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APPLICATION OF PROTEINASES FOR DNA ISOLATION OF CHALLENGED BONE SPECIMENS

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ABSTRACT

Forensic analysis of DNA from bone can be important in investigating a variety of cases involving violent crimes and missing person cases. To remove the potential presence of co-mingled remains and to eliminate contaminants that interfere with forensic DNA analysis, the outer surface of the bone fragment must be cleaned, which is a labor-intensive and a time-consuming step. Thus, a simple and reliable processing method is highly desired. This study is to address this issue and evaluate two methods for processing bone specimens prior to DNA isolation. Mechanical sanding and enzymatic trypsin methods were compared in this study.

The effects of these methods on the yield of DNA isolated and the quality of DNA analysis were studied. It was revealed that comparable values of DNA yields between the two methods were observed. Additionally, to evaluate the capabilities of the cleaning effect of the bone processing methods, the presence of environment-borne inhibitors in the DNA extracts was monitored using the internal positive control. Similar C_t values of the internal positive control were observed as the DNA extracts of the trypsin method compared with that of the sanding method. This suggests that the cleaning effect of the trypsin method was consistent with that of the sanding method. The characterization of the effects of the trypsin treatment on the quality of DNA profiling was also carried out. To evaluate the integrity of the nuclear DNA isolated, the number of allele calls and the peak-height values of alleles of the short tandem repeat profiles were compared between the two methods. To evaluate the quality of mitochondrial DNA isolated, the amplified mitochondrial DNA at the HVI and HVII regions were quantified and compared. Comparable levels of amplification success of mitochondrial DNA fragments were observed between the two methods. A paired-sample t-test revealed no significant difference between the two methods, overall.

In this study, our data suggested that the trypsin method can be used as an alternative cleaning method to mechanical cleaning methods. This method can be used to process multiple samples simultaneously. This can be very important for achieving high throughput DNA isolation through potential automation, which can be extremely valuable for situations such as the forensic DNA analysis of skeletal remains, namely, from mass fatality incidents. Additionally, this cleaning method may have a low risk of cross contamination between samples from airborne bone powder dust. Additionally, it provides a safe method of bone cleaning to prevent exposure to bone powder dust, which may carry blood-borne pathogens.

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EXECUTIVE SUMMARY

The State of the Problem

Human skeletal remains are useful specimens for forensic DNA analysis in a variety of situations when other tissue types are not available, such as when analyzing buried or decomposed skeletal remains (Alonso *et al*., 2005; Budowle *et al*., 2005; Holland *et al.,* 2003; Lawler, 2001). Prior to isolating DNA from bone samples, the cleaning of the bones is required to remove the potential of having co-mingled remains and contamination by physical contact (Sampietro *et al.,* 2006; von Wurmb-Schwark *et al*., 2008; Zehner, 2007). Additionally, polymerase chain reaction (PCR) inhibitors and bacterial contamination that interfere with forensic DNA analysis must be removed during the cleaning process (Calacal *et al.,* 2005). The outer surface of the bone fragment usually is cleaned using manual mechanical cleaning methods, such as sanding the outer surface of the sample.

However, the bone dust generated by sanding can potentially increase the risk for cross-contamination between samples (Kitayama *et al*., 2010). Especially when the bone surface is porous and fragile, surface cleaning using a mechanical method is not possible. Moreover, the sanding method cannot easily be used to process multiple samples simultaneously, and it is difficult to adapt for automation. Therefore, the processing of bone specimens is more labor-intensive and time-consuming than other types of specimens, such as bodily fluids and soft tissues. Thus, developing a high-throughput bone processing method, a "gap" in forensic DNA testing, is highly desired.

The Purpose of the Study

To address these issues, an enzymatic cleaning method using trypsin solution was evaluated. Trypsin, secreted in the digestive system, is a proteolytic enzyme which degrades various types of proteins (Buck *et al*., 1962; Walsh, 1970). In our previous study, the trypsin technique was characterized and adapted to the sample cleaning method prior to DNA isolation from fresh bone samples (Li *et al*., 209; Li & Liriano, 2011). Our data suggest that this method can be potentially used in the initial sample preparation for cleaning the outer surface of human bone samples, which results in a powder-less method and can process multiple samples at the same time. In this study, the application of the trypsin cleaning method for DNA isolation was studied in samples that are more typically encountered in actual forensic cases. The yield and the quality of DNA extracted from challenged bones was compared between the mechanical (sanding) and enzymatic (trypsin) method side-by-side.

Our *goal* of this proposed study is to develop a high throughput, low cost, less time-consuming and labor-intensive method for bone processing than existing methods. Additionally, this improved method should avoid cross-contamination and have low health risks. Thus, the method should improve the effectiveness of processing of skeletal remains from mass fatality incidents and missing-person casework for the forensic community and law enforcement agencies. Our *objective* is to make an improvement to existing methods of processing bone samples by developing a simple

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trypsin method for processing challenged bone specimens prior to DNA isolation. In particular, the study consisted of two parts: first, to characterize the effect of trypsin treatment on the yield and the quality of DNA isolated and, second, to characterize the effect of trypsin treatment on the quality of DNA profiling.

Method and Research Design

Sample Preparation. In this study, challenged human bone specimens were used. Specimens (a similar sample size as in Loreille *et al*., 2007) were selected and represented a variety of bone quality for this study. For instance, aged bones (including buried bones) were included. Bones ranging in age from 50 to over 100 years post-mortem were selected for this study. Different types of bones were included (such as cranium, rib, and tibia). Additionally, bones exposed to potential insults (such as exposed to high heat, humidity, potential bleaching or boiling) were included.

A pair of bone fragments (approximately 1 g each) was dissected from each bone specimen. A pair of bone fragments was then processed using the sanding and trypsin method separately for pair-wise comparisons. A paired-sample t-test (two-tail) was conducted to compare the data from the sanding and trypsin methods in this study.

The Characterization of the Effects of Trypsin Treatment on the Yield of DNA Isolated. To characterize the effect of trypsin treatment on the yield and the quality of DNA isolated, DNA extracts were compared side-by-side: 1) DNA extracts isolated using the sanding method, and 2) DNA extracts isolated with the trypsin treatment. To find out if this trypsin method will achieve sufficient DNA yield for forensic DNA profiling, total DNA, was isolated and quantified. PCR inhibitors cause inhibitions in PCR-based forensic DNA analysis. To evaluate the capabilities of the cleaning effect of the bone processing method, the presence of environment-born inhibitors in the DNA extracts was measured by monitoring the amplification of the internal positive control (*IPC*).

The Characterization of the Effects of Trypsin Treatment on the Quality of STR Analysis. To find out if this processing method causes any adverse effect on DNA, such as DNA degradation, the quality of DNA profiles was evaluated. DNA samples were examined using short tandem repeat (STR) analysis. In highly degraded specimens, STR analysis is often difficult due to the high degree of DNA fragmentation. To increase success in STR analysis, we utilized AmpFℓSTR® MiniFiler™ amplification kit (Applied Biosystems) with primer pairs that produce shorter amplicons.

To evaluate the integrity of the DNA isolated, the number of allele calls was compared. The integrity of the DNA samples was also examined by comparing the average peak height values of each allele at each locus between sanded and trypsin-treated samples.

The Characterization of the Effects of the Trypsin Treatment on the Amplification of Mitochondrial DNA (mtDNA). The characterization of the effects of the trypsin method on the quality of mtDNA analysis results was carried out. To amplify the

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hypervariable regions of mtDNA from highly degraded specimens, a mini-primer set (Gabriel *et al*., 2001) at HVI region with an amplicon size of 170 bp was utilized. Furthermore, a midi-primer set for amplification of the longer fragment (275 bp) at HVII region and HVI (443 bp) as well as HVII primer set (414 bp) were utilized. The quantity of amplification products of trypsin-treated samples was compared to that of the sanded samples.

Findings and Conclusions

Discussion of the Findings. It was revealed that comparable values of DNA yields between the two methods were observed. Additionally, similar IPC C_t values were observed as the DNA extracts of the trypsin method were compared with that of the sanding method. This suggested that the cleaning effect of the trypsin method was consistent with that of the sanding method. The characterization of the effects of the trypsin method on the quality of DNA profiling was also carried out. In the evaluation of the integrity of the genomic DNA isolated, the number of the allele calls of STR profiles and the peak height values of STR alleles were comparable between the two methods. To evaluate the quality of mtDNA isolated, the amplified mtDNA was quantified and compared. Comparable levels of the amplification success of mtDNA fragments were observed between the two methods. The results suggested that the trypsin method was consistent with the sanding method. This study suggested that the trypsin method can be used as an alternative to mechanical cleaning methods for human bone samples.

Implications for Policy and Practice. The identification of human skeletal remains is often necessary in a variety of cases: mass fatality incidents, remains recovered from war, fires, explosions, and remains found at crime scenes. A number of methods can be used to identify human remains. The most common of these methods includes the identification of facial characteristics; the recognition of individualizing scars, marks, or other special features; the matching of dentition with premortem dental X-rays; or by the comparison of fingerprints. In many situations, these methods cannot be used because of extensive putrefaction or the destruction of the remains. The mass fatality terrorist attack on the World Trade Center provides an example of which common methods for identification may not be useful. Large quantities of compromised human skeletal fragments were recovered at the fatality site. In this type of case, the analysis of DNA from bone is a powerful tool for identifying human remains as the other types of identification methods available would not be applicable (Holland *et al*., 2002; Lawler, 2001).

One of the challenges when attempting to analyze DNA from the bones of victims of mass fatality incidents is the initial processing of bone samples. Prior to isolating DNA from bone samples, the cleaning of the bones is required to remove potential contaminants. The outer surface of the bone fragment usually is cleaned using manual mechanical cleaning methods. The mechanical methods cannot easily be used to process multiple samples simultaneously, and it is difficult to adapt for automation.

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Therefore, the processing of bone specimens is more labor-intensive and time-consuming than other types of specimens.

In this study, our data suggested that the trypsin method can be used as an alternative cleaning method to mechanical cleaning methods. This method can be used to process multiple samples simultaneously. This can be very important for achieving high throughput DNA isolation through potential automation, which can be extremely valuable for situations such as the forensic DNA analysis of skeletal remains, namely, from mass fatality incidents. Additionally, this cleaning method may have a low risk of cross contamination between samples from airborne bone powder dust. Additionally, it provides a safe method of bone cleaning to prevent exposure to bone powder dust, which may carry blood-borne pathogens.

The results from this study can have the potential to make a significant impact on the processing of skeletal evidence for the forensic community and law enforcement agencies. This can be potentially achieved by establishing a bone-processing method that will be less time-consuming and labor-intensive than existing mechanical methods, thus improving the speed of testing. Additionally, the labor cost for this method will be reduced. Thus, the overall cost of this method should be lower than physical methods. Lastly, this method has a low potential risk of cross-contamination between samples, therefore, improving the accuracy of testing.

Implications for Further Research. Experiments are needed to further study the feasibility of the adaptation of this trypsin method to an automated platform, such as a liquid-handling and a tissue-lysis device, to improve the throughput to process bone samples for DNA isolation. Such devices can simultaneously process multiple biological samples at the same time. The protocol for processing bone samples using appropriate devices, accessory sets, apparatus parameters, and experimental conditions needs to be developed.

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INTRODUCTION

Human skeletal remains are useful specimens for forensic DNA analysis in a variety of situations when other tissue types are not available, such as when analyzing buried or decomposed skeletal remains (Alonso *et al*., 2005; Budowle *et al*., 2005; Holland *et al.,* 2003; Lawler, 2001). Prior to isolating DNA from bone samples, the cleaning of the bones is required to remove the potential of having co-mingled remains and contamination by physical contact (Sampietro *et al.,* 2006; von Wurmb-Schwark *et al*., 2008; Zehner, 2007). Additionally, PCR inhibitors and bacterial contamination that interfere with forensic DNA analysis must be removed during the cleaning process (Calacal *et al.,* 2005). The outer surface of the bone fragment usually is cleaned using manual mechanical cleaning methods, such as sanding using sandpaper (Anslinger *et al*., 2001; Miazato *et al*., 2005) and sanding discs attached to a rotary tool (Davoren *et al*., 2007; Edson *et al*., 2004; Loreille *et al*., 2007).

However, the bone dust generated by sanding can potentially increase the risk for cross-contamination between samples (Kitayama *et al*., 2010). Especially when the bone surface is porous and fragile, surface cleaning using a mechanical method is not possible. Moreover, the sanding method cannot easily be used to process multiple samples simultaneously, and it is difficult to adapt for automation. Therefore, the processing of bone specimens is more labor-intensive and time-consuming than other types of specimens, such as bodily fluids and soft tissues. Thus, developing a high-throughput bone processing method, a "gap" in forensic DNA testing, is highly desired.

To address these issues, an enzymatic cleaning method using trypsin solution was evaluated. Trypsin, secreted in the digestive system, is a proteolytic enzyme which degrades various types of proteins (Buck *et al*., 1962; Walsh, 1970). Trypsin has been utilized in enzymatic maceration methods for processing bone samples in anthropological laboratories (Hangay & Dingley 1985; Hendry, 1999). In our previous study, the trypsin maceration technique of bone samples was characterized and adapted to the sample cleaning method prior to DNA isolation from fresh bone samples (Li *et al*., 209; Li & Liriano, 2011). Microscopic studies demonstrated that this trypsin method is effective for the removal of outer surface materials such as the mineralized bone connective tissue of fresh human bone samples. The yield of genomic DNA isolated from trypsin-treated fresh bone samples was consistent with the average DNA yield from fresh bone samples that were cleaned using razor blades and sandpaper, as reported by Hochmeister et al. (Hochmeister *et al*., 1991). Thus, the yield of DNA isolated from trypsin-treated samples was sufficient for subsequent STR analysis. The STR analysis revealed that no adverse effect on the DNA profile was detected after trypsin treatment. Our data suggest that this method can be potentially used in the initial sample preparation for cleaning the outer surface of human bone samples, which results in a powder-less method and can process multiple samples at the same time.

In this study, the application of the trypsin cleaning method for DNA isolation was studied in samples that are more typically encountered in actual forensic cases. The

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yield and the quality of DNA extracted from challenged bones was compared between the mechanical (sanding) and enzymatic (trypsin) method side-by-side.

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MATERIALS AND METHODS

Sample Preparation

Precautions were followed to eliminate possible DNA contaminants. Sampling was carried out in a sterilized laminar flow cabinet. Consumables were DNA free. Disposable laboratory coats, gloves, and masks were used throughout the procedure.

Sampling. In this study, challenged human bone specimens (non probative) were used (Figure 1, Table 1). A total of 14 bone specimens (a similar sample size as in Loreille *et al*., 2007) including cranium, rib, and tibia from different individuals were selected. Aged bones including buried bones unearthed from archaeological sites were included. Bones ranging in age from approximately 50 to over 100 years post-mortem were selected for this study. Bones exposed to potential insults (such as the possibility of maceration using bleaching or boiling, or buried under high heat and humidity) were included.

A pair of bone fragments (approximately 1 g each) was dissected from each bone specimen. A pair of bone fragments was then processed using the sanding and trypsin method separately for pair-wise comparisons. A paired-sample t-test (two-tail) was conducted to compare the data from the sanding and trypsin methods in this study.

Surface Cleaning. The trypsin treatment was carried out as developed previously (Li *et al*., 2009). It was initiated by adding 5 ml of fresh trypsin (Fisher Scientific) solution (30µg/µl, 10 mM Tris, pH7.5) to the bone fragment and then was incubated at 55°C with gentle agitation for 2 hr. After incubation, the supernatant was removed. The sanding was carried out using a current sanding method (Davoren *et al*., 2007) with single-use sanding discs attached to a rotary sanding tool (Dremel, Racine, WI). The outer surfaces were sanded thoroughly. The cleaned bone fragments using both methods were further processed by inversion for 30 s in distilled water, 10% bleach, and 96% ethanol as described in Davoren et al. (2007). The bone fragments were then air dried.

Bone Tissue Disruption. The pulverized bone powder from each fragment was prepared using the freezer mill method (Loreille *et al*., 2007) that utilizes a cryogenic impact grinder (SamplePrep 6770 Freezer Mill, SPEX, Metuchen, NJ). The procedure was programmed according to the manufacturer's protocols: 10 min pre-cooling followed by 2 cycles (2 min grinding at rate of 20 impacts/s and 2 min cooling).

DNA Extraction

Demineralization of pulverized bone powder was carried out as described in Loreille *et al*. (2007). For each sample, 0.2 g of pulverized bone powder was decalcified by incubating in 3.2 ml of extraction buffer (0.5 M EDTA, 1% lauryl-sarcosinate) and 200 µl of 20 mg/ml proteinase K overnight at 56°C with gentle agitation.

The DNA from each sample was extracted using the method adapted from Courts & Madea(2011) with slight modifications. The volume of the demineralized sample was reduced to 300-450 µl using an Amicon Ultra-4 (30 kD) column. The small molecular weight cutoffs of the centrifugal device retains a greater number of short DNA elements, thereby may increase the potential for successful DNA amplification

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(Gabriel *et al*., 2001). DNA was extracted using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocols. The final volume of eluted DNA was 60 µl. Extraction negative controls were employed to monitor the potential contaminations. DNA profiles from laboratory personnel were available for the detection of laboratory DNA contaminations.

DNA Quantification of Nuclear DNA Using Real Time PCR

DNA quantitation was performed using the Quantifiler Human DNA Quantitation kit (Applied Biosystems, Foster City, CA) on an ABI 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Internal positive controls were used to monitor PCR inhibitors present in the DNA extracts. For the 2 bone samples (buried for over 100 years) that had IPC C_t values greater than 30, additional purification was carried out to remove inhibitors using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocols. Negative controls and reagent blanks were included to monitor potential contamination. DNA yield was expressed as ng DNA/g bone.

STR Analysis

STR analysis was performed using the AmpFℓSTR® MiniFiler™ amplification kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. A positive control with genomic DNA of a known STR profile was included. Extraction and PCR negative controls were also amplified to monitor potential contamination. Amplified products were separated on an Applied Biosystems 310 Genetic Analyzer and results were analyzed with GeneMapper Id software. A 50 relative fluorescent units (rfu) threshold for allele designation was used, thus peaks below 50 rfu were disregarded.

Amplification of mitochondrial DNA

The amplification of specific fragments of the mtDNA hypervariable region was done as described (Figure 2, Table 2). Samples showing sufficient DNA quantities as detected in DNA quantitation assays were amplified. The target amount of DNA amplified was 0.1 ng as described (Roche Applied Science). For samples that yielded low amplification products, a total of 0.1 - 0.5 ng of DNA template was used. For samples that showed no detectable DNA in the quantitative PCR assay, a total of 10 µl of DNA extract was used. PCR reactions were carried out in 25 µL volumes using 1X AmpliTaq polymerase buffer with $MgCl_2$ (Applied Biosystems, Foster City, CA), 200 µM each dNTP, 0.025 mg/ml bovine serum albumen, 0.4 µM each forward and reverse primer, and 5 units of AmpliTaqGold polymerase (Applied Biosystems, Foster City, CA). The reactions were initiated with a 10 min activation step at 96 °C. For each cycle, the cycling parameters include a 20 s denaturation step at 94 °C, a 20 s primer annealing step, and followed by a 30 s extension step at 72 °C. The PCR was performed for a total of 38 cycles. As a positive control, amplification with 0.1 ng of genomic DNA of known mtDNA sequence was carried out. To monitor contamination, PCR negative controls were included with each amplification experiment.

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To identify and to quantify the PCR products, DNA separations were performed using the DNA 1000 Lab-on-Chips Assay kit with an Agilent Bioanalyzer 2100 (Agilent Technologies) following the manufacture's protocol. The Agilent DNA 1000 ladder (Agilent Technologies) was used as a sizing standard. The data were analyzed to determine fragment size based on the sizing ladder and internal standards. The quantitation of each PCR product was performed using the manufacturer's software provided with the Agilent Bioanalyzer 2100 system.

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RESULTS

The Characterization of the Effects of Trypsin Treatment on the Yield of DNA Isolated

To characterize the effect of trypsin treatment on the yield and the quality of DNA isolated, DNA extracts were compared side-by-side: 1) DNA extracts isolated from the sanding method, and 2) DNA extracts isolated with the trypsin method.

To find out if this trypsin method achieved sufficient DNA yield for forensic DNA profiling, total DNA, including nuclear DNA (nDNA) and mtDNA, was isolated and quantified. Among 14 bone specimens, DNA was detected from 9 bone specimens. The negative quantitation results from 5 buried bones were most probably caused by highly degraded template DNA. The DNA yield from different bone specimens, ranged from 2.25 – 7341 ng DNA/g bone, was highly variable (Table 3). The DNA yields of trypsin-treated samples were slightly higher than that of sanded samples. The average ratio of DNA yield isolated from trypsin treated versus sanded bone samples was 1.66 (with a standard deviation of the average ratio at 1.87). A paired-sample t-test (two-tail) was conducted to compare the yields of the sanding and trypsin methods. There was no significant difference in the yields of the two methods (p value > 0.05).

PCR inhibitors cause inhibitions in PCR-based forensic DNA analysis. To evaluate the capabilities of the cleaning effect of the bone processing method, the presence of environment-born inhibitors in the DNA extracts was measured by monitoring the amplification of the IPC. Our results revealed that similar IPC C_t values were observed as the DNA extracts of the trypsin method compared with that of the sanding method. The average IPC C_t values were 28.49 for the sanding method and 28.24 for the trypsin method, respectively. The average ratio of IPC C_t value from trypsin treated versus sanded bone samples was 0.99 (with a standard deviation of the average ratio at 0.011). A paired-sample t-test (two-tail, comparing the sanding and trypsin methods) indicated that there was no significant difference in the IPC C_t values of the two methods (p value > 0.05). No PCR inhibiting substances were present.

The Characterization of the Effects of Trypsin Treatment on the Quality of STR Analysis

To find out if this processing method causes any adverse effect on DNA, such as DNA degradation, the quality of DNA profiles was evaluated. DNA samples were examined using STR analysis. In highly degraded specimens, STR analysis is often difficult due to the high degree of DNA fragmentation. Since the bone specimens used in this study were exposed to acidic soils and to potential insults such as bleach and boiling, these conditions may lead to DNA template degradation (Gabriel *et al*., 2001). To increase success in STR analysis, we utilized AmpFℓSTR® MiniFiler™ amplification kit (Applied Biosystems) with primer pairs that produce shorter amplicons. Amplicons were detected on a total of 18 DNA samples (9 pairs of sanded versus trypsin-treated samples). In contrast, no amplicon was detected on samples that showed no detectable DNA in the quantitative PCR assay.

To evaluate the integrity of the DNA isolated, the number of allele calls was compared. Only peaks with a peak height of 50 rfu or more were included. Profile

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results were compared between sanding and trypsin-treated samples side-by-side. Successfully typed loci were observed in both sanded and trypsin-treated samples (Figure 3). Some samples (4 pairs of samples), particularly for larger size alleles at D7S820, D18S51, D21S11, and FGA loci, exhibited locus drop-outs, probably due to template degradation. Overall, the number of allele calls of the trypsin method was similar as that of the sanding method (Figure 4). The ratio of the number of allele calls from trypsin treated versus sanded bone samples was 1.02 (with a standard deviation of the ratio at 0.069). A paired-sample t-test revealed no significant difference between the two methods (p value > 0.05). The integrity of the DNA samples was also examined by comparing the average rfu values of each allele at each locus between sanded and trypsin-treated samples. Only alleles showing successful amplification in both sanded and trypsin-treated samples were compared. The rfu values for both sanded and trypsin-treated samples are shown in Figure 5. The rfu values of the trypsin-treated samples were comparable to those of the sanded samples (Table 4). A paired-sample t-test revealed that no significant difference in peak heights was detected in rfu between two methods (p value > 0.05).

The Characterization of the Effects of the Trypsin Treatment on the Amplification of Mitochondrial DNA

The characterization of the effects of the trypsin method on the quality of mtDNA analysis results was carried out. mtDNA HVI and HVII fragments were amplified and quantified using an Agilent Bioanalyzer 2100.

To amplify the hypervariable regions of mtDNA from highly degraded specimens, a mini-primer set (Gabriel *et al*., 2001) at HVI region with an amplicon size of 170 bp was utilized. For the pair-wise study, the pairs of extracts (sanded versus trypsin treated samples) were tested side-by-side (Figure 6). The amplified mtDNA HVI fragments were quantified and compared using an Agilent Bioanalyzer 2100. Of the 9 pairs totaling 18 DNA samples that showed detectable DNA in the quantitative PCR assay, successful amplification was detected in 17 of the DNA samples while no amplification product was observed in 1 sanded sample. No amplicon was detected on 5 pairs of samples that showed no detectable DNA in the quantitative PCR assay. Next, 5 pairs of DNA samples showing the highest signal in the mini-primer set PCR assay was selected for further analysis. A midi-primer set for amplification of the longer fragment (275 bp) at HVII region and HVI (443 bp) as well as HVII primer set (414 bp) were utilized (Figure 6). Successful amplification was detected in all 5 pairs of DNA samples tested. A cycle sequencing reaction usually requires approximately 5 ng of amplified product (Schwark *et al*., 2011). Thus, all successful amplified samples yielded sufficient quantities of PCR products for subsequent sequencing analysis. Overall, the quantity of amplification products of trypsin-treated samples was slightly higher than that of the sanded samples (Figure 7). A paired-sample t-test revealed no significant difference between two methods (p value > 0.05) in amplification with mini, midi, and HVI primer sets. However, the quantity of amplification products of trypsin-treated samples was significant higher (p value < 0.05) than that of the sanded samples with the HVII primer set.

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CONCLUSIONS

Discussion of the Findings

In this study, the feasibility of using the enzymatic trypsin method for cleaning bones prior to DNA isolation was evaluated. The effects of the cleaning methods on the yield and the quality of DNA extracts isolated were compared side-by-side: 1) DNA extracts isolated from the sanding method, and 2) DNA extracts isolated with the trypsin method. It was revealed that comparable values of DNA yields between the two methods were observed. Additionally, to evaluate the capabilities of the cleaning effect of the methods, the presence of environment-born inhibitors in the DNA extracts was monitored using IPC. In this study, similar IPC C_t values were observed as the DNA extracts of the trypsin method were compared with that of the sanding method. This suggested that the cleaning effect of the trypsin method was consistent with that of the sanding method.

The characterization of the effects of the trypsin method on the quality of DNA profiling was also carried out. In the evaluation of the integrity of the genomic DNA isolated, the number of the allele calls of STR profiles and the rfu values of STR alleles were comparable between the two methods. To evaluate the quality of mtDNA isolated, the amplified mtDNA at the HVI and HVII regions was quantified and compared. Comparable levels of the amplification success of mtDNA fragments were observed between the two methods.

The results suggested that the trypsin method was consistent with the sanding method. A paired-sample t-test revealed no significant difference between the two methods, overall. This study suggested that the trypsin method can be used as an alternative to mechanical cleaning methods for human bone samples.

Implications for Policy and Practice

The identification of human skeletal remains is often necessary in a variety of cases: mass fatality incidents, remains recovered from war, fires, explosions, and remains found at crime scenes. A number of methods can be used to identify human remains. The most common of these methods includes the identification of facial characteristics; the recognition of individualizing scars, marks, or other special features; the matching of dentition with premortem dental X-rays; or by the comparison of fingerprints. In many situations, these methods cannot be used because of extensive putrefaction or the destruction of the remains. The mass fatality terrorist attack on the World Trade Center provides an example of which common methods for identification may not be useful. Large quantities of compromised human skeletal fragments were recovered at the fatality site. In this type of case, the analysis of DNA from bone is a powerful tool for identifying human remains as the other types of identification methods available would not be applicable (Holland *et al*., 2002; Lawler, 2001).

One of the challenges when attempting to analyze DNA from the bones of victims of mass fatality incidents is the initial processing of bone samples. Prior to isolating DNA from bone samples, the cleaning of the bones is required to remove

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potential contaminants. The outer surface of the bone fragment usually is cleaned using manual mechanical cleaning methods. The mechanical methods cannot easily be used to process multiple samples simultaneously, and it is difficult to adapt for automation. Therefore, the processing of bone specimens is more labor-intensive and time-consuming than other types of specimens.

In this study, our data suggested that the trypsin method can be used as an alternative cleaning method to mechanical cleaning methods. This method can be used to process multiple samples simultaneously. This can be very important for achieving high throughput DNA isolation through potential automation, which can be extremely valuable for situations such as the forensic DNA analysis of skeletal remains, namely, from mass fatality incidents. Additionally, this cleaning method may have a low risk of cross contamination between samples from airborne bone powder dust. Additionally, it provides a safe method of bone cleaning to prevent exposure to bone powder dust, which may carry blood-borne pathogens.

The results from this study can have the potential to make a significant impact on the processing of skeletal evidence for the forensic community and law enforcement agencies. This can be potentially achieved by establishing a bone-processing method that will be less time-consuming and labor-intensive than existing mechanical methods, thus improving the speed of testing. Additionally, the labor cost for this method will be reduced. Thus, the overall cost of this method should be lower than physical methods. Lastly, this method has a low potential risk of cross-contamination between samples, therefore, improving the accuracy of testing.

Implications for Further Research

Experiments are needed to further study the feasibility of the adaptation of this trypsin method to an automated platform, such as a liquid-handling and a tissue-lysis device, to improve the throughput to process bone samples for DNA isolation. Such devices can simultaneously process multiple biological samples at the same time. The protocol for processing bone samples using appropriate devices, accessory sets, apparatus parameters, and experimental conditions needs to be developed.

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REFERENCES

Alonso, A., Martin, P., Albarrãn, C., Garcia, P., Fernandez De Simon, L., Iturralde, M. J., et al. (2005). Challenges of DNA profiling in mass disaster investigations. *Croatian Medical Journal, 46*(4), 540-548.

Anslinger, K., Weichhold, G., Keil, W., Bayer, B., & Eisenmenger, W. (2001). Identification of the skeletal remains of Martin Bormann by mtDNA analysis. *International Journal of Legal Medicine, 114*(3), 194-196.

Buck, F. F., Vithayathil, A. J., Bier, M., & Nord, F. F. (1962). On the mechanism of enzyme action. 73. Studies on trypsins from beef, sheep and pig pancreas. *Arch Biochem Biophys, 97*, 417-424.

Budowle, B., Bieber, F. R., & Eisenberg, A. J. (2005). Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification. *Legal Medicine, 7*(4), 230-243.

Courts, C., & Madea, B. (2011). Full STR profile of a 67-year-old bone found in a fresh water lake. *J Forensic Sci, 56 Suppl 1*, S172-175.

Davoren, J., Vanek, D., Konjhodzić, R., Crews, J., Huffine, E., & Parsons, T. J. (2007). Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves. *Croatian Medical Journal, 48*(4), 478-485.

Edson, S. M., Ross, J. P., Coble, M. D., Parsons, T. J., & Barritt, S. M. (2004). Naming the dead-confronting the realities of rapid identification of degraded skeletal remains *Foren. Sci. Rev., 16*, 63-90.

Gabriel, M. N., Huffine, E. F., Ryan, J. H., Holland, M. M., & Parsons, T. J. (2001). Improved MtDNA sequence analysis of forensic remains using a "mini-primer set" amplification strategy. *J Forensic Sci, 46*(2), 247-253.

Hangay, G., & Dingley, M. (1985). *Biological museum methods* (Vol. 1). Orlando: Academic Press.

Hendry, D. (1999). Vertebrates. In D. Carter & A. K. Walker (Eds.), *Care and conservation of natural history collections* (pp. 1-36). Oxford: Butterworth-Heinemann.

Holland, M. M., Cave, C. A., Holland, C. A., & Bille, T. W. (2003). Development of a quality, high throughput DNA analysis procedure for skeletal samples to assist with the identification of victims from the World Trade Center attacks. *Croatian Medical Journal, 44*(3), 264-272.

This document is a research report submitted to the U.S. Department of Justice. This report has not been published by the Department. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

Kitayama, T., Ogawa, Y., Fujii, K., Nakahara, H., Mizuno, N., Sekiguchi, K., et al. (2010). Evaluation of a new experimental kit for the extraction of DNA from bones and teeth using a non-powder method. *Legal Medicine, 12*(2), 84-89.

Lawler, A. (2001). Terrorism. Massive DNA identification effort gets under way. *Science, 294*(5541), 278-279.

Li, R., Chapman, S., Thompson, M., & Schwartz, M. (2009). Developing a simple method to process bone samples prior to DNA isolation. *Legal Medicine, 11*(2), 76-79.

Li, R., & Liriano, L. (2011). A bone sample cleaning method using trypsin for the isolation of DNA. *Leg Med (Tokyo), 13*(6), 304-308.

Loreille, O. M., Diegoli, T. M., Irwin, J. A., Coble, M. D., & Parsons, T. J. (2007). High efficiency DNA extraction from bone by total demineralization. *Forensic Science International: Genetics, 1*(2), 191-195.

Miazato Iwamura, E. S., Oliveira, C. R. G. C. M., Soares-Vieira, J. A., Nascimento, S. A. B., & Muñoz, D. R. (2005). A qualitative study of compact bone microstructure and nuclear short tandem repeat obtained from femur of human remains found on the ground and exhumed 3 years after death. *American Journal of Forensic Medicine and Pathology, 26*(1), 33-44.

Roche Applied Science (2005). Linear array mitochondrial DNA HVI/HVII region-sequence typing kit product brochure.

Sampietro, M. L., Gilbert, M. T. P., Lao, O., Caramelli, D., Lari, M., Bertranpetit, J., et al. (2006). Tracking down human contamination in ancient human teeth. *Molecular Biology and Evolution, 23*(9), 1801-1807.

Schwark, T., Heinrich, A., Preusse-Prange, A., & von Wurmb-Schwark, N. (2011). Reliable genetic identification of burnt human remains. *Forensic Sci Int Genet, 5*(5), 393-399.

von Wurmb-Schwark, N., Heinrich, A., Freudenberg, M., Gebühr, M., & Schwark, T. (2008). The impact of DNA contamination of bone samples in forensic case analysis and anthropological research. *Legal Medicine, 10*(3), 125-130.

Walsh, K. A. (1970). Trypsinogens and trypsins of various species. *Methods Enzymol, 19*, 41–63.

Zehner, R. (2007). "Foreign" DNA in tissue adherent to compact bone from tsunami victims. *Forensic Science International: Genetics, 1*(2), 218-222.

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Table 1. Bone fragment specimens investigated in this study.

Sample	Bone Fragment	Description
1	Rib	Buried bones (buried for approximately 50 years) with possible maceration using bleach after unearthing were stored at room temperature.
2	Rib	
3	Rib	
4	Rib	
5	Rib	
6	Rib	
7	Rib	
8	Parietal bone	Autopsy specimens, possible maceration using boiling, were stored at room temperature for approximately 65 years.
9	Mandibular condyle bone	
10	Temporal bone	
11	Cerebral cranium	
12	Occipital bone	
13	Tibia	Buried for over 100 years under high heat and humidity, stored at room temperature.
14	Cerebral cranium	

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Table 2. mtDNA primer sets, names, and designations.

Primer Set	Ampicon Size (bp)	Range of DNA Amplified	Primer	Primer Sequence	Annealing Temperature	Cycle Number	Reference
Mini	170	15989-16158	F15989	5’-CCC AAA GCT AAG ATT CTA AT-3’	50	38	Edson *et al*., 2004
			R16158	5’-TAC TAC AGG TGG TCA AGT AT-3’	50	38	Edson *et al*., 2004
Midi	275	154-429	F154	5'-TTA TTT ATC GCA CCT ACG TTC AAT-3'	58	38	Edson *et al*., 2004
			R429	5'-CTG TTA AAA GTG CAT ACC GC-3'	58	38	Roche Applied Science*
HVI	443	15975-16418	F15975	5'-CTC CAC CAT TAG CAC CCA A-3'	58	38	Roche Applied Science*
			R16418	5'-ATT TCA CGG AGG ATG GTG-3'	58	38	Roche Applied Science*
HVII	414	15-429	F15	5'-CAC CCT ATT AAC CAC TCA CG-3'	58	38	Roche Applied Science*
			R429	5'-CTG TTA AAA GTG CAT ACC GC-3'	58	38	Roche Applied Science*

Primer designations are labeled according to the 5’-end of each primer as it relates to the revised Cambridge Reference Sequence.
* Linear Array Mitochondrial DNA HVI/HVII Region-Sequence Typing Kit product brochure

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Table 3. Summary of pair-wise comparisons of DNA quantitation results.

Sample	Surface Cleaning	DNA Yield (ng DNA/g bone)
1	Sanding	79.5
	Trypsin	34.8
2	Sanding	28.71
	Trypsin	22.8
3	Sanding	9.09
	Trypsin	4.95
4	Sanding	20.64
	Trypsin	41.1
5	Sanding	13.17
	Trypsin	20.85
6	Sanding	5541
	Trypsin	7341
8	Sanding	2.253
	Trypsin	14.61
9	Sanding	11.61
	Trypsin	10.5
10	Sanding	3.69
	Trypsin	3.24

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Table 4. Comparisons of average peak heights at each locus of the AmpFℓSTR® MiniFiler™ in rfu.

Locus	Average rfu Ratio (Trypsin/Sanding)	Standard Deviation	*p* Value (Pair-wise t-test)	Number of Allele Pairs
AMEL (amilogenin)	2.35	2.63	0.691	11
CSF1PO	1.33	0.87	0.571	11
D13S317	2.27	2.6	0.887	13
D16S539	1.73	1.77	0.5	11
D18S51	1.49	1.14	0.137	14
D21S11	1.49	0.81	0.809	7
D2S1338	1.8	1.62	0.081	12
D7S820	0.9	0.37	0.385	7
FGA	1.59	1.53	0.996	10

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Figure 1. Typical bone fragment specimens investigated in this study. A) Rib, buried for approximately 50 years with possible maceration using bleach after unearthing, stored at room temperature; B) parietal bone, autopsy specimen, possibly macerated using boiling, stored at room temperature for approximately 65 years; and C) tibia (no DNA detected), buried for over 100 years under high heat and humidity, stored at room temperature.

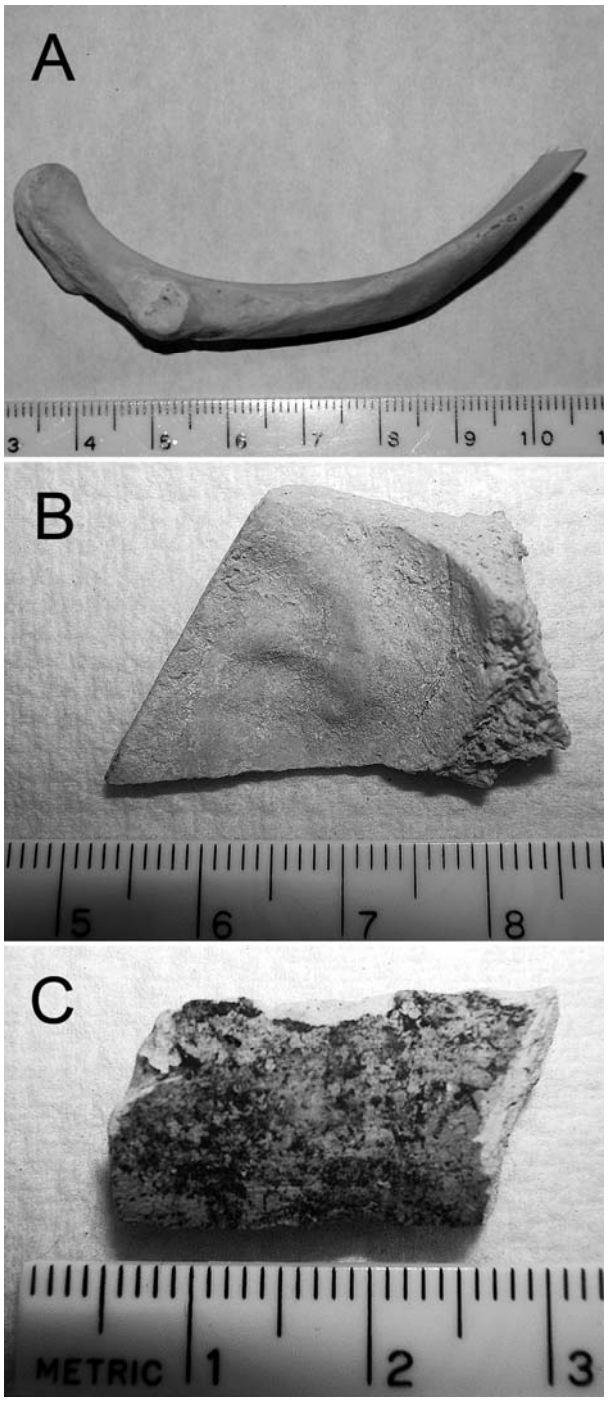

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Figure 2. The layout of the amplicons of mtDNA at hypervariable regions. Nucleotide designations are labeled according to the revised Cambridge Reference Sequence.

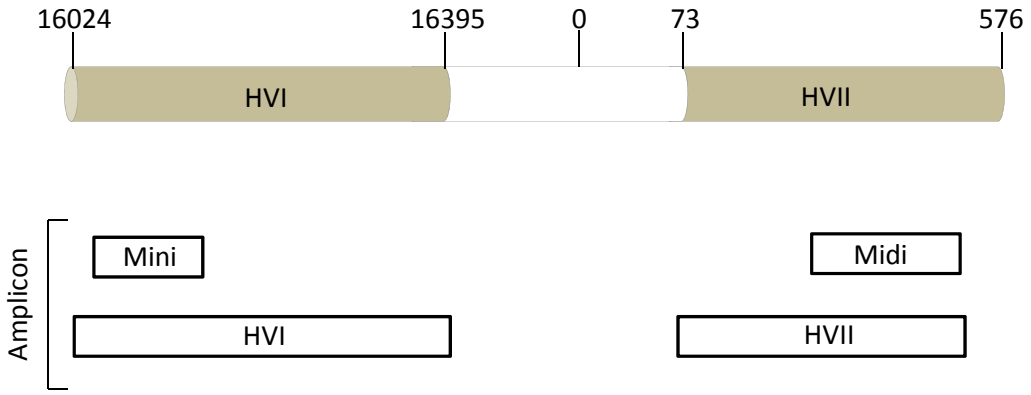

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Figure 3. The effect of trypsin treatment on STR profiles. STR profiles were obtained with the AmpFℓSTR® MiniFiler™ amplification kit. The electropherograms of A) sanded and B) trypsin-treated sample.

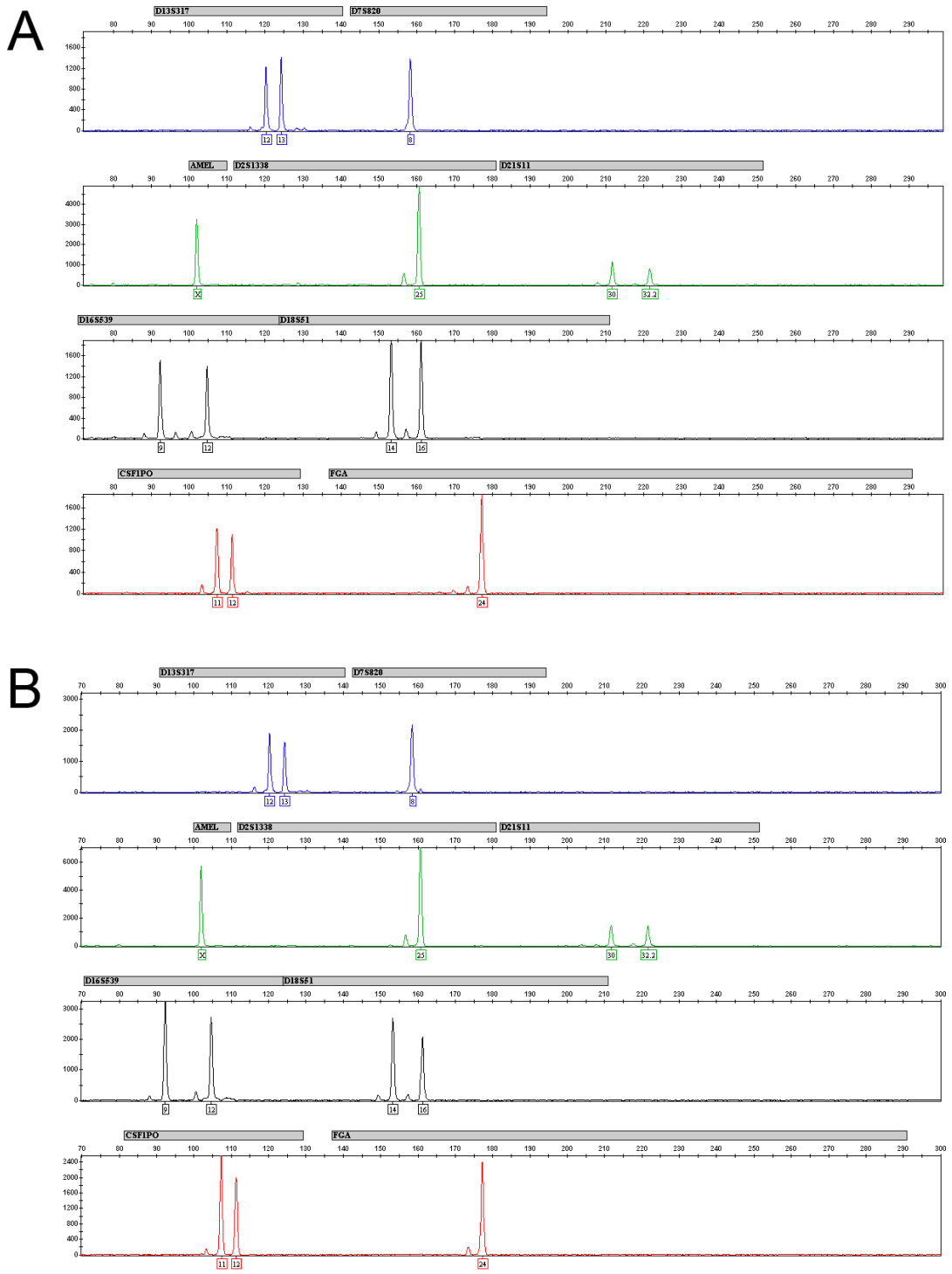

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Figure 4. The comparasion of the number of allele calls at each locus of the AmpFℓSTR® MiniFiler™ in the ratio of the number of allele calls.

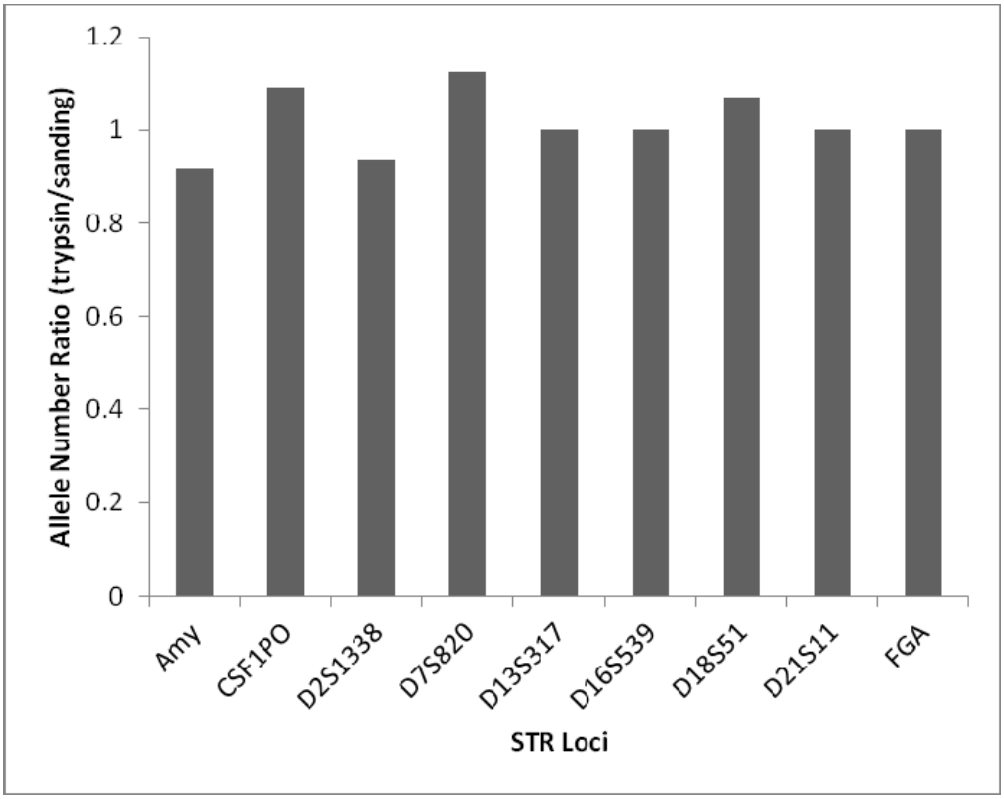

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Figure 5. Average peak heights at each locus of the AmpFℓSTR® MiniFiler™ in rfu.

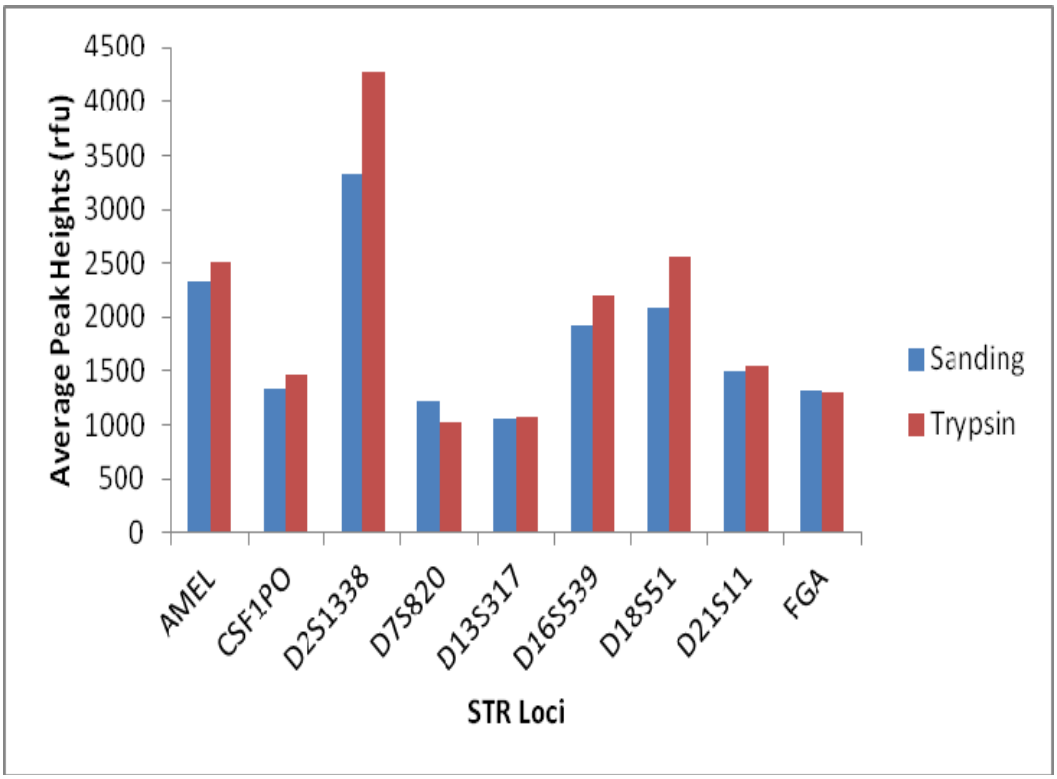

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Figure 6. Results from the Agilent Bioanalyzer 2100 showing an electropharogram with the amplification of mtDNA fragments (arrows): mini-primer set, sanded (A) versus trypsin-treated (B); midi-primer set, sanded (C) versus trypsin-treated (D); HVI and HVII-primer sets, sanded (E) versus trypsin-treated (F).

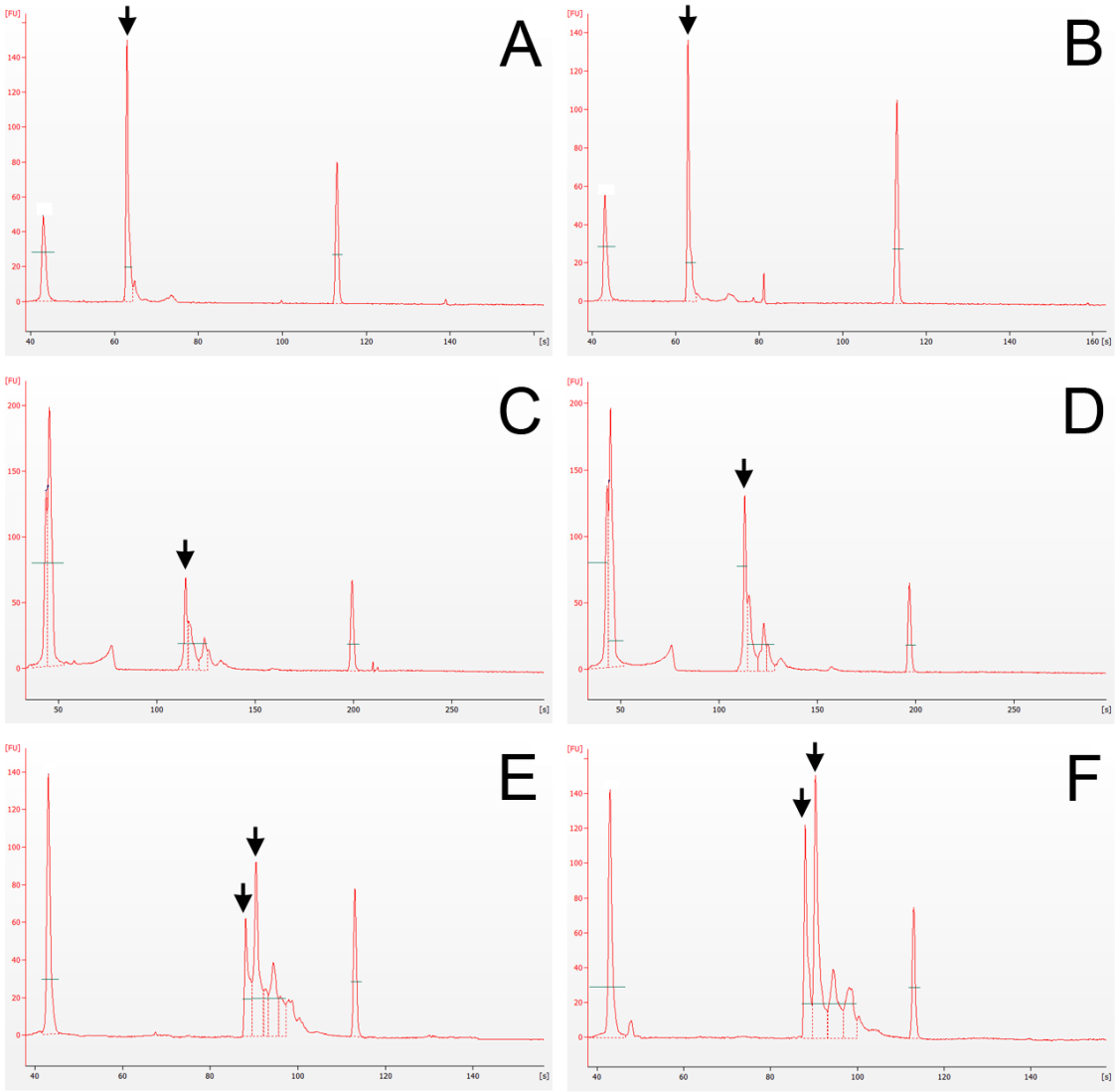

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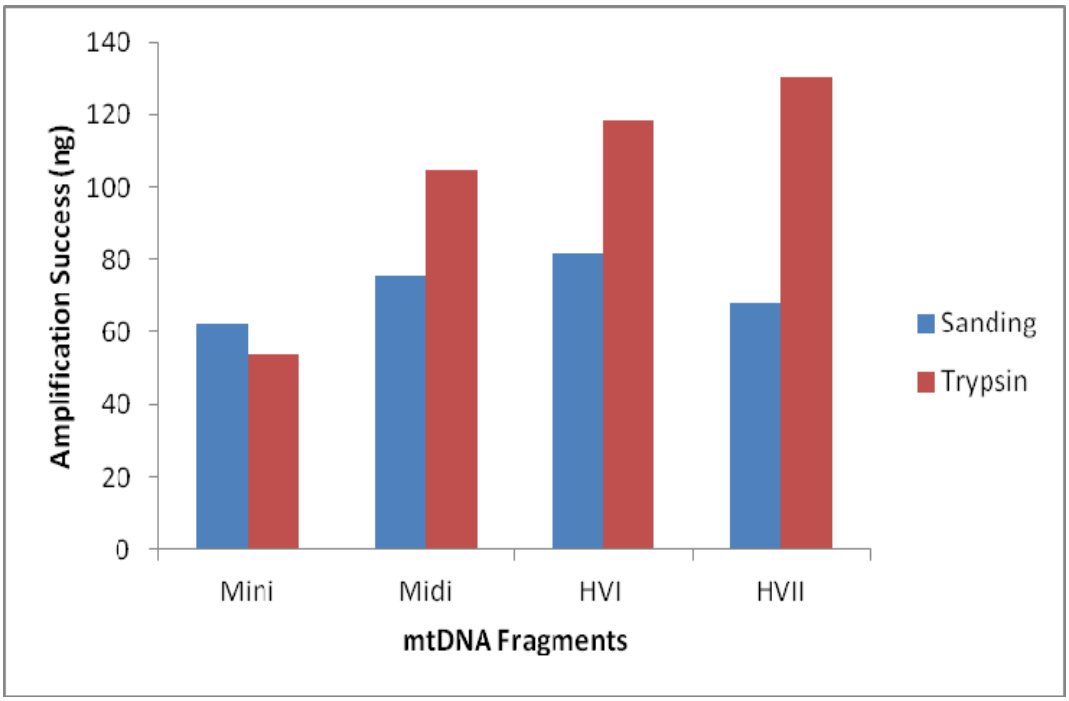
Figure 7. The amplification success of the different specific mtDNA fragments expressed as the quantities of amplified fragments.



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