MOLECULAR SEX IDENTIFICATION OF BURNT TEETH SAMPLES BASED ON NESTED PCR AMPLIFICATION OF AMELOGENIN (AMEL) AND SEX-DETERMINING REGION Y (SRY) GENE REGIONS

by

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percent
°C	Degree Celsius
µg	Microgram
µg/µL	Microgram per microlitre
µL	Microlitre
A_{260}	Absorbance at wavelength 260 nm
$A_{260/230}$	Absorbance ratio of wavelength at 260 nm to 230 nm
$A_{260/280}$	Absorbance ratio of wavelength at 260 nm to 280 nm
A_{280}	Absorbance at wavelength 280 nm
AME2F-AME2R	Amelogenin gene first internal primers
AMEF-AMER	Amelogenin gene external primers
AMEL	Amelogenin gene
AMELX	Amelogenin gene on X chromosome
AMELY	Amelogenin gene on Y chromosome
AMIF-AMIR	Amelogenin gene second internal primers
bp	Base pairs
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDI	Federation Dentaire Internationale
g	Grams
HMW	High molecular weight

Kbp	Kilobase pairs
M	Molar
mg	Milligram
mg/mL	Milligram per millilitre
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolal
ng/μL	Nanogram per microlitre
nm	Nanometre
PCR	Polymerase Chain Reaction
pmol	Picomole
rpm	Revolutions per minute
SRY	Sex-Determining Region Y
SRYEF-SRYER	SRY gene external primers
SRYIF-SRYIR	SRY gene internal primers
STR	Short Tandem Repeat
STS	Steroid sulfatase
TBE	Tris-borate EDTA
TE	Tris-EDTA
TSPY	Y-encoded testis-specific protein
U	Activity units
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Volts

ABSTRAK

Penentuan seks merupakan salah satu komponen asas dalam pengenalpastian mangsa. Terdapat banyak kaedah yang sedia ada seperti antropologi forensik dan kaedah DNA konvensional. Dalam kajian ini, teknik *nested PCR* telah digunakan untuk proses pengenalpastian seks daripada gigi terbakar sebagai sumber DNA melalui penanda seks *amelogenin* (AMEL) dan *sex-determining region Y (SRY)*. Sejumlah 17 sampel gigi telah dibakar pada suhu antara 100 °C hingga 500 °C, pada jangkamasa 2 – 10 minit. Pengekstrakan DNA dilakukan dengan menggunakan seluruh gigi melalui kaedah fenol-kloroform. Penentuan seks yang tepat telah dicapai dalam 13 sampel pada kedua-dua penanda seks. Gen *SRY* telah mencapai sensitiviti yang lebih tinggi berbanding AMEL. Sensitiviti kedua-dua penanda bertambah baik setelah menggunakan kaedah *nested PCR*. Faktor-faktor seperti kekurangan DNA dan gigi berkaries banyak mempengaruhi keputusan penentuan seks. DNA yang tidak mencukupi selepas kaedah *PCR* boleh menghasilkan keputusan yang tidak tepat semasa gel elektroforesis. Keputusan positif palsu pada penanda *SRY* boleh disebabkan oleh kehadiran kontaminasi DNA yang terdapat dalam sampel. *Nested PCR* terbukti kaedah yang baik untuk mengamplifikasi bahan DNA terhad kerana telah menambah baik dengan menggandakan DNA, membuatkan pengenalpastian seks berkemungkinan. Gigi kekal menjadi sumber DNA yang baik walaupun sampel dalam keadaan rosak ataupun terbakar.

ABSTRACT

Sex determination one of the basic components in victim identification. There are many available methods such as forensic anthropology and conventional DNA typing methods. In this study, nested PCR technique was employed in sex typing of burnt teeth through amelogenin (AMEL) and sex-determining region Y (SRY) sex markers. All 17 teeth samples were burnt at temperatures ranging from 100 °C to 500 °C at 2 – 10 minutes. The whole tooth was used for DNA extraction by phenol-chloroform method. Accurate sex determination was achieved in 13 samples by both AMEL and SRY markers. The SRY marker achieved higher sensitivity compared to AMEL marker. The sensitivity of both markers were improved upon nested PCR. Factors such as degraded DNA materials and the presence of tooth caries greatly affects sex typing results. Insufficient DNA can produce inconclusive results during gel electrophoresis even after PCR amplification. False positive results by SRY marker can be caused by exogenous DNA contamination. Nested PCR proved to be a good method to amplify highly degraded DNA material as it greatly increases the DNA copy, making sex typing possible. Teeth remain as a good source to obtain DNA even the samples are badly damaged or burnt.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND STUDY

Sex determination is the basis in establishing identities of victims in forensic cases, while the most direct method to achieve this is by observing the genitals to differentiate between male and female. However, this is not always applicable in the case of explosion, burnt corpse for crime concealment or comingled remains in mass graves when a complete body or skeletal remain is not intact. In routine forensic casework, the fingerprint is the most common and direct method for identification by comparison with the national database. This method can be inaccessible in cases of fire and explosion where the ridges on the fingers are destroyed. In these instances, the sex and the identity of the victims have to be identified through different methods.

Traditionally, the sex of a body can be identified based on the observation of the clothing worn if the genitals are unidentifiable, such as highly decomposed bodies or victims of mass disasters. However, this does not necessarily prove the biological sex of the individual. This is because the biological sex in identification documents does not necessarily matches with the perceived gender which can be seen in transsexual individuals (von Wurmb-Schwark *et al.*, 2007). This will cause a great problem in forensic relevant cases.

Forensic anthropology is another approach which can aid in sex determination of a body based on skeletal remains that can produce accurate and relatively fast results especially by using the pelvis as it is the single most reliable area apart from using the skull (Đurić

et al., 2005; Guyomarc'h and Bruzek, 2011). However, both visual assessment and measurements of bones only provide accuracy of 80% to 90% from a single bone, whereas slightly improved result was achieved when using whole skeleton on sex determination (Quincey *et al.*, 2013). A study conducted shows that the mean ratio of accurately sexing the pelvis was 93.49%, while the skull was only 45.53% (Đurić *et al.*, 2005). This indicates that intra- and inter-population variations can affect the accuracy of sex determination (Đurić *et al.*, 2005). A similar study also showed that the anthropologists' experience is likely to affect the accuracy of sex determination (Đurić *et al.*, 2005).

Sex identification based on bone morphology can be problematic when bones found were to be in small fragments whereby the identifying criteria cannot be examined and scored. The sex of juvenile or infant remains can be difficult to be determined as they have yet to reach maturation and form identifying characteristics on the bones (Zagga *et al.*, 2014). In cases of burnt corpses or bones, they will disintegrate upon touch and physical assessment may not be possible. Combustion and incineration can also further increase the fragility of bones in addition to cause shrinkage of the bones (Imaizumi *et al.*, 2014; Schwark *et al.*, 2011). Thus, molecular analysis using deoxyribonucleic acid (DNA) for sex determination is preferred as it provides a more objective result upon analysis.

The DNA profile is usually generated in cases of mass disaster and missing person by comparison with the living next-of-kin, including a sex marker of amelogenin. Conventional forensic DNA analysis utilises short tandem repeat (STR) to determine the sex based on the homologous amelogenin genes on X and Y chromosomes (AMELX and AMELY), which is usually incorporated into the DNA typing kit (Butler and Li, 2014). In a case report to establish an identity of a burnt corpse in a forest fire, the use of

conventional STR failed to generate a complete DNA profile including sex marker due to degraded DNA materials (Fondevila *et al.*, 2008b).

Since sex determination based on amelogenin gene alone presents its own major problems especially in degraded or ancient samples (Butler and Li, 2014), it is important to further explore other sex typing markers to achieve the same objective at a higher accuracy (Morikawa *et al.*, 2011; Naik *et al.*, 2012). In a review conducted by Butler and Li (2004), apart from amelogenin, there are many other genetic markers which has been utilised for sex determination. This includes sex-determining region Y (SRY), Y-encoded testis-specific protein (TSPY), DXYS156 gene and steroid sulfatase (STS) gene (Butler and Li, 2014). Among these genes, SRY gene is most closely linked to the male sexual phenotype and activation of SRY is required for testis formation (Berta *et al.*, 1990).

In burnt samples, the quantity and quality of DNA are often compromised and is always problematic for individual identification as well as sex identification. The degradation of DNA upon heating produces very short fragments of the double helix structure and cannot be amplified in subsequent analysis due to the loss of primer binding sites as one of the factors (Fondevila *et al.*, 2008a). The DNA in these samples are usually present in a highly fragmented form, rendering conventional STR unsuccessful (Fondevila *et al.*, 2008a). Since the DNA was fragmented, primer sets that will produce a significantly smaller length of amplicon can be considered as an alternative to conventional polymerase chain reaction (PCR) (Imaizumi *et al.*, 2014). This means that nested PCR can be an option as it reduces the error in amplification when dealing with ancient or highly degraded samples, thus saving time and cost.

In a case whereby a burnt femur was examined for its identity using STR approach, result shows that the alleles in STR analysis failed to be amplified including the amelogenin sex marker (Fondevila *et al.*, 2008b). They hypothesised that this phenomenon might be caused by the size of amplicon in conventional STR which is too long for ancient DNA analysis. In the same study, the use of different analytical kits with shorter amplicons showed successful amplification and subsequent interpretation of results (Fondevila *et al.*, 2008b). This suggests that while analysing highly fragmented DNA, the targeted sequence should be shorter by using an alternative primer set during PCR amplification such as in mini-STR (Fondevila *et al.*, 2008a).

1.2 PROBLEM STATEMENT

Victim identification is the basic underlying tenet in the field of forensic science as to acknowledge the passing on of the victim and also to give a closure to the family members of the deceased. Sex is one of the aspects to be determined while establishing the profile of the victim. Methods of sex identification include direct observation of genitals, anthropology approach and conventional DNA typing have their own difficulties. Moreover, fire and explosion scenes give rise to complications in achieving sex identification when the body is badly damaged by the heat and fire. The heat in usual house fires is capable of destroying soft tissues whereas the shock wave in explosion can cause the body to break into smaller parts depending on the type and amount of explosives used.

It has been reported that sex of an individual could be wrongly identified using amelogenin gene alone (Butler and Li, 2014). Based on a study conducted by Zagga *et al.*

(2013), the success rate for sex identification using amelogenin gene was only 33.3% for samples subjected to heating between 100°C to 300°C. Apart from that, mistyping of male subjects as female has also been reported due to the deletion of AMELY in the Y chromosome (Morikawa *et al.*, 2011). Primer site mutation of both AMELX and AMELY gene was theorised as one of the reasons for sex typing failure, causing the lack of an amplicon which will lead to confusion while interpreting DNA mixtures especially in sexual assault cases (Butler and Li, 2014). Thus, it is important that alternative genes are studied to know its reliability and to aid in forensic investigations. By incorporating alternative sex markers of SRY into DNA typing, the probability of mistyping can be largely reduced in addition to saving time and cost of the analysis.

In conclusion, the problems mentioned suggest that alternative sex markers should be employed in forensic casework to reduce errors in mistyping. Furthermore, highly degraded or ancient biological samples can be examined using nested PCR with higher accuracy and confidence in the results. These factors imply that it is important that alternative methods to be studied to improve current techniques. Therefore, the purpose of this study is to determine the sex of the teeth samples, using genetic sex markers of amelogenin and SRY genes, treated at different temperature and time duration.

1.3 OBJECTIVE

GENERAL OBJECTIVE

This study aims to study the effects of different exposure temperatures and duration on sex determination by nested PCR amplification technique on burnt teeth samples.

SPECIFIC OBJECTIVES

a. To perform a nested PCR amplification of teeth samples exposed at different temperature using specific primers of AMEL and SRY.

b. To perform a nested PCR amplification of teeth samples exposed at different time duration using specific primers of AMEL and SRY.

c. To assess the viability of burnt teeth samples as a source of DNA materials by nested PCR amplification.

CHAPTER 2: LITERATURE REVIEW

2.1 DNA YIELD FROM TEETH

DNA has been known to completely degrade at temperature above 190°C which can cause a problem to determine the DNA profile of a burnt corpse (Karni *et al.*, 2013). The DNA present in biological samples such as hair, skin and blood could not withstand at this temperature and is not useful for DNA analysis. Teeth are then considered to be the most suitable sample for DNA analysis of sex identification from fragmented, highly decomposed and burnt corpses because they are highly mineralized and highly resistant to heat and decomposition (Zagga *et al.*, 2014). The DNA in the tooth is also preserved in the sealed pulp cavity and protected from the harsh environmental condition (Higgins *et al.*, 2013), thus making it a reliable source for sex determination even after decomposition.

Based on a study conducted by Adler *et al.* (2011), it was proven that different anatomical parts of the tooth used for DNA extraction give variation in yield. The pulp of the teeth consists of nerve supply, odontoblasts, fibroblasts, undifferentiated mesenchymal cells and endothelial cells together with blood supply that makes the dental pulp a good source of human DNA (Girish *et al.*, 2010). Although dental pulp is a type of loose connective tissue and degrades more easily compared to other dental tissues, they are able to extract DNA from the dental pulp of all the deciduous teeth samples for sex determination (Battepati and Shodan, 2013). Another study found that DNA material of 6 µg to 50 µg can be obtained from the pulp tissue of a single tooth and was not affected by type of tooth, donor's sex and age (Potsch *et al.*, 1992).

Malaver and Yunis (2003), concluded that dentine and cement also contain enough DNA materials for identification. However, based on the histological study conducted by Higgins *et al.* (2013), there are no visible nuclei containing DNA in dentine samples and the DNA yield could be resulted from pulp tissue that is closely associated with dentine, or from the postmortem cellular breakdown that causes DNA to penetrate into the dentine tissue (Figure 2.1). Teeth cementum has more mitochondrial DNA compared to dentine since it has higher cell density (Adler *et al.*, 2011). Root tip should be preferred over dentine as it contains larger amount of cementum providing a higher chance to obtain genetic material (Adler *et al.*, 2011).

In order to sample to the dental pulp tissue without including other hard tissues, the tooth had to be drilled from the external surface using a dental burr. Studies have shown that the higher drill speed significantly decreases the yield of genetic material compared to pulverizing the whole tooth, due to heat built up during the drilling process (Adler *et al.*, 2011). According to Garcia *et al.* (1996), similar DNA yield from tooth was obtained from different methods such as crushing the whole tooth, endodontic access and transverse sectioning of the tooth.

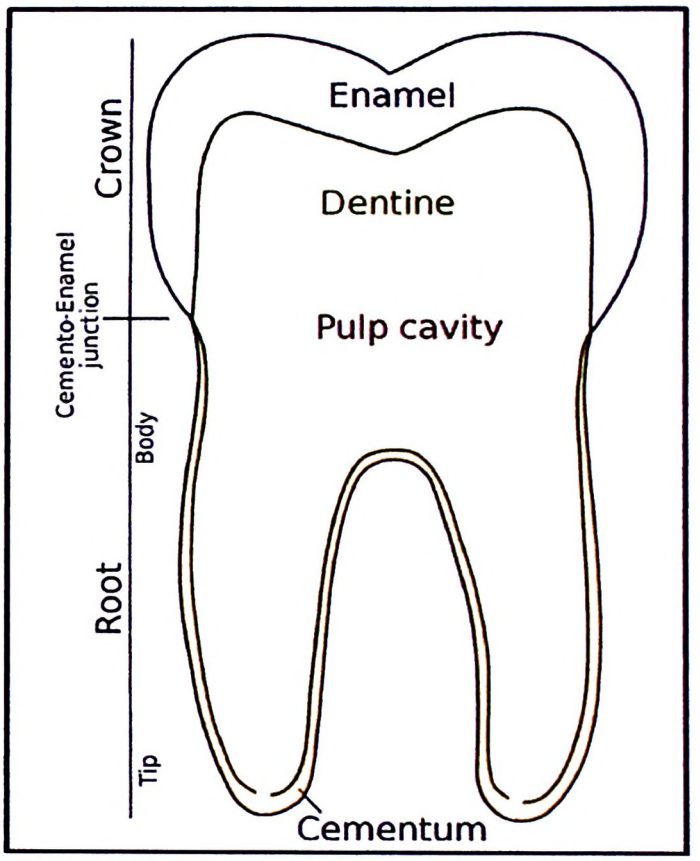

Figure 2.1: Schematic diagram of human molar showing different tooth regions

2.2 DNA EXTRACTION METHODS

The organic phenol-chloroform method of DNA extraction from most of the biological samples such as blood or buccal swab was routinely used as it is the fundamental to understand the theories and principles of the process. There are a few literatures that employed the classical phenol-chloroform method in extracting DNA from tooth samples and proved to be successful (Adler *et al.*, 2011; Battepati and Shodan, 2013; Praveen Kumar and Aswath, 2016; Zagga *et al.*, 2014). Another approach that is commonly applied in DNA extraction from tooth is the silica matrix isolation technique (Higgins *et al.*, 2015; Luptáková *et al.*, 2011; Rohland *et al.*, 2010). Both the methods on DNA extraction have been proven useful when extracting from degraded teeth samples.

The classical phenol-chloroform extraction method involves a few basic steps which are sample digestion in extraction buffer that contains proteinase K for at least 16 hours at 37°C. Samples are then extracted with phenol/chloroform/isoamyl alcohol mixture followed by precipitation with ethanol. Protocols may vary depending on the investigators. Results show that DNA yield from a single tooth based on the classical method range between 55 µg to 86 µg with high purity of 1.5-1.8 (Praveen Kumar and Aswath, 2016).

In the silica based extraction kits, the DNA is released and adsorbed on the silica matrix. The DNA was then eluted in a slightly alkaline buffer after all the components were washed off and ready for subsequent analysis. Rohland *et al.* (2010), found that employing the silica column-based extraction technique produced high DNA yield after slight modification to the protocols. The study conducted by Naik *et al.* (2012) shows that

DNA extracted from the silica based extraction method has an average concentration of 25-27 μg/μL from 25 mg of tooth pulp tissue.

Currently, there is no suitable DNA extraction technique that can achieve a good yield from degraded samples. A comparison study between different methods of DNA extraction was conducted to find out the DNA yield among phenol-chloroform method, crystal aggregation and extraction through total demineralization (Jakubowska *et al.*, 2012). The results showed that good DNA yield was obtained from total demineralization and phenol-chloroform methods (Jakubowska *et al.*, 2012).

2.3 SEX MARKERS

Sex markers are genetic loci that are related to the sex of an individual which can be differentiated by applying molecular techniques. The human sex chromosome X and Y will determine the sex of an individual, thereby developing sexual characteristics of male and female upon reaching puberty. By using suitable primers, sex markers located in the X and Y chromosome can be amplified and visualized by combining techniques such as PCR and gel electrophoresis. The presence and absence of the band on the gel will be able to indicate the sex of an individual based on the sex markers used. Many sex markers had been studied to find out their usefulness such as amelogenin, SRY, STS, alphoid repeats on sex chromosomes, and DYZ1 locus (Fazi *et al.*, 2014; Morikawa *et al.*, 2011; Zagga *et al.*, 2013).

2.3.1 AMELOGENIN (AMEL) GENE MARKER

Amelogenin is a type of protein that plays a major role in early enamel formation in tooth development, which is coded by amelogenin gene. This gene was found to be homologous for both sex chromosomes X and Y in humans with 88.9% homology, located in p22.1 – p22.3 region in X chromosome; whereas located at p11.2 region near the centromere of the short arm in the Y chromosome (Sasaki and Shimokawa, 1995). Amelogenin is the major protein secreted by ameloblast cells of the inner enamel epithelium of the tooth (Sivagami *et al.*, 2000) and is responsible for the development and the unique properties of enamel in dental tissues (Snead, 2003). According to Sasaki and Shimokawa (1995), disruption of AMEL protein development, resulting in amelogenesis imperfecta which is an inherited disease affecting the formation of dental enamel in primary and permanent teeth.

Amelogenin gene has been used as a sex marker for forensic samples since there is a length variation of amplified fragments between X and Y amelogenin (Akane *et al.*, 1991). This advantage of AMEL gene made it possible to amplify using one set of primers targeting both chromosomes X and Y. However, a study by Akane *et al.* (1991) showed that this marker was unable to determine the sex of a drowned victim and dead for more than a month.

The first known case of amelogenin failure was reported by Michael and Brauner (2004), where the blood sample of a male soldier in Egypt was typed as a female using amelogenin gene. However, the karyotype of that soldier showed to be of a normal male with the presence of both X and Y chromosomes (Michael and Brauner, 2004). In

Malaysia, based on a study conducted by Chang *et al.* (2007), results showed that AMELY negative males has the frequency of 3.2% in Malaysian Indians while 0.6% in Malaysian Malays, whereas none in the Malaysian Chinese group. This shows that there is a significant number of AMELY-null individuals present in Malaysia. They suggested that sex typing based solely on amelogenin is insufficient in routine forensic work (Chang *et al.*, 2007).

2.3.2 SEX-DETERMINING REGION Y (SRY) GENE MARKER

An alternative marker for sex determination is the sex-determining region Y (SRY) which is located at p11.31 on the Y chromosome (Butler and Li, 2014). It is a part of the testis-determining factor, TDF gene and was proved to be necessary for male sex determination. The mutation in this gene will cause sex reversal in XY females (Berta *et al.*, 1990). Currently, SRY gene is the only sex marker that has products which directly affects sex development in males. The strong association of SRY products and male phenotype suggests that SRY gene is the most accurate marker for male phenotype appearance when unreliable results were obtained from AMEL analysis (Butler and Li, 2014).

According to Naik *et al.* (2012), sex determination of human dental pulp through SRY gene showed 100% accuracy for all 20 tooth samples. Bands in length of 50 base pairs (bp) show positive results for males while negative amplification for female samples (Naik *et al.*, 2012). However, without incorporating the positive control to be analysed simultaneously, the negative result of SRY amplification is inconclusive that the sample is of a female due to other external factors causing false negative result (Morikawa *et al.*,

2011). Therefore, with the incorporation of internal positive control, the SRY gene is able to serve as a highly accurate marker for sex determination.

2.3.3 OTHER SEX MARKERS

Sex determination also can be performed using other sex markers including steroid sulfatase (STS) and alphoid repeats genes. The STS gene encodes steroid sulfatase enzyme that plays a role in synthesis of estrogens, androgens and cholesterol (Reed *et al.*, 2005). This gene is located at p22.31 on the human X chromosome with the length of approximately 146 Kbp. Mutations such as deletions and point mutations in the STS gene can cause X-linked ichthyosis that affects male individuals (Shapiro *et al.*, 1989). The STS pseudogene is present on Yq, designated as STSP1 with the length of 100 Kbp that has homology of 84.5% (Morikawa *et al.*, 2011). Sex determination using STS gene is very scanty and the only related study was conducted by Morikawa *et al.* (2011).

The alpha satellite DNA or alphoid satellite are repeating DNA sequences found at the centromeric region in human chromosomes and covers up to distance as large as few megabases (Zagga *et al.*, 2014). However, the DNA which contributes to all human chromosome specificity is found at the peri-centromeric region. It was found that the sequence on Y chromosome has higher repeating organisation compared to X chromosome which contributes to the sex typing using this DNA family. Part of the X chromosome-specific alphoid repeats was also found in the Y chromosome, thus, producing a more accurate result with the presence of positive control from the X chromosome (Zagga *et al.*, 2014). The findings from the Zagga *et al.* (2014) showed that this marker is useful for sex estimation involving dry human teeth samples.

2.4 POLYMERASE CHAIN REACTION

The invention of the PCR technique has been a great advancement in molecular science whereby small amounts of genetic material can be amplified up to a billion times. The PCR technique has been proven to be an ideal approach to amplify minute amount of DNA especially in ancient or degraded samples (Paabo *et al.*, 1989). Different sets of primers for different markers also can be included into the PCR mix for multiplex PCR that saves time and cost for the analysis (Palmirotta *et al.*, 1997). The advantage of PCR is the lack of capacity for repairing which can alter the DNA sequence of interest (Palmirotta *et al.*, 1997). However, greatly degraded DNA template cannot be amplified at all due to reasons such as baseless sites that can generate replication errors (Palmirotta *et al.*, 1997). The success of PCR amplification is affected by the size of amplicon. Despite presence of disadvantages, PCR technique was still proved to be useful in identification of decayed bodies (Battepati and Shodan, 2013).

2.4.1 NESTED POLYMERASE CHAIN REACTION

Nested PCR is a two-step PCR protocol modified from conventional PCR in terms of numbers of primer set, whereby two or more primer pairs are being employed for the amplification of the targeted sequence. The first round of PCR utilises the conventional PCR primer set also known as the external primer and followed by second amplification using internal primer. For the amplification with internal primer, PCR product from the first amplification will be used as a template. The internal primer binding site will be situated within external primer binding site, producing PCR products with a shorter length.

Through the application of nested PCR technique, the small degraded DNA fragments can be amplified with lower probability for error (Luptáková *et al.*, 2011).

A study conducted by Luptáková *et al.* (2011) on ancient bone samples suggest that amplification specificity, amplification sensitivity and nuclear DNA recovery can be increased by utilising nested PCR technique. They also concluded that nested PCR is still more effective in amplifying ancient DNA even though the number of cycles have been increased in the conventional PCR (Luptáková *et al.*, 2011). In modern DNA typing, the product length of 204 bp is usually able to produce satisfying results. However, in ancient or degraded DNA samples, the short fragmented template generally has difficulty to amplify PCR amplicons longer than 150 bp (Paabo *et al.*, 1989). As stated by Palmirotta *et al.* (1997), nested PCR technique increases the sensitivity and specificity of the analysis as this allows typing with low amounts of degraded DNA as the starting material. Apart from that, it also reduces the risk of introducing contaminants or artefacts.

CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS AND REAGENTS

All the chemicals, reagents, commercial kits and consumables used in this study are listed in Appendix A.

3.1.2 INSTRUMENTS AND APPARATUS

All the instruments and apparatus used in this study are listed in Appendix B.

3.1.3 BUFFER PREPARATIONS

3.1.3.1 10% Sodium dodecyl sulphate (SDS)

Ten grams (10 g) of SDS powder was dissolved in 90 mL of distilled water. This was done on the hot plate to assist dissolution. The pH of the solution was adjusted to 7.2 using 1 M HCI. The final volume of solution was adjusted to 100 mL by adding sterile distilled water.

3.1.3.2 1 M Tris-Hydrochloride (Tris-HCl) pH 8.0

A total of 121.14 g of Tris-base was dissolved in 800 mL of distilled water. The pH was adjusted to 8.0 by adding 1 M HCl solution. The final volume of the solution was adjusted to 1000 mL by adding distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.3 0.5 M Ethylenediaminetetraacetic acid (EDTA) pH 8.0

A total of 186.1 g of ethylenediaminetetraacetic acid, disodium salt, dihydrate (Na_2EDTA) powder was dissolved in 800 mL of distilled water on a hot plate. The pH of the solution was adjusted to 8.0 by adding sodium hydroxide (NaOH) pellet of approximately 20 g. The final volume was adjusted to 1000 mL. The solution was then autoclaved and stored at room temperature.

3.1.3.4 5 M sodium chloride (NaCl) solution

A total of 73.05 g of sodium chloride powder was dissolved in 250 mL of distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.5 Digestion buffer

The digestion buffer was prepared by adding 1 mL of 1 M Tris-HCl (pH 8.0) solution, 2 mL of 0.5 M EDTA (pH 8.0) solution, 20 mL of 10% SDS solution, and 1 mL of 5 M

NaCl solution. The mixture was then adjusted to a final volume of 100 mL by adding sterile distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.6 10X Tris-borate EDTA (TBE) buffer

A total of 107.8 g of Tris-base and 7.44 g of EDTA powder were weighed and dissolved in 800 mL distilled water using a magnetic stirrer on a hot plate. The pH of the mixture was adjusted to 8.3 by adding approximately 27.5 g of boric acid powder. The final volume of the solution was adjusted to 1000 mL by adding distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.7 1X Tris-EDTA (TE) buffer

The buffer was prepared by adding 10 mL of 1 M Tris-HCl solution, 2 mL of 0.5 M EDTA solution and the mixture was adjusted to 1000 mL by adding distilled water. The solution was aliquot in 100 mL and subjected to autoclave. The solution was then stored at room temperature.

3.1.3.8 3 M Sodium acetate (CH_3COONa)

A total amount of 102.025 g sodium acetate powder was dissolved in 200 mL of distilled water. The pH was adjusted to 5.2 by adding glacial acetic acid. The final volume was adjusted to 250 mL by adding distilled water. The solution was then autoclaved and stored at room temperature.

3.1.3.9 Chloroform:isoamyl alcohol (24:1)

A total of 24 mL of pure chloroform was mixed with 1 mL isoamyl alcohol in an amber bottle wrapped with aluminum foil. The mixture was stored at room temperature.

3.1.3.10 1 M Dithiothreitol (DTT)

A total of 1.5 g DTT powder was dissolved into 10 mL of sterile distilled water. The solution was then stored at -20 °C.

3.1.3.11 Proteinase K (20 mg/mL)

A total of 20 mg of proteinase K powder was dissolved in 1 mL of sterile distilled water. The solution was then stored at -20 °C.

3.1.3.12 Orange G loading dye

A total of 0.125 g orange G powder was added to 15 g of glycerol solution and the total volume was adjusted to 50 mL by adding sterile distilled water. The solution was aliquot into 1.5 mL microcentrifuge tube and stored at -20 °C.

3.2 METHODS

3.2.1 TEETH SAMPLING

Extracted teeth samples were obtained from School of Dental Sciences, Health Campus, Universiti Sains Malaysia and stored in a dry condition. Prior to collection of teeth samples, ethic approval was obtained from Human Research Ethics Committee USM (USM/JEPeM/16020081) (Appendix C). All the teeth samples were scraped to remove the remaining gingival tissue. The Federation Dentaire Internationale (FDI) notation and condition of teeth will be recorded. A blind study was adapted in this project whereby the researcher has no information about the sex of the teeth owner prior to analysis. A total of 17 teeth samples were collected. All collected samples were labelled with a unique identification number.

The following criteria were applied for teeth sampling:

Inclusion criteria:

1. Tooth samples of known sex
2. Tooth samples with caries and without caries
3. Permanent tooth samples

Exclusion criteria:

1. Grossly destructed tooth samples
2. Fractured root

3.2.2 SAMPLE PREPARATION AND TREATMENT

All the teeth were washed by immersing in 10% bleach solution for 5 minutes followed by soaked in 70% ethanol for 5 minutes. The teeth were then exposed to ultraviolet (UV) light for 15 minutes to eliminate exogenous DNA. The weight of the cleaned teeth samples was then recorded using an analytical balance up to four decimal places.

All 17 teeth were separated based on designed experiment. Table 3.1 shows the specific temperature and exposure time applied for each tooth. The burning process of teeth was carried out in a furnace. The conditions of each tooth before and after treatment were shown in Appendix D. After the treatment of temperature, the teeth samples were ground into powder using a pestle and mortar for the DNA extraction process.

Table 3.1: Teeth samples information with the respective exposure temperature and duration

Sample ID	FDI Notation	Temperature (°C)	Duration (mins)
A1	18	100	2
A2	13	200	2
A3	17	300	2
A4	47	400	2
A5	37	500	2
B1	12	100	4
B2	45	100	6
B3	23	100	8
B4	13	100	10
C1	17	200	4
C2	36	200	6
C3	26	200	8
C4	38	200	10
D1	25	300	4
D2	47	300	6
D3	37	300	8
D4	38	300	10

3.2.3 DNA EXTRACTION

In this study, the organic method of phenol-chloroform was employed for DNA extraction procedure as suggested by Presecki *et al.* (2000).

Approximately 0.15-0.20 g of tooth powder was weighed and placed in the 1.5 mL microcentrifuge tube, added with 1 mL of sterile ddH_2O. The samples were then subjected to centrifugation at 13,000 revolutions per minute (rpm) for 15 minutes. The liquid was then removed and 500 µL of digestion buffer, 25 µL of proteinase K (20 mg/mL) and 5 µL of 1 M DTT were added to the samples and incubated overnight in a shaking water bath at 37 °C. The mixture was centrifuged at 15,000 rpm for 15 minutes. The aqueous phase was transferred to a new tube and 1 volume of Tris-buffered phenol was added and mixed by turning the mixture up and down. The samples were left at room temperature for 5 minutes, followed by centrifugation at 7,000 rpm for 10 minutes.

The supernatant was then transferred to a new tube, then half volume of phenol and half volume of chloroform:isoamyl alcohol were added. The samples were mixed thoroughly and subjected to centrifugation at 7,000 rpm for 10 minutes. The supernatant was transferred to a new tube and 1 volume of pure chloroform was added, mixed thoroughly and spun at 7,000 rpm for 10 minutes. The supernatant was then transferred and added with 500 µL of chilled ethanol and 50 µL of 2 M sodium acetate. The mixture was spun at 7,000 rpm for 20 minutes.

The supernatant was discarded and then 700 µL of 70% chilled ethanol was added to the tube and centrifuged at 7,000 rpm for 10 minutes. The ethanol was then discarded and