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**Artikel Asli/*Original Article***

# Identification of Amelogenin Gene on Burnt Teeth Samples through Nested Polymerase Chain Reaction Amplification for Sex Identification

(Pengenalpastian Gen Amelogenin pada Sampel Gigi Terbakar melalui Amplifikasi Tindak Balas Berantai Polimerase untuk Pengenalpastian Seks)

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ABSTRACT

*Sex determination is one of the basic components in victim identification. This study aims to ascertain the sex of an individual from burnt teeth samples exposed at different temperature and time through nested polymerase chain reaction (PCR) on the amelogenin (AMEL) sex marker, to calculate the specificity and sensitivity, and to compare with previous relevant studies. A total of 17 teeth samples was subjected to burning at different temperatures ranging from 100°C to 500°C, at 2 to 10 minutes. The whole tooth was used for deoxyribonucleic acid (DNA) extraction by phenol-chloroform method. All samples were quantified for DNA concentration and then analyzed with nested PCR using two pairs of AMEL primer and results of sex typing were recorded. Out of 17 samples, genomic DNA extracted from 6 samples have concentrations ranging from 27.3 – 130.6 ng/µL. Nested PCR could amplify 16 samples for AMEL gene. Sex typing using AMEL gene showed 76.47% accuracy. Sensitivity of AMEL primer was increased from 6.67% to 63.64% using nested PCR technique; specificity of both external and internal primer was reported at 100%. Nested PCR of AMEL gene proved to be a suitable method for unequivocal determination of sex from degraded DNA samples.*

*Keywords: Amelogenin gene; burnt teeth; genomic DNA; nested PCR; sex determination*

ABSTRAK

*Penentuan seks merupakan salah satu komponen asas dalam pengenalpastian mangsa. Kajian ini bertujuan untuk mengenal pasti seks individu daripada gigi terbakar yang terdedah pada suhu dan masa yang berbeza melalui nested PCR penanda seks amelogenin (AMEL), mengira ketepatan dan kepekaan primer dan membandingkan hasil kajian dengan kajian terdahulu. Sebanyak 17 sampel gigi telah dibakar pada suhu berbeza antara 100°C hingga 500°C, pada jangka masa 2 hingga 10 minit. Seluruh gigi digunakan untuk pengekstrakan DNA yang menggunakan kaedah fenol-kloroform. Kesemua sampel diukur kepekaan dan kemudian dianalisa dengan nested PCR menggunakan dua pasang primer dan keputusan penentuan seks direkodkan. Daripada 17 sampel, DNA genomik yang diekstrak dari 6 sampel menunjukkan kepekatan antara 27.3 – 130.6 ng/µL. Nested PCR mengamplifikasikan 16 sampel untuk gen AMEL. Ketepatan pengenalpastian seks menggunakan gen AMEL mencapai 76.47%. Kepekaan primer AMEL bertambah baik kepada 63.64% dengan menggunakan teknik nested PCR; ketepatan kedua-dua primer mencapai 100%. Nested PCR pada gen AMEL terbukti merupakan kaedah yang sesuai untuk penentuan seks dari sampel DNA yang terdegradasi.*

*Kata kunci: Gen amelogenin; gigi terbakar; DNA genom; nested PCR; pengenalpastian seks*

## INTRODUCTION

Sex identification is a basic element in establishing a victim profile and methods include observation of genitals, forensic anthropology and conventional deoxyribonucleic acid (DNA) typing. However, these methods present difficulties especially in cases of explosion, burnt corpse for crime concealment or commingled remains in mass graves when a complete body or skeletal remain is not intact (Jakovski et al. 2010). Under these circumstances, the sex and identity of victims must be identified through different methods.

In burnt samples, the quantity and quality of DNA are often compromised and are always problematic for individual identification as well as sex identification (Imaizumi et al. 2014). Degraded DNA upon heating will be further fragmented and cause difficulty in amplification due to the loss of primer binding sites, thus rendering conventional short tandem repeat (STR) typing unsuccessful (Fondevila et al. 2008a). Nested PCR is a method using two sets of primer; the first set of primer is the outer primer that generates the PCR amplicon to act as the template for the second set of primer, amplifying specific product and this method was mainly used to reduce nonspecific binding in PCR products due to amplification of unexpected primer

binding site (Imaizumi et al. 2014). This means that nested PCR will reduce amplification error and able to detect a short segment of gene in ancient DNA or degraded samples.

Palmirotta et al. (1997) suggested that nested PCR technique increased the sensitivity and specificity of the analysis, permitting DNA typing of small amount of degraded DNA to undergo typing as the starting material. In another study identifying a charred femur, standard STR analysis was unsuccessful. However, the use of different primer pairs with shorter amplicons showed successful amplification and interpretation of results (Fondevila et al. 2008b). This suggests that when dealing with damaged and fragmented DNA, the targeted sequence should be shorter by using alternative primer set (Fondevila et al. 2008a).

In this study, amelogenin gene was used as the sex typing marker. Amelogenin is a protein required in early enamel formation during tooth development, encoded by the amelogenin gene. It was found to be homologous for both sex chromosomes X and Y in humans with 88.9% homology (Sasaki & Shimokawa 1995). This gene has been used as a sex marker for forensic samples since it was located in both X and Y chromosome with a length variation of amplified fragments (Akane et al. 1991).

Teeth are considered the most suitable sample for DNA analysis of sex identification from fragmented, highly decomposed and burnt corpses as they are highly mineralized and highly resistant to heat and decomposition (Zagga et al. 2014). The DNA in tooth is also preserved in the sealed pulp cavity and protected from harsh environmental conditions (Higgins et al. 2013), making it a reliable source for sex determination even after decomposition. A study found that DNA material of 39.5 µg/mL to 60 µg/mL can be obtained from the tooth samples exposed to temperatures of 100°C to 300°C for 10 to 15 minutes, proving tooth to be suitable as forensic evidence material (Devaraju et al. 2014; Praveen Kumar & Aswath 2016). Since sex typing was determined on untreated tooth samples using amelogenin gene (Praveen Kumar & Aswath 2016), and DNA has been successfully extracted from incinerated tooth followed by sex typing using Y-specific actin gene (Shrishail et al. 2011), thus, this study aims to conduct sex typing on incinerated tooth at different temperatures and duration using conventional sex marker, amelogenin gene through nested PCR method for sex determination.

## MATERIALS AND METHODS

### SAMPLING

A total of 17 extracted teeth samples was obtained from patients who received therapeutic extraction from Dental Department, Universiti Sains Malaysia from April 2015 to April 2016. Prior to collection of teeth samples, ethical approval was obtained from the Human Research Ethics Committee (USM/JEPeM/16020081). All collected tooth samples must fulfill the inclusion criteria such as permanent teeth, teeth with or without caries and teeth with known sex. However, grossly destructed teeth were excluded from this study. Patients who disagreed to provide consent were excluded from this study.This experiment was designed as a blind study where the sex information of the teeth sample was unknown to the researcher.

The apparatus used for sample preparation were all rinsed and cleaned with 70% ethanol solution before and after each tooth was processed to prevent cross-contamination. All the teeth with the gingival tissues scraped off were then immersed in 10% bleach solution for 5 minutes followed by soaking in 70% ethanol for 5 minutes. The teeth were then exposed to ultraviolet light for 15 minutes to eliminate exogenous DNA. As stated by Higgins and Austin (2013), bleach and UV light has been routinely used in decontamination procedure for teeth samples. All teeth samples were grouped as shown in Table 1with the specific temperature and exposure time applied for each tooth. Samples in group A were kept at temperature 100°C to 500°C for 2 minutes. Teeth samples for group B, C and D were incinerated at temperatures 100°C, 200°C and 300°C, respectively for duration ranging from 2 to 10 minutes. The incinerated teeth were displayed in Figure 1. The tooth was placed directly in a crucible without the lid and burnt in a burnout furnace (Manfredi, OVMAT 2007) at the specified duration and temperature. The whole teeth samples were then ground into powder using pestle and mortar for DNA extraction. The powdered teeth samples were then individually stored in separate containers.

TABLE 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 |

*Abbreviations = ID - Identification number;
FDI - Fédération dentaire internationale

### GENOMIC DNA EXTRACTION

In this study, the organic method of phenol-chloroform was used for DNA extraction as suggested inprevious

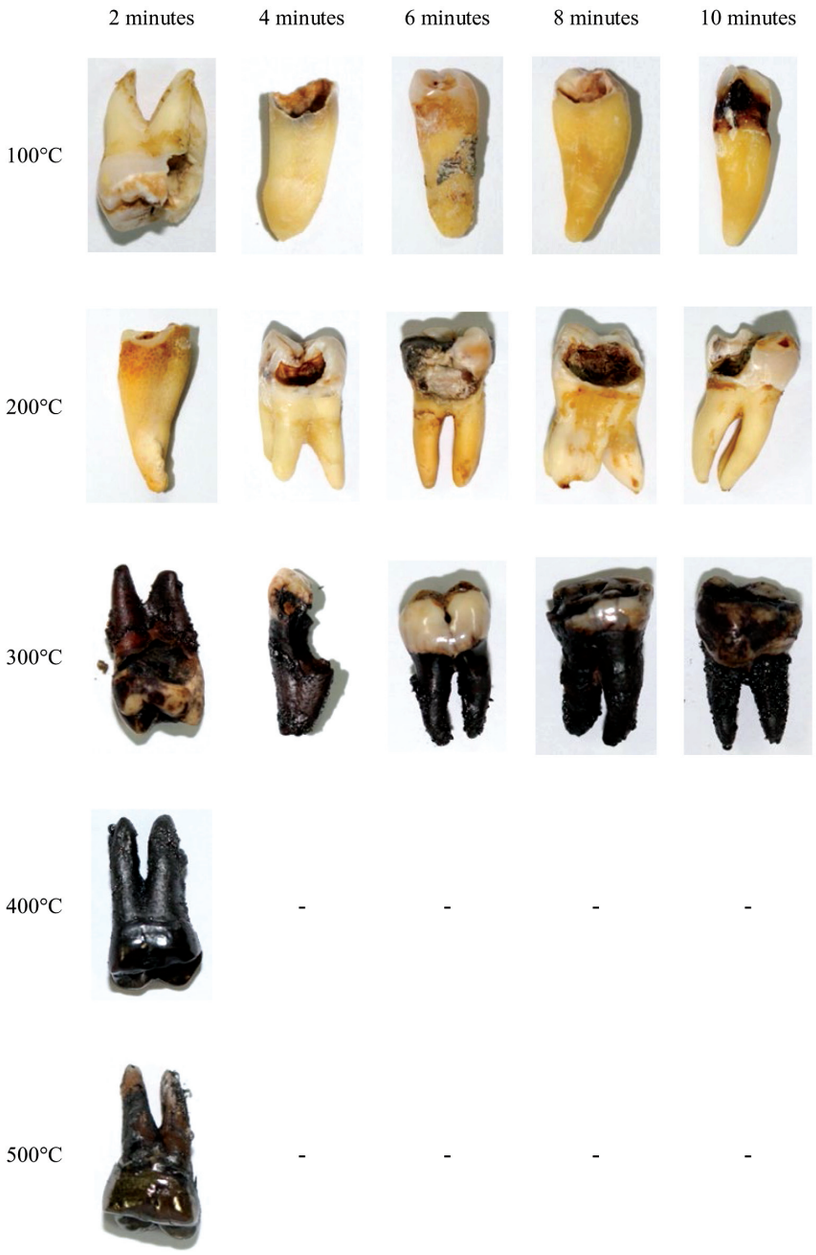


FIGURE 1. Photographs of teeth after incinerated at different temperatures and durations

literature with slight modification (Presecki et al. 2000). Approximately 0.15 – 0.20 g of tooth powder was used as a starting material for DNA extraction. To increase the efficiency of DNA extraction, 0.01 M of Dithiothreitol was added to the tooth powder mixture. The extracted DNA samples were then quantified by Thermo Scientific NanoDrop™2000 Spectrophotometer and stored at -20 °C for subsequent analysis.

### POLYMERASE CHAIN REACTION AMPLIFICATION

In this study, the published primers were used for PCR amplification of AMEL gene (Devaraju et al. 2014) (Table 2). The PCR reaction mix was prepared in 20 µL which consist of 1 µL of 10 pmol of each forward and reverse primer (Sigma, USA), 0.32 µL of 10 mM deoxyribonucleotide triphosphate mixture (Bioline, USA Inc), 1X PCR buffer (Bioline, USA Inc), 2 µL of 25 mM magnesium chloride solution (Bioline, USA Inc), 1 U of Taq DNA polymerase (Bioline, USA Inc) and added with double distilled water resulting in 20 µL of total reaction mixture.

Both the external and internal amplifications have the same components in the PCR mix with the difference in volume of DNA template whereby the external PCR mix contains 2 µL of template while the internal PCR mix contains 10 µL DNA templates. The PCR conditions applied

TABLE 2. Details of primer sequences

| Amplification | Primers | PCR products (bp) |
|---|---|---|
| External | AMEF<br>ACC ACC TCA TCC TGG GCA C<br>AMER<br>TTA CGG CCA TAT TTA GGA | 281 (X) and 287 (Y) |
| Internal | AMIF<br>ACC TCA TCC TGG GCA CCC TGG<br>AMIR<br>AGG CTT GAG GCC AAC CAT CAG | 212 (X) and 218 (Y) |

*Abbreviations = AMEF - Amelogenin external forward primer; AMER - Amelogenin external reverse primer; AMIF - Amelogenin internal forward primer; AMIR - Amelogenin internal reverse primer; bp - base pairs; X - Human X chromosome; Y - Human Y chromosome.

for external and internal amplification were as follows: 95°C for 7 minutes followed by 40 cycles of 95°C for 1 min; 56/66°C for 30 seconds; 72°C for 45 seconds and the final elongation at 72°C for 7 minutes. For monitoring of the contaminations, negative (without DNA) and positive (male and female DNA) controls were simultaneously amplified with samples.

All the amplified products were then analyzed using 2% agarose gel, pre-stained with final concentration 0.4 µg /mL of ethidium bromide solution (10 mg/mL). A total of 5 µL of PCR products was mixed with 2 µL of Orange G dye and was loaded for analysis. The electrophoresis was performed at 100 V for 1 hour 30 minutes.

## RESULTS

The analysis of extracted genomic DNA showed clear bands of high molecular weight DNA, visualised in 6 out of 17 samples, with concentration ranging from 27.3 – 130.6 ng/µL after quantitating using NanoDrop™ spectrophotometer. Other samples were not successfully visualised, ranged from 3.8 – 24.0 ng/µL.The absorbance ratio of 260 nm to 280 nm ($A_{260/280}$) reading on spectrophotometer showed average purity of extracted DNA with values ranging from 1.30 – 1.88.

The external PCR amplification shows only 6 samples yielded amplicon of single bandof 281 base pairs (bp) (X) indicating samples of female origin, while only 1 sample labeled as A1 shows amplicon of two bands at 281 bp (X) and 287 bp (Y), indicating sample of male origin. All 17 samples were then amplified with internal primer and results show 16 samples were successfully amplified with single band at 212 bp (X) identified as female origin, and double band 212 bp (X) and 218 bp (Y) identified as male origin (Table 3). One tooth sample (D2), failed for both external and internal amplifications.

In the present study, the sensitivity and specificity of the sex marker were calculated based on formula from Reddy et al. (2011).

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100\% \quad (1)$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100\% \quad (2)$$

The true positive was assigned as male samples that were correctly identified as males; true negatives are female samples correctly identified as females; false positives are female samples incorrectly identified as males; and false negative are those male samples incorrectly identified as females. All samples with no amplification were considered as false negative. The sensitivity of AMEL gene was increased from 6.67% to 63.64% by nested PCR amplification technique while the specificity of both external and internal primers was 100%. The sex from DNA typing was then compared with the collected data and it was found that 13 samples were correctly identified, shows accuracy of using AMEL gene in sex typing was 76.47%.

## DISCUSSION

In this study, 76.47% of samples were accurately identified with AMEL gene shows that using AMEL gene alone is not reliable for sex typing in degraded samples, which was less accurate compared to other studies. Results of AMEL typing from another study showed 83.33% success rate for teeth burnt at 500°C for 2 minutes, and 66.67% success rate at 200°C for 10 minutes (García et al. 1996). In another study where samples were burnt for 30 minutes, they reported 50% success rate of sex determination at 250°C; while at 300°C, all samples failed to be identified (Murakami et al. 2000). The sensitivity of AMEL gene in sex typing was increased by 9.5 folds in this study based on the ratio after and prior to nested PCR on AMEL, thus proving that nested PCR is indeed a reliable method in amplifying degraded DNA. This shows that sensitivity of amplification can be improved using nested PCR (Luptakova et al. 2011).

TABLE 3. Compilation of polymerase chain reaction results of sex from DNA typing with known sex of tooth owner

| Samples | Presence or absence of DNA product from specific primer | | | | Sex from DNA typing | Sex of tooth owner |
|---|---|---|---|---|---|---|
| | External primer pair | | Internal primer pair | | | |
| | 281 bp (X) | 287 bp (Y) | 212 bp (X) | 218 bp (Y) | | |
| A1 | √ | √ | √ | √ | M | M |
| A2 | × | × | √ | √ | M | M |
| A3 | × | × | √ | × | F | F |
| A4 | × | × | √ | × | F* | M |
| A5 | × | × | √ | √ | M | M |
| B1 | √ | × | √ | √ | M | M |
| B2 | √ | × | √ | √ | M | M |
| B3 | × | × | √ | × | F | F |
| B4 | √ | × | √ | √ | M | M |
| C1 | × | × | √ | × | F | F |
| C2 | √ | × | √ | × | F | F |
| C3 | √ | × | √ | √ | M | M |
| C4 | √ | × | √ | × | F | F |
| D1 | × | × | √ | × | F | F |
| D2 | × | × | × | × | - | F |
| D3 | × | × | √ | × | F* | M |
| D4 | × | × | √ | × | F* | M |

**Samples that are wrongly typed with respective primer*

†The presence of band of interest was marked "√" while the absence of band of interest was marked "×". The "-" shows indeterminate results.

*Abbreviations = M - Male; F - Female

The low sensitivity results in the AMEL primer shows that there is a high number of false negative results from the test. This is caused by the high failure rate of amplification in using the external AMEL primer which was possibly affected by the lack of primer binding site (Fondevila et al. 2008a) or the extremely fragmented DNA template (Pääbo et al. 1989). Upon conducting the nested PCR procedure with internal primer, most of the amplicons were detected and thus increased the sensitivity of AMEL gene. The specificity of both primers were at 100%, showing that there are no false positive result obtained from the AMEL primer.

Three male samples (A4, D3, D4) were wrongly identified as female in origin due to the absence of amelogenin, Y-linked (AMELY) amplicon after comparing with the collected data (Table 3). This shows that by relying on AMEL marker alone, sex can be wrongly identified in routine forensic cases. The lack of amplification of AMELY product can be caused by mutation at the primer binding site in the AMEL gene (Shadrach et al. 2004). A study showed 5 out of 6 samples have primer site mutation which was not amplified by amelogen in primer, was successfully amplified when using Y-STR kit (Steinlechner et al. 2002). This suggests that, AMELY-deleted individual can be correctly identified through alternative sex markers such as sex-determining region Y (SRY) marker and steroid sulfatase (STS) marker as showed in another study (Morikawa et al. 2011). The AMELY-deleted frequency as high as 3.2% was reported among Malaysian population based on race (Chang et al. 2007), however, this cannot be further investigated in this study as the information on the race of tooth owner was not collected.

Furthermore, the shortest amplicon used in this study was 212 bp, which is still considered as a large PCR product in analyzing degraded DNA samples. The amplicons of smaller size can also be considered while analyzing similar samples as they are more readily detected and amplified through PCR. A study showed that fragmented DNA template is difficult to be amplified for products longer than 150 bp (Pääbo et al. 1989). Thus, AMEL primers with amplicons shorter than 150 bp can be utilized in future for a more promising result.

The failure to visualize the extracted genomic DNA could be caused by very low concentration of DNA or fragmented genomic DNA on agarose gel electrophoresis. As stated by Mülhardt and Beese (2007), the detection limit of agarose gel is about 5 ng of DNA per band.This result was supported by the quatitation results from the spectrophotometry, showing low concentrations. In this study, although bleach and UV light were used for decontamination, this procedure do not influence the amount of DNA obtained since its removes exogenous DNA and at the same time retaining the integrity of endogenous DNA (Kemp & Smith 2005). However, this suggests that phenol-chloroform method is still reliable and sufficient DNA can be obtained from the burnt and degraded teeth samples.

In the external amplification, 7 samples exposed at temperatures 100°C and 200°C were successfully amplified and samples A2, B3 and C1 failed to amplify might be due to caries condition of the tooth samples which can affect the DNA materials by degrading the DNA due to the presence of bacteria as the pulp is exposed to

the external environment in caries tooth. Devaraju et al. (2014) has revealed tooth with more caries has less amount of DNA. Other previous studies stated that dental disease includes dental caries and periodontitis have a negative impact on the human DNA content of teeth (Higgins et al. 2011; Higgins & Austin 2013; Dogan et al. 2017). Teeth pulp contains a loose connective tissue rich with nerve supply, odontoblasts, fibroblasts, undifferentiated mesenchymal cells and endothelial cells together with blood supply, thus caries in teeth can lead to acomplete loss of pulp or tooth loss (Battepati & Shodan 2013; Higgins & Austin 2013).

Upon internal amplification, the sex of all these samples were correctly identified. All samples exposed at 300°C, 400°C and 500°C showed no amplification of both X and Y amplicons since the PCR product size might influence the successfulness of the amplification involving degraded DNA samples. After the internal amplification, most of the samples were successfully amplified proving the presence of degraded yet amplifiable extracted DNA, except for sample D2, where amplification failed using both primersand it may be due to damage of pulp structure caused by caries and presence of calcination in the root teeth which considerably influence the variability of DNA. As stated by Reesu et al. (2015), there are several factors influencing the effects of fire on teeth include the duration of exposure, temperature applied and the presence of materials between the teeth and fire. Although root is resistant to heating but increasing temperature as well as duration of exposure will have effect on the DNA yield especially nuclear DNA than mtDNA due to low copy number (Urbani et al. 1999; Reesu et al. 2015; Ramlal et al. 2017).

The absence of PCR product suggests the presence of PCR inhibitor in the samples, such as collagen and calcium in abundance especially in tooth samples (Opel et al. 2010). These PCR inhibitors must be completely removed to avoid interference with the amplification process. Apart from that, the failure of amplification can be caused by the degraded genetic material into very small fragments. A previous study showed that fragmented DNA template is difficult to be amplified for product longer than 150 bp (Pääbo et al. 1989). Even though after PCR amplification, insufficient DNA product can still cause absence of detection via ethidium bromide stained agarose gel electrophoresis. A study reported that the minimum concentration for detection of AMEL product is approximately 8.3 ng/µL as no products were detected at lower concentrations (Akane et al. 1991).

Since this study involves degraded and fragmented DNA samples, hence, several limitations were accounted for, such as the burning temperatures and duration are very much lower compared to a commercial cremation, which can go up to 1200ºC (Ubelaker 2009), thus it does not simulate a usual cremation. The exposure at higher temperature of 500ºC to 1200ºC, and longer duration up to 60 minutes can be studied in the future. Apart from that, the use of only one sex marker has low accuracy and will result in wrong identification of sex samples. More sex markers can be coupled with AMEL to produce a more accurate result. The PCR product in this research might be too long thus are not detected in degraded product. Primers can be redesigned to generate a shorter amplicon which can increase the success rate of amplification in the future.

## CONCLUSION

From this research, it was proven that genetic material can be assessed and sex typing was successful for burnt samples. Apart from that, nested PCR can be a useful technique in amplifying limited and degraded genetic material in teeth samples. Sex markers with shorter PCR products can be considered when samples are burnt and degraded. It is also suggested that alternative sex markers should be included during sex identification on degraded samples for unequivocal determination of sex.

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