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Molecular Mitochondrial DNA and Radiographic Approaches for Human Archaeology Identification

(Pendekatan Molekular DNA Mitokondria dan Radiografi untuk Pengenalpastian Arkeologi Manusia)

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ABSTRACT

Ancient remains are considered very valuable artefacts, as they allow for the study of ancient cultures, phylogeny, evolution and the reconstruction of demographic history. To obtain all the information contained within remains, the investigation of such samples requires the expertise and various techniques from multiple fields of study. The present review focuses on the molecular biology and radiographic approaches used to identify ancient samples. Studies of ancient remains face various limitations; for example, the quality and quantity of the ancient samples can affect the difficulty of the investigations. Due to these limitations, new sophisticated techniques are being introduced to replace the earlier conventional techniques. A search was conducted using PubMed, Scopus, Science Direct and Science Finder to provide a new and timely review on the molecular mitochondrial DNA and radiographic analysis for human archaeology identification. The present review has determined that molecular biological approaches are very accurate and useful for the use in the ancestral determination of incomplete specimens, whereas observations of the dental pulp chamber are suitable for age at death estimations in both adults and children. However, these techniques are expensive and require expert personnel. Therefore, conventional approaches remain the favourite methods of most institutions, especially in Asia.

Keywords: Ancestral and age; human archaeology; molecular analysis; radiographic approach

ABSTRAK

Sampel purba dianggap artifak yang sangat berharga kerana ia membolehkan kajian mengenai budaya purba, filogenetik, evolusi dan penyusunan semula sejarah demografi. Bagi mendapatkan semua maklumat yang terkandung di dalam tinggalan, kajian sampel tersebut memerlukan kepakaran dan pelbagai teknik daripada pelbagai bidang kajian. Penyelidikan ini memfokus kepada pendekatan biologi molekul dan radiografi yang digunakan untuk mengenal pasti sampel purba. Kajian tinggalan purba berdepan dengan pelbagai batasan; sebagai contoh, kualiti dan kuantiti sampel purba boleh memberi kesan kepada tahap kesukaran kajian. Akibat daripada batasan ini, teknik baru yang canggih telah diperkenalkan untuk menggantikan teknik konvensional. Pencarian telah dilakukan menggunakan PubMed, Scopus, Science Direct dan Science Finder bagi memberikan penilaian yang terbaru terhadap analisis molekul DNA mitokondria dan radiografi untuk mengenal pasti arkeologi manusia. Penilaian ini mendapati pendekatan terkini biologi molekul dalam menentukan keturunan adalah sangat tepat dan berguna terhadap spesimen yang tidak lengkap manakala pemerhatian bagi ruang pulpa gigi adalah sesuai untuk menganggar umur semasa kematian terhadap kedua-dua orang dewasa dan kanak-kanak. Walau bagaimanapun, teknik tersebut adalah mahal dan memerlukan individu pakar. Oleh itu, pendekatan konvensional kekal sebagai kaedah yang digemari dalam kebanyakan institusi terutamanya di Asia.

Kata kunci: Analisis molekul; arkeologi manusia; keturunan dan umur; pendekatan radiografi

INTRODUCTION

Human archaeology is the study of the human past. Ancient human remains can be found in various forms, including complete skeleton, fragmented bones or mummified remains. Human bones are attached by cartilage; however, over time, the cartilage will degrade, leaving the skeleton dismembered. Ancient samples can provide important information regarding human evolution, prehistoric customs and certain ancient diseases (Ambrose & Krigbaum 2003). The study of human identification is referred to as forensic anthropology (Cattaneo 2007). Ancient remains can be analysed to identify gender (Shahrul Hisham et al. 2009), ancestry (Durtschi et al. 2009), age at the time of death (Sebecic et al. 2010), diseases (Klaus et al. 2010) and sometimes trauma (Baustian et al. 2012). In the modern technological world, multiple approaches can be applied to ancient samples to obtain such information as mentioned previously. The methods used for anthropological examinations include morphological examinations, anthropometric measurements, radiographic examinations and biochemical and DNA profiling (Blau & Briggs 2011).

The most fundamental question when examining an ancient specimen is whether it is of human origins. The morphology of the cranial bone or skull can be used

to compare between humans and animals (Whitcher et al. 2009). However, scattered bones, for example the phalanges of monkeys, can be confused with those of human origins. Furthermore, ancient bones, especially long bones, are often mistaken for sticks due to their yellowish to brownish colour, as bone generally lose their whitish colour over time. Saulsman et al. (2010) used a morphometric approach on long bones to compare human bones to those of quadrupedal (sheep, dog and pig) and bipedal (kangaroo and emu) animals. They found that there was sufficient variation among species to correctly assign an unknown bone as being human or non-human, with a 63-99% rate of accuracy. Remains are said to be of archaeological importance if the samples are more than 50 years of age (Cattaneo 2007). However, determining the origins of remains constitutes a preliminary examination and the remains must be further analysed. The objective of the present study was to review and discuss the approaches used to make ancestral determinations and age at death estimations for the identification of ancient specimens.

Quantity and Quality of Archaeological Samples

Quantity of ancient specimens for analysis

The valuable archaeological specimens are preferred to be saved in a country's archives, which limits the quantity of samples that can be further examined. To overcome this situation, investigations of ancient samples often utilise non-destructive methods, which can involve radiographic analyses, morphological observations and anthropometric measurements that can provide valuable information. For example, age can be estimated by the morphological analysis of the dental third molar (Sitchon & Hoppa 2005), race can be predicted by dental morphology (Edgar 2005), sex identification can be predicted from the cranial capacity of adult human skulls (Lalwani et al. 2012) and trauma (Outram et al. 2005) and certain diseases (Wess et al. 2002) can also be determined using these methods. However, these analyses require nearly complete skeletal remains with well-preserved surfaces. Unfortunately, archaeological samples are often found in the forms of incomplete or fragmented specimens.

Limited quantities of samples or fragmented ancient bones make it difficult to obtain information using only physical or morphological tests, due to the lack of certain morphology that may be required for radiographic analysis, morphological observations and anthropometric measurements. Chemical test may be required to overcome this problem; however it may cause us to sacrifice a small quantity of ancient samples. These destructive types of analyses include radiocarbon analysis (Zazzo & Saliege 2011), molecular biology analysis (Mohandesan et al. 2004) and other chemical tests such as aspartic acid racemisation analysis (Fernandez et al. 2009). Special pretreatments of the archaeological samples, which involve the cleaning and decontamination of the samples, are required prior to utilising these types of analyses.

Quality of ancient specimen: degree of contamination

The degree of contamination and degradation of ancient DNA (aDNA) in these samples is the primary obstacle to perform chemical tests on ancient samples (Deguilloux et al. 2011). Contamination from the samples may affect the results of analyses and the degradation of DNA may complicate the process of obtaining a complete DNA sample (Koon et al. 2008). The specimen may not only have been contaminated with exogenous contaminants from the burial site but could also be continuously introduced to contaminants during the process of DNA recovery (Bandelt 2005). In the process of DNA extraction, there are three sources of contamination: surface contamination of the ancient specimens; contamination from the equipment, work bench, reagents and lab ware during their manufacture or packaging and DNA transfer between tubes during the process of polymerase chain reaction (PCR) amplifications (Shanks et al. 2005).

Quality of ancient specimen: post-mortem DNA damage

Post-mortem DNA damage can cause aDNA sequences to exhibit small amounts of modification such as double-strand breaks and oxidative dinucleotide modifications, both of which can inhibit subsequent enzymatic replications (Capelli et al. 2003). Minor sequence modifications may also be present, due to the hydrolytic deamination of cytosine to uracil or adenine to hypoxantine. Post-mortem damage rates are higher in control regions than in coding regions in human mitochondrial DNA (mtDNA) (Gilbert et al. 2003). This is due to the secondary structural conformation of the mtDNA control region that promotes post-mortem damage, in contrast constrain mutational rates in coding region (Gilbert et al. 2003).

Preservation of Specimens

Natural preservation

In their study on 291 prehistoric cattle remains from Europe, Bollongino et al. (2008) showed that DNA preservation is primarily influenced by geographic and climatic conditions. Humidity is the primary degradative factor, as it dissolves the bone apatite, allows for the growth of microorganisms and leads to the hydrolytic and oxidative damage of DNA (Jans et al. 2004). Increased temperatures enhance the decomposition and proliferation of microorganisms (Bollongino et al. 2008). The composition and condition of the soil also contributes to sample preservation. Acidic soils can dissolve calcium phosphate which destroys the bone apatite, whereas alkaline conditions can cause the formation of bicarbonate and carbon dioxide, which increases the degradation of the bone apatite (Karkanas 2010). The climatic conditions in the caves of the Himalayas, which are low in temperature and lack water, provide good preservation conditions for

mummified tissues and can potentially support the equally good preservation of aDNA (Alt et al. 2003).

PRESERVATION DURING SPECIMENS HANDLING

Tools used to excavate or clean the specimens are often reused or do not undergo decontamination between samples. Some of the favourite tools for cleaning specimens are brushes that have been reused and that can provide an immediate source of human and bacterial DNA (Fortea et al. 2008). Tap water is not free from modern DNA; therefore, tap water can contribute contamination to the ancient samples. Gilbert et al. (2003) suggested that the simplest remedy for human DNA contamination would be to control the excavation of human samples and to prevent the direct handling and washing of samples that are to be used for aDNA analyses. Yang and Watt (2005) offered practical guidelines for archaeologists when collecting specimens at burial sites, prior to moving the samples to the laboratory for further pretreatment. They suggested that the dirt on the surface of the samples should not be cleaned, as it may serve as protection against contaminations entering the bone tissues and that samples should not be washed, as water may cause contaminant DNA to penetrate into the bones. They also recommended storing specimens in cool and dry conditions and that gloves should be pretreated and changed between samples to avoid cross contamination.

METHODS OF DECONTAMINATION

Regular techniques used to decontaminate specimens include the application of bleach and exposure to UV light (Deguilloux et al. 2011). These methods are able to remove contaminants from the outer surfaces of specimens, such as soil and modern DNA, as these contaminants may compete with endogenous aDNA during PCR amplification reactions, thus yielding false positive results (Dissing et al. 2008). Sodium hypochlorite (NaOCI) is generally used as a bleach. Kemp and Smith (2005) conducted a test to observe the effect of different concentration of NaOCI range between 0.6 and 6.0% and the time of immersion into NaOCI range between 10 min and 21 h on bones that has been introduced to contaminant. They found that the elimination of surface contamination from bone requires immersion into 3.0% (w/v) NaOCI for 15 min. In addition, endogenous DNA proved to be stable even at extreme levels of NaOCI treatment (6% for 21 h).

Other methods for eliminating surface contamination from ancient bone include the physical removal of contaminants from the surface of the bone, exposing the bone with highly concentrated ethanol, washing the surface of the bone with acid (Kemp & Smith 2005) and using a trypsin solution (Li & Liriano 2011). A special work space for the decontamination and handling of archaeological samples is strictly required to reduce cross contamination by modern DNA or other contaminants (Seco-Morais et al. 2007). UV irradiated lab coats and face masks should be used and gloves should be frequently changed. The use of DNase in reagents, lab ware or equipment appears to be helpful for eliminating modern DNA contamination (Eshleman & Smith 2001). In most cases, contamination can still occur, even when strict protocols have been followed. Obviously, decontamination methods are not 100% efficient and contamination remains a serious threat to the validity of aDNA studies. Positive and negative controls should be run concurrently with samples to monitor and detect the existence of contamination during DNA extraction and handling.

TYPES OF ARCHAEOLOGICAL SAMPLES

HARD TISSUES: BONE AND TEETH

Bone and teeth are the most enduring physical evidence of humans that are found at archaeological sites (Campos et al. 2012). Morphological analyses and anthropometric measurements of bones can determine ancestry (Durtschi et al. 2009) and gender (Gupta et al. 2012). Teeth can be used to estimate the age at the time of death (Brkic et al. 2006). Teeth are a better sample than bones for obtaining aDNA (Gilbert et al. 2003) due to the protection of cells by enamel in teeth (Hernandez et al. 2003), which have a lower porosity and a higher resistance to contamination when compared to bone (Dissing et al. 2008). Human bone is porous, and its porosity has been found to increase post-mortem due to the chemical decomposition of collagen and the microbial destruction of the collagen/apatite composite (Ambrose & Krigbaum 2003). These changes in porosity may allow liquid-borne contaminants to penetrate inside the specimens. Although histological studies have identified greater numbers of DNA containing cells per unit area of bone when compared to teeth (Turner-Walker et al. 2002), Adler et al. (2011) have reported better DNA yields when using tooth cementum.

SOFT TISSUES AND HAIR SHAFT SAMPLES

Soft tissues and hair shaft samples can also be found in mummified ancient samples. Hair has been shown to be resistant to contaminant DNA due to the hydrophobic and impermeable nature of the keratin structures forming the hair shaft (Gilbert et al. 2006). mtDNA remain relatively intact inside the hair shaft, on the other hand hair root could be used in both nuclear DNA and mtDNA analysis (Bengtsson et al. 2012). However, the recovery of hair root in ancient sample is extremely rare as compared to hair shaft (Ho & Gilbert 2010). Therefore, hair shaft samples represent an important source of mtDNA in aDNA studies. mtDNA has been successfully extracted not only from modern hair shaft samples (Graffy & Foran 2005) but also from 100-year-old degraded hair shaft samples (Ricaut et al. 2006). Hair shaft naturally contains low amounts of mtDNA, suggesting that hair shaft may be particularly vulnerable to contamination (Gilbert et al. 2006). A previous study by Gilbert et al. (2006) found that degraded hair shafts are either naturally resistant to contamination

with exogenous DNA, easy to decontaminate or both. The decontamination of hair shaft samples can be performed using a commercial bleach solution (Clorox) for 30 s, followed by a rinse in sterile ethanol (Gilbert et al. 2006).

Naturally mummified specimens can be found in climatic environments; for example, studies performed by Alt et al. (2003) found 30 mummified bodies in the cold and dry environment of a cave in the Himalayas. Analyses of mummified tissues showed the diet history of the remains (Turner et al. 2012), as well as a variety of bacterial, protozoa and viral infections, such as *Mycobacterium tuberculosis,* which causes Tuberculosis, and *Trypanosoma cruzi,* which causes Chagas disease (Donoghue et al. 2009; Mitchell et al. 2005).

Methods for Ancestral Determination

Physical examination: non-destructive analysis

Ancestral determination is a crucial part of any investigation of ancient specimens that can provide information regarding the ancient culture, human behaviours and human migration associated with location where the samples were found. The usual anthropological methods used to determine ancestry involve observing the morphology and taking anthropometric measurements of the cranial bone (Cattaneo 2007). These approaches are non-destructive and can therefore preserve the valuable ancient samples. The oral and maxillofacial regions have been shown to be particularly defining regions of variability among different ethnic groups (Durtschi et al. 2009). The nasal bone is the most useful feature to be observed (Bruner & Manzi 2004). Caucasians have a high and narrow root, a high bridge, a pronounced spine and a narrow nose width. In Negroids, the root and bridge are low and rounded, the spine is small and the nose width is wide. In Asians, the nose width is rounded, the root is low and ridged and the spine is small (L'Abbe et al. 2011).

The other observable features in the cranial bone that can be used for ancestral determination include the shape of the cranium (Figure 1(a)) and eye orbits, the face profile, and the lower eye border (L'Abbe et al. 2011; Sholts & Warmlander 2011). The jaws and teeth can also be used to provide additional information for the ancestral determination (Mavrodisz et al. 2007). The jaws are usually small in Caucasians and large in both Negroids and Asians (Harris et al. 1977). The palatal region is parabolic in shape in Caucasians, hyperbolic in Negroids and elliptical in Asians (Gill 1995; Holobinko 2012) (Figure 1(b)). The upper incisors differ in their basic shapes, which are shovel-shaped in Asians and spatula-shaped in Caucasians and Negroids (Bollini et al. 2008) (Figure 1(c)). However, the distinctiveness of the different racial and ethnic categories may be evolving as the number of interracial offspring and immigrants are increasing (Waters 2002). Furthermore, the majority of characteristics that aid in the assessment of ancestry apply only to adults, as most bone development is still occuring during childhood (Cattaneo 2007). Finally, all of these morphologic assessments for ancestral determination require a complete bone. To overcome these limitations, most of the current methods used for ancestry determination are derived from the field of molecular biology and require only minute amounts of samples (Loreille et al. 2007).

Ancient DNA analysis: destructive analysis

Mitochondrial DNA Ancestral determination involving aDNA has traditionally focused on the mitochondrial genome (Adachi et al. 2004). One of the earliest studies involving mitogenomes was performed in the mammoth, which supported the morphological analysis that concluded mammoths were more closely related to Asian elephants than to African elephants (Paijmans et al. 2013). A single mitochondrion has a high copy number of its DNA, unlike the single copy nucleus and the availability of modern mitogenomic sequences has made it easier for mtDNA to be analysed (Ho & Gilbert 2010). Mitochondria are small in size, circular in shape and their genome contains 16569 bp, which is not easily degraded over time, in contrast with the larger sized nDNA (approximately 3×10^9 bp), which is linear in shape (Fernandez-Silva et al. 2003; Snustad & Simmons 2006). A mother passes mtDNA to her offspring, known as maternal inheritance. Therefore, two persons who share the same maternal lineage carry the same mtDNA (Ramakrishnan & Hadly 2009), whereas Y-chromosome nDNA is contributed by the father's sperm, representing paternal lineage (Manjunath et al. 2011). These special characteristics allow for the use of mtDNA as identification tools for ancestral determination. Furthermore, mtDNA has a high degree of sequence variability (Adachi et al. 2004). These variations can be used to determine individual possible origins or haplogroups. For example, Malhi et al. (2007) discovered that prehistoric specimens from the North Americas carried Haplogroup M variation in their mtDNA with variation at positions 16093, 16213 and 16223.

The regions that appear to mutate most easily in human mtDNA are control regions, which contain Hypervariable regions (Tsutsumi et al. 2006). The hypervariable regions consist of three segments: Hypervariable 1 (HV1; 16024-16365), Hypervariable 2 (HV2; 73-340) and Hypervariable 3 (HV3; 438-574) (Goncalves et al. 2011; Lian & Koh 2005). These genes are located in the non-coding region or the displacement (D)-loop region that is approximately 1100 base pairs long (Maruyama et al. 2003). Although a molecular biologically approach can result in the destruction of valuable ancient samples, only a minute amount of the bone samples are required for the analysis. The earliest study that successfully obtained mitochondrial sequence fragments from ancient human specimens performed by Higuchi et al. (1993), using 150-year-old specimens (Ho & Gilbert 2010). There is an ongoing debate in the field of human evolution regarding the possible contributions of Neanderthals (*Homo neanderthalensis)* to the modern human (*Homo sapiens*) gene pool (Krings

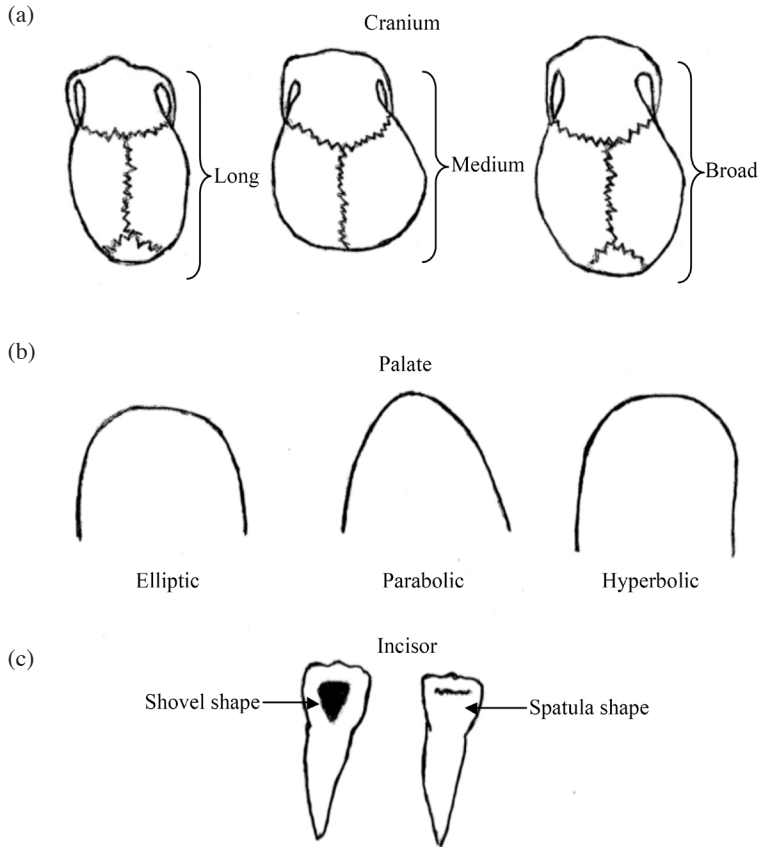

FIGURE 1. Morphological analysis of (a) cranium, (b) palate and (c) incisor for ancestral determination
Cranial morphology is Long in Negroid (elongated and narrow), Medium in Caucasian (similar to Asian but not long) and Broad in Asian (wider than Negroid) (a). Palate shape is Elliptic in Asian (rounded), Parabolic in Caucasian (U-shaped with focus point) and Hyperbolic in Negroid (rectangular like shaped) (b). Incisor is concave shovel-like features in Asian and spatula-shaped in both Caucasian and Negroid (c)

et al. 1997). Analyses of mtDNA from Neanderthals have demonstrated differences in their mtDNA sequences when compared to those of modern human (Fortea et al. 2008; Green et al. 2006). However, studies by Reich et al. (2010) demonstrated that ancient samples found in Denisova cave in Siberia shares common origin with Neanderthals and contributed 4-6% of it's genetic material to modern humans of Melanesian. Therefore, these findings have led to an extensive debate over the evolution of modern humans and have not been completely elucidated.

ISOLATION OF ANCIENT DNA

Commonly, aDNA is isolated using the traditional procedure of phenol-chloroform extraction and proteinase K treatment (Chelomina 2006; Hernandez et al. 2003; Shahrul Hisham et al. 2007). Shahrul Hisham et al. (2007) used approximately 6 cm^2 or 5 g of powdered ancient bone whereas, Adler et al. (2011) used smaller amount, i.e. 0.1 – 0.2 g of bone and tooth powder for phenol-chloroform extraction to isolate the ancient mtDNA. However, phenol-chloroform extraction requires a degree of technical proficiency (Takayanagi et al. 2003). Hernandez et al. (2003) used a centricon filter as a ready-made DNA concentrator, in addition to phenol-chloroform extraction, to enhance the yield of aDNA. A simple and effective method for aDNA extraction has been proposed that is based on ethanol precipitation, with the addition of Dextran Blue (Kalmar et al. 2000). On the other hand, Walsh et al. (1991) extracted aDNA using a chelex based method. Although the chelex method was simple and fast, in most cases, the inhibitory substances had not been eliminated (Kalmar et al. 2000). Therefore, silica extraction and magnetic separation methods produce high amounts of extracted DNA with the minimal presence of inhibitors (Anderung et al. 2008).

Currently, commercial DNA extraction kits were used to isolate genomic DNA from various biological samples with high DNA recovery rates and can shorten the time required for isolation (Manjunath et al. 2011). Some examples of commercially available genomic extraction kits that could also been used in ancient samples include Invisorb Forensic Kit (Wurmb-Schwark et al. 2008, 2004), Rapid QIAquick (Anderung et al. 2008), QIAamp DNA Micro Kit (Li & Liriano 2011) and Investigator Kit (Vural & Tirpan 2010).

Invisorb Forensic Kit 1 by Invitek, Germany is a genomic DNA isolation kit specific for forensic samples,

which also include ancient bone. Furthermore, Wurmb-Schwark et al. (2008, 2004) used Invisorb Forensic Kit to isolate DNA from archaeological specimens in their studies. We found that the same DNA isolation kit able to extract aDNA and successfully yielded approximately 2 – 18 ng of aDNA for every 0.5 g powdered ancient bone. In addition, Li and Liriano (2011) used QIAamp DNA Micro Kit from Qiagen, Germany to isolate DNA from bone samples. On the other hand, Anderung et al. (2008) used Rapid QIAquick (Qiagen, Germany) to isolate aDNA from bone powder treated with EDTA, SDS and Proteinase K. Finally, Vural and Tirpan (2010) used Investigator Kit (Qiagen, Germany) to isolate aDNA and successfully obtained high amount of aDNA recovery. The fact that so many different extraction methods have been used indicates that no single procedure has emerged as the standard method. However, the conventional phenol-chloroform DNA extraction method is still used regularly, as it is cheap and does not require any special equipment (Shahrul Hisham et al. 2007).

POLYMERASE CHAIN REACTION (PCR)

The invention of the PCR technique in modern molecular biology has made it possible to analyse aDNA (Ho & Gilbert 2010). The PCR technique is an extremely sensitive and specific method that can detect a minute amount of DNA molecules and amplify these molecules billions of times in a few hours (Willerslev & Copper 2005). The hypersensitivity of the PCR technique also allows contaminant DNA to be easily amplified, which can generate false positive results. A highly efficient polymerase that is used with a high cycle number during the PCR procedure may result in strong amplification, but negative controls can also be unexpectedly amplified (Yang et al. 2003). Numerous substances have been demonstrated to inhibit PCR and result in the failure of the PCR technique, such as humic acid, fulvic acid, tannins, porphyrin products, phenolic compounds, hematin and collagen type 1 (Kemp et al. 2006). Caramelli et al. (2007) was able to amplify and analyse mtDNA extracted from the skeletal remains of Francesco Petratca, who died in year 1374.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR)

Although the PCR technique is sensitive enough to allow for the amplification of only a few DNA molecules, the PCR analysis of ancient remains is prone to contamination by modern DNA and can be impeded by polymerase inhibitors present in fossil extracts (Alonso et al. 2003). A new method of PCR quantification has been invented called Real-Time PCR or quantitative PCR (qPCR) because it allows us to view the increase in the amount of DNA as it is amplified (Alaeddini 2012). This new technique, developed by Higuchi et al. (1993) is a refinement of the earlier PCR technique developed by Mullis and Faloona (1987). qPCR is a more precise and more informative approach for the determination of the initial number of molecules in the DNA sample and of the inhibitory effects of contaminants (Pruvost & Geigl 2004). qPCR uses a fluorescence detection system that monitors the DNA amplification during the exponential phase of the reaction, in contrast to traditional PCR, which monitors only the end point or plateau of the reaction (Alonso et al. 2003). Many PCR reactions using aDNA extracted from ancient specimens have failed, not due to a lack of DNA but due to the presence of inhibitors (Vural 2011). In qPCR, the presence of inhibitors in the reaction will produce an altered curve shape in the quantification analysis (Kontanis & Reed 2006). The efficiency of the amplification can be improved by adding either bovine serum albumin (BSA) or increased levels of DNA polymerase (Andreasson et al. 2002). Furthermore, qPCR offers another key benefit to aDNA research by determining the number of starting template molecules in the PCR reaction. The system can be used to pre-screen specimens prior to further analysis to select the best analysis methods (Andreasson et al. 2002). Research by Vural (2011) has demonstrated that the extraction of aDNA from 3000 B.C. buried remains in Turkey was successfully improved when using the qPCR technique. Due to its increased precision and the increased sensitivity to the accumulation of amplicons in real time during each cycle of the PCR amplification, it has been suggested that qPCR is the best method for amplifying low levels of aDNA.

AGE ESTIMATION ANALYSIS

Age estimation is one of the important techniques used in ancient specimen investigations (Cunha et al. 2009). It is common for the teeth and bones of specimens to remain undamaged for a longer time than other body parts, making them suitable samples for use in age determination.

AGE ESTIMATION BASED ON BONE MORPHOLOGY

Bones undergo relatively regular changes due to growth and development up to 21 years of age, depending on human variations (Sharma & Srivastava 2010). The normal, conventional, non-destructive techniques for age estimation using ancient bones are morphological and radiological analyses (Willems et al. 2002). The types of bones that can be used for age estimation include the skull, pelvis, ribs and long bones. Cranial sutures close with increasing age, based on observation of ectocranial, endocranial and maxillary suture closure (Key et al. 2005). Morphological changes on the sternal end of the forth ribs have been reported as markers for age estimation (Martrille et al. 2007). Other popular methods include the Suchey-Brooks scoring system of morphological changes on the pubic symphysis (Sitchon & Hoppa 2005) and the auricular surface (Osborne et al. 2004). The Greulich and Pyle method (Buken et al. 2007) and the Tanner and Whitehouse method (Schmidt et al. 2008) estimated human age based on human growth and development, using long bones. However, all of the methods mentioned earlier require a complete part of the particular bone for age estimation.

Age Estimation Based on Tooth Sample

DENTAL DEVELOPMENT

The dental age assessment for children is based on the growth and developmental phases of the deciduous and permanent dentition (Maber et al. 2006). Different types of dentition allow for the differentiation between children and adults. Children below three years of age carry 20 deciduous teeth, unlike adults (17-21 years old), who have 32 permanent teeth. Mixed dentition that includes both deciduous and permanent dentition can be observed between five and eleven years of age (Cunha et al. 2009). The existence of the third molar in dentitions demonstrates that the persons were adults (Mani et al. 2009). However, the presence of the third molar is not suitable for age estimations in adults, as it is dependent on human variation and is unpredictable in its time of eruption (Li et al. 2012). Age assessment in adults is based on changes in the structure of hard dental tissue caused by aging, such as attrition, periodontal disease, root translucency, cementum apposition, secondary dentin deposition, root resorption, colour changes, increases in root roughness (Brkic et al. 2006; Singh et al. 2004) and incremental lines of dental cementum (Aggarwal et al. 2008). However, not all teeth experience wear at the same rate throughout life and a number of studies have shown that tooth wear patterns and rates vary widely among different populations due to lifestyle and dietary habits, making it difficult to estimate adult dental age (Bartlett et al. 2011).

The first systematic, statistical and widely recognised approach for dental age estimation was presented by Gustafson in Swedish (1947) and in English (1950) (Sebecic et al. 2010). In their studies, six changes associated with age were observed on ground sections: attrition (occlusal wear), periodontosis (gingival recession), secondary dentin development within the pulp cavity, cementum apposition on the root, root resorption from the apex and transparency of the apical portion of the root (Singh et al. 2004). Most types of dentition analyses require a complete set of dental specimens, which includes both the maxillary and the mandible. Tooth enamel is the hardest tissue in the human body, making teeth more likely to survive over time (Malaver & Yunis 2003). In ancient remains, the teeth may be scattered or detached from the cranium. Therefore, it can be difficult to determine whether the dentition is deciduous or permanent. More current techniques have been proposed to investigate or estimate the age of individuals by using a single tooth sample.

SINGLE TOOTH SAMPLES

Secondary dentin deposition can also be used for the age estimation of individuals. The pulp chamber is a cavity inside the tooth that was once filled with dental pulp tissues, cells (fibroblast, odontoblast and undifferentiated mesenchymal cells) and extracellular matrix (Shahrul Hisham et al. 2009; Tranasi et al. 2009) (Figure 2). The size of the pulp chamber is much larger in young individual than it is in adults. This change in size is due to the deposition of secondary dentin on the walls of the pulp chamber (Paewinsky et al. 2005). Secondary dentin begins to form while the tooth is still embedded in the jaws and it continues after the tooth has erupted (Mjor 2009). A study performed by Tranasi et al. (2009) found out that the expression profile analyses of human dental pulp represent a sensible and useful tool for the study of mechanisms involved in the differentiation, growth and aging of human dental pulp during physiological and pathological conditions. Several researchers have shown that measuring the height of the coronal pulp cavity is an accurate age estimation method (Bosmans et al. 2005; Drusini 2008). A study on secondary dentin deposition, performed by Philippas (1961), showed that the decrease in the size of the pulp chamber of the tooth that was observed with increasing age was greater vertically than it was mesiodistally (Willershausen et al. 2012). Sharma and Srivastara (2010) found that the coefficient of determination was strongest for the reduction in pulp size in the mandibular first premolar when compared with incisors and canines. All of the previously discussed methods used radiographic analyses that usually involved one or two dimensional (2D) techniques.

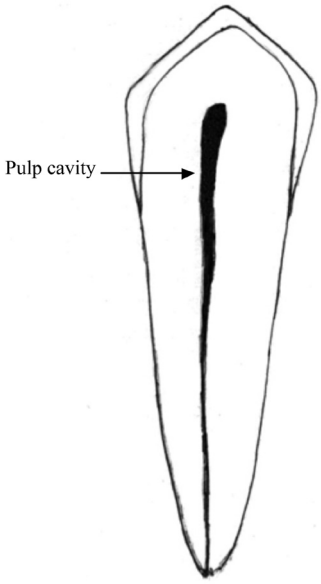

FIGURE 2. Dental pulp cavity

The method of measuring the pulp chamber can be enhanced by the use of three dimensional (3D) radiographs, which are more accurate and can exclude intra-examination error (Oi et al. 2004). However, this sophisticated type of approach is expensive and the equipment is less available. The application of three dimensional images by using a micro-computed tomography (micro-CT) scanner has been well documented in dental research (Swain & Xue 2009). 3D micro-CT analyses have also been used for tooth abrasion analysis (Kofmehl et al. 2010), enamel thickness,

TABLE 1. Summary of methods in ancestral determination and age at death estimation

Researcher	Methods	Advantages	Disadvantages
		Ancestral determination	
Bruner & Manzi 2004 L'Abbe et al. 2011 Bollini et al. 2008 Harris et al. 1977 Mavrodisz et al. 2007 Holobinco 2012	Nasal bone Shovel-shaped incisors, cusps of carabelli Jaw shape	• Is a non-destructive morphological approach • Is cheap and does not require chemicals • Can differentiate between 3 major ethnic groups (Caucasoid, Negroid and Asian)	• Is difficult to differentiate in interracial offspring • Requires a complete and undamaged skeleton • Relies on the observer's experience • Is only applicable in adults
Sholts & Warmlander 2012	Face profile		
Adachi et al. 2004 Loreille et al. 2007 Goncalves et al. 2011	mtDNA	• Is more accurate in regard to non-adult and mixed samples • Does not require complete skeleton • Survives in ancient specimens • Can be compared to known samples of the same maternal lineage • Is able to determine ancestors based on gene mutations	• Is destructive • Requires trained personnel • Is expensive • Requires sample pre-treatment • Requires a special laboratory for sample processing.
		Age at death estimation	
Maber et al. 2006	Type of dentition	• Is non-destructive • Can differentiate between children and adults (17-21 years old)	• Is not applicable when dentition is fully erupted • Requires a complete set of dental specimens
Key et al. 2005	Cranial suture	• Is non-destructive • The suture completely closes in adults	• Requires a complete skull • Is not suitable for aging adults with complete cranial suture closure
Osborne et al. 2004 Sitchon & Hoppa 2005 Martrille et al. 2007	Sternal end of 4th rib, auricular surface and Suchey-Brooks scoring system of Pubic symphysis	• Is non-destructive • The surface changes due to aging	• Is difficult to differentiates between phases • Requires experienced personnel
Buken et al. 2007 Schmidt et al. 2008	Development of long bones	• Is non-destructive	• Is based on the growth and developmental phases of bones • Is influence by individuals' diets
Mani et al. 2009 Li et al. 2012	3rd molar eruption	• Is non-destructive • 3rd molar eruption occurs at the age of 19-21 years old	• Depends on human variation • The time of eruption is unpredictable
Singh et al. 2004 Brkic et al. 2006 Aggarwal et al. 2008	Dental attrition, color changes, periodontal disease	• Is non-destructive	• Depends on lifestyle and dietary habits
Drusini 2008 Tranasi et al. 2009 Sebecic et al. 2010 Willershausen et al. 2012	Pulp chamber narrowing	• Is non-destructive • Narrowing of pulp chamber occurs throughout life • Chambers can be measured at multiple angles • Is a systematic and statistical approach for estimating age • Is applicable in adults • Does not require a complete set of dentition • A well-preserved pulp chamber is enclosed within the tooth	• Is not applicable in systemic disease patients and periodontal diseases

root canal morphology, craniofacial skeletal structure, dental tissue engineering, mineral density of dental hard tissues and dental implants (Swain & Xue 2009). Measurements of the pulp chamber images are performed using specially designated software. Agematsu et al. (2010) investigated the decrease in the volume of the pulp chamber caused by age-related secondary dentin formation using micro-CT. They found that the decrease in the size of the pulp chamber was significantly greater in females when compared to males, which was in contrast to the work previously performed by Drusini (2008), who calculated the coronal pulp cavity index using 2D radiographs and showed that the sex of the individual appeared to have no significant influence on age determination. Research performed by Someda et al. (2009) has supported the findings of Agematsu et al. (2010); they reported that there was a different regression analysis between females and males using mandibular central incisors, in which a higher correlation was observed in females than in males. It has been demonstrated that there are variations in the measurements of the pulp chambers in different types of teeth. Several other researchers have calculated the pulp:tooth volume ratios for various regions of different types of teeth, such as premolars (Cameriere et al. 2012) and canines (Babshet et al. 2010; Cameriere et al. 2009) and they found that age estimation using the pulp:tooth volume ratio was relatively accurate.

However, certain diseases should be taken into consideration when estimating dental age using the pulp chamber; patients with renal disease have been observed to experience an enhancement in dental pulp narrowing (Summers et al. 2007). Among the renal patients, the dental pulp chamber was significantly narrowed in patients who had received renal transplants compared with patients who had not received transplants. The transplantations procedures included the use of higher doses of corticosteroids and total plasma steroid clearance was lower in the patients who had received transplants than in those who had not (Galili et al. 1991). The amount of this drug taken by the patients and its pharmacokinetics appeared to be an essential factor in the initiation of narrowing in the dental pulp chamber (Nasstrom et al. 1985). Other pathological conditions, such as caries and traumatic occlusions, may change the structure of dentin due to tertiary dentin production (Cooper et al. 2010). Tertiary dentin can be deposited on the wall of the pulp cavity along with secondary dentin and decrease the size of the pulp chamber even further. Modifications in secondary dentin could be the result of post-mortem changes in mineral composition (Tutken & Vennemann 2011). The population composition has been shown to affect the estimation of age using teeth samples. Using the Cameriere calculation formula to age humans using the tooth:pulp ratios in the canines of Italian and Portuguese populations (Cameriere et al. 2009) appears to provide different regression models than when applied to an Indian population (Babshet et al. 2010). This difference suggests that a population-specific equation should be used when performing age estimations using human tooth samples.

Conclusion

The current techniques introduced to analyse ancient samples have broadened the findings and the investigations into late human culture. These techniques have been demonstrated to enhance human archaeological exploration. However, some of the latest techniques might be expensive and inaccessible. Furthermore, some of these techniques require expert personnel to handle sophisticated machines. mtDNA analysis is a reliable and accurate method for ancestral determination when examining incomplete ancient specimens (Table 1). Although mtDNA analysis is a destructive method, only a minute amount of sample is needed for the analysis. Conventional methods of DNA extraction are time consuming and involve many steps. These steps may cause the valuable samples to be lost during the process, reducing the quantity of the targeted product and making it impossible to reproduce the method using the same samples. However, in Asia, most of the institutions still rely on traditional methods, as they can produce comparable results to those produced by current advance technology. Furthermore, the availability of equipment and chemicals used for traditional techniques make them the methods of choice. Dental assessment is the preferred type of method used for age at death estimations. However, most of these methods have been based on dental growth and development, which are not applicable when the growth has stopped. The dental pulp chamber decreases in size due to secondary dentin deposition, an aging process that continues throughout life. Measuring the size of the dental pulp chamber is therefore a suitable method for the age at death estimation. Furthermore, this method is applicable even with an incomplete dental specimen (Table 1).

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