS) variables with temperature and duration [4]. It was shown that duration has no significant effect on the variable changes, and WI, YI and CS are reliable to predict temperature-exposure [4]. As a result, the heating duration in this study was fixed to 15 min.

The data obtained for each of the response variables (L, a*, b*, WI, YI and CS) showed weak to moderate correlations with temperature (*r* between -0.73 to 0.5) (Figure 2 and supplementary data, S2). This suggested that all of the variables can be used to develop the temperature prediction tool. However, a*, b* and YI demonstrated strong correlations to each other (*r* between 0.76 and 0.91). The strong correlations suggested that a*, b* and YI have a similar discriminative power. As both a* and b* were measures of chromaticity, whilst YI was a measure of the degree of the yellowness [45], it was decided that b* should be omitted. Thus, L, a*, WI, YI and CS were selected for the subsequent analysis (ANOVA). The post-hoc Tukey's multiple comparison tests confirmed that the selected response variables (L*, a*, WI, YI and CS) were able to significantly discriminate the temperature groups of 27°C, 300°C, 600°C, 800°C and 1000°C.

Multinomial regression

We employed a multinomial regression analysis to develop a prediction model for temperature-exposure. The idea was to fit a regression model that predicts

temperature categorically. At first a linear regression model was used as an attempt to develop a temperature prediction model. Despite the temperature intervals used in the experiment, the model predicts a temperature in a continuous manner. In addition, L, a*, WI, YI and CS have the ability to discriminate the temperature groups. In this study, teeth were incinerated at categorical and discrete temperature groups, thus the application of a linear regression model did not fit the data. Previously, Krap *et al.* [26] proposed a model to predict temperature of heat-exposed bones. This model was constructed from the cluster analysis of colourimetric data. However, the maximum temperature used was 900°C and the colourimetric data were indirectly obtained using a digital image analysis. Moreover, this model is only ideal to be used for bone. In contrast to the model developed by Krap *et al.* [26], a multinomial regression analysis was used to construct the temperature prediction tool. This is because a multinomial regression allows the estimated temperature to fall into a definite temperature category. A multinomial regression model was found to fit the data perfectly and has shown to successfully categorise burned teeth into the respective temperature groups with a substantial accuracy (79%).

The initial model that was developed by fitting the multinomial regression model into the observed data of L, a, WI, YI and CS variables appeared to do well (refer the details in the supplementary data, S2), but the accuracy was 72%. This means the generalisability was not satisfactory. The addition of the qualitative variable 'burned' has successfully improved the accuracy of the model to 79%. It was a subjective interpretation of the teeth appearance: "Based on visual observation, can you tell if this tooth has been burned?" "Uncertain" or "definitely burned". This approach was similar to the three-way classification proposed in the previous studies which were, unburnt, incompletely incinerated and completely incinerated, or non-incinerated [3, 46, 47].

Although the accuracy of the model with added qualitative variable increased to 79%, it still can be refined. Realistically, an expert would be able to tell a tooth that has not been burned. Therefore, the expertise of the researcher was incorporated into the model. Thus, the model was set with categorising any unburnt tooth into the 27°C group. This model revealed a much higher accuracy of 95%. The validation of the model using a different set of data demonstrated a considerably a high degree of

generalisability at 79%. This was excellent considering that the different data set was obtained from teeth that were incinerated for 30mins.

Characterisation of incinerated teeth and comparison against bone

Considering teeth are rarely found isolated in a fire forensic case, it is important to be able to distinguish teeth and bones because although these skeletal structures share the same major compositions (HA and collagen), the compositional ratio is different [7, 48] and so is the reaction to heat exposure [16, 30]. Thus, bones were included in qualitative analyses with an aim to verify structural differences between teeth and bones. The results showed that the heat-induced transformation stages in the teeth and bones were concordant with stages described by Mayne-Correira [9] and Thompson [49]:

1. Dehydration (100°C to 600°C); loss of hydroxyl bonds and loosely bound water;
2. Carbonisation or decomposition (300°C to 800°C); the combustion of the organic matter;
3. Inversion (500°C to 1100°C); the loss of carbonate; and
4. Calcination (>700°C); the coalescing of the crystal matrix.

Visual observation: Teeth vs Bone

The observation of colour and structural changes in teeth and bones heated at the same temperature was concordant with results of previous studies [16, 50]. The alteration of colour in teeth and bone is attributed to the loss of organic content [7, 9, 17]. The neutral colour of unheated teeth was in various shades because enamel and dentine have different ratio of mineral-organic components [16, 50]. The tooth colour is predominantly determined by the enamel thickness and the reflection of the underlying dentine [16, 51]. In contrast to teeth, the neutral colour of all bone samples prior to incineration was light yellowish beige.

When heated to 300°C, no morphological alteration and only a slight colour change were noted on teeth and bones. This was due to the dehydration process that occurred between 100°C and 300°C in the skeletal structure [52]. At 600°C, enamel begun to crack and detach from the crown because of the brittleness of enamel (96% inorganic) [50]. The black colour in teeth and bone was contributed by the combustion

of carbon and collagen resulting in carbonisation [10, 16]. Spots of metallic colour seen in teeth were due to the glossy nature of the enamel [50].

Following heat treatment at 800°C, the colour of teeth and bone became lighter. This is an indicator of the increased degree of organic matter decomposition [9]. The tooth crown and bone became extremely brittle and demonstrated an extensive fracturing because at this point the organic matter has mostly combusted. Fracturing occurs when the stress on a material exceeds its strength [53, 54]. By 1000°C, both teeth and bone had turned white. The colour and fracture patterns of teeth and bone were almost similar to the pattern seen in samples heated at 800°C. This was because no organic matter is left for combustion and HA crystals were already fused with each other at <800°C inhibiting further fracturing [7, 47]. These features marked the occurrence of the calcination process in both teeth and bone [9].

SEM: Teeth vs Bone

This study supports the suggestion that viewing under SEM is useful for diagnostic purposes of incinerated teeth and bones [7]. The use of SEM is one such method to examine the surface texture alterations of heat-treated teeth and bone [7, 20, 55-57]. SEM can provide microstructural details on differences of the surface texture between incinerated teeth and bone that are otherwise not available in XRD analysis.

At 300°C, only the tooth surface exhibited structural disintegration because enamel is of high brittleness and low tensile strength [50]. Also, the loss of water in the tooth structure accentuates shrinkage and fracturing [57-60]. At 600°C, the pitted and increased granularity observed on the tooth and bone surfaces concurred with the pitted and "frothy" appearance in a previous study [7]. The striated fissures of the bone confirmed the disintegration of the bone lining [56, 61].

The porosity of the tooth crown and bone at 800°C was the manifestation of the inversion phase in which their carbonate content was depleted or complete combustion has been achieved [49]. The stalactite pattern in bone indicated the onset of recrystallisation [61]. At 1000°C, the porosity on the tooth surface was augmented and it has a honey-comb appearance. A "melting ice" pattern on the bony surface was a result of the poorly coalesced crystals [7, 62].

Diffraction patterns: Teeth vs Bone

Overall, the diffraction patterns confirmed that HA is the major mineral phase in teeth and bones. Broad diffraction peaks observed on the diffraction patterns in unheated tooth and bone samples were due to their nanocrystalline nature and poor crystal arrangement [13]. However, the width of the diffraction peaks in the unheated teeth were sharper than in the unheated bone because the highly mineralised enamel structure consists of highly crystallised HA [63]. The narrowing of observed peaks in the diffraction patterns at 800°C suggests thermal decomposition that include the reduction of organic matter [64] and the increase of the crystal size [29]. In the study of heated teeth by Vargas-Becerril *et al.* [64], enamel and dentine showed the maximum increment of diffraction intensity at 1000°C which corresponds to our finding.

Mineral phase identification and crystallite size changes: Teeth vs Bone

The Rietveld analysis revealed that WHI was identified only in tooth samples and none in the bone samples. This finding was consistent with the studies by Kohutová *et al.* [63] where WHI was only found in heated teeth and not heated bones. The reduction of HA quantity and the appearance of WHI as the temperature was increased have confirmed the transformation of HA into WHI. It was described that HA in teeth begins to undergo dehydroxylation process when heated at 300°C and above, in which hydroxyl ions (OH^-) were removed from the structure [29, 65]. When the OH^- have been completely removed from the structure, HA is reduced to a by-product form such as WHI [65].

Slight reduction of HA crystallite size at 600°C was potentially attributed by the contraction of the enamel due to the loss of lattice water [66]. The sudden increased of crystallite size and the appearance of WHI at 800°C corresponds to the study by Etok *et al.* [28] in which they found that the crystallite structure changed most significantly between 500°C and 700°C. The HA crystallite expanded to the largest size at 1000°C after the complete depletion of carbonate ions at 800°C [67].

Real casework application

This study introduced a tool to predict temperature-exposure of incinerated pig teeth that has potential to be applied in forensic casework. The prediction model

enables a burned tooth to be categorised into a specified temperature group of 300℃, 600℃, 800℃ or 1000℃. A qualitative variable that defines the certainty of the tooth having been burned was added to the model. This helps to improve the prediction accuracy of the model. The qualitative variable is also practical because it allows an expert to determine whether the tooth has been burnt with certainty or not, based on observation. The application of the tool offers a solution to the previous issue of the lack of consensus on the appropriate methodology to be used for the interpretation of heat-induced changes in teeth [2].

To substantiate the temperature prediction that is obtained from the multinomial regression model, supplementary analyses should be performed on the teeth to characterise the incinerated teeth. The analyses include visual observation, SEM, X-ray diffraction pattern and mineral identification are important because the structural changes are the secondary-level manifestations of the incinerated skeletal microstructure [49]. For example, the use of SEM may be beneficial when presumably small fragments of teeth are recovered in isolation [16]. In addition, supplementary analyses such as SEM and diffraction pattern can identify other substances associated with the skeletal remains that may have formed during post-mortem intervals [68, 69]. Evidently the interpretation through visual observation and SEM is subjective and ambiguous. Sole application of the qualitative methods is insufficient for an evidence assessment in a real forensic casework as the practice demands an objective, reliable and robust method.

This temperature prediction tool was developed exclusively for teeth because of the lack of comprehensive temperature-estimation studies for teeth. Numerous approaches to predict the maximum temperature-exposure of burned remains [11, 14, 26, 70] and many research studies to substantiate a correlation between morphological alterations of skeletal tissues with heating temperature and duration have been published [7, 11, 15-17, 20, 47, 50, 55, 57]. However, only some of the studies used and included teeth in their experiments [7, 11, 16, 17, 50]. The results of the qualitative analyses in this study has confirmed that teeth and bone do not respond to heat with the exact same manner and the observed morphological changes between teeth and bones occurred at different rates. In addition, many studies incinerated individual isolated teeth that had the roots left bare without bony protection [17, 50]. This condition was far from resembling teeth in a real fire situation. Campbell and

Fairgrieve [71] have suggested that the ideal way to incinerate teeth is by retaining teeth in the tooth sockets of maxilla or mandible because the roots of teeth are naturally protected from direct heat exposure. Thus, in this study teeth were retained in its natural position, that were in the tooth sockets of the mandibular bone which was similar to a previous study [4].

This study provides insights on the evidentiary value of teeth for estimating the temperature-exposure of burned human remains. Although pig teeth were used, the results are relevant for human teeth because the heat-induced colour and HA crystal size changes were consistent with similar investigations using human teeth [16, 30, 50]. However we acknowledged that it is also a limitation for real casework applications involving human teeth. The validity of this tool on human teeth has yet to be tested and so it will require additional research involving *in situ* human. Hence an additional research to assess the efficacy of the tool on human teeth and to validate its applicability on burned human remains is warranted.

Conclusion

A rigorous approach was undertaken to develop a reliable tool to predict the maximum temperature-exposure of incinerated pig teeth using colorimetric and hydroxyapatite crystal size data. The multinomial regression-based tool not only can be used to estimate the temperature at which teeth were exposed to, but also to determine whether the teeth have been burned. This tool is a robust approach considering that supplementary qualitative analyses to assess the heat-induced structural changes were included to support the resultant estimated temperature. This tool would be useful for investigators as it can provide valuable intelligence in a fire investigation. Further research is needed to test the applicability of the model to human teeth and to forensic case work involving burned human remains.

The tool for predicting temperature of incinerated teeth was built online and it can be found at https://melsapps.shinyapps.io/Predicting_temp_of_burned_teeth/

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Ethical standards

The handling of the animal remains in this study was conducted according to the University of Adelaide Animal Ethics.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary data, S1

The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data

Name and formula

Reference code:	01-073-6113
Mineral name:	Hydroxylapatite
Compound name:	Calcium Phosphate Hydroxide
Common name:	pentacalcium tris(phosphate(V)) hydroxide
Empirical formula:	$Ca_5HO_{13}P_3$
Chemical formula:	$Ca_5\ (PO_4)_3\ (\ OH\ )$

Crystallographic parameters

Crystal system:	Hexagonal
Space group:	P63/m
Space group number:	176
a (Å):	9.43
b (Å):	9.43
c (Å):	6.88
Alpha (°):	90.00
Beta (°):	90.00
Gamma (°):	120.00
Volume of cell (10^6/m^3):	530.14
Z:	2.00
RIR:	1.06

Status, subfiles and quality

Status:	Alternate Pattern
Subfiles:	Ceramic
	Common Phase
	Excipient
	Forensic
	ICSD Pattern

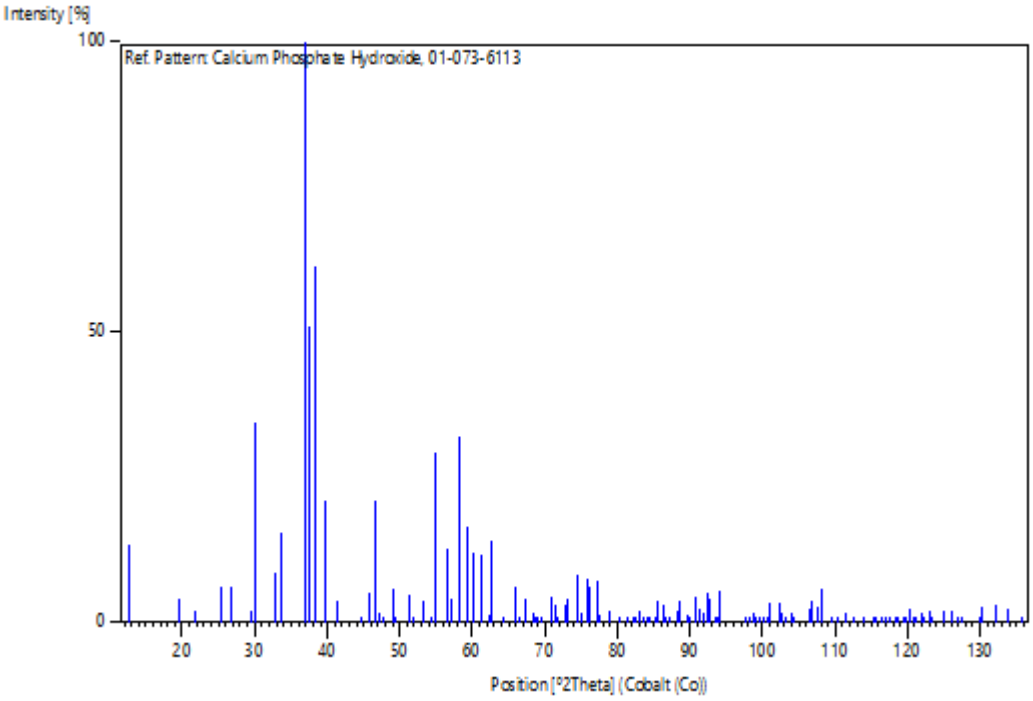
Inorganic

Mineral

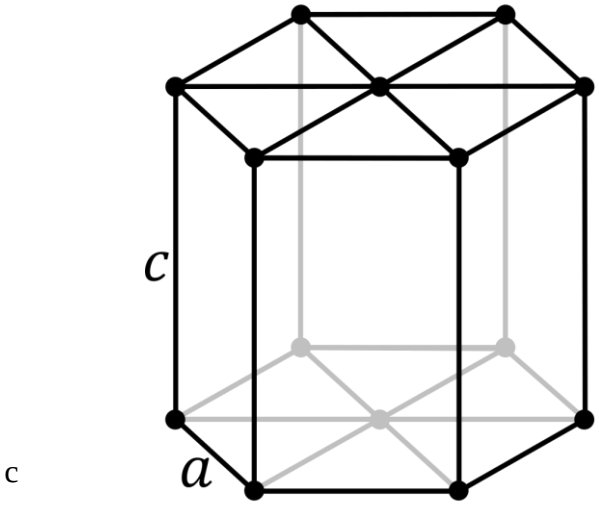
Pharmaceutical

Quality: Indexed (I)

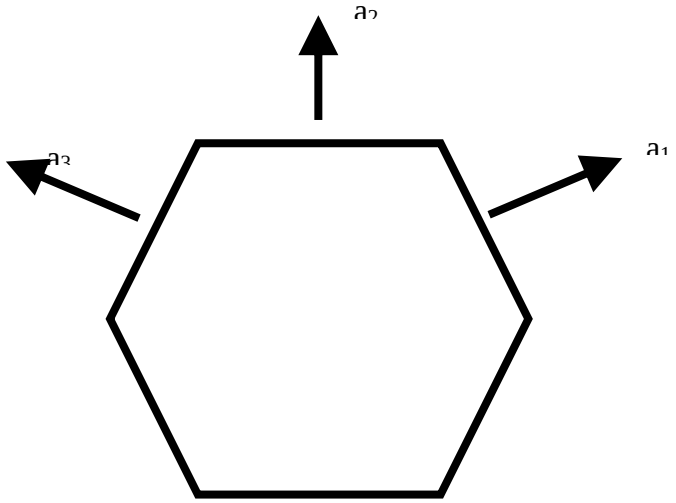
Stick Pattern of hydroxyapatite



3-Dimensional view of hydroxyapatite



Cross-sectional view of hydroxyapatite



References

Primary reference: Calculated from ICSD using POWD-12++

Structure: Kay, M.I., Young, R.A., Posner, A.S., Nature (London), 204, 1050, (1964)

Supplementary data, S2

The development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data

i. Correlation coefficients

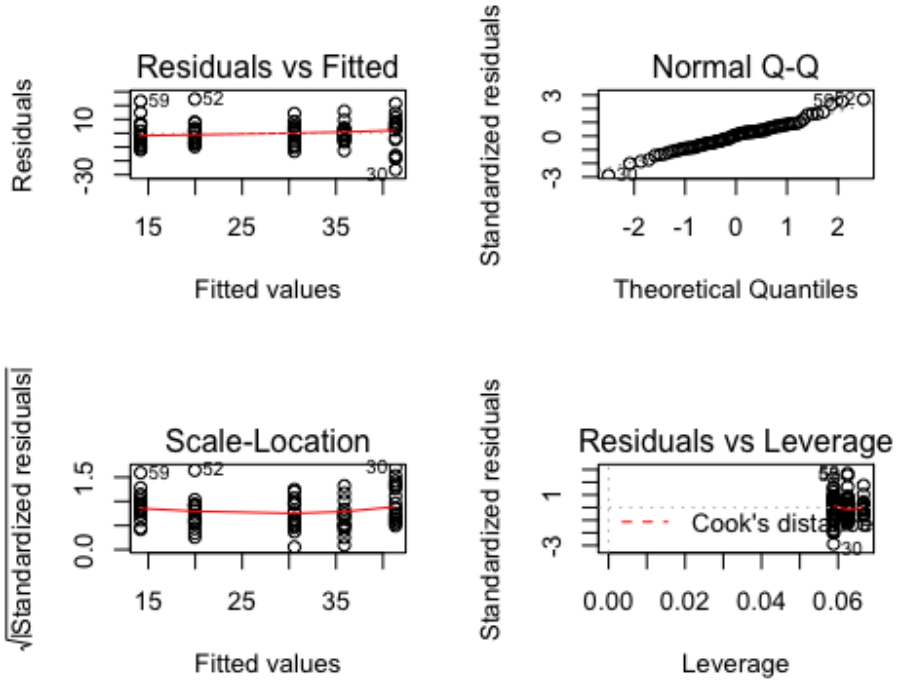
S 1—Correlation coefficients between the values of response variables including CS, L, a*, b*, WI and YI.

Response variables	Temperature	L	a*	b*	WI	YI	CS
Temperature	1	-.04	-.70	-.73	.18	-.67	.50
L		1	.12	.22	.53	.00	.28
a			1	**.76***	-.02	**.76***	-.15
b				1	-.08	**.91****	-.22
WI					1	-.04	.59
YI						1	-.18
CS							1

L=Lightness; a=chromaticity of redness-greenness; b=chromaticity of yellowness-blueness; WI=Whiteness index; YI=Yellowness Index; CS=Crystallite Size; Correlation is highly significant at **p < 0.01; Correlation is moderately significant at *p < 0.05.

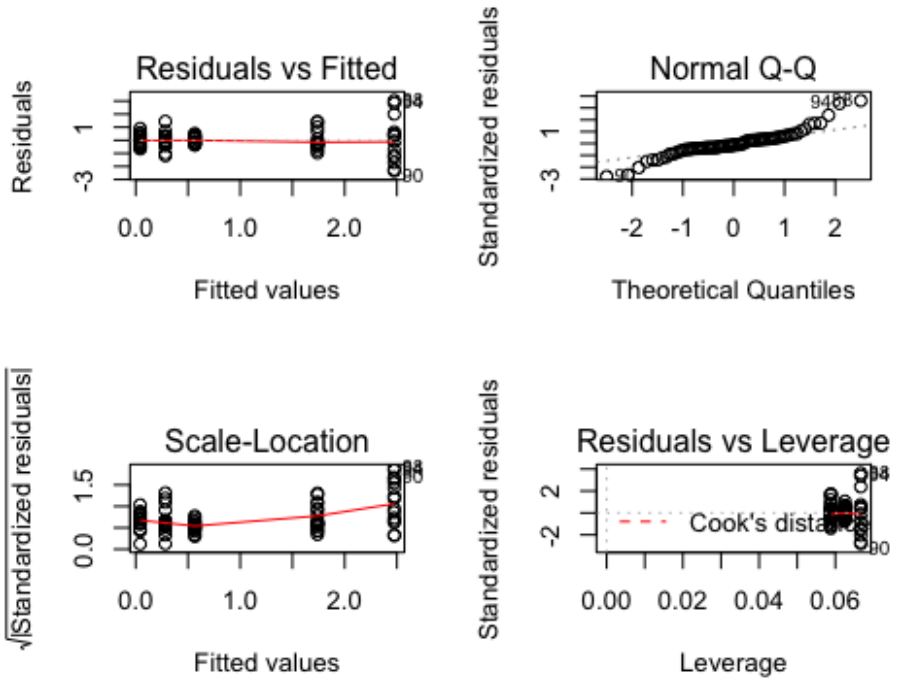
ii. Normality tests

L



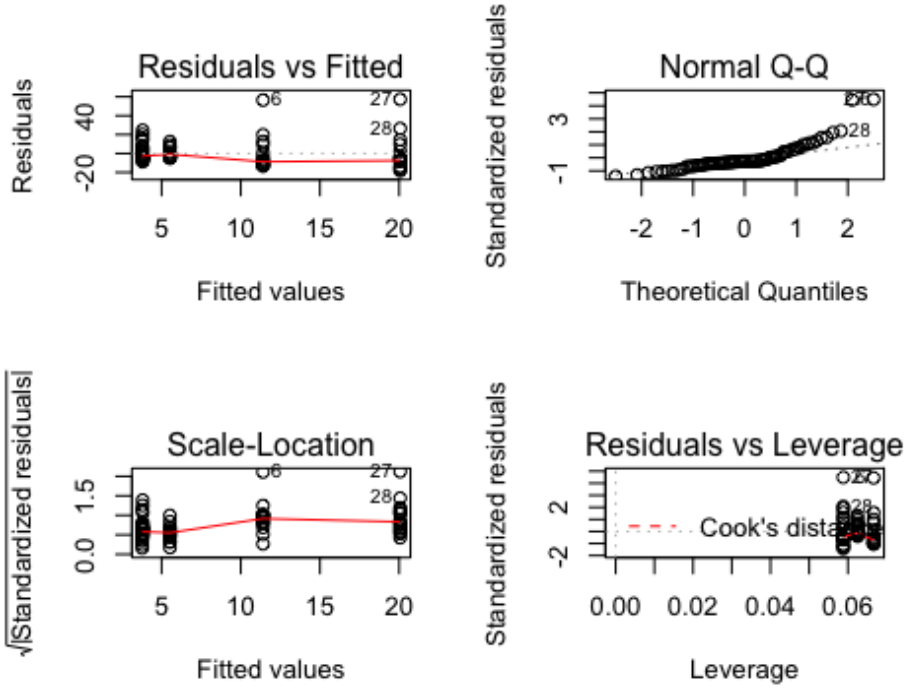
S 2—Assessment L* using model diagnostics shows the assumption of normality was reasonable. Top left: Residuals versus fitted plot. Top right: Normal QQ plot of the residuals. Bottom left: Scale-location diagnostic plot. Bottom right: Diagnostic plot of standardised residuals against leverage.

a*



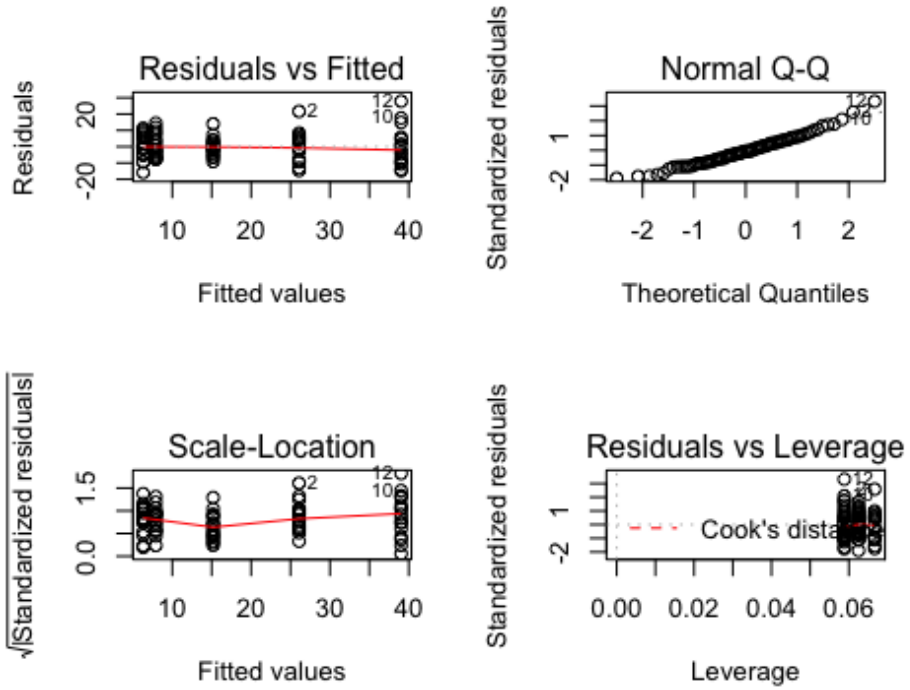
S 3—Assessment a* using model diagnostics shows the assumption of normality was reasonable. Top left: Residuals versus fitted plot. Top right: Normal QQ plot of the residuals. Bottom left: Scale-location diagnostic plot. Bottom right: Diagnostic plot of standardised residuals against leverage.

WI



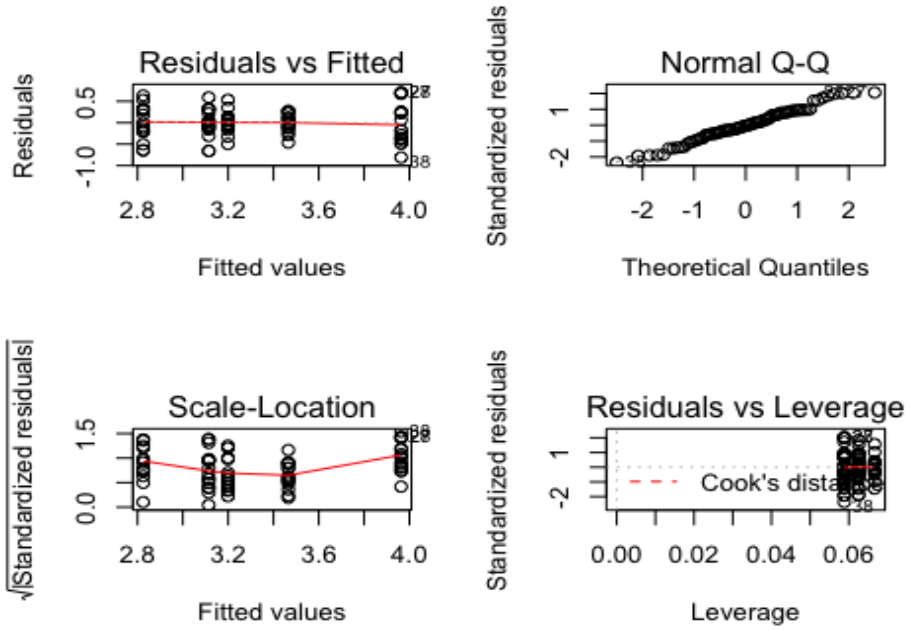
S 4—Assessment WI using model diagnostics shows the assumption of normality was reasonable. Top left: Residuals versus fitted plot. Top right: Normal QQ plot of the residuals. Bottom left: Scale-location diagnostic plot. Bottom right: Diagnostic plot of standardised residuals against leverage.

YI



S 5—Assessment YI using model diagnostics shows the assumption of normality was reasonable. Top left: Residuals versus fitted plot. Top right: Normal QQ plot of the residuals. Bottom left: Scale-location diagnostic plot. Bottom right: Diagnostic plot of standardised residuals against leverage.

CS



S 6—Assessment CS using model diagnostics shows the assumption of normality was reasonable. Top left: Residuals versus fitted plot. Top right: Normal QQ plot of the residuals. Bottom left: Scale-location diagnostic plot. Bottom right: Diagnostic plot of standardised residuals against leverage.

ANOVA

S 7—Results of the pairwise comparisons with a Tukey HSD correction for lightness (L).

Temperature		Lower	Upper	Adj p
27	300	-14.658	4.027	0.508
	600	-31.182	-12.225	< 0.001
	800	-25.405	-6.438	< 0.001
	1000	-3.883	14.802	0.481
300	600	-25.574	-7.201	< 0.001
	800	-19.797	-1.424	< 0.001
	1000	1.729	19.821	0.015
600	800	-3.548	15.101	0.421
	1000	17.977	36.349	< 0.001
800	1000	12.200	30.573	< 0.001

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 8—Results of the pairwise comparisons with a Tukey HSD correction for chromaticity a*.

Temperature		Lower	Upper	Adj P
27	300	-1.595	0.125	0.130
	600	-2.782	-1.036	< 0.001
	800	-3.305	-1.559	< 0.001
	1000	-3.051	-1.331	< 0.001
300	600	-2.020	-0.328	< 0.001
	800	-2.543	-0.851	0.002
	1000	-2.289	-0.623	< 0.001
600	800	-1.382	0.335	0.439
	1000	-1.128	0.564	0.884
800	1000	-0.605	1.087	0.931

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 9—Results of the pairwise comparisons with a Tukey HSD correction for whiteness (WI).

Temperature		Lower	Upper	Adj P
27	300	-20.61	5.41	0.482
	600	-20.83	5.57	0.493
	800	-19.08	7.33	0.726
	1000	-4.36	21.67	0.348
300	600	-12.82	12.77	> 0.999
	800	-11.07	14.52	0.996
	1000	3.66	28.86	0.005
600	800	-11.24	14.74	0.996
	1000	3.49	29.08	0.006
800	1000	1.74	27.33	0.018

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 10—Results of the pairwise comparisons with a Tukey HSD correction for yellowness (YI).

Temperature		Lower	Upper	Adj P
27	300	4.377	21.520	< 0.001
	600	-19.595	-2.203	0.006
	800	-28.466	-11.074	< 0.001
	1000	-26.739	-9.596	< 0.001
300	600	-32.276	-15.420	< 0.001
	800	-41.147	-24.291	< 0.001
	1000	-39.415	-22.817	< 0.001
600	800	-17.426	-0.317	0.038
	1000	-15.696	1.160	0.124
800	1000	-6.825	10.031	0.984

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 11—Results of the pairwise comparisons with a Tukey HSD correction for crystallite size (CS)

		Lower	Upper	Adj P
27	300	0.647	1.310	0.961
	600	0.482	0.980	0.034
	800	0.914	1.859	0.233
	1000	1.507	3.034	< 0.001
300	600	0.533	1.062	0.142
	800	1.010	2.014	0.043
	1000	1.665	3.287	< 0.001
600	800	1.336	2.691	< 0.001
	1000	2.203	4.393	< 0.001
800	1000	1.162	2.316	0.001

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

Multinomial regression analysis

i. First model, fitNN (L, a, WI, YI and CS)

fitNN ‹- multinom (Temperature ~ a+YI+Crystallite.size+WI+L,data=ccG, trace=F)

```
## Call:
## multinom(formula = Temperature ~ a + YI + Crystallite.size +
##     WI + L, data = ccG, trace = F)
##
## Coefficients:
##      (Intercept)           a        YI Crystallite.size
WI
## 300   -144.32677 -42.9885061  4.638253       0.38269796 -1.27968
91
## 600    114.39349   7.7231814 -2.958824      -0.56371013  0.90394
04
## 800     97.27861   0.2510139 -2.853957      -0.01505241  0.56935
95
## 1000    81.53921  -0.3801883 -2.709737       0.16157642  0.42432
49
##              L
## 300   1.798279
## 600  -2.067105
## 800  -1.671671
## 1000 -1.385169
##
## Residual Deviance: 25.58815
## AIC: 73.58815
```

ii. K-fold cross validation of fitNN

```
k ‹- 5
sub ‹- sample(1:k,nrow(ccG),replace=T)
pred ‹- ccG$Temperature
for (i in 1:k) {
```

Delete the i-th subset

```
 fit ‹- multinom(Temperature ~
a+YI+Crystallite.size+WI+L,data=ccG[sub!=i,],trace=F)
```

Predict the i-th subset

```
 pred[sub==i] ‹- predict(fit, newdata=ccG[sub==i,],type="class")}
```

Confusion matrix

```
table (ccG$Temperature,pred)
```

Prediction

	27	300	600	800	1000
27	10	1	0	1	3
300	3	14	0	0	0
600	2	0	10	4	0
800	0	0	2	11	3
1000	1	0	0	3	13

Accuracy: ((81-23)/81,3)*100 = **71.6%**

iii. Second model, fitNN 2 (L, a, WI, YI, CS and ‘burned’) with the hard classifier)

fitNN2 ‹- multinom (Temperature ~Burned + a + YI + Crystallite.size + WI + L, data = ccG, trace = F)

```
## Call:
## multinom(formula = Temperature ~ Burned + a + YI + Crystallite.size +
##     WI + L, data = ccG, trace = F)
##
## Coefficients:
##      (Intercept)    Burned         a         YI Crystallite.size
## 300  -164.412920  34.27447 -28.65153  4.2763678        0.3173723
## 600    70.272526 113.91290  13.54686 -0.1203838       -2.5443812
## 800     1.472616  68.83680 -37.68898  1.1792768        0.6550232
## 1000  -48.970755 104.36452 -38.50670  1.3385372        0.8157505
##               WI         L
## 300  -1.61980954  2.203021
## 600   2.22987150 -4.225182
## 800   0.21489685 -1.316050
## 1000  0.05190196 -1.032370
##
## Residual Deviance: 22.76557
## AIC: 78.76557
```

iv. Cross validation of fitNN2

Generate k random subsets or groups

sub ‹- sample(1:k,nrow(ccG),replace=T)

pred ‹- ccG$Temperature

for(i in 1:k) {

Delete the i-th subset

fit ‹- multinom(Temperature ~ Burned + a + YI + Crystallite.size + WI + L, data = ccG [sub!=i,], trace = F)

Predict the i-th subset

pred[sub==i] ‹- predict(fit,newdata=ccG[sub==i,],type="class")}

Confusion matrix

table(ccG$Temperature,pred)

Prediction

	27	300	600	800	1000
27	13	1	0	0	1
300	1	16	0	0	0
600	0	0	13	3	0
800	0	0	3	9	4
1000	0	0	0	2	15

Accuracy: ((81-17)/81,3)*100 = **79%**

v. Second model with the hard classifier, fitNN 2 (fitNN2 with the hard classifier)

```
ccG$NewPred ‹-  double(dim(ccG)[1])
for (i in 1:dim(cct)[1]){
  if(ccG$Burned3[i]==0){
    ccG$NewPred[i] ‹-  "27"
  } else {
    ccG$NewPred[i] ‹-  as.numeric(as.character(predict(fitNN2,type="class")[i]))
  }
table(ccG$Temperature,factor (ccG$NewPred,
levels=c("27","300","600","800","1000")))
```

	27	300	600	800	1000
27	15	0	0	0	0
300	0	17	0	0	0
600	0	0	15	1	0
800	0	0	1	13	2
1000	0	0	0	1	16

Accuracy: Round ((81-4)/81,3)*100 = **95.1%**

vi. Model validation

```
d2$NewPred ‹- double(dim(d2)[1])
for (i in 1:dim(d2)[1]){
 if (d2$Burned3[i]==0){
  d2$NewPred[i] ‹- "27"
 } else {
  d2$NewPred[i] ‹- as.numeric(as.character(predict(fitNN2,type="class")[i]))
 }
}
table(d2$Temp,factor (d2$NewPred, levels=c("27","300","600","800","1000")))
```

Prediction

	27	300	600	800	1000
27	5	0	0	0	0
300	0	5	0	0	0
600	0	0	5	0	0
800	0	0	0	0	0
1000	0	0	1	3	0

Accuracy: ((19-4)/19,3)*100 = **78.9%**

Chapter 5

Part I

Part II

Part III

Part I

An improved method of preparing teeth to optimise DNA yield

Statement of Authorship

Title of paper	An improved method of preparing teeth to optimise DNA yield
Publication Status	Unpublished and unsubmitted work written in a manuscript style
Publication Details	Rahmat, R. A., et al. (2020). An improved method of preparing teeth to optimise DNA yield, The University of Adelaide.

Principal Author

Name of Principal Author (Candidate)	Rabiah Al-Adawiyah Binti Rahmat		
Contribution to the paper	Research planning, project administration, sample preparation and investigation, data curation, data analysis and interpretation, created figures and tables, wrote manuscript and acted as a corresponding author.		
Overall percentage (%)	80%		
Signature		Date	04/05/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above).
ii. permission is granted for the candidature in include the publication in the thesis.
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Melissa A. Humphries
Contribution to the paper	Statistical analysis and interpretation

Signature		Date	21/04/2020

Name of Co-Author	Jeremy J. Austin		
Contribution to the paper	Resources, supervision, manuscript evaluation and funding acquisition		
Signature		Date	23/04/2020

Name of Co-Author	Adrian M. T. Linacre		
Contribution to the paper	Supervision and manuscript evaluation		
Signature		Date	21/04/2020

Name of Co-Author	Arif Malik		
Contribution to the paper	Facilitated the DNA laboratory work and manuscript evaluation		
Signature		Date	04/05/2020

Manuscript prepared for submission

An improved method of preparing teeth to optimise DNA yield

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Abstract

The common method of preparing teeth for DNA analyses involve a process of cleaning, decontamination, drying and pulverisation. In this study, a freeze-drying step was introduced into the process. The purpose was to remove moisture from the tooth surfaces and the loose water molecule in the intrinsic structure of the biological entities. Moist tooth surfaces can promote bacterial growth that might eventually speed up the sample degradation. Meanwhile, the loose water in the tooth structure helps to maintain the aqueous-gel state of the mineral-organic matrix. The removal of the intrinsic water disrupts the matrix network resulting in the release of the DNA that is attached to it. Thereby, this method can improve the likelihoods of obtaining more DNA from the samples. Here we utilised two methods of DNA extraction (with- and without freeze-drying) for pig tooth samples (n = 21). Quantitative real-time polymerase chain reaction (qPCR) was used to quantify an 83 bp mitochondrial DNA fragment and two nuclear DNA fragments of 82 bp and 150 bp. The results showed that the sample preparation with freeze-drying can result in a better DNA yield without compromising the DNA quality. This study highlights the advantage of incorporating the freeze-drying to improve the DNA yield and minimising the loss of DNA during sample preparation of teeth.

Keywords

Teeth; Sample preparation; Freeze-drying; DNA extraction; Quantitative polymerase chain reaction

Introduction

Teeth are long-standing structures of the body composed primarily of an intertwined matrix of hydroxyapatite minerals (HA), collagen and water [1]. Enamel, the outermost layer of the tooth crown is composed of 97% HA and 3% water, making it the most mineralised structure in the body [2]. This enables DNA in teeth to survive prolonged exposure to extreme conditions such as humidity, elevated temperature and trauma [3, 4]. Thus, teeth are a valued source of DNA in forensic and archaeological casework involving skeletonised, fragmented or burned remains [2, 5]. A robust protocol for DNA analysis of teeth is critical to optimise the DNA yield for subsequent analyses.

Studies have focussed on the optimisation of DNA extraction protocols for skeletal samples [6-9] because the quantity and quality of DNA extracts will determine the success of the downstream analyses. However, the pre-DNA extraction or sample preparation is also important as it is the initial steps in the DNA analysis. The preparation of teeth typically consists of cleaning, decontamination, drying and pulverisation [10]. Many studies have focused on the effects of sample cleaning, decontamination and pulverisation on DNA yield from teeth and bones [6, 10-18]. However, the drying step aspect in the sample preparation protocol has often been overlooked. Many studies have performed air-drying or incubation in an oven or an incubator at 30° – 50 °C [15, 19-22].

Aiming to improve the method of drying the teeth, we introduced a freeze-drying step prior to the pulverisation of teeth. Dryness and low temperature are known factors to favour the preservation of DNA [23-25]. Freeze-drying or lyophilisation has been commonly used in other fields such as pharmaceutical, reproductive, food and other industrial area [26, 27]. Biological products containing proteins such as drugs, reproductive cells and fresh food can be preserved for long term by means of dehydrating the protein biomolecules [27].

In this study, the effects of freeze-drying on DNA recovery from pig teeth were tested. This was achieved by comparing two sample preparation methods on the DNA yield from teeth; (1) air-drying; (2) freeze-drying. Quantitative real time PCR (qPCR)

was used to quantify the DNA yield. Nuclear DNA (nDNA) fragments of different sizes (150 bp and 82 bp) and 83 bp-mitochondrial DNA (mtDNA) fragment were targeted and amplified. Furthermore, the DNA yield between molar and premolar teeth was analysed to gain a better understanding on the influence of the tooth type in the drying treatment.

Materials and methods

Sample preparation

Twenty-one pig teeth including molar and premolar teeth (n = 21) were sourced from fresh adult domestic pigs carcasses (*Sus scrofa domesticus*). Only roots of the teeth were used for DNA analysis. The roots were separated from the crowns in a dedicated laboratory using a set of sterilised cutting instruments. All work surfaces were cleaned with 4% sodium hypochlorite followed by 70% ethanol. Roots of each tooth were stored individually in a sterile container and assigned with a sample code for reference.

Drying method 1 (air-drying): Nine samples consist of five molars and four premolars (n = 9) were cleaned with distilled water to remove any tissue remnants. The samples were wiped with 70% alcohol wipe and allowed to air dry under the UV light for approximately 15 to 30 min, or until completely dry at room temperature (27°C). Then, each sample was pulverised using pestle and mortar. Liquid nitrogen was dispensed at an interval to minimise heat generation. The sample powder was kept in a sterile container and kept in a −20°C freezer until the DNA extraction procedure.

Drying method 2 (air-drying plus freeze-drying): The second method is similar to the first method (described above), however, a freeze-drying step was incorporated in between air drying and pulverisation. Twelve samples consist of six molars and six premolars (n = 12) were cleaned with distilled water to remove any tissue remnant. Then teeth were wiped with 70% alcohol wipe and air dried at room temperature (27°C) for 15 min under the UV light.

The caps of the containers were removed prior to the freeze-drying process. Freeze-drying was performed for four hours using an Alpha 1-2 LDplus freeze-dryer (Martin Christ, Osterode am Harz, Germany), following the manufacturer's operating manual. Upon the completion of the freeze-drying process, the containers of freeze-

dried samples were immediately airtight to avoid any contact with the moisture in the air and any external contaminants.

DNA analyses

DNA analysis was performed vigilantly by adhering to a strict contamination protocol to minimise the risks of cross-contamination. The protocol incudes:

- Dedicated laboratories for DNA extraction, pre- and post-PCR procedures.
- All workbenches were wiped with 4% sodium hypochlorite and followed by Decon 90™ before and after use.
- All instruments and hoods were wiped with 70% ethyl-alcohol and UV irradiated for 20 minutes before and after use.
- Negative controls consisting of DNA-free water were included in every DNA extraction and qPCR.
- qPCR of all samples was performed in triplicates.

Starting with a decalcification process, each pulverised sample of 70-130 mg was suspended in 1800 µL of 0.5 M ethylenediamine tetra-acetic acid (EDTA) pH 8.0 and placed on a shaking incubator at 56°C for 24 hours. After incubation, the decalcified samples were subjected to a silica-column based DNA extraction using the DNeasy® blood and tissue kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. All purified DNA extracts were quantified using a Quantus™ fluorometer (Promega Corporation, Madison, WI), according to the manufacturer's protocol. DNA extracts were stored at - 20°C.

qPCR was performed on all DNA extracts to quantify nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). Each sample was run in triplicate on Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific, Foster City, CA) and detected using an Applied Biosystems™ PowerUp™ SYBR® green master mix. Three primer sets of different type and fragment lengths were used to target nDNA (short and long fragments) and mtDNA (Table 1). The first two porcine primer sets targeting the mitochondrial *ATPase* gene and the nuclear *Melanocortin-1-Receptor (MC1R)* gene were from Antinick *et al.* [28] while the third primer set targeting the nuclear locus, *Actin cytoplasmic 1,* was designed based on the complete *Sus scrofa* nDNA sequence (BLAST Accession

NC_010445.4). Porcine DNA standards were created by serially diluting a DNA stock from porcine kidney DNA in DNA-free water at 1:10 dilution. Standards were at concentrations of 1, 0.1, 0.01, 0.001, 0.0001 ng/µL.

Table 1— Porcine primer sequences used in real-time quantitative PCR assay that were targeting and amplifying sequences of different type and length.

Gene name	Primer	Primer sequence (5' – 3')	Type (bp)	Tm(°C)
Actin_150	Forward	CTCTGACCTGAGTCTCCTTT	Nuclear (150)	55
	Reverse	CGGCTTTGTCACACGAG		
MC1R [28]	Forward	GCCCGGTTCCTACGTG	Nuclear (82)	60
	Reverse	AGAGGGTCCAGCGTCCATA		
ATPase [28]	Forward	AGCTCTGATCCAAGCTTATGTGT	Mitochondrial (83)	60
	Reverse	GCATGTGTTTGGTGGGTCA		

The cycling condition was as described in Rahmat *et al.* [29]. A standard curve was produced using the prepared DNA standards for each of the primer pairs. A melting curve was created using QuantStudio™ Real-Time PCR Software v1.3 (ThermoFisher Scientific, Foster City, CA) to verify one gene-specific peak and any primer-dimer formation.

DNA concentration of each sample (ng/µL) was calculated using the average Ct of the sample triplicate. The baseline adjustment method was used to determine the threshold cycle (Ct) in qPCR assay of each sample. DNA yield for each sample was standardised in ng DNA per milligram of tissues sample (ng/mg) by multiplying the quantified DNA (ng/µL) by the elution volume (µL) and dividing by the mass of powdered sample (mg).

Statistical analyses

Statistical analyses were completed using the statistical software R version 3.5.3. (R Core Team, Vienna, Austria) [30] (for details see supplementary data, S3). Descriptive statistics including mean and standard deviation were used to summarise the data on the DNA extracts and the DNA quantification.

Correlations between the nDNA and mtDNA fragments (response variables) were analysed using the built-in 'cor' function. This was necessary to ascertain if multivariate analysis was required. Response variables which are both theoretically related and moderately correlated should be fit in a multivariate model. If response

variables are weakly correlated than separate univariate analyses can be completed. Alternately, extremely high correlations (for this study we consider $r>0.9$ to be high) between response variables suggest redundancy and the number of response variables can be reduced to a representative set.

ANOVA (analysis of variance) was fit to the chosen response variable using the 'aov' function to investigate the interaction between the drying method and the tooth type. Comparisons between molar and premolar tooth groups were performed using Welch's two sample t-test. The normality of the residuals was checked for the linearity. To address any non-linearity of the data, the response variable was log transformed. The value was then back transformed to provide the confidence interval on the original scale. Statistical significance was accepted at $p \leq 0.05$.

Results

The total DNA concentration of purified DNA extract for all samples are presented in supplementary data, S1. Simple statistical analysis was used to compare the DNA concentration between the freeze-dried teeth and not freeze-dried teeth. Of 12 teeth, the highest DNA concentration was obtained from a freeze-dried tooth (32 ng/µL), and the lowest DNA concentration was retrieved from a not freeze-dried tooth (0.34 ng/µL). The average DNA concentration from the freeze-dried samples (6.42 ng/µL±2.64) was two-fold higher than from the air-dried samples (3.06 ng/µL±1.51).

To assess the amplification efficiency of the primer pair for each qPCR, the mean threshold cycle (Ct) derived from the standard curve (for details see supplementary data, S2) was used in the calculation. The amplification efficiency calculated for all the qPCR assays were within the range of optimum efficiency 85 – 110%. The dissociation curves revealed a single-peak indicating no contamination nor primer-dimer formation (for details see supplementary data, S2).

Table 2 compares the breakdown of the average of the DNA yield for three target amplicons according to the drying treatment. It is apparent from this table that the teeth that were subjected to freeze-drying treatment harvested notably higher DNA yield than the teeth were only air-dried.

Table 2—Descriptive statistics summarising the quantified DNA of the teeth between two different sample preparation methods (freeze-dried and not -freeze-dried) (Total N = 21).

Amplicon	Freeze-drying treatment	Mean (ng/mg) ±SD
ATPase	No	0.55±0.89
	Yes	5.14±8.40
MC1R	No	0.96±1.34
	Yes	9.66±14.56
Actin_150	No	0.40±0.98
	Yes	7.84±12.88

ATPase = 83 bp mtDNA. MC1R = 82 bp nDNA. Actin_150 = 150 bp nDNA. ng/mg = ng DNA per mg tooth SD = Standard deviation.

Two nDNA fragments and a mtDNA fragment, all of different sizes were selected to represent different DNA-typing methods such as miniSTR, SNP and mtDNA typing. The correlations between these three fragments were tested and high correlations were found between them ($r > 0.80$) (for details refer to supplementary data, S3). The high correlations indicate the likeness in the respond of nDNA (150 bp and 82 bp) and mtDNA (83 bp) to the specific drying treatment.

Despite being highly correlated, ANOVA for nDNA (82 bp) and mtDNA (83 bp) showed different results. ANOVA for quantified nDNA fragments (82 bp) revealed that there was no significant interaction of drying treatment and the tooth type on the DNA yield ($p = 0.100$). In contrast to that ANOVA for quantified mtDNA fragments (83 bp) showed that the interaction between the freeze-dry treatment and the tooth type has a significant effect on the DNA yield ($p < 0.05$).

Furthermore, Welch's two sample t-tests were performed on molar teeth and premolar teeth to compare the effects of the tooth group on the DNA yield (for details refer to supplementary data, S3). There was significant higher DNA yield in molars that had been freeze-dried than those that had not ($p = .034$). However, the difference of mean yield between freeze-dried not freeze-dried premolars was not significant ($p = .863$). This finding suggests the effect of drying treatment was different according to the tooth type. The effects of drying treatment on nDNA (82 bp) and mtDNA (83 bp) are shown in the boxplots (Figure 1).

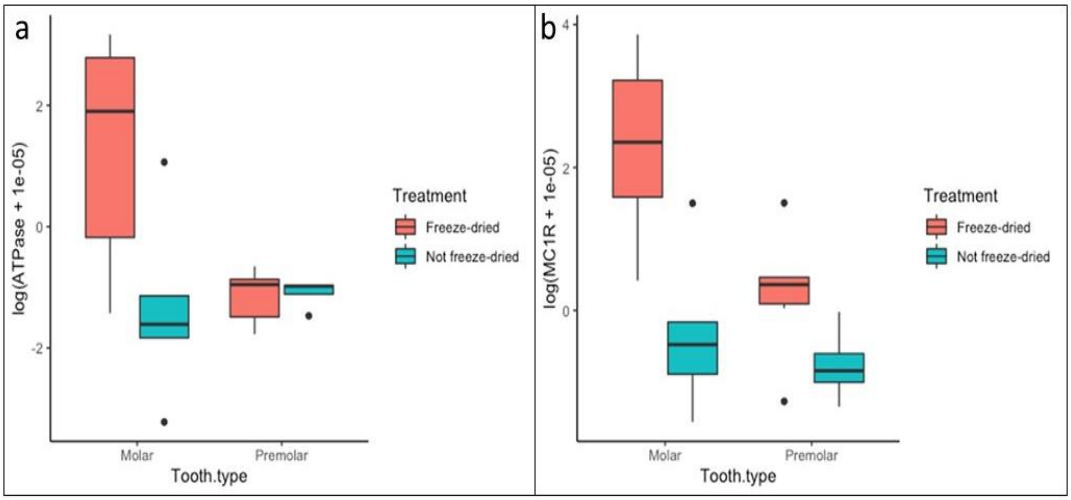

Figure 1— Boxplots show the comparison between the DNA yield from freeze-dried and not freeze-dried samples of molar and premolar groups. (a) In the molar group, the distribution of mitochondrial DNA (ATPase) in the freeze-dried samples was notably more widespread than the not freeze-dried samples. In the premolar group the distribution of mitochondrial DNA (ATPase) in the freeze-dried samples was larger than the not freeze-dried samples, but less spread than the molar group. (b) More nuclear DNA recovered (MC1R) from both the freeze-dried samples of molar and premolar groups than from the not freeze-dried samples. Nonetheless, the freeze-drying effect was greater for the molar group.

Discussion

The present study was to determine the effect of freeze-drying on the DNA yield in teeth. Two drying methods, were compared, one was with freeze-drying and another was only air-drying. The results indicate that the DNA yield was remarkably improved when teeth were subjected to the freeze-drying treatment. Initial analysis of the concentrations of purified DNA extracts in the freeze-dried teeth were higher than the not freeze-dried teeth. To further validate the efficiency of these two methods, qPCR of nDNA and mtDNA fragments was performed.

The effects of freeze drying were found to be different according to the tooth type. In molars, freeze-drying has shown to greatly increase the DNA recovered. However, the freeze-drying has no significant effect on the DNA yield in premolar teeth. Evidently, the statistical analysis suggests that mtDNA was easier to be recovered from freeze-dried premolars, but a larger data set is needed to confirm this finding. In a previous study, it has been shown that the DNA yield between molar and premolar were widely varied due to the form and size of teeth [2]. A molar has a larger pulp volume and more roots than a premolar, hence greater number of dental cells including odontoblasts and cementocytes are available. This increases the likelihood of retrieving DNA in molar in comparison to the DNA in premolar [31, 32].

An overview of the physicochemical properties of teeth can help to better understanding on how freeze-drying the teeth can improve the DNA yield. The major compositions of teeth are hydroxyapatite minerals and collagen type I [1]. Collagen interlaced with the HA minerals forming an aqueous-gel mineralised matrix, in which DNA containing cells such as odontoblasts and cementocytes are embedded [10, 33, 34]. The mineralised matrix is considered as physical challenge for DNA extraction [35]. The function of water in the matrix is to maintain the aqueous-gel state and it is present in two forms [36, 37]: 1) loosely present in the mineral-collagen matrix (loose water); 2) firmly bound to the surfaces of mineral and organic. The loosely bound water is presumed to be present in the pores, adsorbed on the HA crystallite surfaces in enamel, or associated with the organic matrix [38]. In the second method, an additional step of freeze-drying was added to the process in between the air-drying and the pulverisation steps (Figure 2). Freeze-drying enhances the sample dehydration by removing extrinsic and intrinsic water molecules of the skeletal structure. When loose water is reduced, the link between collagen and hydroxyapatites begins to break. As a result of the broken network, DNA containing cells are unbound and free from the skeletal matrix.

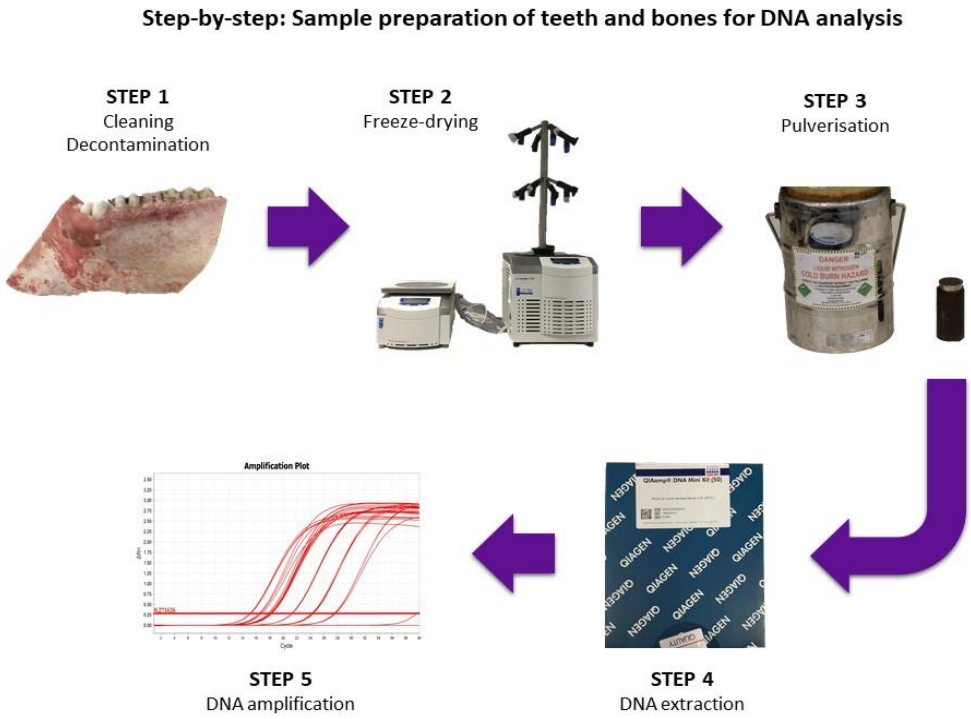

Figure 2—The proposed workflow for preparing teeth and bones used in a DNA analysis which include the application of freeze-dryer to intensify the sample drying.

Another benefit of using freeze-drying is that it can reduce the risk of microbial contamination. The removal of excess water from tooth samples prevents or delays the putrefaction process that is caused by microbial contamination [24]. Elevated level of moisture in post-mortem biological tissue promotes microbial growth particularly in skeletal samples that were recovered from soil [24, 39]. Moisture environment encourages the growth of bacteria and also provides an activation medium for hydrolytic enzymes [39]. These enzymes denature proteins to their basic form or amino acids [39], which promotes tissue decomposition and interfere with the integrity of DNA. Conversely, a dry environment would greatly reduce the rate of DNA degradation [24].

In addition, the inclusion of the freeze-drying step makes the sample preparation less time-consuming. The immediate used of sterile, airtight containers following the freeze-drying step can prevent cross-contamination DNA from the laboratory equipment and the surrounding environment [26, 40]. In this study, all tooth samples were freeze-dried simultaneously for 4 hours duration to achieve satisfactory dryness. This was in contrast to a previous study that applied a time consuming drying method, in which bones and teeth were dried in an oven at 50°C for 28 hours to yield DNA, or two days to dry the samples at room temperature [20].

Conclusion

This study has confirmed that freeze-drying the teeth in sample preparation can optimise the DNA yield. It also helps to minimise the microbe contamination that may accelerate sample degradation that can inhibit the downstream analyses. Moreover, freeze-drying is feasible and straightforward. This robust method has a potential to largely benefit the DNA analyses of human teeth for forensic identification and archaeological work. However the data of this study were derived from pig teeth, therefore the applicability of this method on human teeth has to be tested. This recommendation has been highlighted in a recent study [41]. Hence an additional research to assess the efficacy of the method on human teeth is warranted.

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Conflict of interest

The authors declare no conflict of interest with this research.

Ethics

The handling of the animal remains in this study was done in line with the University of Adelaide Animal Ethics.

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Supplementary data, S1

An improved method of preparing skeletal remains to optimise DNA yield

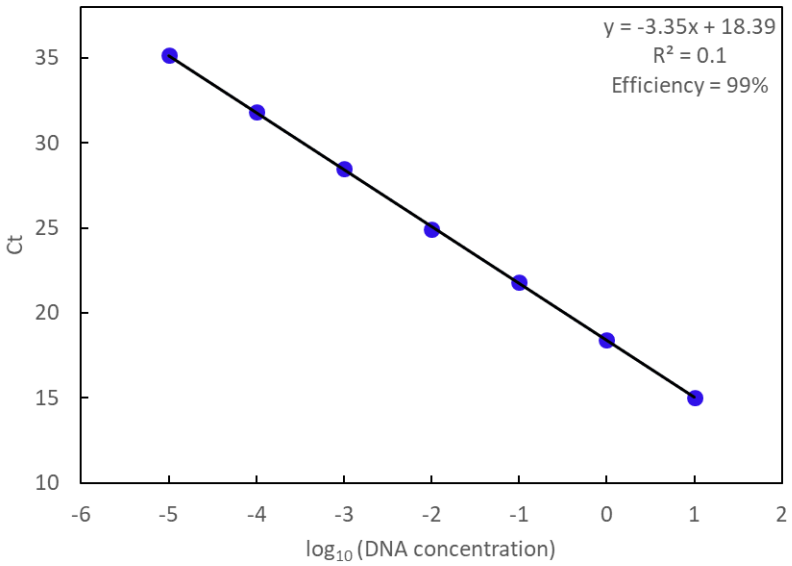
S 1—Concentrations of DNA extracts quantified using a Quantus™ fluorometer.

Sample No	Freeze-dry Treatment	Tooth type	DNA extracts (ng/µl)
58	Not Freeze-dried	Premolar	1.58
59	Not Freeze-dried	Premolar	1.79
60	Not Freeze-dried	Premolar	2.72
61	Not Freeze-dried	Molar	15
63	Not Freeze-dried	Molar	1.65
66	Not Freeze-dried	Premolar	1.03
68	Not Freeze-dried	Molar	0.35
69	Not Freeze-dried	Molar	2.04
70	Not Freeze-dried	Molar	1.41
72	Freeze-dried	Premolar	0.84
73	Freeze-dried	Premolar	1.57
74	Freeze-dried	Premolar	1.24
75	Freeze-dried	Molar	14
76	Freeze-dried	Molar	32
77	Freeze-dried	Molar	4.05
79	Freeze-dried	Premolar	1.09
80	Freeze-dried	Premolar	1.99
81	Freeze-dried	Premolar	1.32
82	Freeze-dried	Molar	6.7
83	Freeze-dried	Molar	11
84	Freeze-dried	Molar	1.2

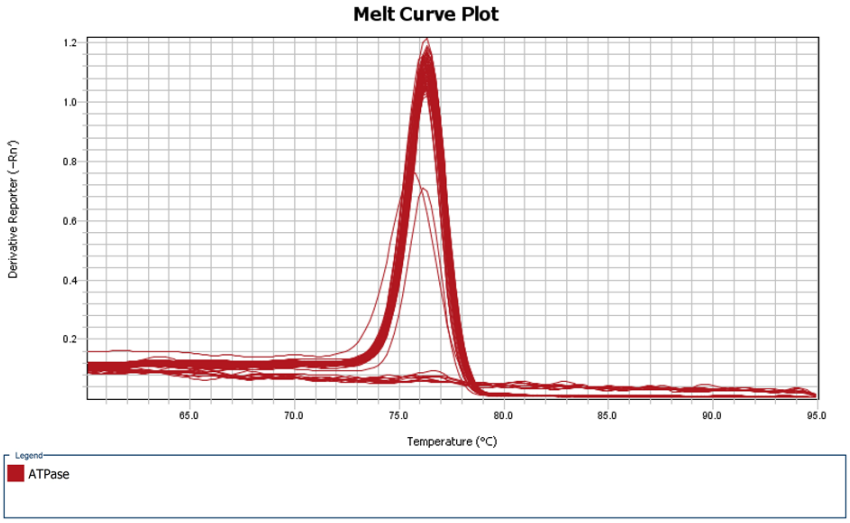
Supplementary data, S2

An improved method of preparing skeletal remains to optimise DNA yield

ATPase Non-freeze-dried

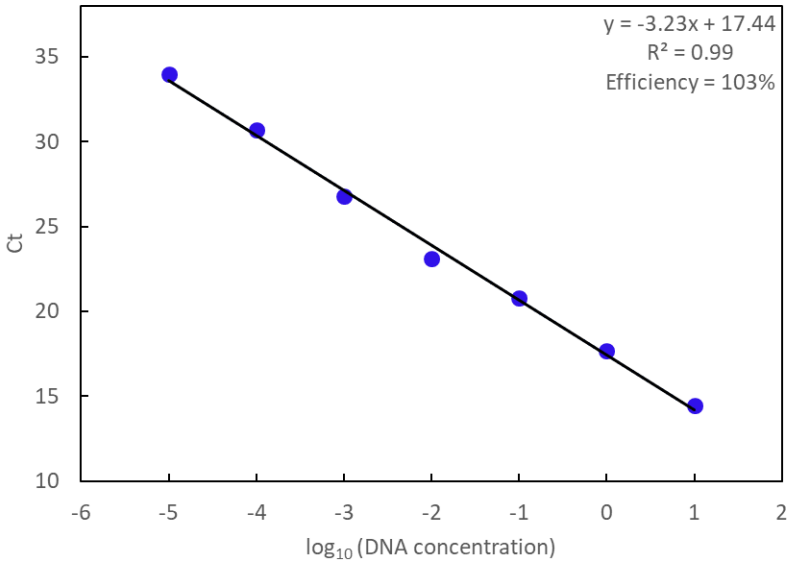


S 1— Standard curve generated from the seven serial diluted standards for the qPCR reaction of ATPase-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, 0.0001 ng/µL, 0.00001 ng/µL, 0.000001 ng/µL.

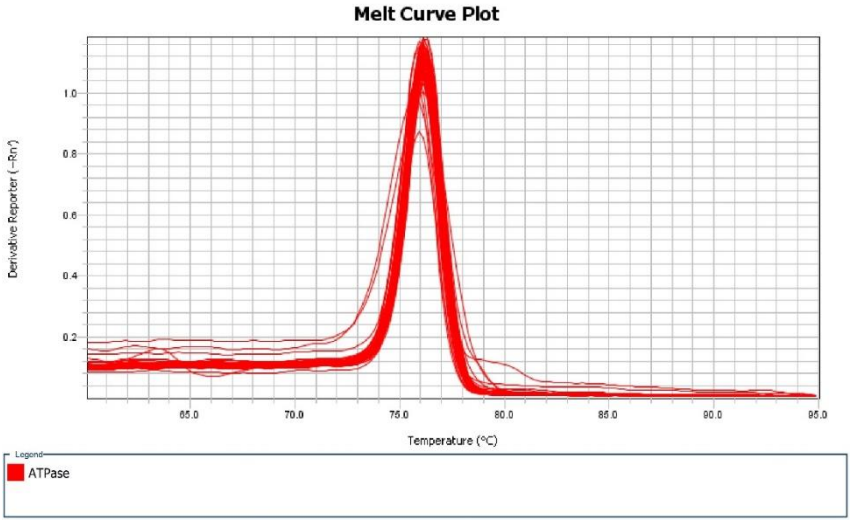


S 2—Dissociation curve shows a single-peak of the ATPase-gene which indicate no contamination or primer-dimer formation.

ATPase Freeze-dried

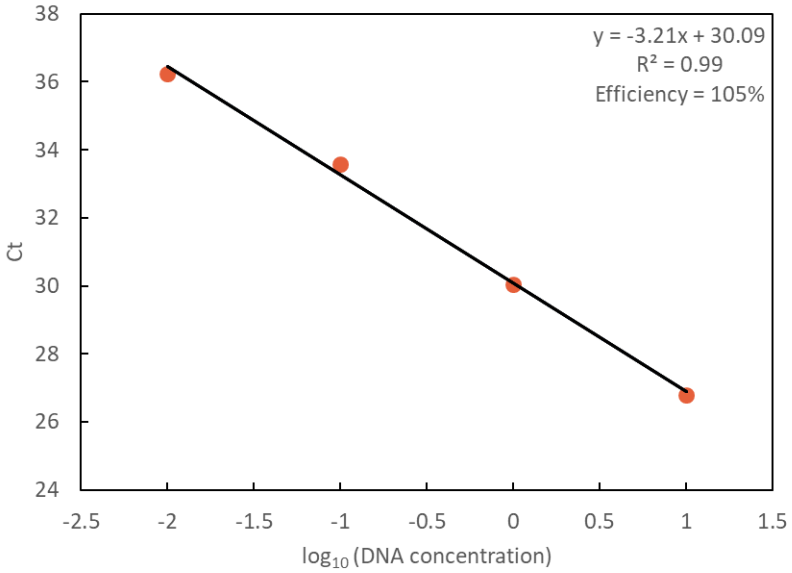


S 3— Standard curve generated from the seven serial diluted standards for the qPCR reaction of ATPase-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, 0.0001 ng/µL, 0.00001 ng/µL, and 0.000001 ng/µL.

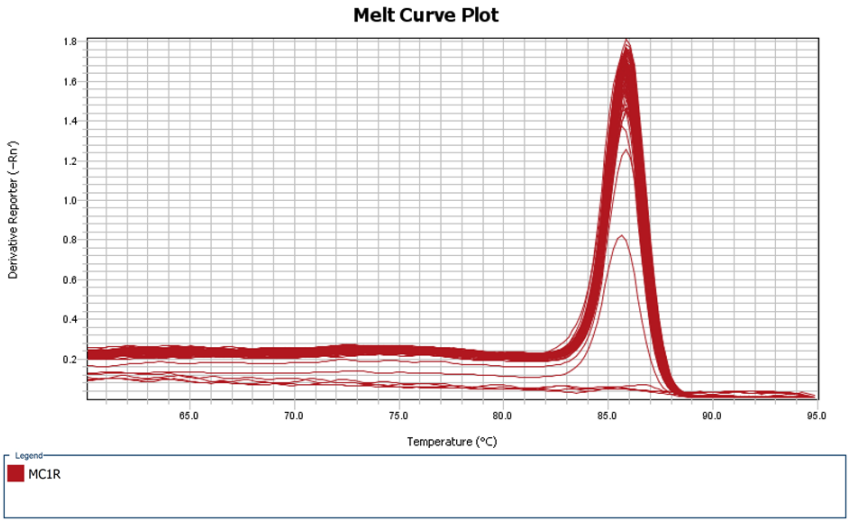


S 4—Dissociation curve shows a single-peak of the ATPase-gene which indicate no contamination or primer-dimer formation.

MC1R Non-freeze-dried

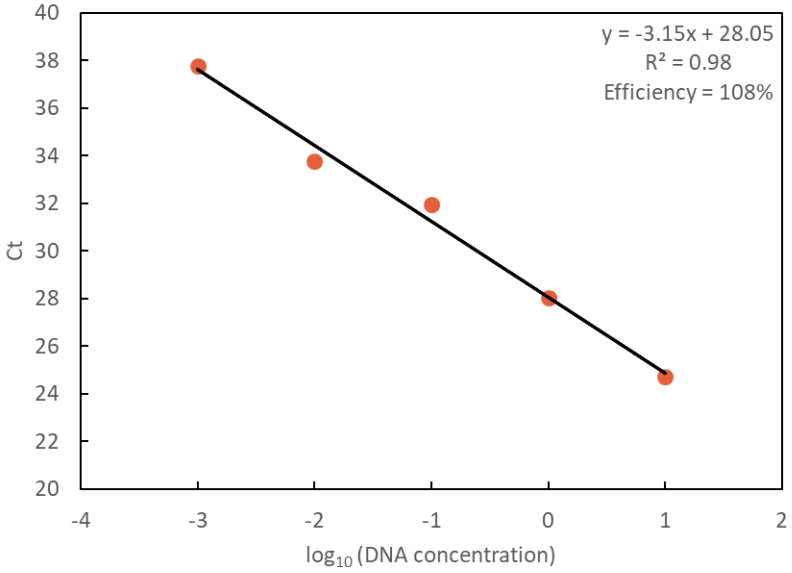


S 5— Standard curve generated from the four serial diluted standards for the qPCR reaction of MC1R-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, and 0.001 ng/µL.

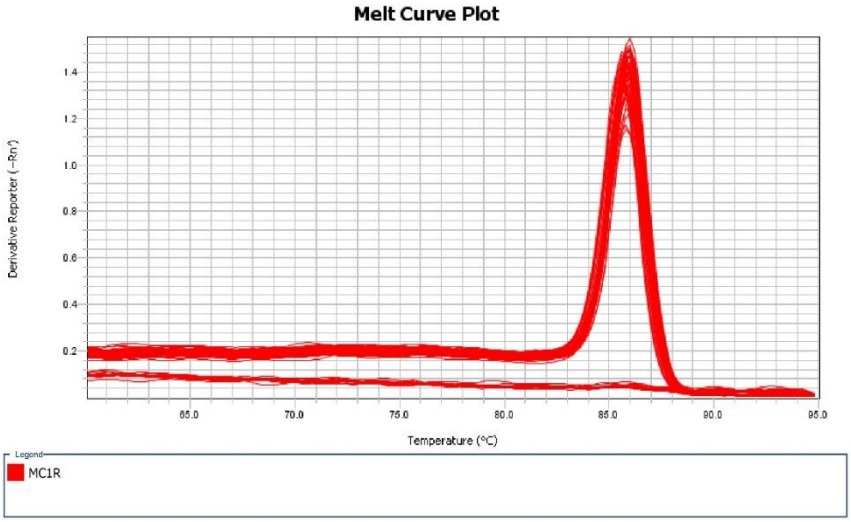


S 6—Dissociation curve shows a single-peak of the MC1R-gene which indicate no contamination or primer-dimer formation.

MC1R Freeze-dried

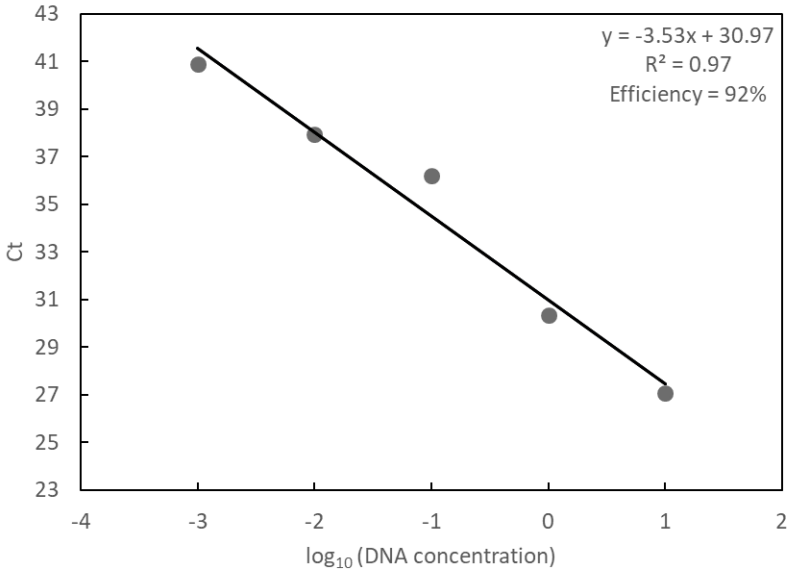


S 7— Standard curve generated from the five serial diluted standards for the qPCR reaction of MC1R-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, and 0.0001 ng/µL.

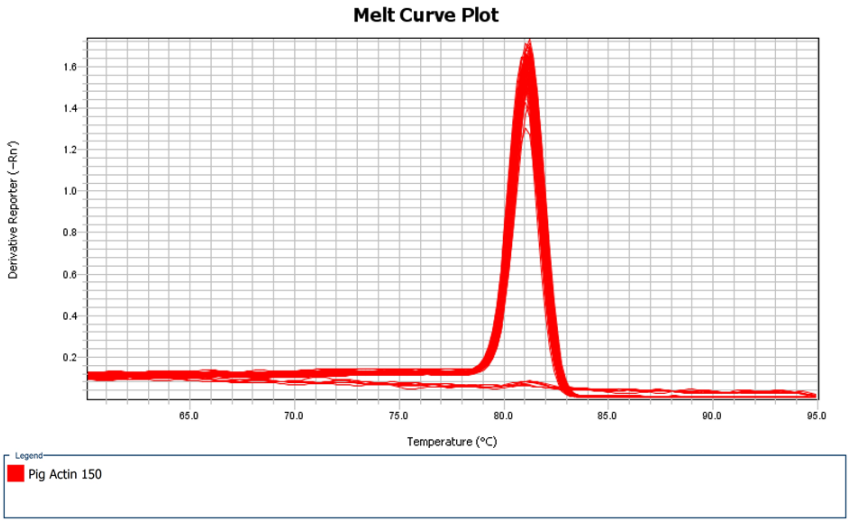


S 8— Dissociation curve shows a single-peak of the MC1R-gene which indicate no contamination or primer-dimer formation.

Actin_150 Freeze-dried

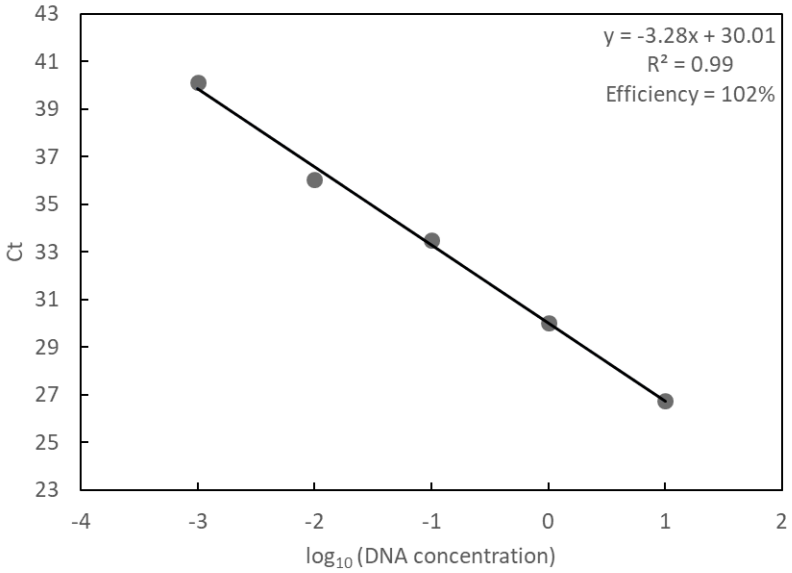


S 9— Standard curve generated from the five serial diluted standards for the qPCR reaction of Actin 150-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, and 0.0001 ng/µL.

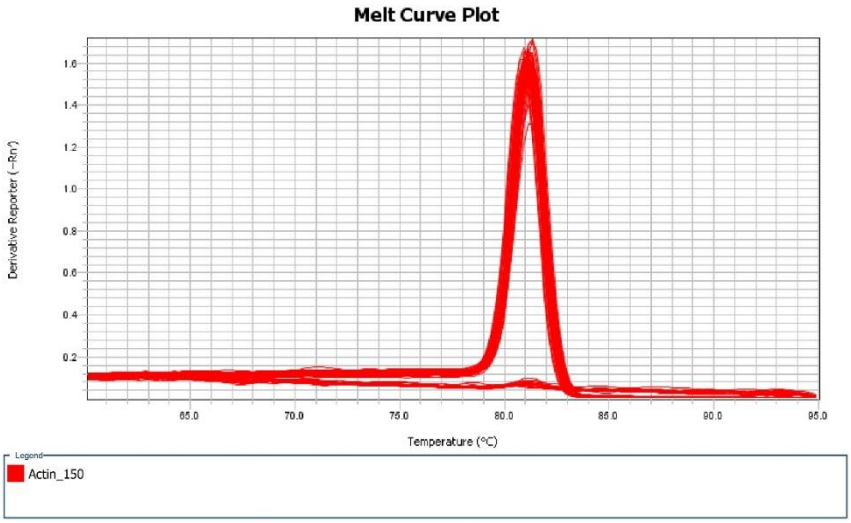


S 10—Dissociation curve shows a single-peak of the Actin 150-gene which indicate no contamination or primer-dimer formation.

Actin_150 Non-freeze-dried



S 11— Standard curve generated from the five serial diluted standards for the qPCR reaction of Actin 150-gene assays. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1 ng/µL, 0.1 ng/µL, 0.01 ng/µL, 0.001 ng/µL, and 0.0001 ng/µL.



S 12—Dissociation curve shows a single-peak of the Actin 150-gene which indicate no contamination or primer-dimer formation.

Supplementary data, S3

An improved method of preparing teeth to optimise DNA yield

i. Descriptive statistics

```
# We start by reading in the data
dteeth <- read.csv("freeze_dry.csv", header=TRUE)
# Look at the summary and check variables have read in properly
summary(dteeth)
```

```
##      Sample                Treatment     Tooth.type     ATPase
##  Min.   :58.0   Freeze-dried    :12   Molar   :11   Min.   : 0.0
40
##  1st Qu.:66.0   Not freeze-dried: 9   Premolar:10   1st Qu.: 0.2
30
##  Median :73.0                                      Median : 0.3
80
##  Mean   :71.9                                      Mean   : 3.1
76
##  3rd Qu.:79.0                                      3rd Qu.: 0.5
60
##  Max.   :84.0                                      Max.   :23.7
60
##       MC1R          Actin_150        X
##  Min.   : 0.210   Min.   : 0.02   Mode:logical
##  1st Qu.: 0.450   1st Qu.: 0.07   NA's:21
##  Median : 1.320   Median : 0.65
##  Mean   : 5.935   Mean   : 4.65
##  3rd Qu.: 4.500   3rd Qu.: 2.25
##  Max.   :47.530   Max.   :41.85
```

```
#Interquartile ranges
rbind(c("ATPhase","MC1R","Actin_150"),c(IQR(dteeth$ATPase), IQR(dteeth$MC1R), IQR(dteeth$Actin_150)))
```

```
##      [,1]      [,2]   [,3]
## [1,] "ATPase" "MC1R" "Actin_150"
## [2,] "0.33"    "4.05" "2.18"
```

ii. Correlation-coefficient analysis

```
cor(dteeth[,4:6])
```

```
##              ATPase      MC1R Actin_150
## ATPase    1.0000000 0.8696657 0.8627851
## MC1R      0.8696657 1.0000000 0.9986345
## Actin_150 0.8627851 0.9986345 1.0000000
```

iii. ATPase-target gene

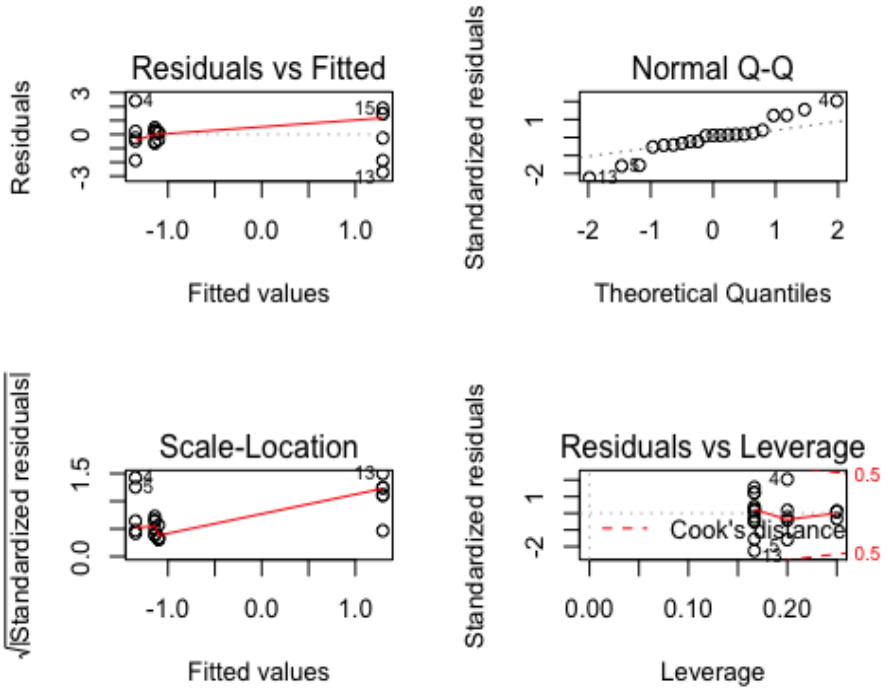
ANOVA

```
fitATl <- aov(log(ATPase+.00001)~Treatment*Tooth.type, data=dteeth)
summary(fitATl)
```

```
##                       Df Sum Sq Mean Sq F value Pr(>F)
## Treatment              1  8.869   8.869   5.054 0.0381 *
## Tooth.type             1  8.716   8.716   4.967 0.0396 *
## Treatment:Tooth.type   1  9.165   9.165   5.222 0.0354 *
## Residuals             17 29.833   1.755
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Look at the residuals:

```
opar <- par(mfrow=c(2,2))
plot(fitATl)
```



T-tests

Molar

```
LogATttest <- t.test(log(ATPase+.00001)~Treatment, data=dteeth[dteeth$Tooth.type
=="Molar",])
LogATttest


##  Welch Two Sample t-test
##
##  data:  log(ATPase + 1e-05) by Treatment
## t = 2.5005, df = 8.9959, p-value = 0.03385
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  0.2513738 5.0269363
## sample estimates:
##     mean in group Freeze-dried mean in group Not freeze-dried
##                       1.292111                      -1.347044

#Let's put these in back-transformed numbers
exp(LogATttest$conf.int)

## [1]   1.285791 152.465190
## attr(,"conf.level")
## [1] 0.95
```

Premolar

```
LogATttest <- t.test(log(ATPase+.00001)~Treatment, data=dteeth[dteeth$Tooth.type
=="Premolar",])
LogATttest

##
##  Welch Two Sample t-test
##
##  ##data:  log(ATPase + 1e-05) by Treatment
## t = -0.17849, df = 7.8158, p-value = 0.8629
## alternative hypothesis: true difference in means is not equal to 0
## 95 percent confidence interval:
##  -0.5615040 0.4811335
## sample estimates:
##     mean in group Freeze-dried mean in group Not freeze-dried
##                      -1.139929                      -1.099743



#Let's put these in back-transformed numbers
exp(LogATttest$conf.int)

## [1]   0.5703506 1.617907
## attr(,"conf.level")
## [1] 0.95
```

iv. MC1R-target gene

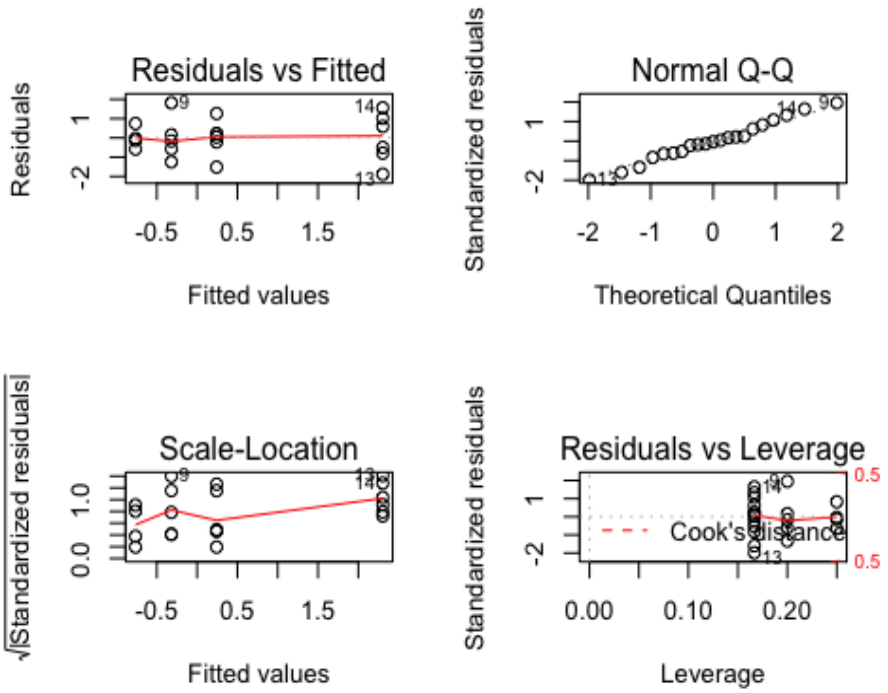
ANOVA

```
fitMCl <- aov(log(MC1R+.00001)~Treatment*Tooth.type, data=dteeth)
summary(fitMCl)
```

```
##                      Df Sum Sq Mean Sq F value  Pr(>F)
## Treatment             1 16.475  16.475  15.206 0.00115 **
## Tooth.type            1  9.861   9.861   9.102 0.00777 **
## Treatment:Tooth.type  1  3.335   3.335   3.078 0.09738 .
## Residuals            17 18.419   1.083
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

First, we need to look at the residuals:

```
opar <- par(mfrow=c(2,2))
plot(fitMCl)
```



T-tests

Molars

```
LogMCtMol <- t.test(log(MC1R+.00001)~Treatment, data=dteeth[dteeth$Tooth.type
=="Molar",])
LogMCtMol
```

```
##
##  Welch Two Sample t-test
##
```

```
## data:  log(MC1R + 1e-05) by Treatment
## t = 3.5808, df = 8.9341, p-value = 0.005995
## alternative hypothesis: true difference in means is not equal to
0
## 95 percent confidence interval:
##  0.9639385 4.2814908
## sample estimates:
##     mean in group Freeze-dried mean in group Not freeze-dried
##                      2.3040995                      -0.3186151

#Let's put these in back-transformed numbers
exp(LogMCtMol$conf.int)

## [1]  2.622003 72.348215
## attr(,"conf.level")
## [1] 0.95
```

Premolars

```
LogMCtpMol <- t.test(log(MC1R+.00001)~Treatment, data=dteeth[dteeth$Tooth.typ
e=="Premolar",])
LogMCtpMol

##
##  Welch Two Sample t-test
##
## data:  log(MC1R + 1e-05) by Treatment
## t = 2.1957, df = 7.9934, p-value = 0.05942
## alternative hypothesis: true difference in means is not equal to
0
## 95 percent confidence interval:
##  -0.05072642  2.06372306
## sample estimates:
##     mean in group Freeze-dried mean in group Not freeze-dried
##                      0.2421766                      -0.7643217

#Let's put these in back-transformed numbers
exp(LogMCtpMol$conf.int)

## [1] 0.9505387 7.8752353
## attr(,"conf.level")
## [1] 0.95
```

Part II

Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires

Statement of Authorship

Title of paper	Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires
Publication Status	Unpublished and unsubmitted work written in a manuscript style
Publication Details	Rahmat, R. A., et al. (2020). Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires, The University of Adelaide.

Principal Author

Name of Principal Author (Candidate)	Rabiah Al-Adawiyah Binti Rahmat		
Contribution to the paper	Research planning, project administration, sample preparation and investigation, data curation, data analysis and interpretation, created figures and tables, wrote manuscript and acted as a corresponding author.		
Overall percentage (%)	80%		
Signature		Date	04/05/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidature in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Melissa A. Humphries		
Contribution to the paper	Statistical analysis and interpretation		
Signature		Date	21/04/2020

Name of Co-Author	Jeremy J. Austin		
Contribution to the paper	Resources, supervision, manuscript evaluation and funding acquisition		
Signature		Date	23/04/2020

Name of Co-Author	Adrian M. T. Linacre		
Contribution to the paper	Supervision and manuscript evaluation		
Signature		Date	21/04/2020

Name of Co-Author	Arif Malik		
Contribution to the paper	Facilitated the DNA laboratory work and manuscript evaluation		
Signature		Date	04/05/2020

Manuscript prepared for submission

Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires

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Abstract

Teeth are a valued DNA source in the identification of severely burned human remains where the body is fragmented, and the soft tissues are completely charred. Forensic fire cases such as motor-vehicle crashes and bushfires can reach temperatures up to 1100°C. We investigated the effect of a wide range of incineration temperatures on DNA recovery and amplification from pig teeth. Fifty-seven pig teeth were incinerated in their natural anatomical position in jaw bones at 300°C – 1000°C for 15 minutes. DNA extraction using a silica-based spin column method and DNA amplification using a quantitative real-time polymerase chain reaction (qPCR) specifically targeting a short nuclear Actin gene (74 bp) were performed on all samples. All DNA extracted from control teeth and teeth incinerated up to 300°C were successfully amplified but no DNA amplification was detected in pig teeth incinerated at 600°C – 1000°C. Two-way ANOVA revealed that temperature had differing effects on DNA yield for molars and premolars ($p = 0.032$). The total DNA extracted from molars being higher than premolars ($p = 0.019$), and significantly more DNA was extracted from premolars heated at 300°C than those that were unheated ($p < 0.001$). This study has shown that the likelihood of obtaining amplifiable DNA from incinerated pig teeth can be predicted based on the temperature of the forensic fire. Teeth exposed to temperature less than 300°C are likely to contain amplifiable DNA.

However, DNA extracted from teeth incinerated at more than 600°C were deemed to be unsuitable for DNA amplification.

Keywords

Forensic science; High temperature; Incinerated teeth; DNA analysis; Polymerase chain reaction; Quantitative polymerase chain reaction

Introduction

The application of genetic analysis is necessary in the identification of burned human remains that are fragmented, commingled and visually unrecognisable [1, 2]. In forensic fire cases where remains can be severely charred and fragile due to exposure to high temperatures, teeth are valuable source of DNA [3-5]. This is because teeth can survive fire, are often recovered at fire scenes and preserve DNA better than soft tissues in such high temperatures (>300°C). For instance, 246 post-mortem samples of teeth and teeth fragments were collected in the MH17 crash, and contributed to positive identifications via DNA analysis [6]. Due to the covering of teeth by the durable enamel, the location of the root in the jaw bone and the thermal insulation afforded from the cheek muscles, DNA in teeth is shielded from direct exposure of the damaging heat [7, 8].

In general, temperature has a major influence on the integrity of DNA structure in biological tissues [9, 10]. Previous studies have investigated the effect of different temperatures on DNA recovery from incinerated teeth [11-16]. Alvarez Garcia *et al*. [11] analysed DNA from premolars and molars heated at 75° – 500°C for 1 – 5 min. Garriga *et al.* incinerated molar teeth at a range of 100°C – 700°C for 1 – 15 min. Rubio *et al*. [13] have investigated the effect of temperature of 100°C – 400°C for 1-hour on permanent teeth that were unspecified. Tsuchimochi *et al.* [14] have specifically incinerated third molars at 100°C – 500°C for 2 minutes. Using a similar range of incineration temperature, Williams *et al.* [15] has studied the effect of heat specifically on deciduous teeth. Overall, all of these studies show DNA yield was inversely proportional to the temperature and DNA can only be retrieved up to 400°C. However, the maximum temperature included in all of these studies was limited to 700°C with different durations of heat-exposure.

Forensic fire cases including house fires, motor vehicle crashes, bombings and explosions, and natural disasters occur at temperatures greater than 700°C [17]. In mass disasters such as in the 2001 World Trade Centre tragedy and the 2009 Victorian bushfires, fragmented remains were exposed to fires that exceeded 1000°C [18, 19]. The effect of temperature up to 1000°C on DNA in premolars and molars was investigated by Maciejewska *et al.*[9], but the teeth used were isolated and not encased in the jaw bone which is their natural anatomical state. Rees and Cox [16] has analysed DNA from teeth that were incinerated in two different conditions; (1) furnace incineration of teeth encased in the jaw bones ; (2) fire ignition using charcoal and wood on porcine heads. However, the maximum temperatures of both conditions were 600°C and 850°C respectively. A lack of studies investigating a wide range of incineration temperatures on DNA yield from teeth situated in jaw bones has led us to conduct this research.

This study was designed such that the temperatures used to incinerate the teeth were typical of the temperatures in forensic fire cases and additionally the physical barrier afforded from the jaw bones was considered. The aims of this study therefore were to investigate the effects of wide-ranging incineration temperatures (300°C – 1000°C) on the DNA extraction and amplification from pig teeth and to analyse the different DNA yield between pig molar and premolar teeth.

Materials and methods

Sample preparation

Fifty-seven pig teeth comprised of 27 molars and 30 premolars attached within mandibular bone segments were collected from pig carcasses *(Sus scrofa domesticus)* (N=57). Each tooth was annotated with a sample code. The freshly slaughtered pigs were obtained from a local abattoir. Only pig mandibles with healthy and intact molar and premolars were included for incineration.

Samples were cleaned with distilled water to remove any outer tissue and allowed to air dry. Control samples consisted of six molars and six premolars. Samples were incinerated at designated temperatures of 300°C, 600°C, 800°C and 1000°C for 15 minutes using an electrical furnace (Ward, Serial No: 12098, South Australia). The buccal surface of the mandible faced upward and was positioned in the centre of the

furnace. Each mandibular segment was put in a crucible to avoid loss of tooth samples during the sample retrieval.

Post-incineration, the roots and root fragments of every tooth were collected in an individual sterile container labelled with the sample code. Tooth roots were snap frozen in liquid nitrogen before being pulverised into powder using metal pestle and mortar. Powdered samples were stored in a –20°C freezer prior to subsequent DNA extraction. All equipment was disinfected using 70% ethyl-alcohol and workbenches were cleaned with 4% sodium hypochlorite before and after sampling procedure.

DNA extraction

Approximately 50 to 100 mg of each powdered sample was utilised for DNA extraction. The DNA extraction method used was silica-column based using DNeasy® blood and tissue kit (QIAGEN, Hilden, Germany), following the protocol for skeletal materials. All DNA extracts were quantified using the Quantus™ fluorometer (Promega Corporation, Madison, WI), according to the manufacturer's protocol for double-stranded DNA. The limit of detection for double-stranded DNA (dsDNA) concentration was 0.01 ng/µL. All DNA extracts were stored at -20°C until further analysis.

DNA amplification

We performed qPCR on all DNA extracts to quantify porcine nuclear DNA. The qPCR specific assay consists of a pair of forward and reverse primers targeting a 74-base pair (bp) fragment of the Actin, cytoplasmic 1 gene (BLAST Accession NC_010445.4) (Table 1). Each qPCR contained 10 µL Applied Biosystems™ PowerUp™ SYBR® green master mix (Thermo Fisher Scientific, Foster City, CA), 10 µM forward and reverse primers, 10 – 50 ng of DNA extract and DNA-free water to make the final volume up to 20 µL. A serial dilution of DNA standards at concentrations of 1, 0.1, 0.01, 0.001, 0.0001 ng/µL were prepared from stock DNA of porcine kidney. Using the DNA standards, a standard curve was constructed for the primer pair. A no template control (NTC) was included in every qPCR run by substituting DNA extract with DNA-free water to check for contamination and primer-dimer formation.

The qPCR of each sample was run in triplicate on an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Foster City, CA). The cycling condition was one cycle of UDG (Uracil-DNA glycosylase) activation at 50°C/2 min and dual-lock DNA polymerase at 95°C/2 min, 50 cycles of denaturation at 95°C/ 15 sec, annealing at 55°C/15 sec and extension at 72°C/1 min followed by one three-steps cycle of product dissociation (95°C/15 sec, 60°C/1 min, 95°C/15 sec). A dissociation curve was generated using QuantStudio™ Real-Time PCR Software v1.3 (Thermo Fisher Scientific, Foster City, CA) to validate one gene-specific peak and any primer-dimer formation. The baseline adjustment method was used to determine the Ct (threshold cycle) in each reaction. The Ct mean of each sample was used to calculate the qPCR concentration (ng/µL) of each sample. For a quality control, PCR products were examined by DNA electrophoresis in 1.5% agarose gel matrix stained with Midori green Direct to verify that only a single product was being amplified and the fragment size was the predicted length. Following the electrophoresis, the agarose gel was visualised and recorded using Axygen® gel Documentation Systems (Corning, NY, U.S.A).

The DNA concentration per mg tissue was calculated based on a previous study [20]; DNA concentration per qPCR (ng/µL) multiplied by total extract volume (µL) and divided by the mass of tooth powder (mg) resulted in nanograms of DNA recovered per milligram tooth powder (ng/mg).

Table 1—Porcine primer sequences used in real-time quantitative PCR assay.

Gene name	Primer	Primer sequence (5’ – 3’)	Product length (bp)	Tm(°C)
Actin, cytoplasmic 1	Forward	CTCTGACCTGAGTCTCCTTT	74	55
	Reverse	CAAACACGAGAAAGACTCCA		

The expected product length was 74 base pairs (bp) and the melting temperature (Tm) was estimated to be 55°C.

Statistical analyses of DNA yields

Statistical analyses were performed using R [21]. Statistical significance for all tests were accepted at $p<0.05$. Two-way analysis of variance (ANOVA) was performed to identify any effects of temperature and tooth type on DNA yield. Pairwise post-hoc t-tests were then conducted to elaborate on the differences found in the ANOVA.

Results

DNA extraction

The quantified concentrations of all DNA extracts using the Quantus™ fluorometer (Promega Corporation, Madison, WI), based on temperature of heat-exposure and tooth type are presented in the Supplementary Data. DNA was extracted from all control teeth and teeth heated at 300°C. Teeth heated at 600°C and 800°C showed less than0.01 ng/µL of DNA concentrations in 64% and 58% of the total samples. Interestingly, 42% of the DNA extracts from the teeth heated at 1000°C resulted in a concentration of more than 0.01 ng/µL.

Quantitative real-time PCR (qPCR)

Amplification efficiency per qPCR was calculated from the mean threshold cycle (Ct) derived from the standard curve (for details see Supplementary Data). The amplification efficiency in this study was 95% which was within the range of optimum efficiency. A single-peak was detected in the dissociation curve which indicated that no contamination nor primer-dimer formation occurred in the qPCR (for details see supplementary data). Using gel electrophoresis, the successful amplification of qPCR products of 74 bp was verified (Figure 5, for details see supplementary data).

The qPCR results show genomic DNA (nDNA) was recovered from all teeth in the control and 300°C groups whereas no DNA was detected in teeth heated at 600°C and above (Figure 1) (for details see Supplementary Data). Molar teeth yielded the highest DNA concentration and showed more widespread variability than premolar teeth. Individually, the greatest quantity of DNA was detected in a molar tooth from the control group with the concentration of 41.14 ng/mg, whereas the lowest value was in premolar of the control group.

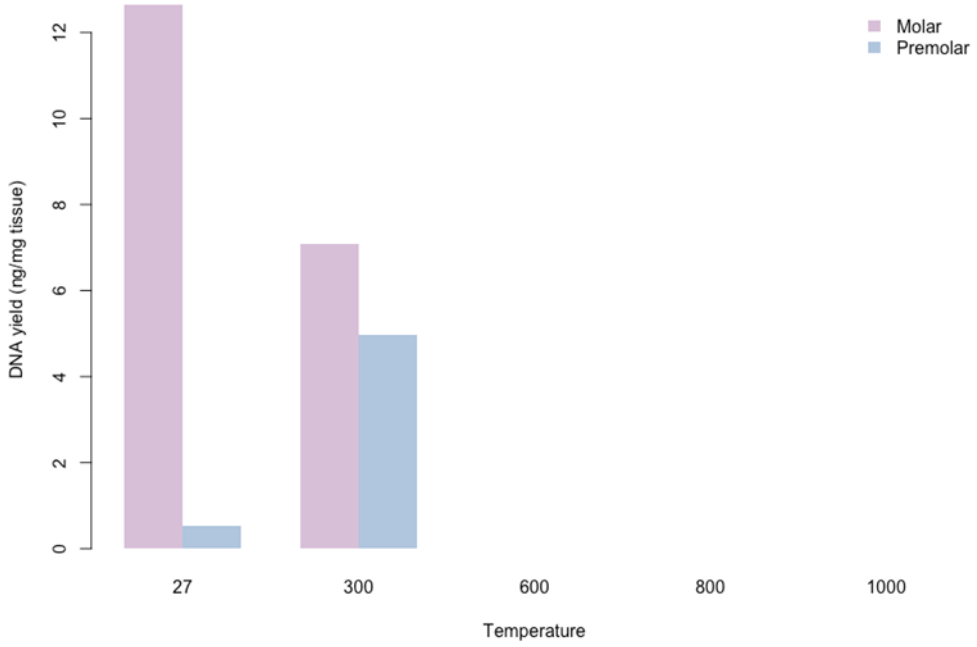

Figure 1—The trend of the average nuclear DNA yield according to the tooth type at an increasing temperature level from 27° to 1000°C (n=57).

The average nDNA yield from tooth samples based on the tooth type are shown in Table 2. The average of DNA recovered from unheated molar group (12.66 ng/mg) was twelve-fold higher when compared to the unheated premolar group (0.53 ng/mg). The average of DNA recovered from molars heated at 300°C was two-fold lower when compared to the unheated teeth. Interestingly, the DNA yield from premolars of 300°C group was 5-fold higher than from the control group. In the 300°C group, the DNA yield from molars (5.97 ng/mg) was comparable to the DNA yield in premolars (5.43 ng/mg). In contrast, no DNA yields were noted in teeth heated at higher temperatures, 600°C – 1000°C.

Table 2— The average DNA yield from control teeth and teeth heated at temperatures from 27°C – 1000°C. These values were calculated based on the results of the qPCR assays.

Temperature (°C)	Tooth type	Mean ng/mg (SD)
27	Molar	12.66 (16.10)
	Premolar	0.53 (0.36)
300	Molar	5.97 (3.81)
	Premolar	5.43 (2.50)
600	Molar	0
	Premolar	0
800	Molar	0
	Premolar	0
1000	Molar	0
	Premolar	0

ng/mg = Nanogram DNA per microgram of sample. SD = Standard deviation

Tooth type vs Temperature

Two-way analysis of variance (ANOVA) revealed a significant interaction between tooth type and temperature on the retrieval of DNA (for additional details see Supplementary Data). Although the DNA yield was, on average, between 1.362 and 5.607 ng/mg tissue higher for molars than premolars, temperature effected the yield for each tooth type differently.

As DNA was only successfully extracted at 300°C (M = 4.20, SD = 1.30), and for the control teeth (M = 2.20, SD = 0.84), follow-up post-hoc analysis considered only the comparison between these two groups. After log transforming DNA (to meet normality assumptions), a t.test revealed the amount of DNA extracted from premolars heated at 300°C was between 0.405 and 4.557 ng/mg tissue higher than the control teeth ($p < .001$). Alternately, there was no significant difference in the amount of DNA extracted between control molars and molars heated at 300°C ($p = .717$).

Discussion

DNA extraction

We incinerated the teeth encased naturally in jaw bone and used only the roots for DNA extraction because:(1) the roots are physically protected from external assaults and contamination by the bone; (2) the morphology of the tooth crown can be preserved for other analytical methods; (3) the high mineral content of enamel in the crown has no DNA, hence it can potentially reduce the efficiency of DNA extraction [22]. Moreover, when exposed to extreme heat, the tooth crown is susceptible to stress-induced fracture or dislodgement, and pulp is vulnerable to desiccation, especially

when teeth are exposed to temperature greater than 450°C [16]. Several studies have highlighted the advantages of using the tooth roots for DNA analysis of teeth exposed to unfavourable conditions for DNA preservation [8, 23].

The silica-based column extraction method proved to be suitable for retrieving DNA from incinerated teeth. Using the Quantus™ fluorometer, 43% of the teeth heated at 600°C - 1000°C showed DNA concentration of 0.01 ng/µL and above. Whilst, all the DNA extracts of teeth heated at 300°C was greater than 0.5 ng/µL with the highest concentration of 6.2 ng/µL measured in a molar (Sample 49). This DNA yield was better when compared to the study by Garriga *et al*. [12], in which the DNA extracted from a molar tooth heated at the same temperature and duration was 0.13 ng/µL. This could because the teeth used and incinerated in the study was isolated and had no bony protection. In this study, the roots of our tooth samples were protected from direct heat insult due to the mandibular bone.

Quantitative real-time PCR (qPCR)

We utilised qPCR due to its high-sensitivity to detect and amplify a specific DNA sequence (amplicon) and simultaneously quantify the amplicon [24]. The concentration of the DNA extracted from incinerated teeth ranged from undetectable to 11.59 ng/mg (sample 55). Our results suggest that DNA can be recovered and amplified from teeth that have been exposed to temperature up to 300°C whilst DNA can only be recovered and not amplified from teeth that have been exposed to temperature at or higher than 600°C (Figure 1). This finding corresponded with what was observed in another study which determined that DNA amplification can successfully be achieved from DNA extracted from teeth heated up to 525°C [16] and no DNA amplification can occur above 500°C [11, 25]. However, it is worth noting that the failure of DNA amplification in this study after 600°C does not necessarily suggest that DNA has degraded. Alvarez Garcia *et al.* [11] suggested that the fragment size is a crucial factor in determining the success of amplification. Further investigation using primers targeting mitochondrial DNA (mtDNA) and different fragment size might be useful to validate this finding. The analysis of mtDNA has shown to be a reliable option in the identification of burned skeletal remains [26] and furthermore, mtDNA in teeth was found to be more stable than nuclear DNA following incineration at high-temperature [9].

Tooth type vs temperature

Our results indicated that the average DNA concentration retrieved from molar teeth is higher than in premolar teeth (Figure 2). Tooth type was shown to be interrelated with the DNA contents [4]. Higher amounts of DNA can be retrieved from tooth with a larger pulp volume and/or with multiple roots than teeth with a small pulp volume and/or a single root [27, 28]. In addition, molar teeth produced a higher DNA yield due to the more cementum coverage on the surface area of the roots [29]. In fact, molar teeth or tooth roots are preferable DNA samples to be collected from severely burned remains in a Disaster Victim Identification (DVI) process as suggested by the INTERPOL and the DNA commission of the International Society for Forensic Genetics (ISFG) [30, 31].

Premolar teeth that were heated at 300°C show a noticeably higher concentration of DNA compared to premolar teeth from the control group (Figure 1). Given the premolar teeth in the control group were not subjected to the heat treatment, the result was unexpected. Rees and Cox [16] have reported a comparable finding in their study where the DNA obtained in teeth heated at 375°C has shown to be greater than in teeth heated at lower temperatures. According their study, the pulp tissue was still intact and it can be retrieved completely with ease because teeth show only minor signs of damage, with only cracks occurring at this temperature.

In the present study, the whole roots including pulp were used for DNA analysis. The roots of premolars were still situated in alveolar sockets following heat-exposure at 300°C. Unlike the control teeth where periodontal ligaments hold tooth roots firmly in alveolar sockets, the soft tissues in teeth exposed to 300°C were already dried. Thus, the roots of the premolar teeth heated at 300°C were removed with lesser force than the force applied upon the removal of the roots of control teeth.

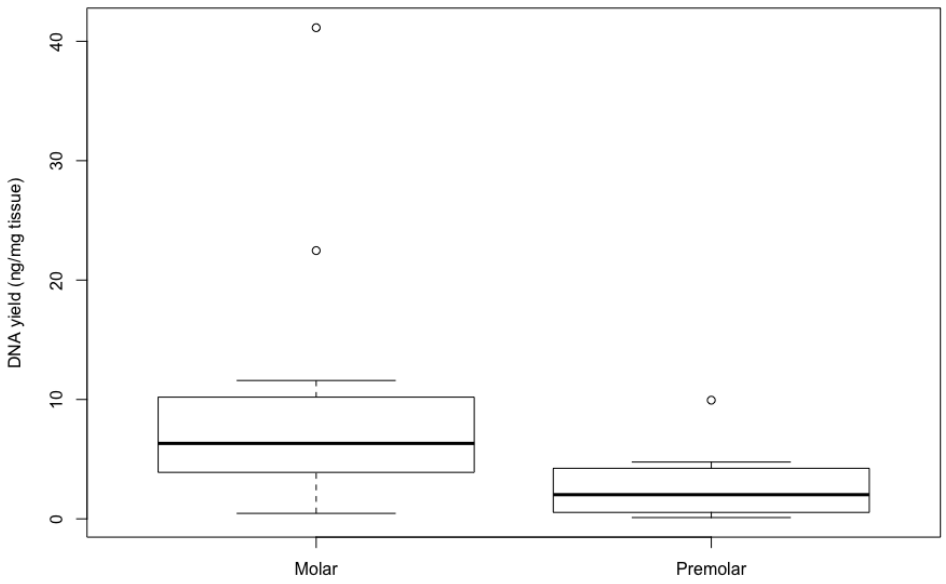

Figure 2—Comparison of the DNA yield between molar and premolar tooth groups for control teeth and those heated to 300°C. The remaining temperature groups are excluded as no DNA was able to be extracted and their inclusion unfairly skews the boxplot. Note that the distribution of DNA yield in the molar group is more widespread than the DNA yield in the premolar group.

Overall, the average DNA yield was inversely associated with increasing temperature. Temperature is well-known to play a critical role in DNA preservation [32]. High temperatures of heat-exposure in fire forensic cases are not favourable conditions for the sustainability of DNA integrity [33]. Our results demonstrated that DNA can be successfully amplified from teeth that were incinerated up to 300°C, while no DNA amplification was observed for the teeth heated at 600°C and above. This finding is supported by a previous study by Rees and Cox [16] in which amount of DNA extracted from pig teeth incinerated above 375°C were found to be inversely associated to the increased heating temperature.

Based on the results, we categorised the temperature exposure according to the likelihood to estimate the success of DNA retrieval from incinerated teeth into two groups: high-temperature (≥600°C) and low-temperature (≤300°C) groups. DNA is more likely to be retrieved and amplified in low-temperature group and is retrievable but less likely to be amplified in high-temperature group. However we acknowledged that the data of this study were derived from pig teeth, therefore the results may not reflect the DNA retrievability from human teeth. Pig teeth were chosen because of the close similarities in terms of size, morphology and composition to the human teeth

[34, 35]. Both pigs and human are omnivores, contributing to the structural resemblance of enamel and dentine in both species [36]. Further validation work involving human teeth is necessary. Validation studies of forensic methods using human cadavers has been recommended in a recent study [37].

Conclusions

This study confirmed that temperature has a statistically significant effect on the amplification of DNA. We proposed a classification based on temperature exposure on incinerated pig teeth namely low-temperature (<300°C) and high-temperature (>600°C), to estimate the likelihood of retrieval and amplification success of DNA sourced from incinerated teeth. Further research is needed to validate the applicability of the classification to incinerated human teeth. We also substantiated claims that molar teeth yield more DNA than premolar teeth and highly suggest that in the future research, teeth should be retained in their bone sockets during incineration for this method mimics the forensic fire situation.

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Conflict of interest

The authors declare no conflict of interest with this research.

Ethics

The handling of the animal remains in this study was done in line with the University of Adelaide Animal Ethics.

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equipment to incinerate the samples. Also, the authors thank Mr. Anthony Wilkes from the School of Animal and Veterinary Sciences, the University of Adelaide, Australia for providing his expert technical assistance to prepare the porcine mandibular bone segments.

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Supplementary data

Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires

S 1—Quantified concentration of each DNA extracts measured in ng/mL using the Quantus™ fluorometer.

Sample code	Temperature (°C)	Tooth type	DNA concentrations (ng/µL)
2	1000	Premolar	0.03
3	1000	Premolar	<0.01
4	1000	Premolar	<0.01
5	1000	Molar	<0.01
6	1000	Molar	<0.01
7	1000	Molar	0.10
9	1000	Premolar	<0.01
10	1000	Premolar	<0.01
11	1000	Premolar	<0.01
12	1000	Molar	0.08
13	1000	Molar	0.01
14	1000	Molar	<0.01
16	800	Premolar	0.01
17	800	Premolar	<0.01
18	800	Premolar	0.02
19	800	Molar	<0.01
20	800	Molar	<0.01
21	800	Molar	0.02
23	800	Premolar	<0.01
24	800	Premolar	<0.01
25	800	Molar	<0.01
26	800	Molar	<0.01
27	800	Molar	0.04
28	800	Premolar	<0.01
31	600	Premolar	<0.01
32	600	Premolar	<0.01
33	600	Molar	0.01
34	600	Molar	<0.01
35	600	Molar	0.01
37	600	Premolar	0.03
38	600	Premolar	0.03
39	600	Premolar	0.01
40	600	Molar	<0.01
41	600	Molar	0.06
42	600	Molar	0.15

44	300	Premolar	0.89
45	300	Premolar	0.65
46	300	Premolar	0.84
47	300	Molar	0.51
49	300	Molar	6.20
51	300	Premolar	0.60
52	300	Premolar	0.30
53	300	Premolar	0.54
54	300	Molar	0.42
55	300	Molar	0.63
56	300	Molar	1.73
72	27	Premolar	0.72
73	27	Premolar	0.84
74	27	Premolar	1.57
75	27	Molar	1.24
76	27	Molar	14.00
77	27	Molar	32.00
79	27	Premolar	2.60
80	27	Premolar	1.09
81	27	Premolar	1.99
82	27	Molar	1.32
83	27	Molar	6.70
84	27	Molar	11.00

<0.01ng/µL = Lower than the detectable quantification limit of the quantification assay.

S 2— DNA yield from individual sample calculated in standardised ng DNA per milligram of tissue sample (ng/mg).

Sample Code	Temperature (°C)									
	27		300		600		800		1000	
	Molar	Premolar	Molar	Premolar	Molar	Premolar	Molar	Premolar	Molar	Premolar
2	.	.	.	.	.	.	.	.	.	.00
3	.	.	.	.	.	.	.	.	.	.00
4	.	.	.	.	.	.	.	.	.	.00
5	.	.	.	.	.	.	.	.	.00	.
6	.	.	.	.	.	.	.	.	.00	.
7	.	.	.	.	.	.	.	.	.00	.
9	.	.	.	.	.	.	.	.	.	.00
10	.	.	.	.	.	.	.	.	.	.00
11	.	.	.	.	.	.	.	.	.	.00
12	.	.	.	.	.	.	.	.	.00	.
13	.	.	.	.	.	.	.	.	.00	.
14	.	.	.	.	.	.	.	.	.00	.
16	.	.	.	.	.	.	.	.00	.	.
17	.	.	.	.	.	.	.	.00	.	.
18	.	.	.	.	.	.	.	.00	.	.
19	.	.	.	.	.	.	.00	.	.	.
20	.	.	.	.	.	.	.00	.	.	.
21	.	.	.	.	.	.	.00	.	.	.
23	.	.	.	.	.	.	.	.00	.	.
24	.	.	.	.	.	.	.	.00	.	.
25	.	.	.	.	.	.	.	.00	.	.
26	.	.	.	.	.	.	.00	.	.	.
27	.	.	.	.	.	.	.00	.	.	.
28	.	.	.	.	.	.	.00	.	.	.
31	.	.	.	.	.	.00	.	.	.	.
32	.	.	.	.	.	.00	.	.	.	.
33	.	.	.	.	.	.00	.	.	.	.
34	.	.	.	.	.00	.	.	.	.	.
35	.	.	.	.	.00	.	.	.	.	.
37	.	.	.	.	.	.00	.	.	.	.
38	.	.	.	.	.	.00	.	.	.	.
39	.	.	.	.	.	.00	.	.	.	.
40	.	.	.	.	.00	.	.	.	.	.
41	.	.	.	.	.00	.	.	.	.	.
42	.	.	.	.	.00	.	.	.	.	.
44	.	.	.	4.76	.	.	.	.	.	.
45	.	.	.	4.74	.	.	.	.	.	.
46	.	.	.	9.94	.	.	.	.	.	.
47	.	.	5.00	.	.	.	.	.	.	.
49	.	.		.	.	.	.	.	.	.
51	.	.	3.73		.	.	.	.	.	.
52	.	.	.	3.09	.	.	.	.	.	.
53	.	.	.	3.56	.	.	.	.	.	.
54	.	.		6.49	.	.	.	.	.	.
55	.	.	11.5	.	.	.	.	.	.	.
56			9							
72	.	.	3.54	.	.	.	.	.	.	.
73	.	0.36	.	.	.	.	.	.	.	.
74	.	0.11	.	.	.	.	.	.	.	.
75	.	0.96	.	.	.	.	.	.	.	.
76	0.46	.	.	.	.	.	.	.	.	.
77	41.1	.	.	.	.	.	.	.	.	.
79	4									

80	22.4	.	.	.	.	.	.	.	.	.
81	7									
82	.	0.19	.	.	.	.	.	.	.	.
83	.	0.72	.	.	.	.	.	.	.	.
84	.	0.85	.	.	.	.	.	.	.	.
	1.29	.	.	.	.	.	.	.	.	.
	4.25	.	.	.	.	.	.	.	.	.
	6.32	.	.	.	.	.	.	.	.	.

Tests of Between-Subjects Effects

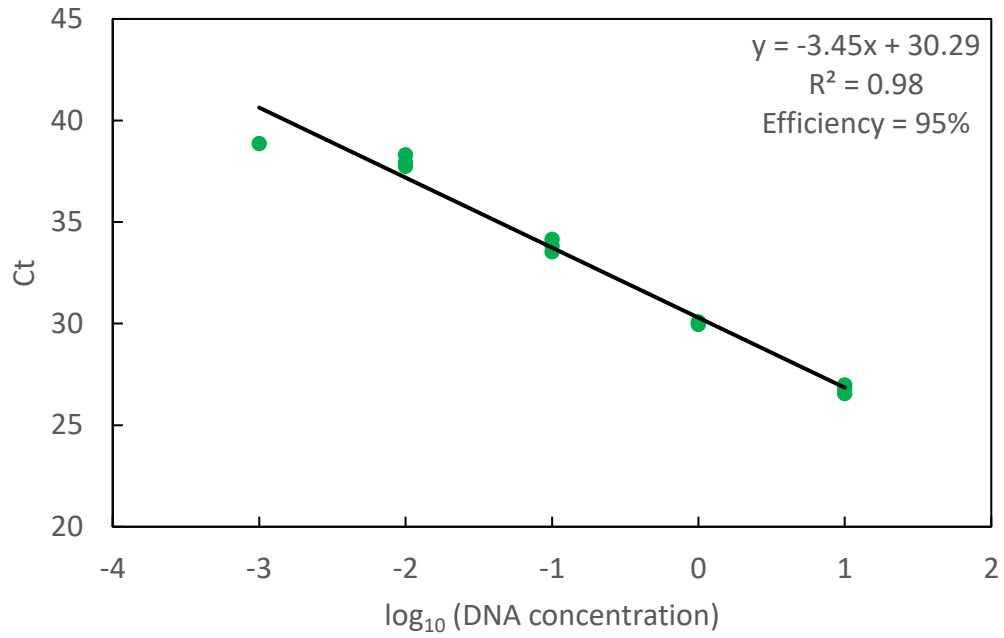
```
fit <- lm(DNA~Temperature*Tooth_type, data = d)
anova(fit)

## Analysis of Variance Table
##
## Response: DNA
##                        Df  Sum Sq Mean Sq F value   Pr(>F)
## Temperature             4  549.29 137.322  4.8134 0.002402 **
## Tooth_type              1  122.83 122.826  4.3053 0.043377 *
## Temperature:Tooth_type  4  330.29  82.572  2.8943 0.031693 *
## Residuals              48 1369.39  28.529
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

T.test, not assuming equal variance, was conducted to compare the effect of temperature treatment on DNA yield from premolars.

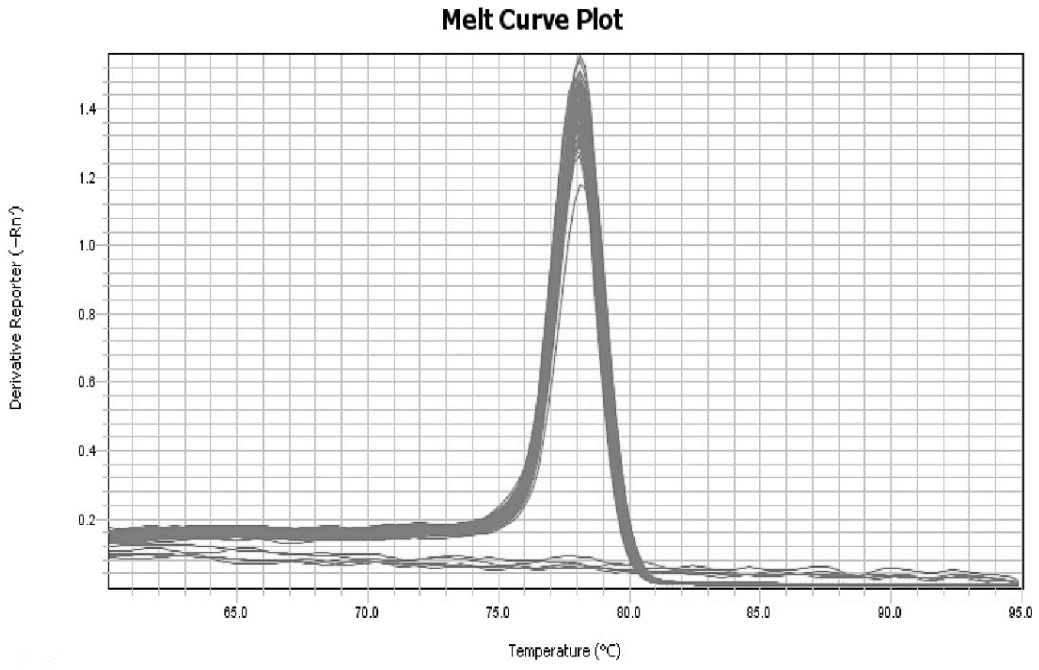
```
t.test(log(d$DNA[d$Temperature=="27" & d$Tooth_type=="Premolar"]),
log(d$DNA[d$Temperature=="300" & d$Tooth_type=="Premolar"]))

##
##  Welch Two Sample t-test
##
## data:  log(d$DNA[d$Temperature == "27" & d$Tooth_type == "Premol
ar"]) and log(d$DNA[d$Temperature == "300" & d$Tooth_type == "Premo
lar"])
## t = -6.078, df = 7.1054, p-value = 0.0004736
## alternative hypothesis: true difference in means is not equal to
0
## 95 percent confidence interval:
##  -3.365302 -1.484263
## sample estimates:
##  mean of x  mean of y
## -0.9035837  1.5211986
```

T.test, not assuming equal variance, was conducted to compare the effect of temperature treatment on DNA yield from molars.

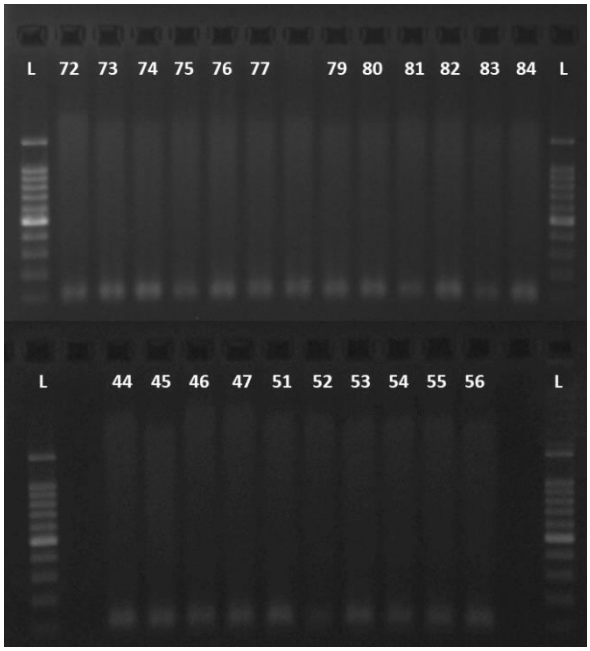
```
t.test(log(d$DNA[d$Temperature=="27" & d$Tooth_type=="Molar"]), log
(d$DNA[d$Temperature=="300" & d$Tooth_type=="Molar"]))

##
##  Welch Two Sample t-test
##
## data:  log(d$DNA[d$Temperature == "27" & d$Tooth_type == "Molar"
]) and log(d$DNA[d$Temperature == "300" & d$Tooth_type == "Molar"])
## t = -0.38003, df = 5.8867, p-value = 0.7173
## alternative hypothesis: true difference in means is not equal to
0
## 95 percent confidence interval:
##  -2.047163  1.498978
## sample estimates:
## mean of x mean of y
##  1.599652  1.873744
```



S 3— Standard curve generated from the five serial diluted standards for the qPCR reaction. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1, 0.1, 0.01, 0.001, 0.0001 ng/µL.



S 4— Dissociation curve shows a single-peak of the 74-bp Actin target gene which indicate no contamination or primer-dimer formation.



S 5— qPCR products of successfully amplified DNA amplicon using 74-bp nuclear target gene from control samples or unheated teeth (lane 1, samples: 72–77, 79–84) and teeth heated at 300°C (lane 2, samples: 44-47, 51-56). L = 100 bp mol. wt. DNA ladder (product no: G2101, Promega Corporation, Madison, WI).

Part III

Novel diagnostic models to predict nuclear DNA and mitochondrial DNA recovery from incinerated teeth

Statement of Authorship

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Name of Principal Author (Candidate)	Rabiah Al-Adawiyah Binti Rahmat		
Contribution to the paper	Research planning, project administration, sample preparation and investigation, data curation, analysis and interpretation, created figures and tables, wrote manuscript and acted as a corresponding author.		
Overall percentage (%)	80%		
Signature		Date	04/05/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidature in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the paper	Facilitated the DNA laboratory work		
Signature		Date	04/05/2020

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Novel diagnostic models to predict nuclear DNA and mitochondrial DNA recovery from incinerated teeth

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Abstract

Teeth are frequently used for human identification from burnt remains, as the structure of a tooth is resilient against heat exposure. The intricate composition of hydroxyapatite (HA) mineral and collagen in teeth favours DNA preservation compared to soft tissues. Regardless of the durability, the integrity of the DNA structure in teeth can still be disrupted when exposed to heat. Poor DNA quality can negatively affect the success of DNA analysis towards human identification. The process of isolating DNA from biological samples is arduous and costly, thus an informative pre-screening method that could help in selecting samples that can potentially yield amplifiable DNA would be of a great value. A multiple linear regression model to predict the DNA content in incinerated pig teeth was developed based on the colourimetry, HA crystallite size and quantified nuclear and mitochondrial DNA. The chromaticity a* was found to be a significant predictor of the regression model. This study proposes the use of the newly developed models to predict the viability of extracting nuclear and mitochondrial DNA from pig teeth that were exposed to a wide range of temperatures (27℃ to 1000℃) with high accuracy (99.5% - 99.7%). This model has a potential to serve as a diagnostic triage for incinerated teeth prior to a subsequent DNA analysis for forensic identification or intelligence purposes.

Keywords

Incinerated teeth; DNA analysis; Colourimetry; X-ray Diffraction; Linear regression model; Quantitative polymerase chain reaction; Mitochondrial DNA; Nuclear DNA

Introduction

In an extreme fire events, in which there are human fatalities, a mixture of whole teeth and tooth fragments are usually recovered. It would be beneficial to be able to predict the ability to effectively isolate DNA suitable for forensic human identification from skeletal samples including teeth and bone, prior to undertaking a DNA analysis. This is because DNA analysis can be expensive, laborious and time-consuming [1, 2]. Without a pre-screening tool, extensive effort and time are required to perform DNA analysis on teeth. It should be noted that the sample preparation prior to DNA analysis including sample cleaning, pulverisation and demineralisation require substantial time and effort [3, 4]. Having a tool to screen the most suitable incinerated teeth for DNA analysis would be a great assistance to the forensic investigators to select samples that are most likely contain amplifiable DNA. This is important to improve the efficiency of the identification process and to minimise the resource wastage.

To date, there have been many published papers on pre-screening methods for assessing DNA availability in skeletal remains, for forensic and archaeology applications [2, 5-12]. In these studies, the applicability of various quantitative methods such as colourimetry [2], collagen preservation [7], crystallinity [12] and proteomic analyses [10] to indicate the DNA preservation in skeletal remains were assessed. Using a proteomic analysis, Wadsworth *et al*. [10] have found that no protein group is apt to be a biomarker to indicate the availability of DNA, but rather the survival of DNA in bone was claimed to be strongly related to its connection with HA crystals.

More recent literature has established DNA predictive models for burnt skeletal remains using quantitative analytical techniques such as colourimetry and FTIR spectroscopy [2, 5]. Bones were used as samples in both studies. Although teeth and bone are skeletal tissues, the biochemistry and histology of both structures are different [13-15]. Therefore, it is important to specify DNA predictive models for teeth

and bone. However, less attention has been paid to the development of such models using incinerated teeth. A recent study by Rubio *et al.* [11] highlights the potential of colourimetry value to predict the feasibility of extracting human DNA. In an ancient DNA study, DNA extractions of tooth samples were shown to be more successful than of bone samples [10].

Moreover, teeth have been widely used for genetic investigations in the identification of burnt human remains. DNA profiling from teeth and bones for the identification in forensic cases or mass disaster situations are important because skeletal tissues are often the only biological materials available [16]. DNA profiling was deployed as a primary method of identifications in many well-known cases such as MH-17, 2002 Victorian Black Saturday and 2001 World Trade Center attack [17-19]. In these cases, the body remains were usually burnt and fragmented. In total, 246 teeth and tooth fragments were recovered and used to source DNA in the MH-17 airplane crash [17].

Teeth can endure high temperatures and are the least of all body parts affected by fire due the heat protection afforded from the surrounding musculoskeletal structures [20, 21]. The structural endurance is attributed to the high mineral contents: enamel is composed of 97 wt.% of HA mineral and dentin is composed of 70 wt.% of HA mineral [22]. There is an increasing volume of published studies describing the role of HA in the preservation of DNA [10, 23-26]. DNA preservation in teeth has been associated to the matrix of hydroxyapatite (HA mineral) and collagen [27, 28]. HA mineral binds to DNA and inactivates nucleases, enzymes that are responsible for DNA fragmentation [25, 29, 30]. In addition, HA mineral acts as a physical boundary that prevents microbes from accessing the collagen [31]. This delays the chemical and biological degradation process of skeletal remains. DNA molecules are tightly bound to HA due to the strong affinity of the negatively charged phosphate groups in DNA towards the positively charged calcium ions (Ca^{2+}) in the HA mineral, $Ca_{10}(PO_4)_6OH_2$ [32, 33]. del Velle *et al.* [33] reported that the structural and chemical changes of HA affect its capacity to bind with DNA. The bond between HA and DNA may be lost during the recrystallisation of the HA crystal.

Pig teeth as human proxy

This study used pig mandibular segments with *in situ* teeth, specifically molars and premolars. Pigs *(Sus scrofa)* have been commonly used as human proxy in forensic experimental studies [34-37]. Pig teeth were chosen to substitute human teeth because the dentition of both species share close similarities in terms of size, morphology and composition [38, 39]. In addition, previous studies have shown that the thermal alterations of teeth and bone of pigs and human were consistent [34, 35, 40].

Aims of the study

The main objective of this study was to develop a model that can be utilised to triage the DNA retrievability in teeth of burnt remains based on the heat-induced colour and HA crystallites size changes. This is achieved using a regression model that correlates the DNA recovered from incinerated teeth with the colourimetric value and HA crystallite size. The colour and HA crystallite size were measured using a portable spectrophotometer and an X-ray diffraction (XRD) system. Both instruments provide an objective and quantitative measurement. Quantitative real time PCR (qPCR) was used to quantify the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in the dental tissue. Additionally, further insight into the variability of colour, HA crystallite size, and DNA type in teeth against the incinerating temperature were explored and reported.

Materials and methods

Furnace incineration

Fifty-eight teeth from ten mandibular jaw segments of adult domestic pigs *(Sus scrofa domesticus)* were randomly selected. Each mandibular segment consisted of premolars and molars. Twelve teeth from two mandibular segment were kept as control samples at room temperature (27°C). Eight mandibular segments were incinerated for 15 minutes, with two segments at each of the following temperature: 300°C, 600°C, 800°C and 1000°C. The mandibles were prepared and incinerated as described in a previous study [41]. This procedure was conducted at CSIRO Land and Water Division, Urrbrae, South Australia. XRD analysis.

Colourimetric analysis

A portable handheld spectrophotometer (CM-700d, Konica Minolta Sensing Americas, Inc., U.S.A) was used to measure colour. The colour was measured following the protocol from a previous study (4). The buccal surface of the tooth crown was targeted for measuring the colour. The colourimetry data collected were CIELAB lightness (L), chromaticity a and b, whiteness (WI) and yellowness indices (YI). This analysis was performed at CSIRO Land and Water Division, Urrbrae, South Australia. XRD analysis.

XRD analysis

The XRD analysis was performed according to the protocol that was used in the previous study [41]. The tooth crowns were pulverised into a fine powder in an agate mortar and pestle. Powder were pressed onto Si low background holders for XRD analysis. A PANalytical X'Pert Pro Multi-purpose Diffractometer using Fe-filtered Co Kα radiation, automatic divergence slit, 2° anti-scatter slit, and fast X'Celerator Si strip detector at a scan rate of 2.43°C two theta per minute was used to process the XRD data. XRD experiments were taken at the X-ray Diffraction Laboratory, CSIRO Land and Water Division, Urrbrae, South Australia.

DNA analysis

DNA analysis was performed at the Advanced DNA, Identification & Forensic Facility (ADIFF), the University of Adelaide, Australia. A strict cross-contamination protocol was followed throughout the steps of DNA analysis [42]. The protocol incudes performing each step of the analysis in a dedicated laboratory, cleaning the workbenches with 4% sodium hypochlorite and followed by Decon 90^{TM}, all instruments and hoods were sterilised with 70% ethyl-alcohol and UV radiation and incorporating negative controls consisting of DNA-free water in every DNA extraction and qPCR.

Only roots of the teeth were used to source DNA. The roots were pulverised in liquid nitrogen using a sterilised pestle and mortar. Each pulverised sample was kept in an individual sterile container labelled with the sample code. The details of the sample preparation was the same as the previous study [43]. Tooth powder was demineralised prior to DNA extraction. 1.8 mL of 0.5 M ethylenediamine tetra-acetic

acid, EDTA (pH 8) was added to each sample and then incubated overnight on a shaker that was set to 56°C.

i. DNA extraction

DNA was extracted from approximately 50 to 100 mg sample powder. A silica-column extracted method was performed using the DNeasy® blood and tissue kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol. All DNA extracts were quantified using the Quantus™ fluorometer (Promega Corporation, Madison, WI), according to the manufacturer's protocol. The limit of detection for double-stranded DNA was 0.01 ng/µL. All DNA extracts were stored at -20°C until further analysis.

ii. qPCR

Three sets of primer pairs were used to target DNA fragments of different lengths: 150 bp, 83 bp and 82 bp [42]. Nuclear actin150 or *Actin cytoplasmic 1* was from a copy of Actin gene (*Sus scrofa;* BLAST Accession NC_010445.4). Nuclear *Melanocortin-1-Receptor (MC1R)* and mitochondrial *ATPase* genes were from Antinick *et al.* [44]. Using serial dilutions of Porcine DNA standards that were created from a stock DNA of porcine kidney a set of DNA standards of known concentration (1, 0.1, 0.01, 0.001, 0.0001 ng/µL) were produced.

The quantification of nuclear and mitochondrial DNA fragments was performed using an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific, Foster City, CA) and detected using an Applied Biosystems™ PowerUp™ SYBR® green master mix (ThermoFisher Scientific, Foster City, CA). Samples were run in triplicate and negative blanks were included in each run. The qPCR condition described in the previous study [43]. A standard curve and a melting curve were produced by QuantStudio™ Real-Time PCR Software v1.3 (ThermoFisher Scientific, Foster City, CA) for every run according to the primer pairs (refer to supplementary data, S1). The melting curve was relied upon to confirm one gene-specific peak and any primer-dimer formation.

Table 1—Details of primer pairs used in RT-qPCR assay that were used to amplify nuclear DNA and mitochondrial DNA fragments of different lengths [42].

Gene name	Primer	Primer sequence (5' – 3')	Type (bp)	Tm(°C)
Actin150	Forward	CTCTGACCTGAGTCTCCTTT	Nuclear (150)	55
	Reverse	CGGCTTTGTCACACGAG		
MC1R [44]	Forward	GCCCGGTTCCTACGTG	Nuclear (82)	60
	Reverse	AGAGGGTCCAGCGTCCATA		
ATPase [44]	Forward	AGCTCTGATCCAAGCTTATGTGT	Mitochondrial (83)	60
	Reverse	GCATGTGTTTGGTGGGTCA		

The average Ct of the sample was used to calculate the qPCR concentration (ng/µL). The threshold cycle (Ct) in each run was determined using an adjustment method. DNA yield for each sample was standardised in ng DNA per milligram of tissues sample (ng/mg) by multiplying the quantified DNA (ng/µL) by the elution volume (µL) and dividing by the mass of powdered sample (mg).

Development of a diagnostic model

All statistical analyses were completed using the statistical software R [45]. Analysis of variance (ANOVA) with Tukey's post-hoc analysis was conducted to investigate value and crystallite size across the heating temperatures. DNA quantification against the temperature groups for each DNA fragment was assessed. Further insight on the tooth type was explored.

The strength of relationships between the multiple predictor variables (L, a*, b*, WI, YI and CS) and multiple response variables (150 bp nDNA, 82 bp nDNA and 83 bp mtDNA) was quantified using correlation coefficient analysis. A correlation plot was fitted using the R 'corrplot-package' to visualise a correlation matrix [46].

For each fragment type (nDNA and mtDNA), a second order multiple linear regression model was fitted to measure the joint effects of temperature and tooth type on predicting the selected continuous response variables (L, a*, WI, YI, and CS) using the 'lm' function. This produced full interaction models for 82bp nDNA and 83bp mtDNA respectively.

A stepwise model selection was applied to each fragment to select the predictors that best fit the model, using BIC (Bayesian information criterion). The predictors have a significant impact ($p < 0.05$) on the DNA yield were retained.

Results

Complete data consisting of the colourimetric value (L, a*, b*, WI and YI), the calculated HA crystallite size and the quantified DNA of nDNA (150 bp and 83 bp) and mtDNA (82 bp) in 57 tooth samples are shown in the Supplementary data, S1.

Colourimetric value and crystallite size vs temperature

The significant differences of L, a*, b*, WI, YI and HA crystallite size across the temperature-exposure groups based on Tukey's post-hoc test are summarised in the Supplementary Data, S2. The data distributions of L, a* and b* in teeth at 27℃, 300℃, 600℃, 800℃ and 1000℃ are shown in Figure 1. The spread of L data across the temperature groups was roughly uniform, except the 600℃ was more variable than others. L value decreased between 27℃ and 600℃ with significant differences at 600℃ versus 27℃ and 300℃. L values significantly increased from 600℃ to 800℃, and 800℃ to 1000℃ ($p < 0.05$).

The distribution of chromaticity a* data was widely spread at 27℃. The variability of a* became notably less when the teeth were heated. The overall values of a* diminished as the temperature increased from 27℃ to 800℃. The reduction of a* value from 27℃ to 300℃, 600℃, 800℃ and 1000℃ was significant ($p < 0.05$). The result indicates that the redness in the teeth became less saturated and the shade was approaching the neutral grey as the temperature increased from 27℃ to 1000℃.

Similar to a*, the chromaticity b* was more variable in the unheated teeth and the data spread became narrower at 300℃ and above. The chromaticity b* increased from 27℃ to 300℃ before significant reduced at 600℃ ($p < 0.05$). The mean differences of 600℃ to 1000℃ were significantly lower than 27℃ and 300℃ ($p < 0.05$). This means the yellow shade of the teeth became remarkably lighter when teeth were incinerated at 600℃ and above.

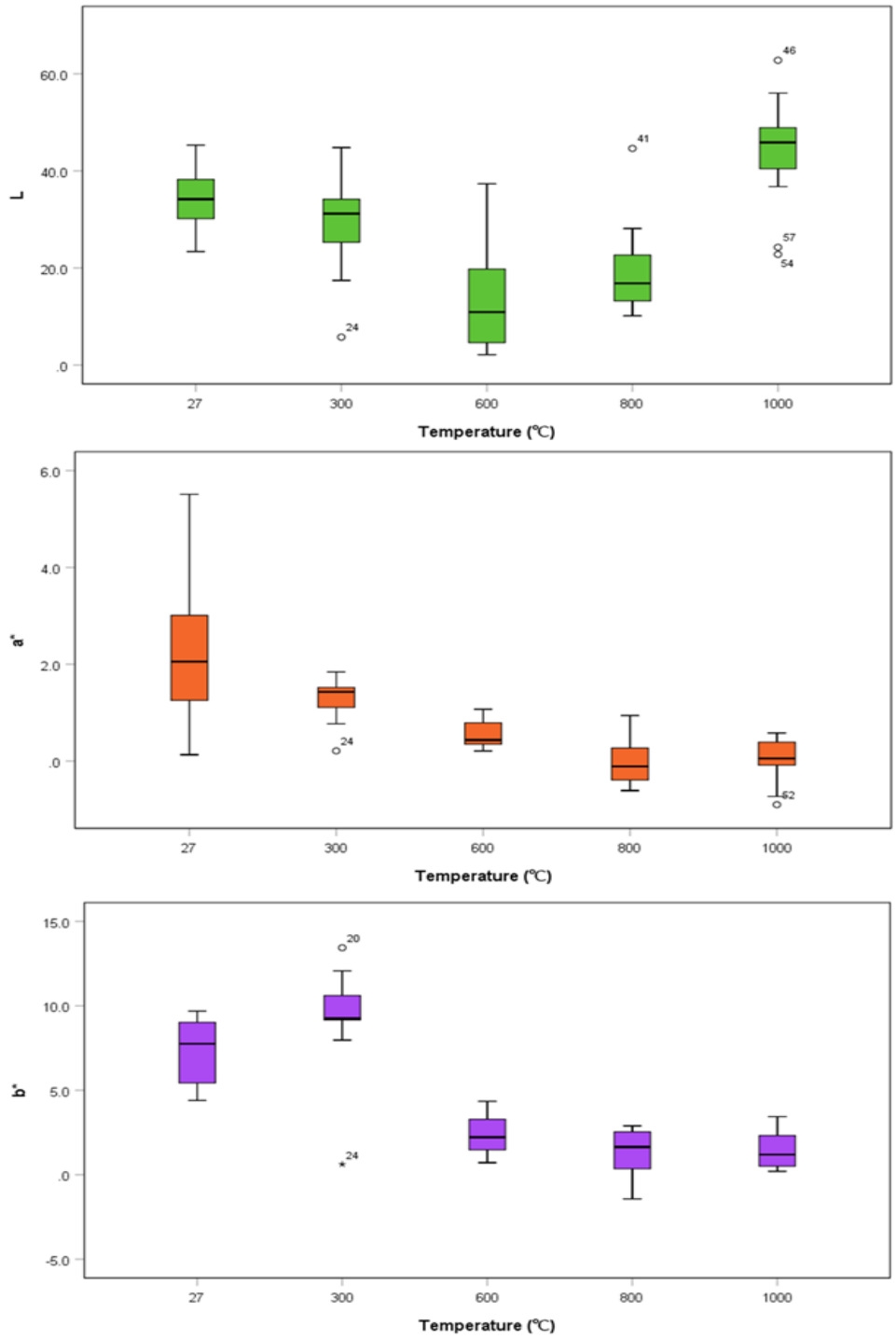

Figure 1—Boxplots showing the data distribution for L (lightness), chromaticity a* (red to green), chromaticity b* (yellow to blue) in control teeth (27℃) and teeth incinerated at 300℃, 600℃, 800℃ and 1000℃. The central line is the median and boxes enclose 50% of the observations. Here outliers are marked with dots lying beyond the tails of the boxes.

The data distributions of WI, YI and CS in teeth at 27℃, 300℃, 600℃, 800℃ and 1000℃ are shown in Figure 2. The distribution of whiteness in unheated teeth (27℃) and teeth incinerated at 1000℃ was greatly variable, in a stark contrast to the whiteness data in teeth incinerated at 300℃, 600℃ and 800℃. Whiteness (WI) in

teeth significantly diminished from 27℃ to 300℃ ($p < 0.05$) before it gradually increased up to 1000℃, with a significant upsurged of WI from 800℃ to 1000℃ ($p < 0.05$). This result was concordant to the whiteness that was observed visually, in which tooth crown turned white calcined when incinerated to 1000℃.

The yellowness data of teeth at 27℃, 300℃ and 800℃ was more variable compared to the distribution seen in 600℃ and 1000℃. The pattern of YI against the increased temperature was in contrast to the pattern observed in WI. Yellowness was intensified when teeth were heated to 300℃. YI exponentially decreased from 300℃ to 1000℃, with significant differences between 300℃ and the higher temperature groups (600℃, 800℃ and 1000℃) ($p < 0.05$).

The spread of the crystallite size data was similar in teeth at 27℃, 300℃, 600℃ and 800℃. However, the data of crystallite sizes in teeth heated at 1000℃ was immensely spread compared the teeth exposed to the lower temperature groups. The crystallite size appeared to be reduced as the temperature increased from 27℃ to 600℃, but the mean differences between these temperature groups were insignificant. The crystallite size expanded exponentially as the teeth were exposed to 800℃ and 1000℃. The size increment from 600℃ to 1000℃ was significant ($p < 0.05$).

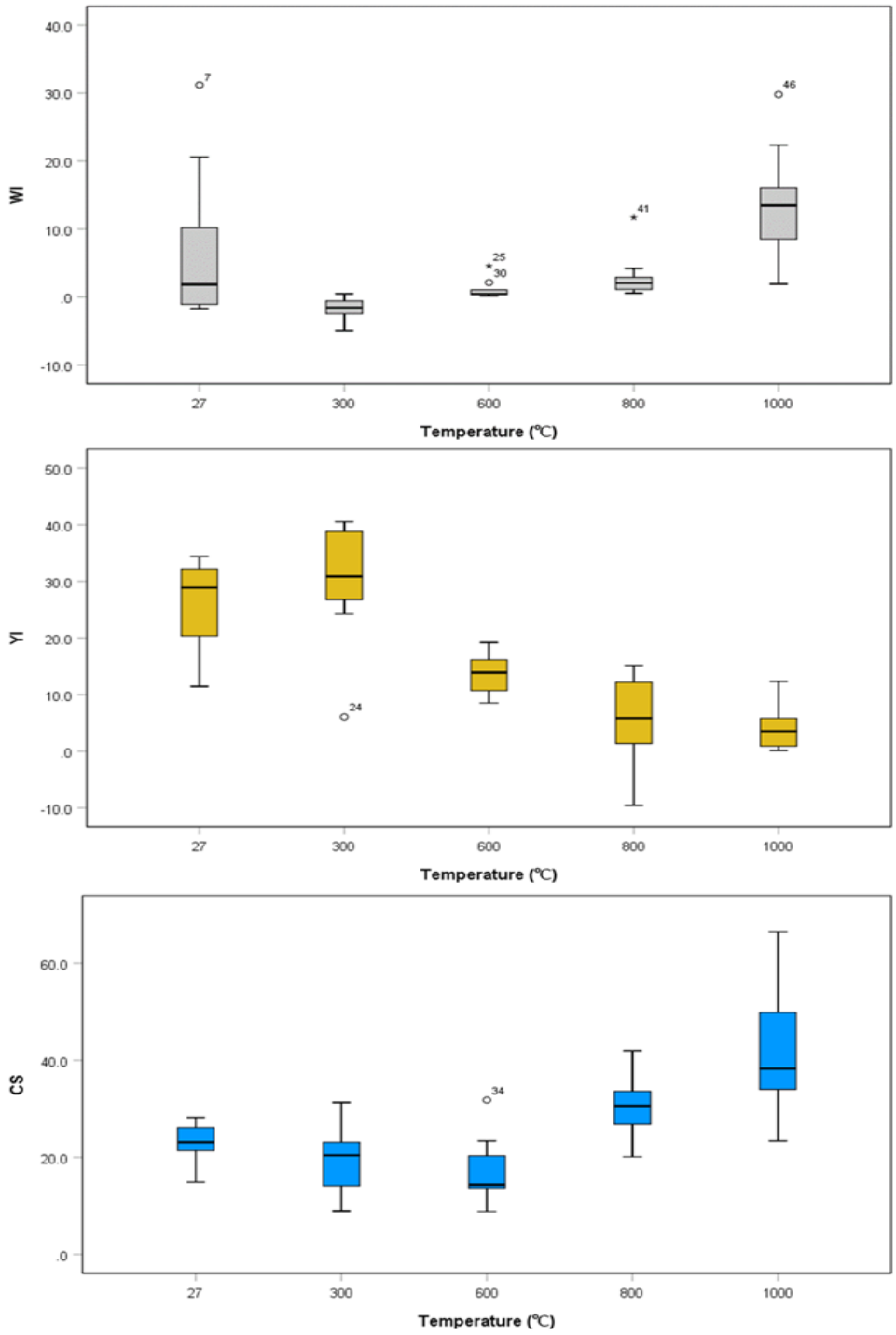

Figure 2—The boxplots of whiteness (WI), yellowness (YI) and crystallite size (CS) indicate the data distribution in control teeth (27℃) and teeth incinerated at 300℃, 600℃, 800℃ and 1000℃. ℃. The central line is the median and boxes enclose 50% of the observations. Here outliers are marked with dots lying beyond the tails of the boxes.

DNA quantification

Figure 3 displays the mean of the quantified 150 bp nDNA, 82 bp nDNA and 83 bp mtDNA targets in control teeth and teeth incinerated at 300℃, 600℃, 800℃ and 1000℃. Overall, the mean of quantified DNA decreased when teeth were exposed

to heat at 300℃ and no DNA was recovered at 600℃ and above. This finding agrees with the preliminary DNA research that DNA fragments are not amplifiable above 600℃ [43]. The 82 bp nDNA and 83 bp mtDNA targets showed a similar trend, with around 30% reduction in DNA concentration. Interestingly, the quantity of the 82 bp nDNA fragment did not show reduction when heated at 300℃.

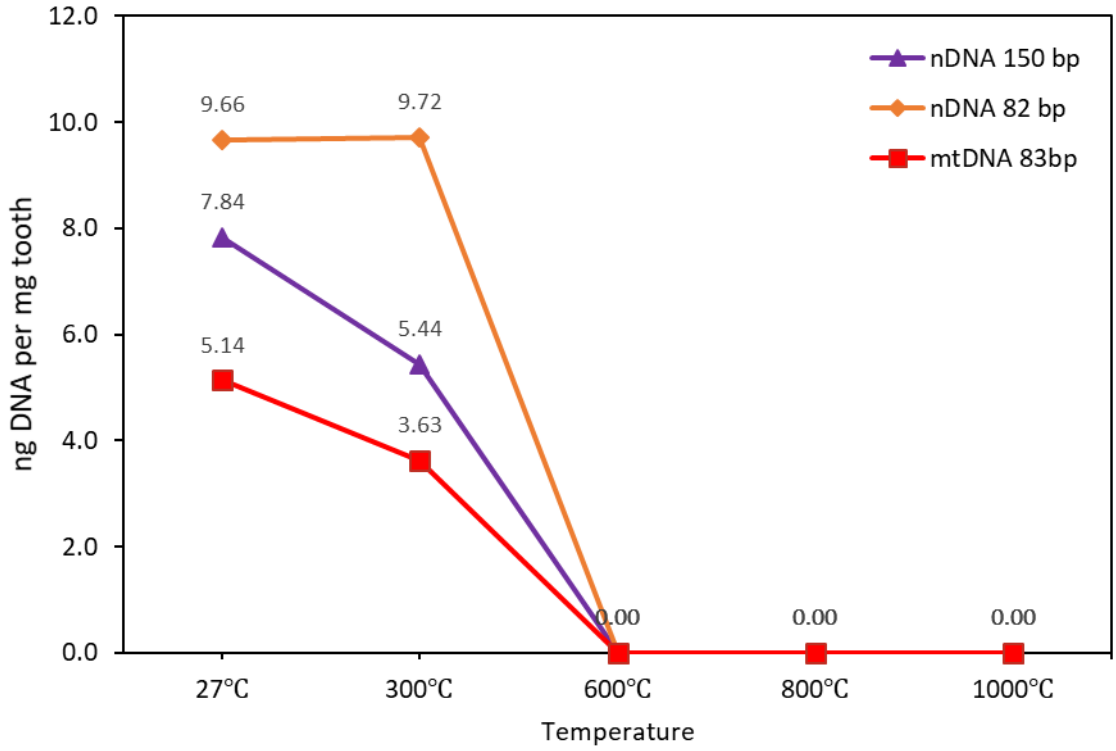

Figure 3—The average DNA yields at each temperature group (27℃ to 1000℃) for two nuclear DNA targets (150bp and 82bp) and one mtDNA target (83bp).

The distribution of the DNA yield in each temperature group for each DNA target between the tooth type was examined. Figure 4 shows the average DNA yields for each DNA target in molar and premolar teeth. At room temperature (27℃), molars yielded remarkably higher DNA than premolars by 90% to 95% for all three DNA targets. At 300℃, the mtDNA yield from molars was higher than from premolars by 91%. Whilst the nuclear DNA recovered from premolars was lesser than from molars by 19% (82 bp nDNA) and 52% (150 bp nDNA).

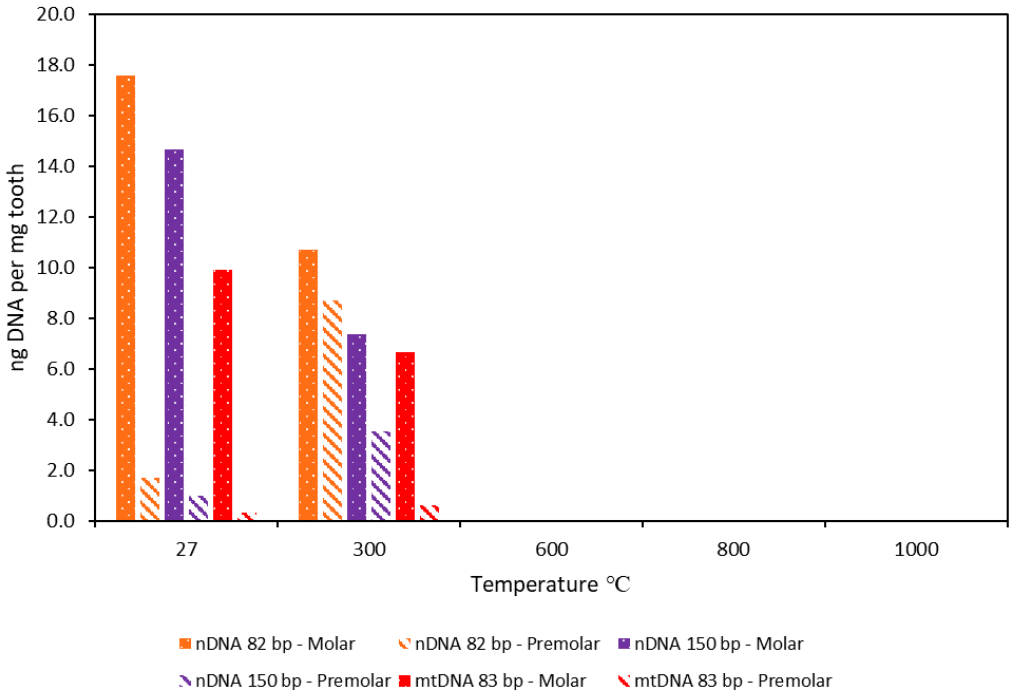

Figure 4—The average of DNA yields for each DNA target in molar and premolar teeth of 27℃, 300℃, 600℃, 800℃ and 1000℃ groups.

Correlations

Figure 4 displays a correlation matrix between all the response variables. Correlation coefficients showed 150 bp nDNA, 82 bp nDNA and 83 bp mtDNA are highly correlated ($p > 0.05$) (refer to supplementary data, S2). The 82 bp nDNA and 83 bp mtDNA fragments were chosen to represent the type of DNA. All DNA fragments demonstrate weak correlations with L, a*, b*, WI, YI and CS ($r < 0.5$), in which positive correlations were noted with L, a*, b* and YI and negative correlations were observed with WI and CS. However, b* was strongly correlated with a* ($r = 0.71$) and YI ($r = 0.94$). Thus, 150 bp nDNA and b* were omitted from further analysis. 82 bp nDNA, 83 bp mtDNA, L, a, WI, YI and CS were used in the regression analysis.

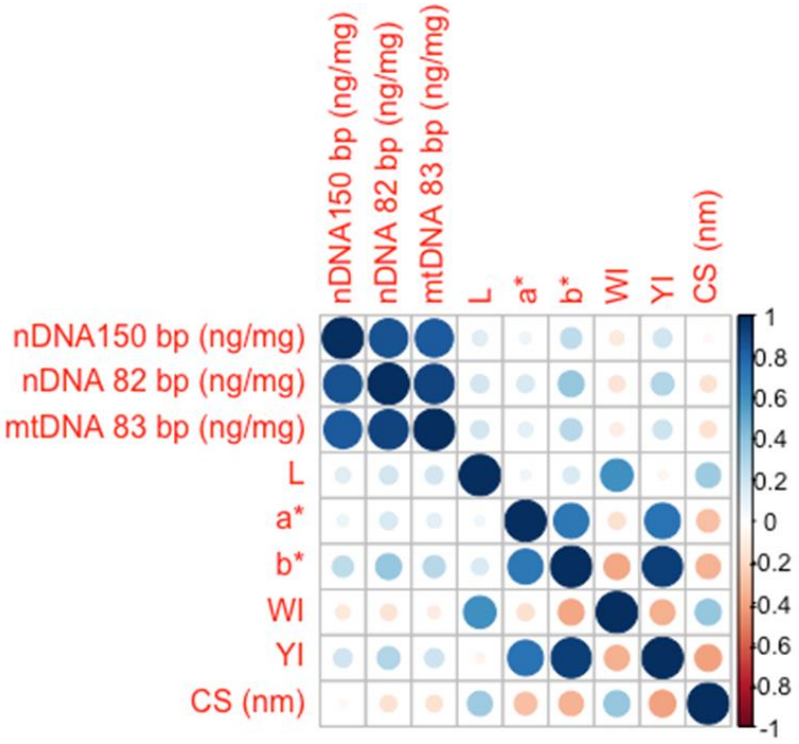

Figure 5— A correlation matrix shows the correlation coefficients of all response variables: 150 bp nDNA, 82 bp nDNA, 83 bp mtDNA, lightness (L), chromaticity a* and b, whiteness (WI), yellowness (YI) and crystallite size (CS). Blue colour indicates a positive correlation and red colour indicates a negative correlation. The intensity of colour and the size of the circle follows the strength gradient of the correlation. A darker and larger circle indicate a stronger correlation. A lighter colour and smaller circle indicates a weaker correlation. Empty box with no circle indicates no correlation.

Regression models

The resulting regression equations for mtDNA include three variables: Temperature, a* and type of tooth (refer to supplementary data, S2). This model explains the variability in mtDNA level with 99.7% accuracy. The regression models that can be used to predict mtDNA in incinerated teeth according to the temperature-exposure are:

Premolars – Room temperature:

$$\boldsymbol{log(mtDNA) = 0.72 - 0.39a*}$$

Premolars – 300°C:

$$\boldsymbol{log(mtDNA) = 1.65 - 0.39a*}$$

Premolars – 600°C:

$$\boldsymbol{log(mtDNA) = -16.03 - 0.39a*}$$

Molars – Room temperature:

$$\boldsymbol{log(mtDNA) = 2.88 - 0.39a*}$$

Molars – 300°C:

$$\boldsymbol{log(mtDNA) = 2.33 - 0.39a*}$$

Molars – 600°C:

$$\boldsymbol{log(mtDNA) = -16.07 - 0.39a*}$$

The resulting regression equations for nDNA include three variables; Temperature, a* and type of tooth. This model explains the variability in nDNA level with 99.5% accuracy. The regression models that can be used to predict mtDNA in incinerated teeth according to the temperature-exposure are:

Premolars – Room temperature:

$$\boldsymbol{log(nDNA) = 0.94 - 0.30a*}$$

Premolars – 300°Cs:

$$\boldsymbol{log(nDNA) = 2.51 - 0.30a*}$$

Premolars – 600°C:

$$\boldsymbol{log(nDNA) = -13.75 - 0.30a*}$$

Molars – Room temperature:

$$\boldsymbol{log(nDNA) = 3.02 - 0.30a*}$$

Molars – 300°C:

$$\boldsymbol{log(nDNA) = 2.14 - 0.30a*}$$

Molars – 600°C:

$$\boldsymbol{log(nDNA) = -13.78 - 0.30a*}$$

When the relationship is strong, the regression equation models the data accurately. The high adjusted R-squared value (R^2_{adj} = 0.997 and 0.995 respectively) indicates the regression equation models the data with high accuracy.

Discussion

The present study was designed to investigate the potential of colourimetric values (L, a*, b*, WI, and YI), and HA crystallite size (CS) to predict DNA in teeth that have been exposed to various temperature groups (27℃, 300℃, 600℃, 800℃ and 1000℃). It is important to account the broad range of temperature in this experiment because in a fire case, the temperature that the recovered teeth or tooth fragments exposed to was varied according to the scenario. For example the temperature of a house fire ranges between 700°C – 900°C [47], a motor-vehicle explosion ranges between 800°C – 1100°C [48, 49], and a fire-storm can increase beyond 1000°C [50].

Multiple linear regression modeling revealed that both temperature and tooth type influence the amount of the DNA recovered from teeth. It was identified that temperature, once it reached 600℃, had a consistent effect on the prediction, so a new variable that categorised the temperature as “room temperature”, “300℃” or “600℃ and higher” was created. This is the variable used in the final model. The value of chromaticity a* was found to be a reliable predictor to assess the retrievability of DNA. The quantity of DNA in the teeth is negatively correlated to increasing value of chromaticity a*. This finding supports previous research which proposed the use of chromaticity a* to indicate the DNA concentration in heat-treated teeth [11].

From the viewpoint of colourimetric and XRD analyses, the trend of L, a*, b*, WI, YI and CS in this study were compared with that of the teeth that were incinerated for 15-min in the recent published study [41]; the results observed in this study substantiate the findings in this preliminary study. This confirms that the pattern of colourimetric value and crystallite size at an increasing heating temperature can be anticipated. This study also emphasised the application of the portable spectrophotometer and X-ray diffractometer because these instruments can provide quantitative data, can be calibrated and easy to use.

The results of the DNA quantification indicate that the quantity of DNA in teeth decreases with the increasing heating temperature, and DNA can be obtained from teeth heated up to 300℃. This finding agrees with the previous studies [11, 36, 51]. Garriga *et al.* [51] retrieved small amounts of DNA from teeth exposed to 300℃. Rubio *et al.* [11] obtained DNA from 20% of the teeth heated at 400℃. However, in the present study, DNA can be amplified from all the teeth incinerated at 300℃. This could be because the previous studies have performed incineration on isolated teeth without accounting the protection offered by the jaw bone [11, 51]. In another study by Rees and Cox [36], teeth were incinerated in the natural position of the jaw segments and demonstrated that a substantial quantity of DNA can be recovered from teeth that were incinerated at 300℃. This is consistent with the DNA obtained in this study. The present study also validated the finding in the previous work in which the amplification of DNA is no longer possible when teeth are heated at 600℃ and above [43]. Approximately at 500°C, combustion of the organic matter begins, especially in collagen-rich dentin [52, 53]. This indirectly causes disruption to the integrity of DNA [54].

An outstanding finding from the DNA quantification was the similar quantity of the nDNA (82 bp) yield in teeth heated at 300℃ and the unheated teeth. Following the incineration at 300℃, teeth had undergone the dehydration process [40, 55]. From 110 – 260°C, dehydration occurs in which hydroxyl bonds break and eventually teeth lose any water molecules [56]. Thus, it was easier to pulverise the teeth. More effort was required when powdering unheated teeth because the sturdy structure. Excessive heat loaded in the sample can lead to the rapid increase of temperature and influence the DNA recovery from teeth [23]. The probability of recovering DNA from teeth rapidly decreases with increasing heat intensity and duration [29, 57].

Tooth type is known to be an important factor influencing the DNA yield [20]. Human and other mammals have four types of teeth, incisors, canines, premolars and molars, all of which are varied in size and number of roots [58]. The dentition of pigs and humans share close similarities in terms of size, morphology and composition [38, 39]. Both pigs and human are omnivore, making the dentitions of both species comparable [59]. The results of this study demonstrate that molar teeth yield a substantial DNA quantity compared to premolar teeth. Molars have greater pulp tissues and more number of roots than the premolars, therefore the likelihood to

retrieve DNA is higher [17, 60]. Molars afford better heat protection because the density of the alveolar process increased towards the posterior direction and the density the bone tissue is positively correlated to the ability to resist taphonomic effects from the surrounding environment [16, 61].

In this study, real-time quantitative PCR (qPCR) was deployed because of the high specificity. qPCR is a method to quantify the targeted DNA fragment retrieved in a sample [62]. Obal *et al.* [63] verified that the DNA quantity is an ideal parameter to assess the DNA preservation in skeletal elements. qPCR allows the quantification of DNA fragments, even in samples with low number of DNA templates [64]. Furthermore, different lengths of nuclear DNA (150 bp and 82 bp) were utilised to quantify both nDNA and mtDNA. This provides the assessment of DNA degradation in the samples [65]. The recovery of 82 bp nDNA was higher than 150 bp nDNA at 300℃. This suggests the reduction in the DNA quality following the incineration of teeth. A further study using a Fourier transform infrared (FTIR) spectroscopy may validate this finding and can provide a better understanding on the effects of heat on the integrity DNA structure. In a study using FTIR spectroscopy, collagen and mineral in bone were shown to be reliable indicators to indicate the DNA integrity [2].

This study highlights an important finding, of which the DNA predictive models were developed for nuclear DNA and mitochondrial DNA respectively. No previous study has individualised DNA prediction model for incinerated teeth according to the DNA type. The individualisation of the model into nDNA and mtDNA will greatly benefit particular forensic DNA applications. Nuclear DNA has been used extensively for short tandem repeat (STR) typing and single nucleotide polymorphisms (SNPs) typing [66-69]. In fire fatalities where the biological samples are severely compromised and DNA is highly degraded, STR-based typing is no longer successful, SNP typing with DNA fragment of 60-80 bp could provide a successful DNA profiling. Alternately, mitochondrial DNA (mtDNA) typing is the preferred method to use in forensic identification of degraded samples that could not produce DNA profiles [70].

We acknowledged that the data to develop the DNA predictive models were derived from pig teeth, therefore the models may not necessarily be applicable to human teeth in real casework situations. Further research to assess the validity of the models on human teeth are certainly required. Validation studies of forensic methods

using human cadavers has been recommended in a recent study [71]. Nonetheless, the outcomes of the study have contributed valuable insight for further work of incinerated teeth involving human teeth.

Conclusion

Selecting which tooth to sample for DNA analysis is crucial. This study proposes a novel and robust DNA predictive model to triage the viability to extract DNA from incinerated teeth. In specific, the colourimetric value of chromaticity a* is a reliable predictor. Evidently, temperature and tooth type were shown to influence the DNA quantity in teeth. This study can have a positive implication in real forensic casework. This model can potentially be used in the identification of burned human remains. However a further validation study to test this model on human teeth with a larger data set is required.

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Conflict of interest

The authors declare no conflict of interest with this research.

Ethics

The handling of the animal remains in this study was done in line with the University of Adelaide Animal Ethics.

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Supplementary data, S1

Novel diagnostic models to predict the nuclear DNA and mitochondrial DNA in incinerated teeth

S 1—Complete data consisting of the colourimetric value (L, a*, b*, WI and YI), the calculated HA crystallite size and the quantified DNA of nDNA (150 bp and 83 bp) and mtDNA (82 bp) in 57 tooth samples

Sample no	Temperature (°C)	Tooth type	nDNA150 bp (ng/mg)	nDNA 82 bp (ng/mg)	mtDNA 83 bp (ng/mg)	L	a*	b*	WI	YI	CS (nm)
1	27	Premolar	0.384	1.035	0.733	29.55	1.31	8.87	-1.08	29.41	25.6
2	27	Premolar	0.187	0.280	0.289	34.44	2.39	8.11	-0.06	25.18	22
3	27	Premolar	0.388	1.558	1.159	35.3	2.38	7.72	0.6	23.29	23
4	27	Molar	0.243	1.519	0.653	33.92	5.51	9.18	-1.08	28.33	14.9
5	27	Molar	16.275	47.529	41.848	45.31	1.2	4.4	8.05	11.44	17.7
6	27	Molar	23.761	27.855	23.562	39.88	0.13	4.58	5.38	13.03	27.5
7	27	Premolar	0.167	1.315	0.501	38.32	3.1	7.78	31.19	34.38	26.6
8	27	Premolar	0.426	1.599	1.255	31.03	2.92	6.31	20.61	33.15	28.2
9	27	Premolar	0.521	4.501	2.050	23.39	1.73	4.92	12.31	30	22
10	27	Molar	0.559	4.531	2.255	30.81	5.31	9.69	-1.67	31.26	20.7
11	27	Molar	2.798	6.100	5.009	38.11	0.77	5.95	3.1	17.43	24.1
12	27	Molar	16.002	18.150	14.755	25.05	1.44	9.26	-1.69	34.37	23.2
13	300	Premolar	1.090	4.873	5.889	33.8	1.38	9.54	-1.55	29.8	16.5
14	300	Premolar	0.389	13.964	2.050	44.8	0.99	9.26	0.46	24.21	20.7
15	300	Premolar	0.914	5.355	6.720	31.84	1.23	9.22	-1.36	29.78	11.6
16	300	Molar	0.790	2.441	6.605	34.02	0.77	7.97	-0.05	25.17	21.3
17	300	Molar	20.749	38.574	14.673	28.65	1.59	9.25	-1.6	31.9	31.3
18	300	Molar	0.385	3.856	4.279	22.35	1.45	10.1	-2.07	39.01	23.7

19	300	Premolar	0.463	8.793	2.303	28.27	1.58	12.07	-3.45	40.16	20.1
20	300	Premolar	0.514	8.877	1.918	35.85	1.46	13.44	-4.95	38.56	20.1
21	300	Premolar	0.302	10.510	2.243	30.54	1.45	11.12	-2.88	35.91	11.7
22	300	Molar	0.821	1.909	3.941	17.43	1.84	9.22	-1.52	40.54	22.5
23	300	Molar	14.955	12.641	11.128	34.27	1.41	9.11	-1.11	28.36	31
24	300	Molar	2.173	4.886	3.573	5.75	0.21	0.62	0.48	6.07	8.9
25	600	Premolar	0.000	0.000	0.000	37.35	1.01	4.35	4.57	13.36	14.7
26	600	Premolar	0.000	0.000	0.000	15.78	0.49	2.38	1.05	12.36	8.8
27	600	Premolar	0.000	0.000	0.000	3.14	0.27	0.79	0.14	14.7	14
28	600	Molar	0.000	0.000	0.000	9.5	0.35	2.53	0.38	16.14	20.3
29	600	Molar	0.000	0.000	0.000	2.11	0.4	2.06	0.39	14.43	23.4
30	600	Molar	0.000	0.000	0.000	20.86	0.37	1.86	2.13	8.51	15.5
31	600	Premolar	0.000	0.000	0.000	19.78	0.79	3.57	1	16.58	13.8
32	600	Premolar	0.000	0.000	0.000	12.31	1.07	3.28	0.34	19.2	10.2
33	600	Premolar	0.000	0.000	0.000	7.97	0.47	1.47	0.51	10.7	13.7
34	600	Molar	0.000	0.000	0.000	4.62	0.21	0.72	0.33	9.14	31.8
35	800	Molar	0.000	0.000	0.000	12.3	0.33	2.65	0.58	15.14	24
36	800	Premolar	0.000	0.000	0.000	10.16	-0.41	0.39	1.07	1.79	20.1
37	800	Premolar	0.000	0.000	0.000	12.53	-0.53	-0.33	1.66	-2.95	29.1
38	800	Molar	0.000	0.000	0.000	13.9	-0.61	-1.43	2.35	-9.57	30.6
39	800	Molar	0.000	0.000	0.000	23.64	0.94	2.89	2.05	12.24	36.3
40	800	Molar	0.000	0.000	0.000	28.13	0.03	1.64	4.2	6	42
41	800	Premolar	0.000	0.000	0.000	44.63	-0.37	1.77	11.69	4.58	27.9
42	800	Premolar	0.000	0.000	0.000	16.64	0.21	2.42	1.16	12.09	25.7
43	800	Premolar	0.000	0.000	0.000	21.02	-0.26	0.32	3.13	0.94	31.6
44	800	Molar	0.000	0.000	0.000	21.67	-0.11	1.39	2.63	5.85	32.6
45	800	Molar	0.000	0.000	0.000	16.84	0.42	2.79	1.02	13.9	34.6
46	1000	Molar	0.000	0.000	0.000	62.78	-0.06	0.67	29.8	1.23	35.3
47	1000	Premolar	0.000	0.000	0.000	49.77	0.12	2.2	14.34	5.38	43.8
48	1000	Premolar	0.000	0.000	0.000	47.98	0.51	2.43	12.64	6.25	43.7

49	1000	Premolar	0.000	0.000	0.000	36.77	0.58	3.44	5.42	10.71	55.9
50	1000	Molar	0.000	0.000	0.000	44.12	0.19	1.55	11.62	4.2	39.7
51	1000	Molar	0.000	0.000	0.000	44.35	-0.01	1.08	12.5	2.83	66.4
52	1000	Molar	0.000	0.000	0.000	46.39	-0.9	0.2	15.48	0.11	23.4
53	1000	Premolar	0.000	0.000	0.000	45.32	-0.73	0.34	14.43	0.58	32.7
54	1000	Premolar	0.000	0.000	0.000	22.8	0.52	2.94	1.91	12.33	32.3
55	1000	Premolar	0.000	0.000	0.000	48.03	-0.11	0.26	16.54	0.42	36.5
56	1000	Molar	0.000	0.000	0.000	56.02	-0.05	0.81	22.36	1.68	36.9
57	1000	Molar	0.000	0.000	0.000	24.25	0.27	1.31	3.3	5.34	65.6

Supplementary data, S2

Novel diagnostic models to predict the nuclear DNA and mitochondrial DNA in incinerated teeth

i. ANOVA

S 1—Results of the pairwise comparisons with a Tukey HSD correction for lightness (L).

Temperature		Lower	Upper	Adj p
27	300	-6.502	16.092	.752
	600	8.569	32.265	< .001
	800	2.076	25.177	.013
	1000	-21.586	1.007	.090
300	600	3.774	27.470	0.004
	800	-2.719	20.382	.211
	1000	-26.381	-3.788	< .001
600	800	-18.881	5.299	.512
	1000	-42.554	-18.858	< .001
800	1000	-35.466	-12.365	< .001

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 2— Results of the pairwise comparisons with a Tukey HSD correction for chromaticity a*

Temperature		Lower	Upper	Adj P
27	300	.080	2.058	.028
	600	.769	2.843	< .001
	800	1.371	3.393	< .001
	1000	1.333	3.311	< .001
300	600	-.300	1.774	.277
	800	.302	2.324	.005
	1000	.264	2.241	.007
600	800	-.483	1.634	.544
	1000	-.522	1.553	.628
800	1000	-1.071	.951	>.999

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 3—Results of the pairwise comparisons with a Tukey HSD correction for chromaticity b*

Temperature		Lower	Upper	Adj P
27	300	-4.232	.207	.093
	600	2.602	7.258	< .001
	800	3.643	8.182	< .001
	1000	3.575	8.015	< .001
300	600	4.614	9.270	< .001
	800	5.656	10.195	< .001
	1000	5.588	10.027	< .001
600	800	-1.393	3.358	.769
	1000	-1.463	3.193	.831
800	1000	-2.387	2.152	>.999

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$

S 4—Results of the pairwise comparisons with a Tukey HSD correction for whiteness (WI)

Temperature		Lower	Upper	Adj P
27	300	.797	15.080	.022
	600	-2.269	12.711	.295
	800	-3.864	10.740	.674
	1000	-14.198	.085	.054
300	600	-10.207	4.773	.843
	800	-11.803	2.801	.418
	1000	-22.136	-7.854	< .001
600	800	-9.426	5.860	.964
	1000	-19.768	-4.788	< .001
800	1000	-17.796	-3.193	.001

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 5—Results of the pairwise comparisons with a Tukey HSD correction for yellowness (YI)

Temperature		Lower	Upper	Adj P
27	300	-13.028	3.328	.457
	600	3.850	21.005	.001
	800	12.122	28.846	< .001
	1000	13.506	29.862	< .001
300	600	8.700	25.855	< .001
	800	16.972	33.696	< .001
	1000	18.356	34.712	< .001
600	800	-.696	16.809	.085
	1000	.680	17.834	.028
800	1000	-7.162	9.563	.994

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

S 6—Results of the pairwise comparisons with a Tukey HSD correction for crystallite size (CS)

Temperature		Lower	Upper	Adj P
27	300	-6.436	12.452	.896
	600	-3.567	16.243	.380
	800	-17.107	2.206	.203
	1000	-29.169	-10.281	< .001
300	600	-6.575	13.235	.876
	800	-20.115	-.803	.028
	1000	-32.177	-13.289	< .001
600	800	-23.897	-3.681	.003
	1000	-35.968	-16.158	< .001
800	1000	-21.931	-2.618	.006

Temperature in degree Celcius (°C). Statistical significant accepted at $p < 0.05$.

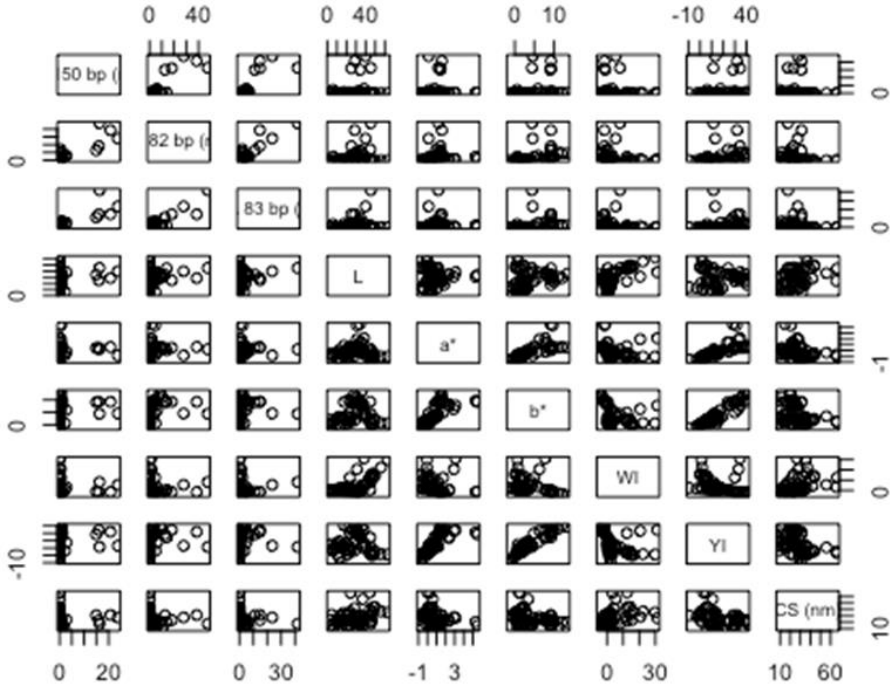
ii. Correlation coefficients

S 7—Correlation coefficients between the values of response variables including 150bp nDNA, 82bp DNA, mtDNA, L, a*, b*, WI, YI and CS.

	150bp nDNA	82bp nDNA	83bp mtDNA	L	a*	b*	WI	YI	CS
150bp nDNA	1	**0.87****	**0.84****	0.13	0.08	0.25	-0.12	0.20	-0.04
82bp nDNA		1	**0.92****	0.19	0.17	0.38	-0.15	0.30	-0.15
83bp mtDNA			1	0.18	0.12	0.28	-0.10	0.21	-0.15
L				1	0.06	0.16	0.60	-0.05	0.35
a					1	**0.71****	-0.16	0.73	-0.29
b						1	-0.38	**0.94****	-0.34
WI							1	-0.36	0.39
YI								1	-0.41
CS									1

L=Lightness; a=chromaticity of redness-greenness; b=chromaticity of yellowness-blueness; WI=Whiteness index; YI=Yellowness Index; CS=Crystallite Size; Correlation is highly significant at **p < 0.01; Correlation is moderately significant at *p < 0.05.

iii. Pairwise variable plot



S 8—A scatterplot matrix with respect to all continuous variables: 150 bp nDNA, 82 bp nDNA, 83 bp DNA, lightness (L), chromaticity a*, chromaticity b*, whiteness index (WI), yellowness index (YI) and crystallite size (CS).

iv. Regression model

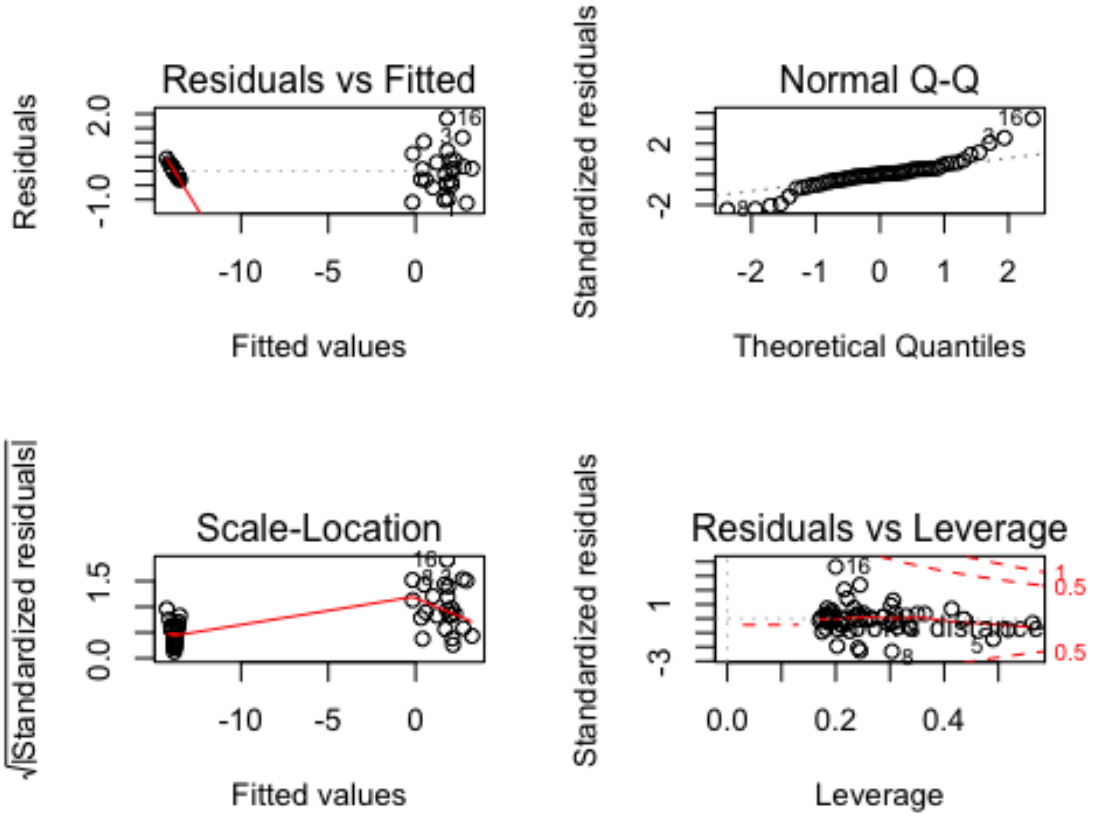
Nuclear DNA

```
fitn82 <- lm(log(`nDNA 82 bp (ng/mg)`+.000001)~.+`Temperature (℃)`*
`Tooth type`, data=d[,-c(1, 4,6,9)])
summary(fitn82)

##
## Call:
## lm(formula = log(`nDNA 82 bp (ng/mg)` + 1e-06) ~ . + `Temperatur
e (℃)` *
##     `Tooth type`, data = d[, -c(1, 4, 6, 9)])
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -1.1182 -0.1886  0.0076  0.1543  1.8461
##
## Coefficients:
##                                             Estimate Std. Error
t value
## (Intercept)                                 2.552096   0.536466
4.757
## `Temperature (℃)`300                       -1.009140   0.389091
-2.594
## `Temperature (℃)`600                      -16.822634   0.491250
-34.245
## `Temperature (℃)`800                      -17.052508   0.465229
-36.654
## `Temperature (℃)`1000                     -17.627638   0.552556
-31.902
## `Tooth type`Premolar                       -2.470541   0.383563
-6.441
## L                                          -0.002279   0.010231
-0.223
## `a*`                                       -0.370840   0.112790
-3.288
## WI                                          0.027128   0.016832
1.612
## YI                                          0.010922   0.014595
0.748
## `CS (nm)`                                   0.019600   0.010887
1.800
## `Temperature (℃)`300:`Tooth type`Premolar    2.978763   0.555073
5.366
## `Temperature (℃)`600:`Tooth type`Premolar    2.777318   0.567388
4.895
## `Temperature (℃)`800:`Tooth type`Premolar    2.438138   0.527080
4.626
## `Temperature (℃)`1000:`Tooth type`Premolar   2.720714   0.528619
5.147
##                                                Pr(>|t|)
## (Intercept)                                    2.33e-05 ***
```

```
## `Temperature (℃)`300                            0.01302 *
## `Temperature (℃)`600                            < 2e-16 ***
## `Temperature (℃)`800                            < 2e-16 ***
## `Temperature (℃)`1000                           < 2e-16 ***
## `Tooth type`Premolar                          9.21e-08 ***
## L                                              0.82482
## `a*`                                           0.00205 **
## WI                                             0.11452
## YI                                             0.45841
## `CS (nm)`                                      0.07901 .
## `Temperature (℃)`300:`Tooth type`Premolar  3.22e-06 ***
## `Temperature (℃)`600:`Tooth type`Premolar  1.50e-05 ***
## `Temperature (℃)`800:`Tooth type`Premolar  3.55e-05 ***
## `Temperature (℃)`1000:`Tooth type`Premolar 6.60e-06 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.5667 on 42 degrees of freedom
## Multiple R-squared:  0.996,  Adjusted R-squared:  0.9946
## F-statistic: 739.8 on 14 and 42 DF,  p-value: < 2.2e-16
```

```
opar <- par(mfrow=c(2,2))
plot(fitn82)
```




```
par(opar)
```

```
fitstepn <- step(fitn82, trace=FALSE, k=log(57))
summary(fitstepn)

##
## Call:
## lm(formula = log(`nDNA 82 bp (ng/mg)` + 1e-06) ~ `Temperature (℃
)` +
##     `Tooth type` + `a*` + `Temperature (℃)`:`Tooth type`, data =
d[,
##     -c(1, 4, 6, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.48739 -0.08994  0.01409  0.14794  1.99815
##
## Coefficients:
##                                            Estimate Std. Error
t value
## (Intercept)                                 3.08211    0.32927
9.360
## `Temperature (℃)`300                       -0.91079    0.35400
-2.573
## `Temperature (℃)`600                      -16.78953    0.42356
-39.639
## `Temperature (℃)`800                      -16.84344    0.39714
-42.411
## `Temperature (℃)`1000                     -16.92796    0.41094
-41.193
## `Tooth type`Premolar                       -2.09071    0.33568
-6.228
## `a*`                                       -0.32510    0.09538
-3.408
## `Temperature (℃)`300:`Tooth type`Premolar   2.45905    0.47507
5.176
## `Temperature (℃)`600:`Tooth type`Premolar   2.20476    0.50511
4.365
## `Temperature (℃)`800:`Tooth type`Premolar   1.94810    0.48745
3.997
## `Temperature (℃)`1000:`Tooth type`Premolar  2.16927    0.47562
4.561
##                                           Pr(>|t|)
## (Intercept)                               3.19e-12 ***
## `Temperature (℃)`300                       0.01338 *
## `Temperature (℃)`600                       < 2e-16 ***
## `Temperature (℃)`800                       < 2e-16 ***
## `Temperature (℃)`1000                      < 2e-16 ***
## `Tooth type`Premolar                      1.32e-07 ***
## `a*`                                       0.00137 **
## `Temperature (℃)`300:`Tooth type`Premolar  4.85e-06 ***
## `Temperature (℃)`600:`Tooth type`Premolar  7.14e-05 ***
## `Temperature (℃)`800:`Tooth type`Premolar   0.00023 ***
## `Temperature (℃)`1000:`Tooth type`Premolar 3.77e-05 ***
## ---
```

```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.5812 on 46 degrees of freedom
## Multiple R-squared:  0.9953, Adjusted R-squared:  0.9943
## F-statistic:   984 on 10 and 46 DF,  p-value: < 2.2e-16
```

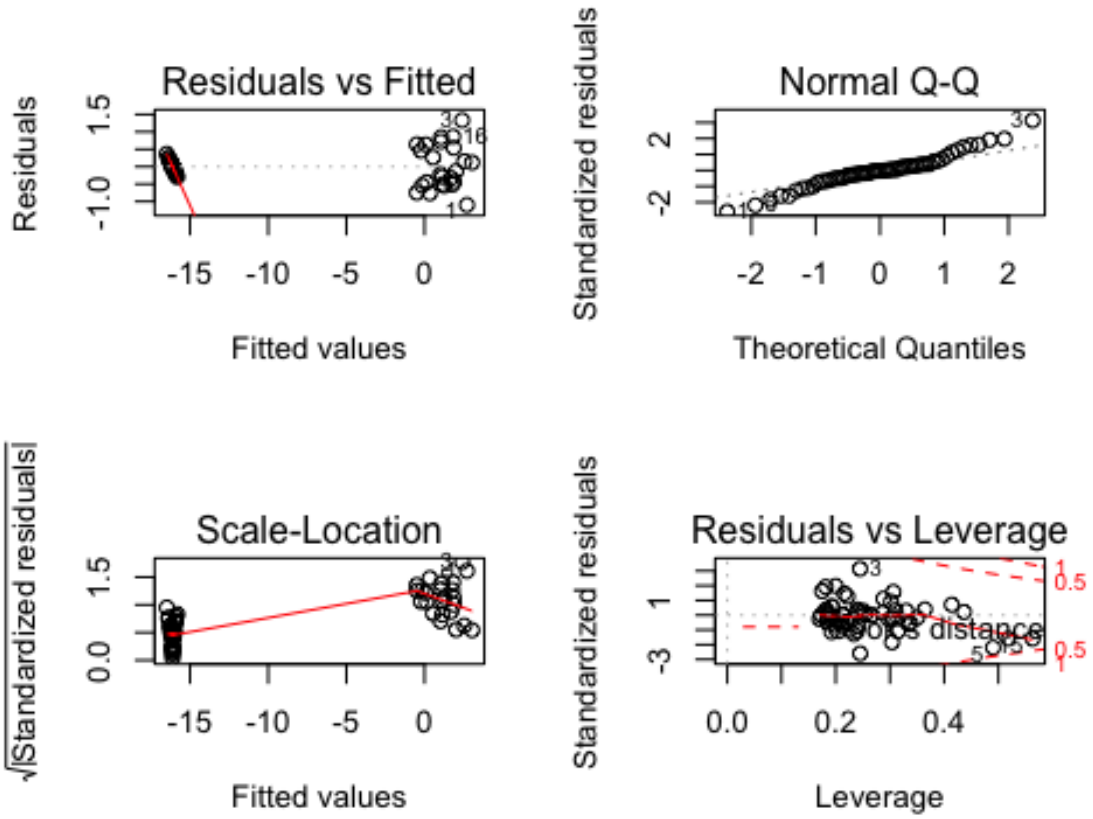
Mitochondrial DNA

```
fit82 <- lm(log(`mtDNA 83 bp (ng/mg)`+.0000001)~.+`Temperature (℃)`
*`Tooth type`, data=d[,-c(1, 4:5,9)])
summary(fit82)

##
## Call:
## lm(formula = log(`mtDNA 83 bp (ng/mg)` + 1e-07) ~ . + `Temperatu
re (℃)` *
##     `Tooth type`, data = d[, -c(1, 4:5, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.09962 -0.21150 -0.01194  0.15224  1.33036
##
## Coefficients:
##                                          Estimate Std. Error
t value
## (Intercept)                               2.379219   0.461809
5.152
## `Temperature (℃)`300                      -0.720614   0.334943
-2.151
## `Temperature (℃)`600                     -18.860374   0.422886
-44.599
## `Temperature (℃)`800                     -19.048215   0.400486
-47.563
## `Temperature (℃)`1000                    -19.509487   0.475661
-41.016
## `Tooth type`Premolar                      -2.439829   0.330185
-7.389
## L                                         0.001628   0.008807
0.185
## `a*`                                     -0.495415   0.097094
-5.102
## WI                                        0.015334   0.014490
1.058
## YI                                        0.015784   0.012564
1.256
## `CS (nm)`                                 0.013628   0.009372
1.454
## `Temperature (℃)`300:`Tooth type`Premolar    1.792855   0.477827
3.752
## `Temperature (℃)`600:`Tooth type`Premolar    2.696437   0.488428
5.521
## `Temperature (℃)`800:`Tooth type`Premolar    2.346176   0.453729
5.171
## `Temperature (℃)`1000:`Tooth type`Premolar   2.640578   0.455055
```

```
5.803
##                                                 Pr(>|t|)
## (Intercept)                                    6.49e-06 ***
## `Temperature (℃)`300                            0.037238 *
## `Temperature (℃)`600                             < 2e-16 ***
## `Temperature (℃)`800                             < 2e-16 ***
## `Temperature (℃)`1000                            < 2e-16 ***
## `Tooth type`Premolar                           4.05e-09 ***
## L                                              0.854278
## `a*`                                           7.63e-06 ***
## WI                                             0.295990
## YI                                             0.215946
## `CS (nm)`                                      0.153354
## `Temperature (℃)`300:`Tooth type`Premolar  0.000532 ***
## `Temperature (℃)`600:`Tooth type`Premolar  1.94e-06 ***
## `Temperature (℃)`800:`Tooth type`Premolar  6.10e-06 ***
## `Temperature (℃)`1000:`Tooth type`Premolar 7.63e-07 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.4878 on 42 degrees of freedom
## Multiple R-squared:  0.9976, Adjusted R-squared:  0.9968
## F-statistic:  1255 on 14 and 42 DF,  p-value: < 2.2e-16

opar <- par(mfrow=c(2,2))
plot(fit82)
```



```
par(opar)

fitstep <- step(fit82, trace=FALSE, k=log(57))
summary(fitstep)

##
## Call:
## lm(formula = log(`mtDNA 83 bp (ng/mg)` + 1e-07) ~ `Temperature (
℃)` +
##     `Tooth type` + `a*` + `Temperature (℃)`:`Tooth type`, data =
d[,
##     -c(1, 4:5, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.02734 -0.17631  0.00746  0.15853  1.29445
##
## Coefficients:
##                                          Estimate Std. Error
t value
## (Intercept)                               2.95145    0.28089
10.507
## `Temperature (℃)`300                      -0.58764    0.30199
-1.946
## `Temperature (℃)`600                     -18.92772    0.36133
-52.384
## `Temperature (℃)`800                     -18.99846    0.33879
-56.077
## `Temperature (℃)`1000                    -19.10936    0.35056
-54.511
## `Tooth type`Premolar                      -2.16002    0.28636
-7.543
## `a*`                                     -0.42655    0.08137
-5.242
## `Temperature (℃)`300:`Tooth type`Premolar    1.48622    0.40526
3.667
## `Temperature (℃)`600:`Tooth type`Premolar    2.30966    0.43089
5.360
## `Temperature (℃)`800:`Tooth type`Premolar    1.97290    0.41583
4.745
## `Temperature (℃)`1000:`Tooth type`Premolar   2.26310    0.40574
5.578
##                                             Pr(>|t|)
## (Intercept)                                8.24e-14 ***
## `Temperature (℃)`300                        0.057790 .
## `Temperature (℃)`600                         < 2e-16 ***
## `Temperature (℃)`800                         < 2e-16 ***
## `Temperature (℃)`1000                        < 2e-16 ***
## `Tooth type`Premolar                       1.41e-09 ***
## `a*`                                       3.88e-06 ***
## `Temperature (℃)`300:`Tooth type`Premolar  0.000634 ***
## `Temperature (℃)`600:`Tooth type`Premolar  2.60e-06 ***
## `Temperature (℃)`800:`Tooth type`Premolar  2.06e-05 ***
## `Temperature (℃)`1000:`Tooth type`Premolar 1.24e-06 ***
```

```
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.4958 on 46 degrees of freedom
## Multiple R-squared:  0.9973, Adjusted R-squared:  0.9967
## F-statistic:  1700 on 10 and 46 DF,  p-value: < 2.2e-16
```

Can we make this cleaner?

Classify temperature as <600℃ or >600℃

```
d2 <- d
d2$NewTemp <- double(dim(d2)[1])
thezeros <- which(d2$`Temperature (℃)`=="27")
theones <- which(d2$`Temperature (℃)`=="300")
d2$NewTemp[theones] <- 1
d2$NewTemp[-c(thezeros,theones)] <- 2
d2$NewTemp <- factor(d2$NewTemp)
```

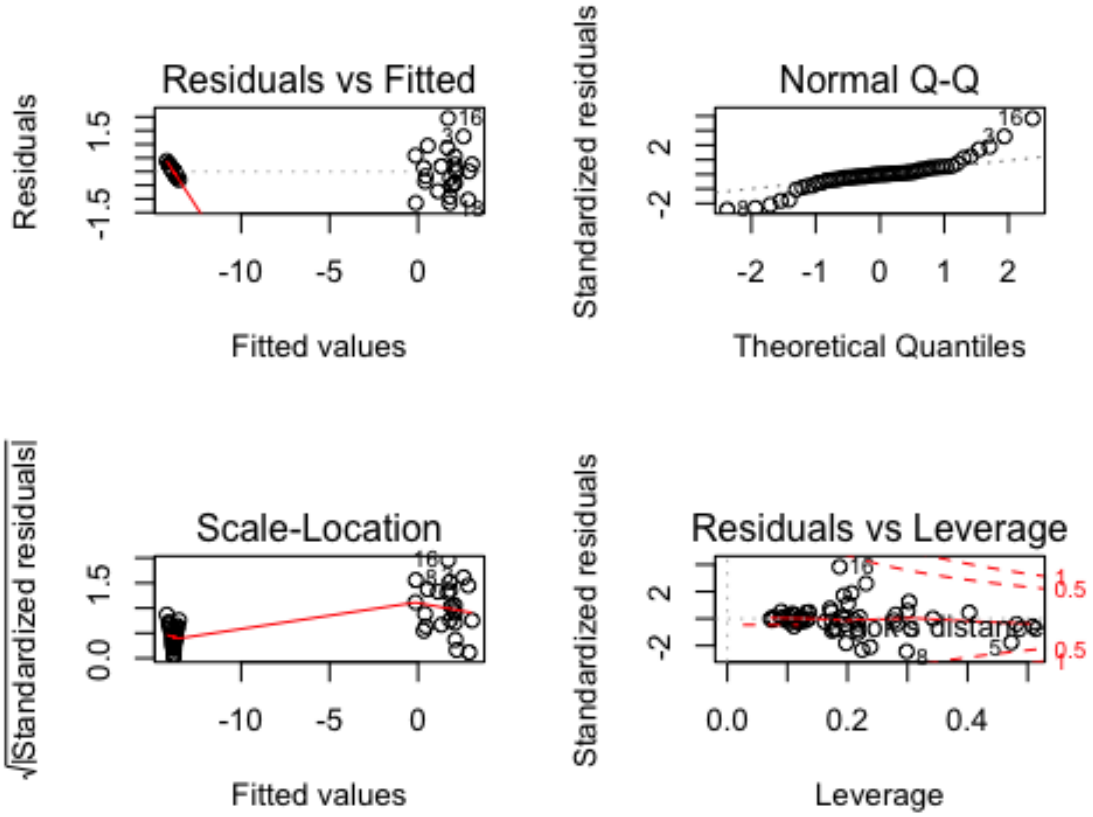
Nuclear DNA

```
fitn82N <- lm(log(`nDNA 82 bp (ng/mg)`+.000001)~.+NewTemp*`Tooth ty
pe`, data=d2[,-c(1,2, 4,6,9)])
summary(fitn82N)

##
## Call:
## lm(formula = log(`nDNA 82 bp (ng/mg)` + 1e-06) ~ . + NewTemp *
##     `Tooth type`, data = d2[, -c(1, 2, 4, 6, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.14773 -0.16418 -0.00497  0.15960  1.94961
##
## Coefficients:
##                                 Estimate Std. Error t value Pr(>
|t|)
## (Intercept)                      2.870303   0.468876   6.122 1.90
e-07 ***
## `Tooth type`Premolar            -2.446267   0.380301  -6.432 6.49
e-08 ***
## L                               -0.007608   0.009251  -0.822  0.4
1509
## `a*`                            -0.376491   0.111842  -3.366  0.0
0155 **
## WI                               0.021114   0.016258   1.299  0.2
0052
## YI                               0.018374   0.013706   1.341  0.1
8663
## `CS (nm)`                        0.006857   0.008171   0.839  0.4
0570
## NewTemp1                        -1.117727   0.380797  -2.935  0.0
0519 **
## NewTemp2                       -16.948015   0.399541 -42.419  < 2
e-16 ***
## `Tooth type`Premolar:NewTemp1   2.888309   0.549147   5.260 3.66
e-06 ***
## `Tooth type`Premolar:NewTemp2   2.543783   0.448390   5.673 8.92
e-07 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
##
## Residual standard error: 0.5638 on 46 degrees of freedom
## Multiple R-squared:  0.9956, Adjusted R-squared:  0.9947
## F-statistic:  1046 on 10 and 46 DF,  p-value: < 2.2e-16

opar <- par(mfrow=c(2,2))
plot(fitn82N)
```




```
par(opar)

fitstepnN <- step(fitn82N, trace=FALSE, k=log(57))
summary(fitstepnN)

##
## Call:
## lm(formula = log(`nDNA 82 bp (ng/mg)` + 1e-06) ~ `Tooth type` +
##     `a*` + NewTemp + `Tooth type`:NewTemp, data = d2[, -c(1,
##     2, 4, 6, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.48945 -0.18758  0.04795  0.17343  1.98898
##
## Coefficients:
##                                 Estimate Std. Error t value Pr(>|
t|)
## (Intercept)                      3.02406    0.31302   9.661 5.07e
-13 ***
```

```
## `Tooth type`Premolar           -2.08857    0.32499  -6.427 4.73e
-08 ***
## `a*`                           -0.30084    0.08884  -3.386  0.00
139 **
## NewTemp1                       -0.88212    0.34143  -2.584  0.01
275 *
## NewTemp2                      -16.80629    0.33718 -49.843  < 2e
-16 ***
## `Tooth type`Premolar:NewTemp1   2.45359    0.45990   5.335 2.32e
-06 ***
## `Tooth type`Premolar:NewTemp2   2.11952    0.37982   5.580 9.75e
-07 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.5627 on 50 degrees of freedom
## Multiple R-squared:  0.9953, Adjusted R-squared:  0.9947
## F-statistic:  1749 on 6 and 50 DF,  p-value: < 2.2e-16
```

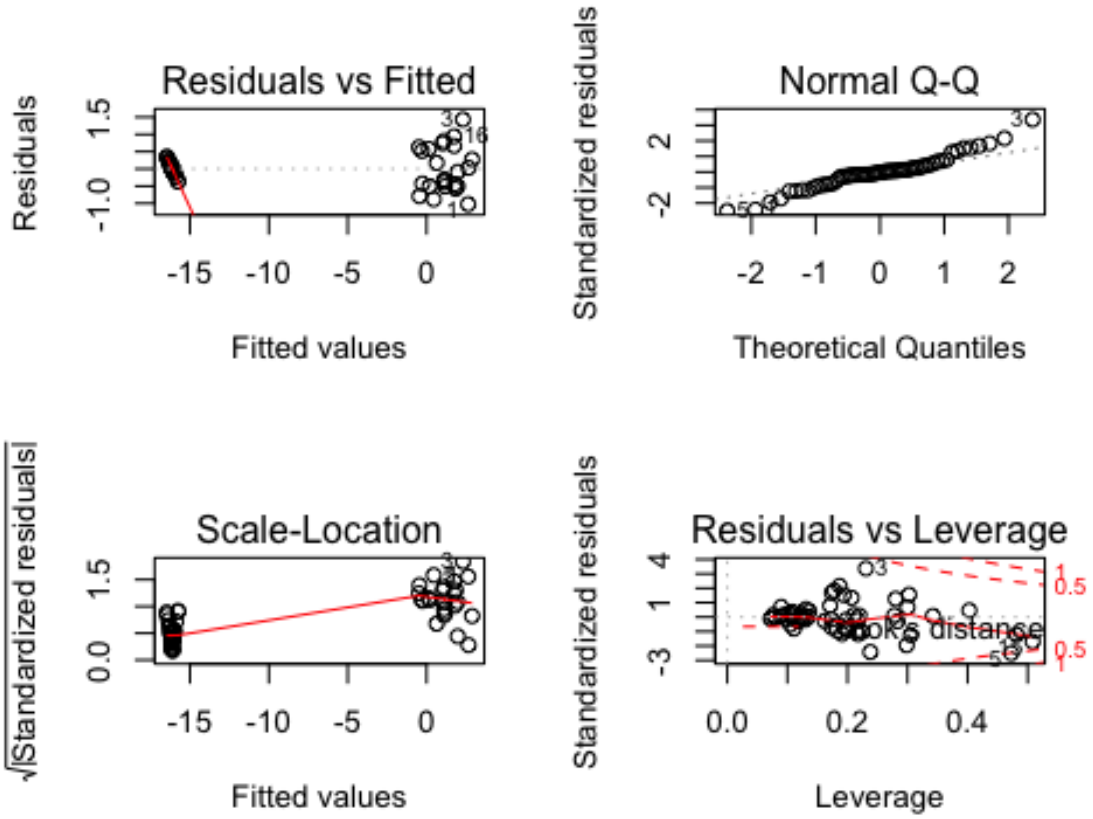
Mitochondrial DNA

```
fit82N <- lm(log(`mtDNA 83 bp (ng/mg)`+.0000001)~.+NewTemp*`Tooth t
ype`, data=d2[,-c(1,2, 4:5,9)])
summary(fit82N)

##
## Call:
## lm(formula = log(`mtDNA 83 bp (ng/mg)` + 1e-07) ~ . + NewTemp *
##     `Tooth type`, data = d2[, -c(1, 2, 4:5, 9)])
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.02501 -0.23836 -0.01491  0.16779  1.43156
##
## Coefficients:
##                                 Estimate Std. Error t value Pr(>
|t|)
## (Intercept)                     2.606847   0.403352   6.463 5.84
e-08 ***
## `Tooth type`Premolar           -2.422311   0.327155  -7.404 2.26
e-09 ***
## L                              -0.002255   0.007958  -0.283 0.77
8182
## `a*`                           -0.498629   0.096212  -5.183 4.75
e-06 ***
## WI                              0.010425   0.013986   0.745 0.45
9799
## YI                              0.022564   0.011791   1.914 0.06
1888 .
## `CS (nm)`                       0.003057   0.007029   0.435 0.66
5690
## NewTemp1                       -0.805882   0.327582  -2.460 0.01
```

```
7707 *
## NewTemp2                        -18.939195   0.343706 -55.103  < 2
e-16 ***
## `Tooth type`Premolar:NewTemp1    1.711991   0.472406   3.624 0.00
0722 ***
## `Tooth type`Premolar:NewTemp2    2.480567   0.385729   6.431 6.53
e-08 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.485 on 46 degrees of freedom
## Multiple R-squared:  0.9974, Adjusted R-squared:  0.9969
## F-statistic:  1777 on 10 and 46 DF,  p-value: < 2.2e-16

opar <- par(mfrow=c(2,2))
plot(fit82N)
```




```
par(opar)

fitstepN <- step(fit82N, trace=FALSE, k=log(57))
summary(fitstepN)

##
## Call:
## lm(formula = log(`mtDNA 83 bp (ng/mg)` + 1e-07) ~ `Tooth type` +
##     `a*` + NewTemp + `Tooth type`:NewTemp, data = d2[, -c(1,
##     2, 4:5, 9)])
##
```

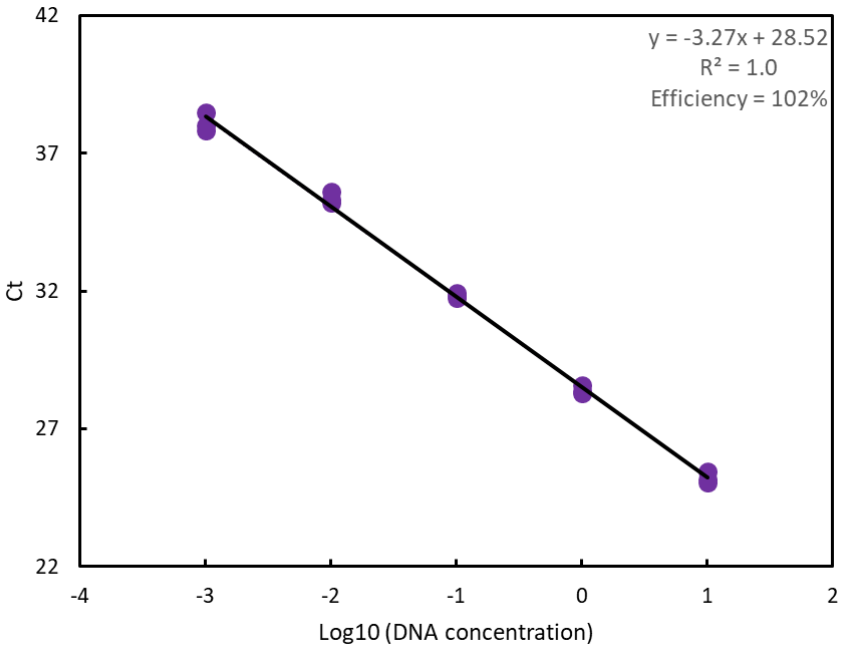
```
## Residuals:
##      Min       1Q   Median       3Q      Max
## -1.12653 -0.23033  0.02229  0.14465  1.33243
##
## Coefficients:
##                                Estimate Std. Error t value Pr(>|
t|)
## (Intercept)                     2.87528    0.27038  10.634 1.95e
-14 ***
## `Tooth type`Premolar           -2.15720    0.28072  -7.685 5.13e
-10 ***
## `a*`                           -0.39472    0.07673  -5.144 4.51e
-06 ***
## NewTemp1                       -0.55003    0.29492  -1.865   0.0
681 .
## NewTemp2                      -18.94971    0.29125 -65.064  < 2e
-16 ***
## `Tooth type`Premolar:NewTemp1   1.47906    0.39725   3.723   0.0
005 ***
## `Tooth type`Premolar:NewTemp2   2.19782    0.32808   6.699 1.77e
-08 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.4861 on 50 degrees of freedom
## Multiple R-squared:  0.9972, Adjusted R-squared:  0.9968
## F-statistic:  2948 on 6 and 50 DF,  p-value: < 2.2e-16
```

Supplementary data, S3

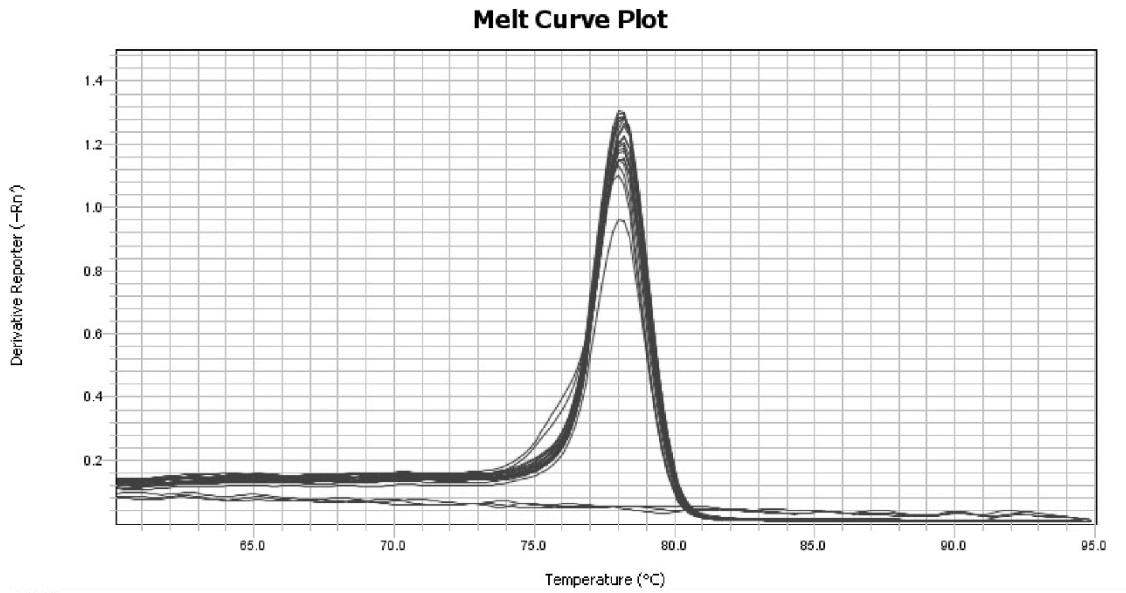
Novel diagnostic models to predict the nuclear DNA and mitochondrial DNA in incinerated teeth

Followings are examples of a standards curve and a melting curves that were produced from a qPCR run for each primer pair:

i. Actin 150 (nuclear DNA, 150 bp fragment)

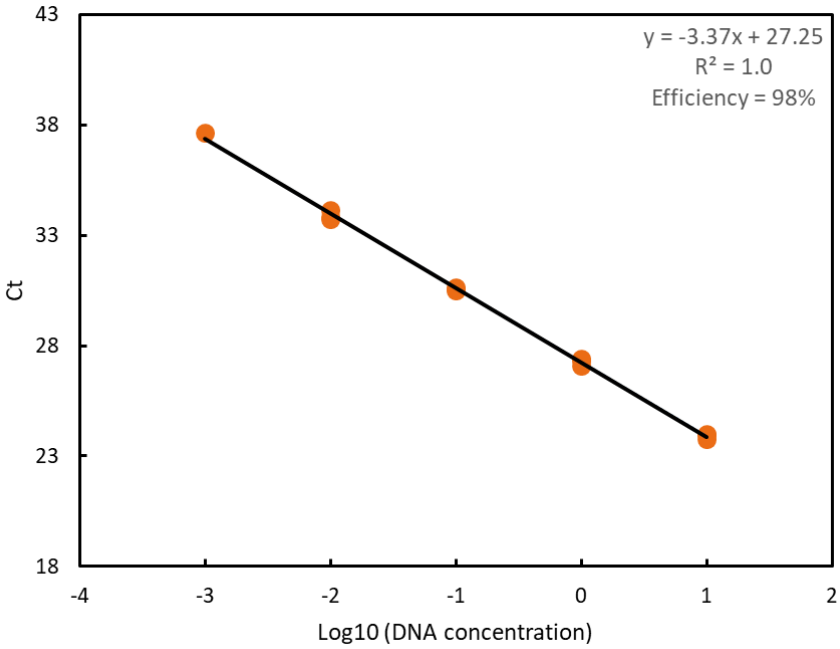


S 1—Standard curve generated from the five serial diluted standards for the qPCR reaction. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1, 0.1, 0.01, 0.001, 0.0001 ng/µL.

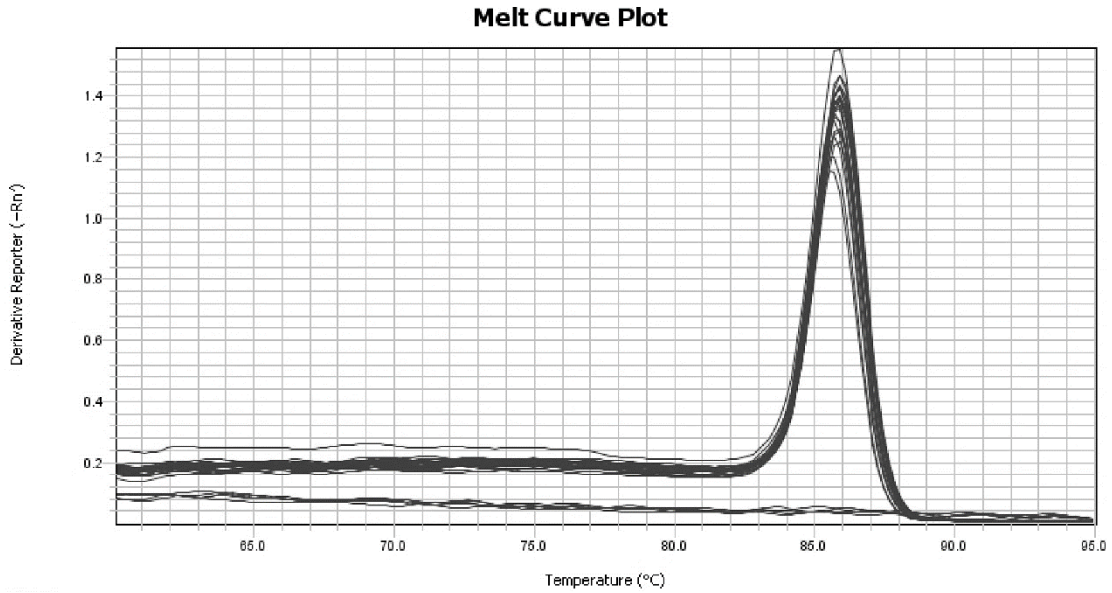


S 2— Melt curve shows a single-peak of the 150bp Actin target gene which indicate no contamination or primer-dimer formation.

ii. MC1R (nuclear DNA, 82 bp fragment)

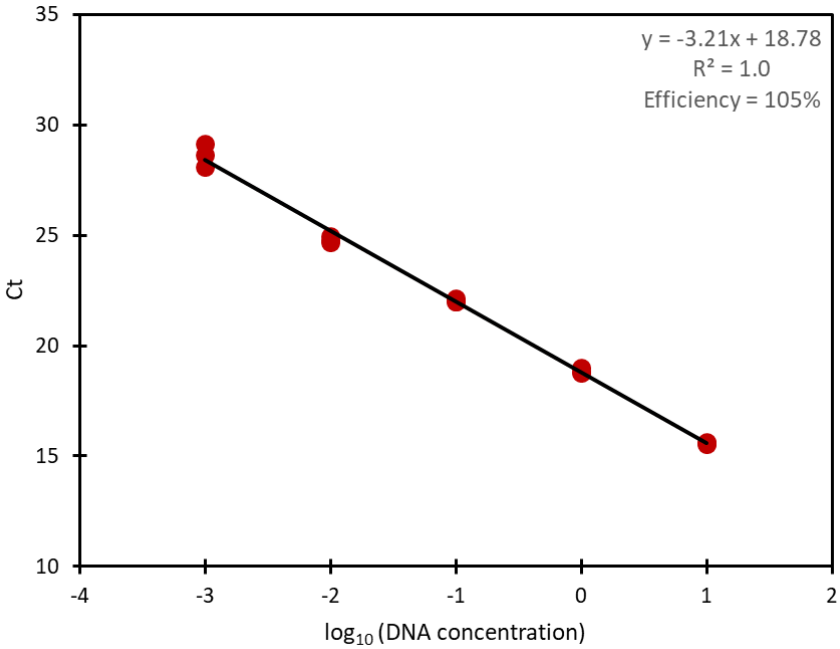


S 3—Standard curve generated from the five serial diluted standards for the qPCR reaction. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1, 0.1, 0.01, 0.001, 0.0001 ng/µL.

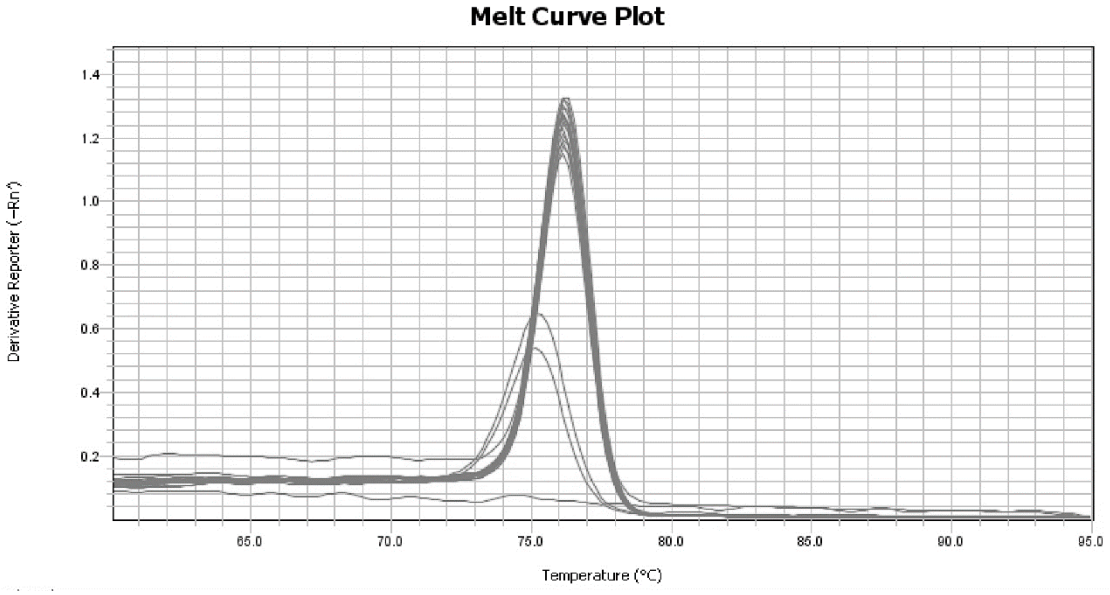


S 4—Melt curve shows a single-peak of the 82 bp MC1R target gene which indicate no contamination or primer-dimer formation.

iii. ATPase (mitochondrial DNA, 83 bp fragment)



S 5—Standard curve generated from the five serial diluted standards for the qPCR reaction. Ct = threshold cycle. The $\log_{10}$ of DNA concentrations of the serially diluted standards at 1, 0.1, 0.01, 0.001, 0.0001 ng/µL.



S 6—Melt curve shows a single-peak of the 83 bp Actin target gene which indicate no contamination or primer-dimer formation.

Chapter 6
Discussion, Future Research & Concluding Remarks

Discussion

From my personal view, teeth remain an important biological evidence in forensic identification of burnt human because of their endurance and remarkable versatility. The characteristic changes in morphology, biochemistry and histology of incinerated teeth are related to the intensity and duration of heat-exposure [1, 2]. Teeth provide a valuable source of DNA for genetic analysis [3, 4] and as such, in fatalities involving fire, teeth are often relied upon as DNA sources for DNA-based identification [5-8]. Here, I discuss all the work that has been completed for this thesis. The discussion includes the aims, findings, significance and limitations of the research work.

Overview of the aims and findings

This study was set out mainly to investigate the value of incinerated teeth as a forensic evidence in a fire fatality and a critical DNA source in the identification of burnt human remains. The research work was undertaken with an intention to develop diagnostic tools that can aid in retrieving valued information including the temperature that the teeth have been exposed given their physical and chemical composition, and the likelihood to obtain DNA from the teeth. Here, I present the overall aims of the thesis and highlight the corresponding key findings from the comprehensive investigation on incinerated teeth in Figure 1.

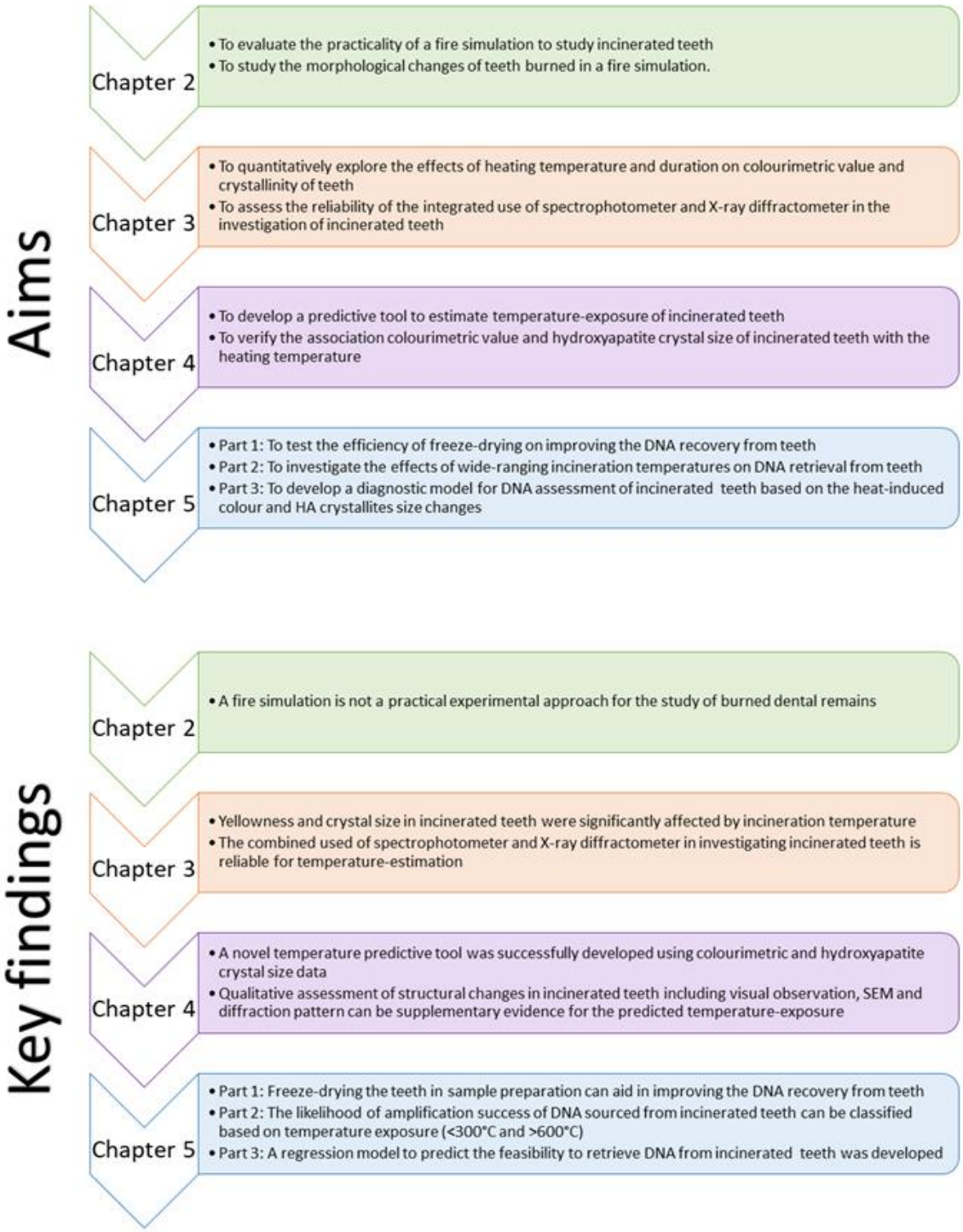


Figure 1—An overview of the aims and the key findings of this research thesis according to the chapter sequence. The boxes were colour coded referring to the respective chapters. Green box: Chapter 2; Orange box: Chapter 3; Purple box: Chapter 4; Blue box: Chapter 5.

The fundamental work of this thesis revolves around the structural changes of heat-treated teeth and the DNA retrievability from the incinerated teeth. Specifically, colour, hydroxyapatite (HA) crystallite size, and DNA yield of teeth were chosen as

the main response variables to the heat-treatment. Brief background and significant findings are synthesised and deliberated by chapter.

Chapter 2: Lessons learned from a fire simulation as an experimental approach to study burned dental remains

The original idea of this research study was to use teeth that are incinerated in a condition that simulate a real fire situation, specifically a building fire. In this study, I have conducted an experimental fire simulation in a collaboration with the South Australian Police (SAPOL), the South Australian Country Fire Service (CFS) and the South Australian Metropolitan Fire Service (MFS). The experiment was performed using the fire equipment and facility at the CFS training ground in Brukunga. Technical support and advice were provided by a group expertise from SAPOL, CFS and MFS.

The use of fire simulation to incinerate teeth proved to be inconvenient because there are many confounding factors that influence the intensity of the fire, which are difficult to control. These factors include the airflow, the level of oxygen and the animal fat that can act as an additional fuel. As a result, I resorted to an alternative strategy of sample incineration to closely mimic the teeth obtained from a real fire situation for the following studies (Chapters 3 to 5). The strategy includes: (1) the used of teeth that were in the natural anatomical position, and were firmly attached in the tooth socket of the jaw; (2) incinerating the teeth using a laboratory furnace at a broad range of temperature that imitates the temperature of forensic fire cases.

Chapter 3: Integrating spectrophotometric and XRD analyses in the investigation of burned dental remains

This is a pilot study that serves as a foundation for the remaining chapters. The aim was to evaluate effects of heat-exposure on colour and mineral structure of teeth. Temperature and duration of heat-exposure were set as dependent variables. In this study, I capitalised the predictable and quantifiable characters of incinerated teeth, specifically the colour and crystallite size. The alterations of colour and crystallite size were empirically measured using a handheld spectrophotometer and X-ray

diffractometer. The data obtained were the colourimetry value of CIELAB colour space (lightness, chromaticity, whiteness and yellowness), and the HA crystallite size.

Using statistical analyses, I associated these structural changes with the temperature and duration of the heat-treatment and I found that duration has no significant effect on the changes of colour and crystallite size. Yellowness (YI) and HA crystallite size emerged as significant variables that can potentially be used to predict temperature. Another finding to be highlighted is the formation of whitlockite mineral in teeth that were heated at 800℃ and a sudden growth of the HA crystallites at 1000℃. These HA mineral changes are distinct features to differentiate incinerated teeth from incinerated bone. This study indicates the value of combined application of spectrophotometric and XRD analyse in estimating the temperature at which teeth are exposed to.

Chapter 4: Development of a tool to predict temperature-exposure of incinerated teeth using colourimetric and hydroxyapatite crystal size data

In this study, the aim was to develop a temperature predictive tool. This study was an extension of the previous study in which colourimetric value and crystallite size were revealed to be reliable predictors for temperature estimation. A rigorous statistical analysis on a dataset of 81 teeth revealed that L*, a*, WI, YI and CS are reliable to discriminate the temperature of incineration into the following category: unburnt, 300℃, 600℃, 800℃ and 1000℃. Then, a multinomial regression analysis was fitted into the dataset in an attempt to build a tool to predict a temperature at which teeth are exposed to. By incorporating a qualitative variable which is the subjective assessment to determine the certainty of burnt based on the tooth appearance, a tool predicting the temperature-exposure of the incinerated teeth, with high accuracy (95%) was developed.

The key strength of this study is that the tool was cross-validated using a different dataset and revealed a substantial generalisability, with 79% estimation accuracy. Qualitative examinations of the teeth including visual observation, images of scanning electron microscopy (SEM) and diffraction patterns were used to corroborate the estimated temperature. This study has successfully developed a reliable temperature predictive tool for incinerated teeth, and also it recognises

qualitative methods to provide supplementary evidence with respect to the incineration temperature. The tool for predicting temperature of incinerated teeth can be found online: https://melsapps.shinyapps.io/Predicting_temp_of_burned_teeth/

Chapter 5

Part 1: An improved method of preparing teeth to optimise DNA yield

This study was designed specifically to determine the effect of freeze-drying on DNA recovery from teeth. Freeze-drying the teeth helps to remove moisture from the external surfaces and the loose water molecule in the tooth matrix. I compared two sample preparation methods on the DNA yield from teeth; air-drying and freeze-drying. DNA fragments of different lengths and types were quantified using real-time polymerase chain reaction (qPCR). This study found that freeze-drying tooth samples can increase the amount of DNA recovered form teeth compared to normal air-drying. Freeze-drying can also minimise the potential loss of DNA during sample preparation of teeth.

Part 2: Retrieving DNA from teeth incinerated at temperatures that simulate forensic fires

This study is a preliminary study to the final part of the Chapter 5. I focussed on investigating the effects of a wide-range of heating temperatures on the DNA retrievability and DNA amplification from teeth. The temperatures of 300℃, 600℃, 800℃ and 1000℃ were selected to simulate the temperature range of real fire situations such as house fires (700°C – 900°C) [9], and motor-vehicle explosion (800°C – 1100°C) [10, 11]. The role of temperature on DNA survival have been established in several studies [12-17]. The findings in this study substantiate the previous studies, in which temperature has a significant effect on DNA yield. Other than temperature, the effect of tooth type on DNA yield is also significant. Incorporating the tooth type in the analysis was essential because DNA contents in molars and premolars are known to be widely varied [18-20]. Interestingly, two-way ANOVA showed that temperature had significantly differing effects on DNA yield for molars and premolars ($p < 0.05$).

This study demonstrates the likelihood of obtaining amplifiable DNA from incinerated teeth can be predicted based on the temperature of the forensic fire. Teeth exposed to temperature less than 300°C are likely to contain amplifiable DNA. However, DNA extracted from teeth incinerated at more than 600°C were deemed to be unsuitable for DNA amplification. This finding confirms that the principal loss of organic constituents occur between 660°C and 825°C [21].

Part 3: Novel diagnostic models to predict nuclear DNA and mitochondrial DNA recovery from incinerated teeth

I finished the research work with a study that aimed to develop a model assessing the feasibility of obtaining DNA from incinerated teeth. The process of isolating DNA from biological samples is arduous and costly [22], thus an informative pre-screening method that could help in selecting samples that can potentially yield amplifiable DNA would be valuable for forensic casework. I fitted a multiple linear regression into a dataset that consists of colourimetric value, HA crystallite size and quantified DNA, separately for nuclear and mitochondrial DNA. The chromaticity a* was found to be a significant predictor of the regression model.

The successful development of the models is another major highlight of this thesis, after the temperature predictive tool. The is the first study reporting models that able to predict the viability of extracting nuclear and mitochondrial DNA from teeth that were exposed to a wide range of temperatures (27°C to 1000°C). An implication of this study is the possibility to apply the models as a diagnostic triage for incinerated teeth prior to a subsequent DNA analysis for forensic identification or intelligence purposes.

The novelty of the research

Following the recovery of teeth of burnt dental remains at a fire scene, a forensic odontologist or a biologist will be consulted to choose teeth or/and dental fragments for DNA analysis. Even as the International Society for Forensic Genetics (ISFG) has comprehensively elaborated on the strategy for DNA-based victim identification, details on processing tooth samples was not specified in the recommendations [23].

Based on the key findings of the thesis from Chapters 2 to 5, I constructed a workflow (Figure 2) that is intended to: (1) Provide guidance on assessing heat-exposed teeth as DNA samples; (2) Offer a tool to estimate the temperature at which the teeth were exposed to; (3) Suggest the use of DNA diagnostic models to select appropriate samples for DNA analysis. Processing teeth samples for DNA analysis require substantial effort in comparison to blood and soft tissues [6]. Thus, determining appropriate samples is important to increase the likelihood of obtaining successful DNA profiling, and to minimise the energy, time and cost spent to isolate DNA from teeth [22]. I strongly propose the application of the workflow as a diagnostic triage in the assessment of incinerated teeth that are deemed to be suitable for DNA analysis.

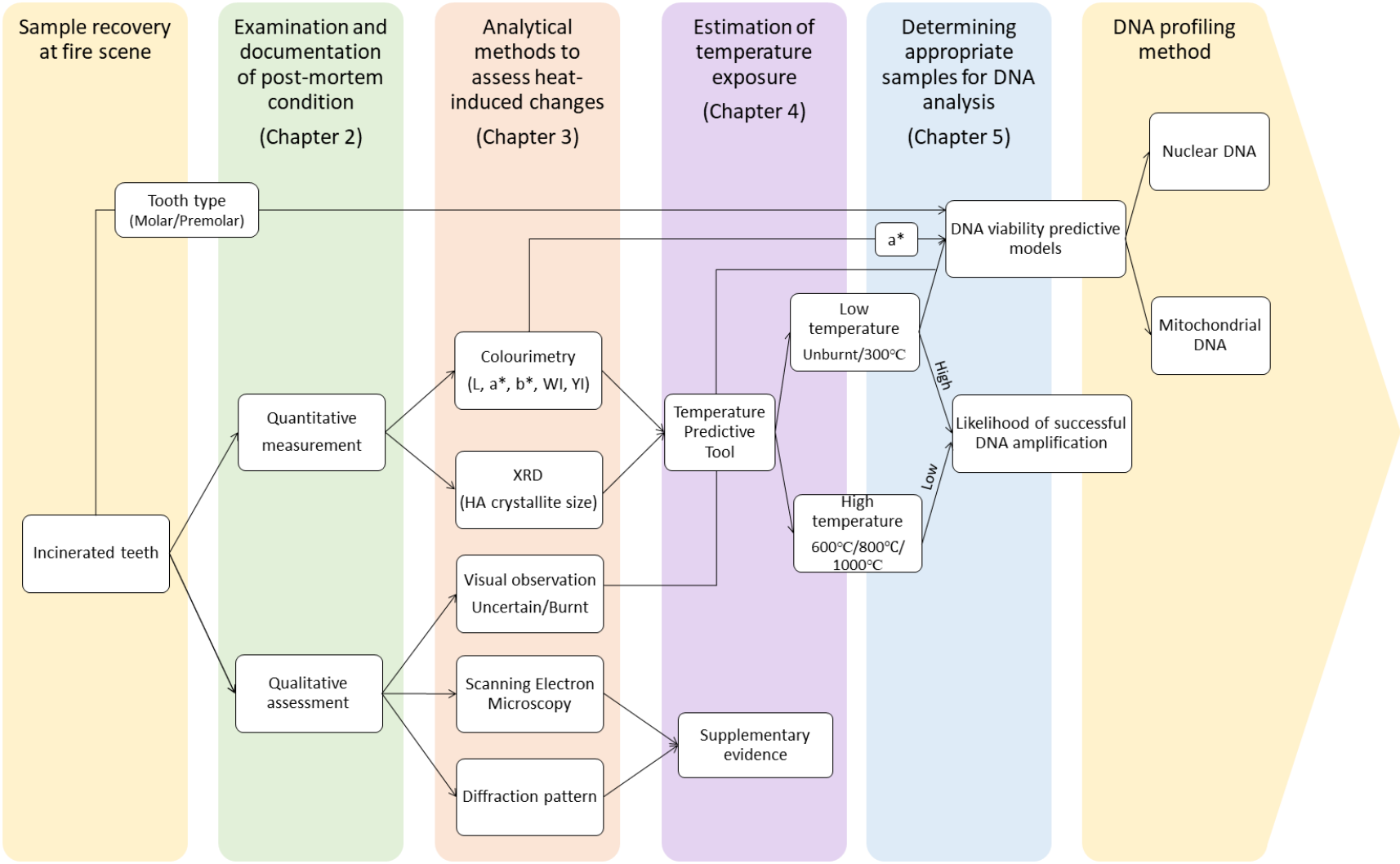


Figure 2—The workflow developed from the key findings of this thesis. It serves as a diagnostic triage in the assessment of incinerated teeth that are deemed to be suitable for DNA analysis. The boxes were colour coded referring to the respective chapters. Green box: Chapter 2; Orange box: Chapter 3; Purple box: Chapter 4; Blue box: Chapter 5.

This workflow is useful to accelerate the DNA-based identification of burnt human remains especially in cases with multiple fatalities. In a mass fire fatality disaster, enormous number of fragmented and commingled remains slowed down the efforts to identify the victims. Unprecedented events that could have benefited from the proposed workflow are:

1. *Grenfell Tower fire:*

On 14th of June 2017, an estimated 71 lives were lost during a fire blaze of the Grenfell Tower of flats in London [24]. Dental fragments were amongst the biological samples that were recovered and used for DNA-based identification [25]. Identification of the victims was difficult and greatly delayed because the burnt remains were commingled underneath the collapsed rubbles [26, 27].

2. *MH17 airplane crash:*

On 17th of July 2014, a total of 298 victims perished in the MH17 airplane crash in Ukraine [28]. DNA-based identification was undertaken in the efforts to identify the victims [6]. Of 4958 recovered body parts that were deemed suitable for DNA retrieval, 246 were tooth samples [6]. It was emphasised that the sample processing of teeth was slow and difficult [6].

3. *World Trade Centre attack*

The victims' identification of the World Trade Centre attack on 11th of September 2001 was the largest DNA-based identification efforts in the world history [29]. More than 21 000 body remains were recovered from the scene [30]. It took years to establish the identity of 1600 out of around 3000 victims [29]. Out of which 1282 positive identification was resulting from DNA profiling [29].

Limitations

Overall, the research work that was conducted throughout the thesis was limited to three major factors. The limitations are as the following:

i. Porcine teeth as proxy models

To obtain an ideal sample from non-formalin fixed human remains was challenging. There was an ethical concern to obtain mandibles from the heads of deceased donors. Thus, pigs were chosen as human proxy because: (1) porcine dentin and human dentin share similarities in microstructural arrangement, such that the thickness of dentin and the rate of the crystallisation rate was indicated by the proportion of calcium and phosphate [31]; (2) pattern alteration of hydroxyapatite crystal structure in incinerated teeth of human and of porcine are comparable [32]; and (3) no evidence to suggest major structural differences among bones of different mammalian species [1]. To closely simulate a real-fire situation, freshly sectioned mandibles of pig heads were used. To minimise the risks of sample degradation, heads of pig carcasses were obtained immediately after the pigs were scavenged at a local abattoir. Later, mandibles were dissected from the heads and properly prepared in a sterilised condition.

ii. Dental diseases and tooth wear

Throughout this study, I have only included teeth of adult pigs (approximately two years of gestational age) that are healthy, intact, and fully erupted and developed. This is because of two reasons. Firstly, healthy and structurally intact teeth are preferred source of DNA in forensic identification [4]. Secondly, dental diseases and age progression of dental tissues have a negative impact of the DNA in teeth [33, 34]. In general, carious teeth and tooth wear are characterised by the loss of tooth structure. Carious lesion is a pathological condition is caused by a harmful bacterial activity that dissolves and destruct the calcified tissue of teeth and eventually form a cavity in teeth [35]. Tooth wear including abrasion, erosion and attrition are characterised by the thinning of tooth surfaces over time [36]. It is a natural condition that occur with increasing age and is augmented by habitual activities such as rigorous brushing, acidic dietary intake or bruxism. The progressive disruption of the mineralised tooth structure in both conditions can lead to the loss of the enamel covering and the

permeability of dentinal tubules [37]. This could expose the dental DNA to external assaults such as bacteria, acidic fluid and direct heat contact and can substantially reduce the DNA contents [33].

iii. Categorical incineration temperature

Although this study has successfully developed a predictive temperature tool, it has a limitation in terms of the paucity of temperature groups for sample incineration. I specifically selected 300℃, 600℃, 800℃ and 1000℃ to represent fire situations such as wooden pyre, house fire and motor-vehicle accident. Thus, the data obtained were deemed to be inadequate to build a tool that predicts a continuous temperature.

iv. Applicability of the tools on forensic casework

Due to time and financial constraint, I had no opportunity to test the newly developed temperature predictive tool and DNA diagnostic models by conducting trials on teeth of burnt human remains. Thus, the applicability of the tools on human remains in forensic casework can potentially be argued. Continuing efforts that consists of multiple validation studies of the tools with large datasets are essential to prove the effectiveness before these tools can be incorporated into the protocols of forensic casework. Should this be the case, the application of the tools can be made available for the forensic investigators to triage the DNA.

Future research

To overcome the limitations addressed in the previous section, I provide recommendations as guidance for the future research work. In addition, the complex structure of teeth offers a vast opportunity to expand the scope of the current study such as to explore the potentials of incorporating collagen assessment into the newly developed DNA diagnostic models. assess collagen in the DNA preservation of teeth.

i. Validation of the developed tools on teeth of burnt human remains

Further work is required to implement the use of the temperature predictive tool and DNA diagnostic models in forensic casework. I suggest that a validation study similar to this one using both tools should be carried out on human teeth, including

healthy and carious teeth. Interestingly, Alia-García E., *et al.* [38] have found that the use of teeth with cavities does not have negative influence in DNA profiling. An opportunity to collect a large dataset from teeth of burnt human remains would support the establishment of the tools. Perhaps a future work can be conducted at a body farm where body donors are available for research use, if permissible.

ii. Application of FTIR analysis

Fourier transform infrared spectrometry (FTIR) has been used to evaluate the changes of mineral crystallinity [39, 40] and to explore its molecular structure [41]. The combination of derivative- and curve-fitting from FTIR analysis was claimed to be a sensitive method to estimate of the maximum temperature-exposure on bone [42]. Perhaps it is worth investigating the impact of FTIR analysis on improving the sensitivity of the current temperature predictive tool.

iii. DNA adsorption on apatite

The application of FTIR is useful in the investigation of the adsorption process on the apatite mineral surface [43, 44]. A report by del Velle *et al.* [45] described the structural and chemical changes of HA (e.g. crystal growth and whitlockite formation) and how this affects its capacity to bind with DNA. The bind may be lost during the recrystallisation of the HA crystal [46]. Moreover, FTIR can also be used to analyse the effects of heat on collagen content in teeth [39].

An advanced research with more focus on the protective role of HA mineral and collagen toward DNA and the effect of heat on the DNA adsorption on its surface is therefore suggested. FTIR can be used to explore the interaction between apatite mineral and collagen in incinerated teeth, and the association between this interaction with the DNA preservation. Previous studies have reported that the SF, C/P and AmI/P ratios from the FTIR analysis can indicate DNA preservation in the skeletal remains [47-50]. Recent evidence suggests that the state of carbonates, Amide I with its secondary structure, and phosphates are to be indicators to assess the endogenous DNA in teeth and bone [41]. Sosa et al. [46] suggested the assessment of both HA mineral and collagen in any study pertaining to DNA degradation.

Concluding Remark

Three major highlights of this thesis are the novel development of: (1) the temperature predictive tool for incinerated teeth, (2) the DNA diagnostic models to predict nuclear DNA and mitochondrial DNA recovery from incinerated teeth, (3) the workflow that serves as a guidance to assess incinerated teeth and to determine which tooth is suitable for DNA analysis.

Overall, this thesis presents rigorous and extensive work investigating the changes to teeth during incineration that influence DNA recovery for forensic investigation. It contributes to an understanding on how structural changes of incinerated teeth can be used to estimate the temperature-exposure and to predict the feasibility to retrieve DNA from incinerated teeth. This will be of value to forensic science to provide triaging of incinerated teeth prior to costly, labour intensive and time-consuming DNA testing.

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Ethics guidelines

Extract from: THE UNIVERSITY OF ADELAIDE ANIMAL ETHICS COMMITTEE
ANIMAL USER'S HANDBOOK - INFORMATION ABOUT YOUR RESPONSIBILITY TO USE ANIMALS HUMANELY AND ETHICALLY 10/2007.
Revised 2/2010, 2/2011, 7/2011

2.2 Acquiring Organs, Tissues or Materials from Animals for Use in Scientific Research or Teaching by Scavenging

In some situations animal tissues and substances are available for collection from discarded **dead** animals**.** Another term used with reference to collection of materials from dead animals is "***scavenging***", or *collection from "**animals killed for other purposes**".* That is, the animals have not been specifically killed for the purpose of obtaining these materials for scientific research or teaching. This definition includes materials sourced from abattoirs.

"Scavenging" tissue from carcases is highly recommended[1] to be used (whenever possible) as an alternative to killing animals specifically for that purpose, because it reduces the number of animals used in research and teaching. Prior approval by the Animal Ethics Committee (AEC) is not a legislative requirement. However, the AEC should be informed when an investigator or teacher is "scavenging", especially if this is occurring on a regular basis. **Investigators are encouraged to inform the AEC promptly**, when practicable.

Subject to the conditions and considerations listed below, AEC approval may not be required prior to obtaining the material.

1. The opportunity for scavenging must not influence the decision to kill the animal, nor the time when this occurs, if this comprises animal welfare.

2. Collection of organs, tissues, materials or substances from a **living** animal for scientific or teaching purposes is a **scientific procedure** and requires prior approval from the AEC.

3. In some situations, animal tissues and substances from living animals are discarded following routine animal husbandry practices, or are discarded from veterinary practices following veterinary surgery or veterinary medical procedures. **The specific details of the particular situation must be provided to the AEC in order to determine whether AEC approval is required prior to collection or use of these materials for scientific or teaching purposes.** As **living** animals are involved there may be legal, ethical or welfare issues (e.g. owner consent). This is a University requirement.

4. In some situations, animal materials and substances (including urine, faeces, feathers and hair) are found discarded by an animal in its environment. **The specific details of the particular situation must be provided to the AEC in order to determine whether AEC approval is required prior to collection or use of these materials for scientific or teaching purposes.** As **living** animals are involved there may be legal, ethical or welfare issues (e.g. tracking wildlife, entering private property).

5. Killing an animal specifically to collect tissues or substances for scientific or teaching purposes is also a scientific procedure, is not considered to be a case of "scavenging", and therefore requires prior approval from the AEC.

What do I have to do if scavenging?

• Scavenging from carcases
AEC approval is not required prior to collecting and using the tissues or materials sourced from carcases. However, the AEC should be informed when an investigator or teacher is "scavenging", especially if this is occurring on a regular basis. Investigators are encouraged to inform the AEC promptly, when practicable.

If considered desirable an Application for AEC approval of a project involving "scavenging" can be submitted.

"Scavenging" is not required to be entered in the *Annual Statistics Return and Progress Report*, however the source of the tissues/materials and the circumstances surrounding their collection must be documented and made available to the AEC upon request. Animal houses and investigators both have responsibilities for documenting the reason for the death of an animal, and the subsequent fate of the carcase.

• **Discarded tissues and substances from living animals**
The AEC must be informed prior to collecting and using the material, as living animals are involved and there may be legal, ethical or welfare issues. The source of the tissues/materials and the circumstances surrounding their collection must be documented and made available to the AEC upon request.

Additional considerations to scavenging:

• When researchers are scavenging tissue from privately owned animals or Veterinary Clinics, written consent of the owner of the animal must be obtained.
• Researchers obtaining tissues (including eggs, hair and feathers) collected from living or dead native wildlife (including road kills) require a wildlife permit from the Department of Environment and Heritage.
• If animal tissues are imported from overseas then additional permits may be required (AQIS, CITES).

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From the NHMRC Australian code of practice for the care and use of animals for scientific purposes, 7th Edition.
[1] "3.3.21 Where practicable tissues from animals being killed should be shared among investigators and teachers in line with the principle of Reduction."