This is to certify that the
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THE UTILITY OF WHOLE GENOME AMPLIFICATION IN FORENSIC DNA ANALYSIS

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M. S. degree in Forensic Science

Major Professor's Signature

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THE UTILITY OF WHOLE GENOME AMPLIFICATION IN FORENSIC DNA ANALYSIS

By

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ABSTRACT

THE UTILITY OF WHOLE GENOME AMPLIFICATION IN FORENSIC DNA ANALYSIS

By

Amy Leigh Barber

Forensic biologists are often confronted with issues of limited DNA which may restrict their ability to generate an identification profile and reserve sample for future analysis. Although PCR is commonly used to amplify specific segments of DNA, whole genome amplification (WGA) is a process which is capable of replicating an entire sample; reducing the likelihood of entirely consuming evidence. WGA of low quantity clinical samples has been studied thoroughly, but there is nothing in the literature describing its use in forensic biology. The objective of this project was to characterize Improved Primer Extension Preamplification (I-PEP) and Multiple Displacement Amplification (MDA), two methods of WGA, on samples commonly seen in forensic laboratories by testing both low quality and quantity DNA. Control DNA, artificially degraded DNA, and DNA from fresh and aged blood, hair, and bone before and after WGA were PCR amplified to test maximum amplicon lengths and to examine product yields. No difference was seen in the maximum PCR product length of DNA from fresh blood, longer product was generated from aged blood, and there was a reduction in amplicon length from hair and aged bone after WGA. Product yield was increased 20 to 2000 fold by I-PEP and 1000 to 10,000 fold by MDA. All MDA tests on low quality samples were unsuccessful and often resulted in extensive non-target DNA amplification. Overall, I-PEP and MDA increased the product yield of high quality DNA, but they were not successful on highly degraded samples.

To my mother, Kay, and my grandparents, Frederick and Irene—

my three biggest fans

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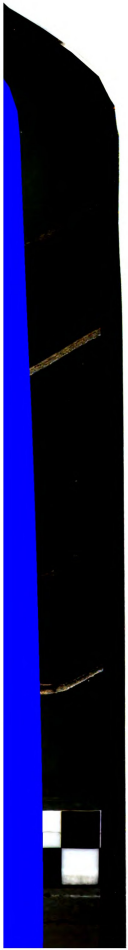
Two companies were kind enough to provide me with free samples for this project. Roche Applied Sciences donated an *Expand High Fidelity PCR System*, and Amersham Pharmacia donated a *GenomiPhi* kit.

What would you do without family? I would like to thank Grammy and Grampy Wellman, all my parents (Kay and Eric, Russell and Mary), siblings (Karin and Nathan), and nieces (Madison and Morgan) for always being here for me, even if only in spirit. And finally, I need to thank Vincent Annese for motivating me, supporting me, and for keeping me laughing.



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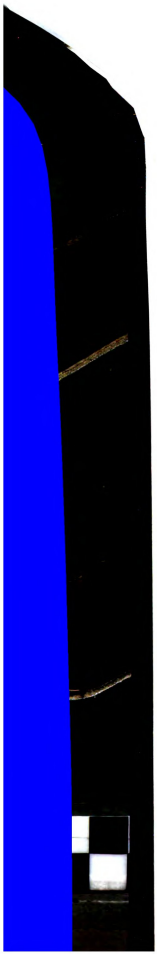

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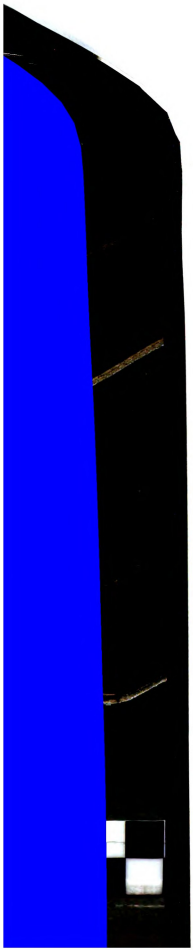

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INTRODUCTION

Overview

DNA analysis has revolutionized forensic identification. Even before DNA was considered in forensic casework, new techniques were being developed and optimized to make DNA analysis easier, faster, and more informative. With advances in molecular biology developing quickly, the use of DNA as a means of individual identification became an obvious choice for the forensic community. The advent of the polymerase chain reaction (PCR) has further revolutionized forensic identification by allowing laboratories to obtain results with ever smaller amounts of DNA. However, while amplification techniques require a relatively small amount of input DNA template, the initial quantity and quality of the extracted DNA can still be a limiting factor. In many instances, only a minute amount of evidence containing DNA is deposited at a crime scene. Likewise, adverse interim storage conditions may compromise the quality of the sample. In these or other cases, obtaining sufficient amounts of DNA to determine a sample's identification profile may be problematic. Moreover, the preservation of a sample for analysis by the defense must be considered; failure to do so may raise further legal issues.

Concerns associated with limited starting quantities of DNA in forensic samples could be alleviated by increasing the overall amount of DNA prior to beginning standard laboratory procedures. Whole genome amplification (WGA) is a procedure used to duplicate DNA, but unlike PCR, which generally amplifies small sections of the genome, WGA is capable of replicating the genome in its entirety. Currently, a handful of WGA

techniques have been developed, including Primer Extension Preamplification (PEP), Improved Primer Extension Preamplification (I-PEP), Multiple Displacement Amplification (MDA), OmniPlex, and Degenerative Oligonucleotide Primed Polymerase Chain Reaction (DOP-PCR). These methods are all backed by authors who claim product yield is increased 1 to 80μg per 100μl reaction with great fidelity (Barker et al., 2004; Dietmaier et al., 1999; Hosono et al., 2003; Kuivaniemi et al., 2002; Lovmar et al., 2003). These studies involved the use of WGA on clinical samples that are typically in good condition but are limited in quantity. Currently, no data have been published characterizing WGA for use with forensic samples in which DNA can be limiting in both quality and quantity.

The goal of this project was to characterize WGA methods for use on samples commonly encountered in forensic casework. This included determining the usefulness of WGA when very small amounts of DNA were present, and when DNA quality was poor (degraded DNA). Two WGA methods, I-PEP and MDA, were tested in detail, as previous work showed they increase DNA yields while requiring less input DNA (Dietmaier et al., 1999; Dean et al., 2002). The three remaining methods were not evaluated as they had characteristics and requirements that made them inappropriate for forensic samples (see below).

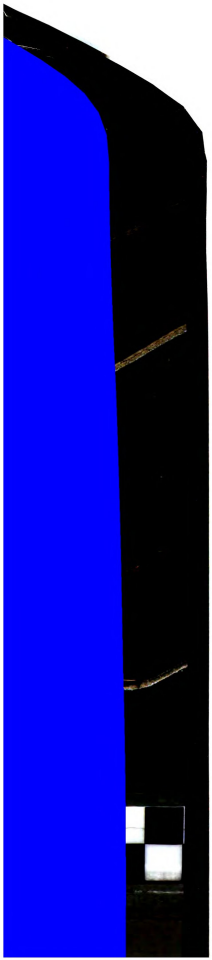
Methods of WGA

The WGA technique DOP-PCR (Telenius et al., 1992) uses a 22-base primer that has six random bases inserted between specific sequences on the 5' and 3' ends (5'-CCGACTCGAGNNNNNNATGTGG-3'). This degenerative oligonucleotide is added to the WGA mixture and cycled through an amplification reaction of 95°C for 5min,

followed by five cycles of 94°C for 1min, 30°C for 90sec, ramping to 72°C at a rate of 3.5°C/15sec and 72°C for 3min, then 35 cycles of 94°C for 1min, 62°C for1min and 72°C for 2min (with a 14sec increase per cycle) and a final extension of 72°C for 7min. Although it has been shown that DOP-PCR has the capability of amplifying greater amounts of DNA than some other methods (Dean et al., 2002), it was not characterized for use with forensic samples as the required amount of input DNA is relatively high, with a minimum of DNA from 10 to 100 cells being necessary for complete amplification (Dietmaier et al., 1999). Further, Grant et al. (2002) showed that preferential amplification of certain regions of the genome may occur due to the partially random nature of the primer. These authors also found that DOP-PCR whole genome amplified product may not be stable even when stored at –20°C, although no explanation for this instability was given.

OmniPlex (Langmore, 2002) is a relatively new method of WGA, marketed by Rubicon Genomics. With this technique, a library of the sample is made from the DNA and then WGA is performed. Very little information exists in the primary literature on this proprietary method, and therefore it was not used in this study. Future investigations will be necessary to determine its usability in forensic casework.

PEP was first introduced by Zhang et al. in 1992, and was one of the earlier methods developed for WGA. PEP is completed under conditions similar to that of typical PCR, except that random 15 base oligonucleotides are utilized. The PEP amplification procedure involves 50 cycles of denaturation of DNA at 95°C for 1 minute, annealing of primers at 37°C for 2 minutes, a 10 sec /degree ramping from 37°C to 55°C, followed by a 4 minute extension step at 55°C. Sun et al. (1999) showed that PEP WGA



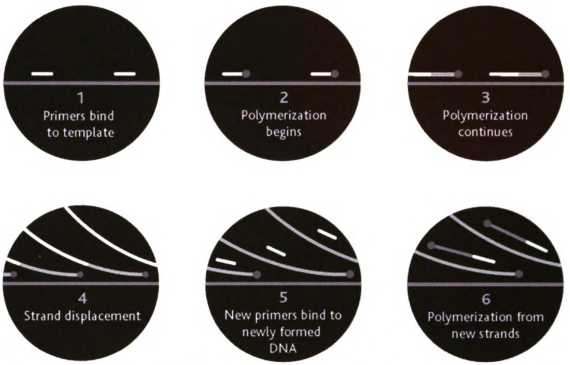
can result in a 30 fold increase in DNA. However, due to improvements made to this technique, it was not investigated for use with forensic samples.

I-PEP (Dietmaier et al., 1999) incorporates three modifications to PEP to allow for more complete DNA amplification. First, the I-PEP method utilizes a particular lysis buffer, (1X Expand High Fidelity PCR buffer (no 3), 4mg/ml proteinase K and 0.5% Tween 20) during the initial DNA extraction phase to release DNA from a sample more efficiently. Second, I-PEP includes an additional elongation step at the end of each cycle; the modified settings are 95°C for 1 minute, 37°C for 2 minutes, a 10sec/degree ramping from 37°C to 55°C, 55°C for 4 minutes, and 68°C for 30 seconds, all repeated 50 times. The final adjustment made by Dietmaier et al. (1999) is the incorporation of both Taq polymerase and a proofreading enzyme in the reaction mixture. The researchers used a product by Boehring Mannheim called Expand High Fidelity PCR System which contained Pwo DNA polymerase (the enzyme has since been substituted by the manufacturer for the enzyme Tgo DNA polymerase, though there is no functional difference between the two enzymes, Roche, 2003). Like Taq, Pwo is a heat stable, 5' to 3' polymerase, which also exhibits a 3' to 5' exonuclease activity which allows the enzyme to remove incorrectly incorporated nucleotides. This proofreading ability results in an error rate 18-fold lower than that of Taq polymerase alone (Roche, 2003). DNA polymerases present in a PEP and I-PEP WGA reactions are, in theory, capable of replicating the genome in an unbiased manner as the random 15-mer oligonucleotides can locate a complimentary sequence anywhere in the sample DNA. Like PEP, I-PEP WGA has been shown to result in a 30 fold increase in product yield (Dietmaier et al., 1999).

MDA, was first described by Dean et al. (2002; see Figure 1 for an illustration of the MDA process) and utilizes the DNA polymerase Phi 29, which has an optimal working temperature of 30°C. This polymerase is very processive, with an average product length of approximately 10kb before becoming dislodged from the template strand, however it is capable of synthesizing up to 70 kilobases (Blanco et al., 1989). In addition, Phi 29 has a proofreading 3' to 5' exonuclease activity and is capable of separating double stranded DNA encountered during synthesis. Separation of the DNA helix in front of the enzyme not only allows continuous access to the template strand, but it also frees the non-template strand of DNA to which more random primers can anneal, allowing for more Phi29 enzyme extension. Despite its recent introduction, this method has been studied extensively. Hosono et al. (2004) determined that up to a 10,000 fold increase in DNA yield can be achieved with MDA.

Two companies offer kits that can simplify the MDA reaction, including the one used in this project called GenomiPhi, manufactured by GE Healthcare Systems/Amersham Pharmacia. The MDA reaction involves denaturating the DNA template in the provided sample buffer, which contains random hexamer primers. The mixture is then chilled, in order to protect the heat sensitive Phi 29 DNA Polymerase. Next, the reaction buffer (containing dNTP's) and the enzyme mix (composed of the Phi 29 DNA polymerase, additional random hexamer primers, and a proprietary buffer) is added, and the reaction mixture is incubated at 30°C for 16 to 18 hours.

Figure 1: Schematic Illustrating the MDA Process



Dark grey dots represent the Phi29 DNA polymerase, white bars represent the random hexamer primers, solid gray lines represent template DNA, lines transitioning from white to gray represent recently amplified DNA. (Image from Amersham Biosciences, 2003)

Problems Associated with WGA

The premise of WGA is that product yield can be increased from limited starting material. Authors have demonstrated that DNA from as little as 1 cell can be amplified (Dietmaier et al., 1999, Lasken et al., 2003, Zhang et al., 1992), but such minute starting material raises concern regarding efficiency rates, loss of heterozygosity and sequence bias. Dietmaier et al. (1999) found that when using the I-PEP procedure, post-WGA testing (sequencing and RFLP-PCR) had a 100% efficiency rate (based on the percentage of successful amplifications) when the DNA from as little as 5 cells was used. This was in comparison to a 10 to 50% efficiency rate for PEP, and a 0 to 30% efficiency rate for DOP-PCR with the same amount of starting DNA. However, they determined that when

DNA was extracted from formalin fixed, paraffin embedded tissues, (a treatment that is expected to adversely affect the quality of the sample) a minimum of 30 cells was needed to achieve an efficiency rate of 100% from I-PEP samples. Dean et al. (2002) claimed that less than 10 cells are necessary to accurately amplify the genome with MDA, but did not indicate the necessary starting quality.

Another concern surrounding I-PEP is the loss of heterozygosity when a minute DNA sample is subjected to WGA. This loss is likely due to stochastic effects resulting from so few copies of each allele being present in the starting material, such that one allele is amplified by chance far more than the other, which could be problematic for any method of WGA. Dietmaier et al. (1999) showed that when a known number of cells (10 to 100) was added to PEP and I-PEP reactions, fewer cells were necessary for I-PEP to accurately amplify both alleles at 6 loci.

Finally, Dean et al. (2002) found that some sequence bias towards certain regions of the genome may occur during PEP, DOP-PCR and MDA. The likelihood of any one region of the genome being preferentially amplified over another can be attributed to priming efficiency, GC content, and proximity to the ends of the chromosome (Dean et al. 2002). In studies investigating the utility of MDA (Paez et al., 2004; Tzvetkov et al., 2005), direct sequencing and SNP analysis were used to determine the degree of amplification bias. These authors showed that MDA often under-represents certain regions of the genome, typically near the ends of a chromosome, but that approximately 99% of the genome is efficiently replicated. Dean el al. (2002) showed that sequence bias experienced during MDA is reduced in comparison to the PEP and DOP-PCR methods.

Forensic Samples Used to Characterize I-PEP and MDA

Understanding the practical limitations of I-PEP and MDA is necessary to recognize when WGA may accurately amplify a forensic sample and when it may be a waste of time, supplies, and most importantly, limited evidentiary material. To recognize each method's benefits and limitations in terms of DNA quantity and quality, high molecular weight DNA was first used to optimize each method and then to test progressively smaller amounts of whole genome amplified DNA. Next, a stock of male DNA was digested and fractionated through gel electrophoresis to evaluate the practical limitations of WGA on degraded DNA.

The use of DNA from human hair, skeletal material, and aged blood made it possible to analyze WGA on samples common to forensic biology laboratories. Head hair samples were those previously extracted by Graffy (2003), using the standard grinding protocol described by the Armed Forces DNA Identification Laboratory (AFDIL) (http://www.afip.org/Departments/oafme/dna/ afdil/protocols.html) followed by an organic extraction. DNA from aged skeletal material was previously extracted by Misner (2004) and Rennick (personal communication, February 18, 2005). One source of skeletal material originated from a 3000 year old burial mound in Kamenica, Albania. Three Albanian samples were utilized; 1 originating from a clavicle and 2 from femur. Rennick (personal communication, February 18, 2005) had previously amplified a 266bp mtDNA product from each of the bones. The second group of samples was extracted from bones (5 femur samples), that had been buried for 140 to 170 years at the Voegtly Cemetery in Pittsburgh Pennsylvania (Ubelaker and Jones, 2003). It had been previously determined that up to 220bp of mtDNA could be amplified from these samples (Misner, 2004). The

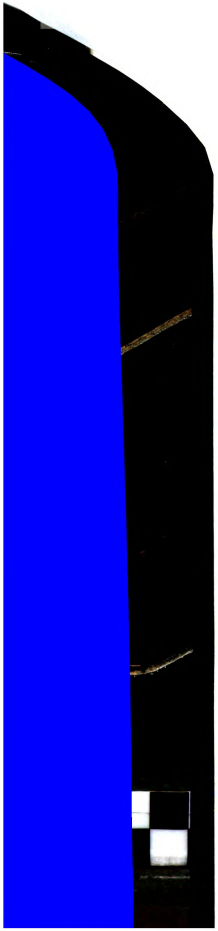
DNAs from the Voegtly Cemetery and Albania burials were isolated using a standard organic extraction which included grinding or drilling of the bone, an overnight incubation of bone particles at 55°C in digestion buffer (20mMTris, 100mMEDTA, 0.1% SDS) and proteinase K, followed by an organic extraction.

MATERIALS AND METHODS

Preparation of Artificially Degraded DNA

DNA was artificially degraded by using the endonuclease DNase I (Promega) to obtain DNA of varying lengths. The enzyme randomly nicks DNA leaving a phosphorylated 5' end, and a hydroxylated 3' end. To determine the length of time needed to digest DNA, 5000ng of male DNA was added to 10µl of 10X digestion buffer (Promega), 5 units of DNase I and 55.5µl of water. Immediately, 5µl of the mixture was removed and placed into a microcentrifuge tube containing 5µl of 20mM EDTA to stop the enzymatic activity; which served as the control, undigested sample. The reaction mixture was incubated at 17°C, where after 1, 2, 5, 10 and 30 minutes 15µl was transferred to a new microcentrifuge tube containing 5µl of 20mM EDTA. Digested DNA was denatured (95°C for 5 minutes), and separated through a 2% agarose gel, using a 100bp ladder (Promega) as a size reference. Based on the outcome of these timed experiments, it was determined that the combination of a 1 and 2 minute digestion would produce degraded DNA ranging from approximately 100 bases to DNA greater than 1500 bases in length.

The artificial degradation of DNA for WGA analysis was completed by mixing 750ng of male DNA, 1.5µl of 10X digestion buffer, 0.75 units of DNase I and water for a total reaction volume of 15µl. The DNA was incubated at 17°C for 1 and 2 minute time increments after which 7.5µl of the reaction mixture was transferred to a new microcentrifuge tube containing 2.5µl of 20mM EDTA. For later trials of DNA digestion, a 30 second incubation was also included; in these, 1125ng of male DNA was added to



2.25µl of 10X digestion buffer and 1 unit of DNase I for a total reaction volume of 22.5µl.

The degraded DNA was pooled into one microcentrifuge tube and denatured for 5 minutes at 95°C then chilled on ice. The entire volume was loaded onto a 2% low melting point agarose gel. An 8cm long gel was used to separate the digested DNA from trials 1 and 2. Greater separation was attempted for trials 3, 4 and 5 by using a 12cm gel. For this longer gel, a 2% gel of standard agarose was poured first and allowed to solidify to act as a supporting gel. Then the middle section of the standard agarose was removed to allow the low melting point agarose to be poured. DNA was separated in a cold room at a constant amperage of 60mAmps, and stained with ethidium bromide. The DNA smear was cut into six fractionated sections of <200, 200 to 400, 400 to 600, 600 to 800, 800 to 1000 and >1000 nucleotides. Each section of gel was placed into labeled micro-centrifuge tubes and heated to 65°C for approximately 5 minutes to liquefy the agarose. See Organic Extraction of DNA below for the extraction of DNA from gel.

Digestion of Fresh Blood

Fresh blood samples of 1, 2, 5, and 10µl were placed into microcentrifuge tubes and allowed to dry overnight. These were suspended in 300µl of digestion buffer (20mM Tris, 100mM EDTA, 0.1% SDS) along with 3µl of 20mg/ml Proteinase K and incubated for 1 hour at 55°C. An organic extraction was then completed on each sample; see Organic Extraction of DNA below.

Digestion of Aged Blood

In October of 2003, a sample of human blood was allowed to dry on a sterile Petri dish and left at room temperature to age in a dark, dry environment. To characterize the efficiency of the WGA methods on aged DNA, a flake of blood approximately 1mm by 1mm was taken from the 16 month old sample, suspended in 300µl of digestion buffer and 3µl of proteinase K, and incubated for 1 hour at 55°C. An organic extraction was then completed; see Organic Extraction of DNA below.

DNA from Aged Skeletal Material and Hair

To characterize the effectiveness of WGA on aged skeletal material, five Voegtly DNA samples (burials 27F, 114F, 249F, 448F, and 704F) and three Albanian samples (burial 76F, 198F and 198C) were tested. Five hair samples (24, 27, 28, 29 and 36) were also used for WGA analysis, see Introduction.

Organic Extraction of DNA

Artificially degraded DNA and DNA from blood were organically extracted. To the digestion buffer (blood) or melted agarose (artificially degraded DNA), an equal volume of phenol was added, vortexed for approximately 10 seconds, then centrifuged for 5 minutes at 12,000rpm. A second phenol extraction was completed if the sample appeared reddish or cloudy. The aqueous portion was transferred to a new microcentrifuge tube, where an equal volume of chloroform was added, vortexed for 10 seconds then centrifuged for 5 minutes at 12,000rpm. The aqueous portion was transferred to a tube where DNA was precipitated using two volumes of cold 95% ethanol and 10% 3M sodium acetate for at least one hour at -20°C. The samples were

centrifuged at 12,000 rpm, 4°C for 15 minutes, ethanol was removed, and pellets were washed with 200 µl of cold 70% ethanol, followed by a final centrifugation (5 minutes at 4°C, 12,000rpm). Ethanol was removed and the pellet was vacuum dried for 10 to 15 minutes. Artificially degraded samples were resuspended in TE (10mM Tris, 1mM EDTA) bringing the final estimated DNA concentration to 10ng/µl, based on the amount of DNA added to the initial digestion and the assumption that DNA was evenly distributed when separated into fractions. Aged and fresh blood samples were resuspended in 15µl of TE.

Whole Genome Amplification

Artificially degraded DNA was whole genome amplified using an estimated 2ng of input DNA. One microliter of DNA from blood (fresh and aged) was whole genome amplified. DNA from hair was whole genome amplified using 1µl of a 20 fold dilution. One microliter of DNA from the Albanian skeletal material was whole genome amplified. DNA from the Voegtly samples was whole genome amplified using 1µl of a 20 fold dilution. The hair and bone dilutions were based on the optimal amounts of DNA described by Graffy (2003), Rennick (personal communication, February, 18, 2005) and Misner (2004), respectively. WGA reactions with no input DNA were processed as negative controls.

Improved Primer Extension Preamplification

I-PEP WGA was performed as described by Dietmaier et al. (1999), with the exceptions of digestion buffers; see Digestion of Fresh Blood above. Using an Expand High Fidelity PCR system (Roche Applied Sciences) each reaction contained sample

DNA, 20μM random 15 base primer, 0.2mM dNTP, 1X Expand High Fidelity Buffer without magnesium, 2.5mM $MgCl_2$, and 0.75 units of Expand High Fidelity Enzyme mix, in a total reaction volume of 10μl. When WGA was performed on DNA from hair or bone, 1μl of BSA (3μg/μl stock) was included in the reaction mixture. The amplification process was performed in an ABI Gene Amp PCR system 2400. Amplification included an initial DNA denaturation for 1 minute at 94°C, followed by 50 cycles of 94°C for one minute, 37°C for 2 minutes, 0.1°C per second temperature ramp from 37°C to 55°C, 4 minutes at 55°C, and 68°C for 30 seconds.

Multiple Displacement Amplification

MDA was performed using a GenomiPhi DNA amplification kit (Amersham Biosciences). DNA was added to 4.5μl of Sample Buffer and then denatured at 95°C for 3 minutes. Samples were immediately cooled on ice, then 4.5μl of Reaction Buffer and 0.5μl of enzyme was added to the denatured sample and incubated at 30°C for 18 hours in an Eppendorf Mastercycler Gradient thermocycler. The enzyme was inactivated by heating the sample to 65°C for 10 minutes.

Purification of Whole Genome Amplified Sample

Excess primer and unincorporated nucleotides were removed from whole genome amplified samples using a Microcon YM-100 column (Millipore). Each sample was transferred to a column along with 300μl of TE and centrifuged for 12 minutes at 500x g. A total of three washes, (300μl of TE per wash), were performed. The final volume was returned to 10μl, the original WGA volume

PCR Amplification

PCR amplification was undertaken on untreated samples and whole genome amplified product, including negative controls, using both mitochondrial and nuclear DNA primers (see Tables 1 and 2). The mtDNA primers are commonly used in forensic DNA analysis, and were designed to amplify sections of the mtDNA control region. The nuclear specific primers were designed to amplify a section of the amelogenin gene—a single copy gene. The first PCR reaction was performed using primers designed to produce a small amplicon. If the amplification was successful additional PCR amplifications were attempted using different primers designed to produce larger product lengths. Tests comparing the quality and quantity of DNA from hair and bone were performed using the same primer pairs utilized by Graffy (2003), Misner (2004), or Rennick (personal communication, February 18, 2005). PCR reactions consisted of 1X Hot Start PCR buffer (Eppendorf), 0.2mM dNTP, 2 µmol of each primer, and 1 unit HotMaster Taq (Eppendorf) to a final reaction volume of 20µl for sequencing reactions or 10µl for all other reactions. Amplification reactions of DNA from bone also included 2µl of 3µg/µl BSA.

Table 1: Nuclear DNA Primers

Primer Name	Primer Sequence
Forward Amel	5' CTCCCCTCCTCCCTGTAAAA 3'
Reverse Amel 496bp	5' AGCAGAGGCAAGCAAGAGAC 3'
Reverse Amel 213bp	5' TAAACTGGGAAGCTGGTGGT 3'

Column 1 displays the primer name. The number following the reverse primer name refers to the size of the PCR amplicon obtained when used with 'Forward Amel'. Column 2 indicates the primer's sequence (Nakahori et al., 1991; Rosen and Skaletsky, 2000).

Table 2: Mitochondrial DNA Primers

Primer Name	Primer Sequence
Forward 15	5' CACCCTATTAACCACTCACG 3'
Forward 82	5' ATAGCATTGCGAGACGCTGG 3'
Forward 155	5' TATTTATCGCACCTACGTTC 3'
Reverse 285	5' GTTATGATGTCTGTGTGGAA 3'
Reverse 485	5' TGAGATTAGTAGTATGGGAG 3'
Forward 16190	5' CCCCATGCTTACAAGCAAGT 3'
Forward 15989	5' CCCAAAGCTAAGATTCTAAT 3'
Reverse 16144	5' TGACCACCTGTAGTACATAA 3'
Reverse 16410	5' GAGGATGGTGGTCAAGGGGAC 3'

Column 1 displays the primer name and location on the human mitochondrial genome where the primer's 5' end anneals (Anderson et al., 1981). Column 2 gives the primer's sequence, (AFDIL, http://www.afip.org/ Departments/oafme/dna/afdil/protocols.html).

Table 3: Primer Pairs Used for PCR Amplification and Sequencing

Artificially Degraded DNA		
Forward Amel/ Reverse Amel 200	213bp	Nuclear
Forward Amel/ Reverse Amel 496	496bp	Nuclear
Forward 16190/ Reverse 16410	220bp	Mitochondrial
Forward 15/ Reverse 285	270bp	Mitochondrial
Forward 15989/ Reverse 16410	421bp	Mitochondrial
Forward 16190/ Reverse 484	863bp	Mitochondrial
Fresh Blood		
Forward Amel/ Reverse Amel 200	213 bop	Nuclear
Forward Amel/ Reverse Amel 496	496bp	Nuclear
Forward 15989/ Reverse 285	1064bp	Mitochondrial
Aged Blood		
Forward Amel/ Reverse Amel 200	213bp	Nuclear
Forward Amel/ Reverse Amel 496	496bp	Nuclear
Aged Skeletal material—Voegtly Cemetery		
Forward 16190/ Reverse16410*	220bp	Mitochondrial
Forward 155/ Reverse 484	329bp	Mitochondrial
Aged Skeletal Material--Albanian Burial Mound		
Forward 16144/ Reverse16410*	266bp	Mitochondrial
Hair		
Forward 82/ Reverse285*	203bp	Mitochondrial
Forward Amel/ Reverse Amel 200	213bp	Nuclear

Column 1 includes the primer pair used to amplify the sample. Column 2 displays the length of the PCR product. Column 3 is the type of DNA amplified by the primer pair. * indicates the primer pair was used in previous studies. (Graffy, 2003; Misner, 2004; Rennick, unpublished)

Whole genome amplified DNA was serial diluted from 10 to 1,000,000 fold to determine the maximum dilution possible while still generating a positive result—an indication of starting DNA quantity. Amplification dilutions which were determined to produce the brightest band (greatest amount of PCR product) were used as a guideline for further analyses, (maximum PCR amplicon length, DNA sequencing, or STR analysis), however if the amplification using these dilution guidelines failed, a second amplification was attempted with more DNA. The quality of whole genome amplified product was tested using a series of primers which are designed to produce progressively longer PCR products. Cycling parameters for nuclear single locus amplifications were 94°C for 2 minutes, followed by 38 cycles of 94°C for 1 minute, 58°C for 45 seconds, and 72°C for 45 seconds. Cycling parameters for mtDNAs were 94°C for 2minutes, followed by 38 amplification cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 45 seconds. A final extension of 72°C for 5 minutes was added to the end of all PCR programs. Five microliters of PCR product was loaded onto an agarose gel; for PCR products of 400 base pairs or less a 2% agarose gel was utilized while a 1.5% agarose gel was utilized for larger PCR products.

STR Analysis of Blood and Artificially Degraded DNA

STR analysis was conducted using an Applied Biosystems Identifiler kit. Reactions consisted of 4µl AmpFlSTR PCR reaction mix, 2µl AmpFlSTR Identifiler Primer set, 1 unit AmpliTaq Gold, and DNA in a final volume of 10µl. For artificially degraded DNA size classes, 1µl of untreated DNA diluted 10 fold, and 1µl of undiluted WGA product was PCR amplified. For the PCR amplification of STR loci from fresh blood, 1µl of untreated DNA diluted 10 fold, 1µl of neat IPEP product, and 1µl of MDA

product diluted 1000 fold was amplified. STR amplification from aged blood was started with 1µl of neat untreated DNA, and 1µl of WGA product diluted 10 fold. The PCR parameters were 95°C for 11minutes, 35 amplification cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute, followed by a final extension step of 60°C for 60 minutes. Five microliters of PCR product was loaded onto a 2% agarose gel for amplification confirmation. For analysis, 1.5µl of amplified product, 24.5µl of formamide and 0.5µl of ABI GeneScan 500 Liz size standard were heat denatured (95°C for 3 minutes) then chilled on ice. STR profiles were generated on an ABI Prism 310 Genetic Analyzer (GS STR POP4 (1ml) G5.md5 module, 5 second injection, 15kV injection, 15kV run voltage, 28 minute run time) using ABI 310 Genetic Analyzer Data Collection Software version 3.0.0. ABI GeneMapper ID, version 3.1 software was used to analyze data.

Mitochondrial DNA Sequencing of Bone and Hair Samples

Amplified mtDNA was purified using a Montage PCR Centrifugal Filter Device (Millipore) (one 400µl rinse of TE, 1000x g). DNAs were sequenced using a CEQ DTCS Quick Start Kit (Beckman Coulter). Sequencing reactions consisted of 4µl DTCS Quick Start Master Mix, 2µM primer and 50 to 100fmol PCR product, with a final volume of 10µl. The sequencing parameters were 30 cycles of 95°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. Each reaction was stopped with 2.5µl of a stop solution (1.2M sodium acetate, 20mM EDTA, 4mg/mL glycogen). The DNA was precipitated in 60µl of 95% ethanol then centrifuged immediately at 14,000rpm for 15 minutes. Ethanol was removed, and pellets were rinsed twice with 200µl of 70% ethanol followed by centrifugation at 14,000rpm for 2 minutes. DNA pellets were vacuum dried for 15

minutes then resuspended in 40µl of deionized formamide. DNA sequences were obtained on a Beckman Coulter CEQ 8000 Genetic Analysis System (120 second denaturation at 90°C, 15 second injection, 20kV injection voltage, 4.2KV run voltage, 60 minute separation time), using CEQ 8000 Genetic Analysis System software, version 8.0. Sequences were aligned using Bio Edit software (Hall, 1999) and the Anderson reference sequence (Anderson et al., 1981). These results were ultimately compared to sequences previously obtained by the original researcher.

RESULTS

The characterization of I-PEP and MDA was completed using various forensic sample types. Whole genome amplified product was compared to untreated DNA in terms of quality, (whether there was a change in the maximum PCR product length), quantity (which dilution factors successfully amplified DNA), and downstream analysis, including DNA sequencing and STR testing. Figure 2 presents an image of an agarose gel of whole genome amplified product. Notice that a smear of DNA is seen in each lane, even when no DNA was added to the WGA reaction. Also, the MDA lanes are brighter than the I-PEP lanes, and the positive lanes are slightly brighter than the reagent blanks.

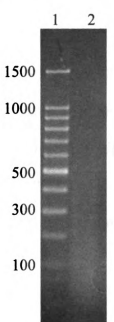
Figure 2: Image of I-PEP and MDA Whole Genome Amplified Product

Lane 1 is a MDA reagent blank. Lane 2 is a MDA reaction with DNA added. Lane 3 is an I-PEP reagent blank. Lane 4 is an I-PEP reaction with DNA added.

Quality of I-PEP and MDA Product from Artificially Degraded DNA

The maximum nuclear and mitochondrial PCR amplicon that could be obtained from artificially degraded fractions was determined. DNA was degraded with DNase I five times, four with DNA from a commercial stock (which was created from a mixture of sources; Trials 1 through 4) and once from a single source of DNA (Trial 5). Figure 3 displays an image of DNase I digested DNA separated by gel electrophoresis. Notice the digested DNA is visible below the 100bp marker and extends to the top of the image, near the 1500bp band, and that the intensity of the DNA becomes progressively weaker as the length of the digested DNA becomes longer. Table 4 displays results for maximum PCR product length experiments (nuclear or mtDNA) between fractionated DNA and fractionated DNA following both methods of WGA.

Figure 3: DNase I Digested Male DNA



Lane 1 is the ladder. Lane 2 is digested DNA.

When testing untreated DNA from trial 1, a 496bp nuclear product was generated from all size classes except the <200 and >1000 fractions. A 496bp nuclear product was only generated from the >1000 size class from trial 2 and the 600 – 800, and 800 – 1000 sized classes from trial 4. The 496bp amplification was unsuccessful from all trial 3 size classes and was not attempted in trial 5. A 213bp nuclear product was amplified from untreated DNA in all but six reactions throughout all trials and size fractions. The amplification success of the 213bp nuclear product across the five trials of DNA does not seem directly related to size class; the 200 – 400, 400 – 600 and >1000 size amplified 5 out of 5 times, the 600 – 800 size amplified 4 out of 5 times, the 800 – 1000 size amplified 3 out of 5 times, and the <200 size amplified 2 out of 5 times. An 865bp mtDNA product was generated in all size classes from trials 1 and 2 but not from subsequent trials. A 270bp mtDNA product was generated from four of the trial 3 size classes while DNA from the <200 and the 600 – 800 size class had a maximum product of 220bp. All size classes from trial 4 had a maximum mtDNA product of 220bp. From trial 5, 270bp was the maximum mtDNA product length generated from all size classes. There does not seem to be a direct relationship between size class and amplification success of a 270bp mtDNA product across the 5 digestion trials; the 200 – 400 class amplified 4 out of 5 times, the 400 – 600, 800 – 1000 and >1000 sizes amplified 3 out of 5 times, the 600 – 800 size amplified 2 out of 5 times, and the <200 size amplified 1 out of 5 times.

When testing I-PEP treated DNA, a 496bp nuclear product was successfully amplified from all trial 1 size classes. A 496bp nuclear product was only generated from the >1000 fraction from trial 2, and the 600 – 800, and 800 – 1000 size classes from trial

Table 4: Nuclear and mtDNA Amplification of Artificially Degraded DNA

Fraction sizes are located at the beginning of each row, followed by the attempted PCR product. 'Nuc' indicates primers were nuclear specific, 'mt' indicates primers were mtDNA specific. Numbers following the DNA type indicate the PCR product size. Treatments (untreated, I-PEP or MDA) and degradation trial numbers are specified across the top of columns. + indicates successful amplification, - indicates PCR failure. [-] indicates amplification was not successful, but multiple nonspecific bands were also present. [+] indicates amplification not successful, but multiple nonspecific bands were also present. No data available is indicated as n.d., largest mt indicated maximum mtDNA amplicon obtained

		Trial 1			Trial 2			Trial 3			Trial 4			Trial 5			Summary Data		
		No WGA	1-PEP	MDA	No WGA	1-PEP	MDA	No WGA	1-PEP	MDA	No WGA	1-PEP	MDA	No WGA	1-PEP	MDA	No WGA	1-PEP	MDA
<200	Nuc-213bp	-	+	+	+	+	+	+	-	-	-	-	[-]	-	+	-	2/5	3/5	3/5
	Nuc-496bp	+	+	[+]	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	1/4	1/4	1/4
	mt-270bp	-	+	+	-	+	+	-	-	-	-	-	-	+	+	n.d.	1/5	3/5	2/4
	largest mt	865	865	270	865	865	865	220	220	220	220	220	220	270	270	n.d.	/	/	/
200-400	Nuc-213bp	+	+	+	+	+	+	+	+	+	+	-	[+]	+	+	-	5/5	4/5	4/5
	Nuc-496bp	+	+	[+]	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	1/4	1/4	1/4
	mt-270bp	+	+	+	+	+	+	+	+	[-]	-	-	-	+	+	n.d.	4/5	4/5	2/4
	largest mt	865	865	270	865	865	865	270	270	220	220	220	220	270	270	n.d.	/	/	/
400-600	Nuc-213bp	+	+	+	+	+	+	+	+	+	+	+	[+]	+	+	-	5/5	5/5	4/5
	Nuc-496bp	+	+	[-]	-	-	-	-	-	-	-	-	-	n.d.	n.d.	n.d.	1/4	1/4	0/4
	mt-270bp	+	+	+	+	+	+	+	+	[-]	-	+	-	+	+	n.d.	3/5	5/5	2/4
	largest mt	865	865	270	865	865	865	270	270	220	220	270	220	270	270	n.d.	/	/	/
600-800	Nuc-213bp	+	+	+	+	+	+	-	+	+	+	+	[-]	+	+	-	4/5	5/5	3/5
	Nuc-496bp	+	+	[+]	-	-	-	-	-	-	-	+	-	n.d.	n.d.	n.d.	1/4	2/4	1/4
	mt-270bp	+	+	+	-	+	+	-	-	[-]	-	+	-	+	+	n.d.	2/5	4/5	2/4
	largest mt	865	865	270	865	865	865	220	220	220	220	270	220	270	270	n.d.	/	/	/
800-1000	Nuc-213bp	+	+	+	+	+	+	-	+	-	-	+	[+]	+	+	-	3/5	5/5	3/5
	Nuc-496bp	+	+	[-]	-	-	-	-	-	-	-	+	-	n.d.	n.d.	n.d.	1/4	2/4	0/4
	mt-270bp	+	+	+	-	+	+	+	+	[-]	-	+	-	+	+	n.d.	3/5	5/5	2/4
	largest mt	865	865	270	865	865	865	270	270	220	220	270	220	270	270	n.d.	/	/	/
>1000	Nuc-213bp	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	5/5	5/5	3/5
	Nuc-496bp	+	+	[+]	-	+	+	-	-	-	-	-	-	n.d.	n.d.	n.d.	1/4	2/4	2/4
	mt-270bp	-	+	+	+	+	+	+	+	[-]	-	+	-	+	+	n.d.	3/5	5/5	2/4
	largest mt	865	865	270	865	865	865	270	270	220	220	270	220	270	270	n.d.	/	/	/

4 (no 496bp nuclear amplifications were attempted from trial 5). A 213bp nuclear product was amplified from I-PEP DNA in all but three reactions throughout all trials and size fractions. The three reactions which did not amplify were from fractions less than 400 nucleotides in length. The ability to replicate nuclear PCR amplification results using I-PEP treated samples from all trials was related to the fraction size. DNA from all fractions 400 bases and larger were successfully amplified from each 213bp nuclear DNA attempt. Successful PCR amplification of a 213bp product from the <200 and 200 – 400 nucleotide size classes occurred 3 and 4 times out of 5, respectively.

An 865bp mtDNA product was generated from all trial 1 and 2 size classes after I-PEP. Size fractions greater than 800 nucleotides from trial 3 had a maximum mtDNA product of 270bp; all other reactions from this trial had a maximum product length of 220bp. Fractions from trial 4 greater than 400 nucleotides had a maximum mtDNA product of 270bp, while all others had a maximum product of 220bp. All size classes from trial 5 had a maximum mtDNA product length of 270bp. The replication of mtDNA PCR amplification results using I-PEP treated samples from all trials was related to the fraction size. All attempted amplifications of a 270bp mtDNA amplicon were successful from the 400 – 600, 800 – 1000 and >1000 size classes. Successful PCR amplification of a 270bp product from the 200 – 400 and 600 – 800 size classes occurred 4 times out of 5 times, and amplifications from the <200 class were successful 3 out of 5 times.

The amplification of a 496bp nuclear product from MDA treated DNA was successful from the <200, 200 – 400, 600 – 800, >1000 size classes from trial 1, and the >1000 size class from trial 2. A 213bp nuclear product was generated from all size classes from trial 1 and 2. A maximum nuclear product length of 213bp was generated

from the 200 – 400, 400 – 600, 600 – 800 size classes from trial 3, and the 200 – 400, 400 – 600, 800 - 1000, and >1000 size classes from trial 4. No nuclear product was generated from the DNA digested in trial 5. There was no direct relationship between MDA size classes and amplification success of a 213bp nuclear product across the 5 trials; a 213bp nuclear DNA product was amplified from the 200 – 400 and 400 – 600 size classes 4 out of 5 times, the <200, 600 – 800, 800 – 1000, and >1000 MDA nucleotide fractions was successful 3 out of 5 attempts. Multiple non-target DNAs were amplified in many of the PCR reactions of MDA product; these bands were not observed in reagent blank PCR reactions.

MDA treated DNA from trial 1 was not tested for an 865bp mtDNA product due to multiple bands in the 213bp nuclear PCR reactions, the largest mtDNA product tested was 270bp. Trial 2 had a maximum mtDNA product length of 865bp in all size classes, while the maximum mtDNA product from trials 3 and 4 was 270bp. No mtDNA reactions were attempted from trial 5 because no nuclear product was obtained. The ability to reproduce mtDNA PCR amplification results using MDA treated samples from all trials was not directly related to the fraction size; each size class was successfully amplified 2 out of 4 times.

One inconsistency observed across trials of digestion was the larger PCR products generated from trials 1 and 2 in comparison to trials 3, 4, and 5. A 496bp nuclear product was amplified in all size classes from trial 1, and as seen in the 'largest mt' rows of Table 4, an 865bp mtDNA was generated in all size classes in untreated and I-PEP samples from trial 1 and 2, and in all size classes of MDA samples from trial 2. DNA digested in trials 3, 4 and 5 had a maximum mtDNA product of either 220 or 270bp.

Quality of I-PEP and MDA Product using DNA from Fresh and Aged Blood

Table 5 displays the results of PCR amplifications from fresh and aged blood samples with and without WGA. A 496bp nuclear product was PCR amplified from untreated and whole genome amplified DNA extracted from 1, 2, 5, and 10µl of fresh blood. As amplification was successful from all blood volumes, all further tests were completed on the 1µl sample. All amplifications using DNA from fresh blood were successful, testing a maximum nuclear DNA product of 496bp and a maximum mtDNA product of 1065bp. The longest nuclear and mtDNA PCR product obtainable from untreated aged blood was 496bp and 865bp, respectively. After I-PEP and MDA, a 496bp nuclear, and 1064bp mtDNA amplicon was amplified. Multiple bands were observed in the MDA nuclear DNA amplification reactions.

Table 5: Amplification of Nuclear and Mitochondrial DNA from Fresh and Aged Blood

		Nuc 213bp	Nuc 496bp	mt 664bp	mt 865bp	mt 1064bp
Fresh	No WGA	+	+	nd	nd	+
	I-PEP	+	+	nd	nd	+
	MDA	+	+	nd	nd	+
Aged	No WGA	-	+	-	+	-
	I-PEP	nd	+	+	+	+
	MDA	nd	[+]	+	+	+

The top row displays the attempted nuclear (nuc) or mitochondrial (mt) PCR product lengths. + indicates successful amplification, - indicates PCR failure. [+] indicates amplification was successful, but multiple nonspecific bands were also present. 'nd' indicates that no data is available since the amplification was not attempted. White rows are results from DNA without WGA, light gray rows are results from I-PEP whole genome amplified DNA, dark gray rows are results from MDA whole genome amplified DNA

Quality of I-PEP and MDA Product using DNA from Hair Shafts

Table 6 displays the results of attempted DNA amplifications from hair shaft samples with and without WGA. No nuclear DNA could be amplified from the untreated or whole genome amplified hair samples. According to Graffy (2003), the five DNA samples extracted from hair had a maximum mtDNA amplicon size of 865bp. After I-PEP, maximum mtDNA PCR product length was reduced for all five samples; DNA from two samples (number 24 and 27) had a maximum amplicon length of 664bp, while three had a maximum product length of 203bp (number 28, 29, and 36). The amplification of mtDNA from all MDA samples was unsuccessful but multiple non-target DNAs were amplified.

Table 6: Amplification of Mitochondrial and Nuclear DNA from Hair Shafts

	mtDNA									nucDNA	
	No WGA *	I-PEP	MDA	No WGA*	I-PEP	No WGA*	I-PEP	No WGA*	I-PEP	No WGA	I-PEP
Sample #	203 bp-mt			664 bp-mt		865bp-mt		1064bp-mt		213 bp-nuc	
24	+	+	[-]	+	+	+	-	-	-	-	-
27	+	+	[-]	+	+	+	-	-	-	-	-
28	+	+	[-]	+	-	+	-	-	-	-	-
29	+	+	[-]	+	-	+	-	-	-	-	-
36	+	+	[-]	-	-	+	-	-	-	-	-

DNA type is indicated at the top of each column, followed by DNA treatment, the third row displays the attempted nuclear (nuc) or mitochondrial (mt) product lengths, + indicates successful amplification, - indicates amplification failure. [-] indicates amplification was unsuccessful, but multiple non-target bands were generated. Sample ID and columns marked with a * indicates data are reproduced from Graffy (2003)

Quality of I-PEP and MDA Product from Aged Skeletal Material

Given the age and highly degraded nature of the skeletal samples, nuclear DNA was not tested. Attempted PCR amplifications of mtDNA from aged skeletal material are

shown in Table 7. Misner (2004) determined that up to a 220bp mtDNA product could be amplified from the Voegtly bones; Rennick (personal communication) successfully amplified a 266bp mtDNA product using nested PCR from the Albanian samples; these results were used to compare maximum product lengths generated from whole genome amplified DNA. Of the eight I-PEP samples, only one was successfully PCR amplified (Voegtly bone sample 704; Table 7, column 4; Figure 4). MDA product was determined to be unusable due to the high degree of non-target bands after PCR amplification.

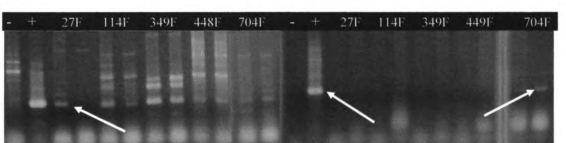
Table 7: Amplification of Mitochondrial DNA from Aged Skeletal Material

		No WGA*	I-PEP	MDA
		220 bp		
Voegtly	27F	+	-	[+]
	114F	+	-	[+]
	349F	+	-	[+]
	448F	+	-	[+]
	704F	+	+	[+]

		No WGA**	I-PEP	MDA
		266bp		
Albanian	76F	N	-	-
	198 F	N	-	-
	198C	N	-	-

Treatment type is displayed on the top of each column followed by the attempted mtDNA PCR product length. * indicates data were obtained from Misner (2004), ** data obtained from Rennick (personal communication), + indicates successful amplification, - indicates amplification failure, N indicates nested PCR was completed to obtain a positive result, [+] indicates sample amplification was successful, but multiple nonspecific bands were present.

Figure 4: 220bp mtDNA Product from MDA and I-PEP Treated Voegtly Cemetery Bone Samples



Lanes 1 through12 are PCR amplified aged bone samples which underwent MDA. Lanes 12 through 24 are PCR amplified I-PEP aged bone samples. Each sample is present twice, the first was PCR amplified at 10 fold dilution, the second at a 100 fold dilution. Arrows denote positive amplifications.

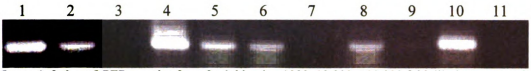
DNA Yield Comparisons using Serial Dilutions

Relative DNA concentrations before and after WGA were examined by conducting 10 fold dilutions until PCR amplifications were no longer successful. Dilution factors presented below are in reference to 1µl of neat (undiluted) untreated DNA. Since WGA was completed in a 10µl reaction (where 1µl of template was added to 9µl of reaction mixture), 1µl of whole genome amplified product from fresh and aged blood began 10 fold more dilute than 1µl of untreated DNA. DNA from hair that was to be treated with I-PEP was first diluted 20 fold, based on Graffy (2003). Taking this and the 10 fold dilution from the WGA procedure into account, I-PEP hair samples ended up being 200 times more dilute than the original hair DNA. No yield comparisons were completed on MDA product from hair because quality experiments were unsuccessful. No yield comparisons were conducted on WGA product from aged skeletal material since only one sample amplified.

Quantity of I-PEP and MDA Product using DNA from Fresh and Aged Blood

Table 8 displays the amplification results of untreated, I-PEP and MDA samples from fresh and aged blood after serial dilutions. PCR amplification of untreated DNA from fresh blood was successful when input PCR DNA was diluted 10 fold. The PCR amplification of I-PEP DNA from fresh blood was successful when DNA was 1000 times more dilute than untreated DNA, while MDA DNA 10,000 times more dilute than untreated DNA was successfully amplified. Figure 5 displays an image of a 496bp nuclear product from serial diluted untreated, I-PEP and MDA DNA.

Figure 5: 496bp Nuclear PCR Product of I-PEP, MDA and Untreated DNA Diluted from Fresh Blood



Lanes 1, 2, 3 are I-PEP samples from fresh blood at 1000, 10,000, 100,000 fold dilutions, respectively. Lanes 4, 5, 6, 7 are MDA samples from fresh blood at 1000, 10,000, 100,000, 1,000,000 fold dilutions, respectively, Lanes 8 and 9 are untreated DNA at 10 and 100 dilutions, respectively, Lane 10 is a positive control, Lane 11 is a negative control.

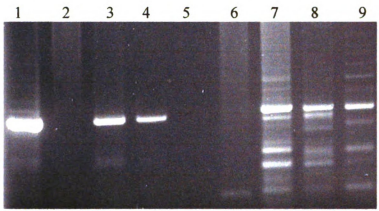
Without WGA on DNA from aged blood, a 496bp nuclear DNA product was amplified using 1µl of sample. After WGA, PCR amplification of I-PEP product 100 times and MDA product 1,000 times more dilute than untreated DNA was successful. As seen in Figure 6, diluted whole genome amplified products were successfully amplified, but extra bands were generated in the MDA reactions.

Table 8: PCR Amplification of Nuclear DNA (496bp product) from Fresh and Aged Blood

		neat	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000
Fresh	No WGA	+	+	-	-	-	-	-
	I-PEP	--	+	+	+	+	-	-
	MDA	--	+	+	+	+	+	-
Aged	No WGA	+	-					
	I-PEP	--	+	+	-			
	MDA	--	[+]	[+]	[+]	-		

The top row displays the attempted dilutions, sample types are displayed in the first column, + indicates successful amplification, - indicates amplification failure. [+] indicates amplification was successful, but multiple nonspecific bands were also present. - -indicates the reaction is not possible due to the 10 fold dilution from the WGA procedure. White rows are results from DNA without WGA, light gray rows are results from I-PEP DNA, dark gray rows are results from MDA DNA.

Figure 6: 496bp Nuclear PCR Product of I-PEP and MDA DNA Diluted from Aged Blood



Lane 1: positive control; lane 2: I-PEP reagent blank; Lanes 3 to 5: I-PEP DNA from aged blood at 1/10, 1/100, 1/1000 fold dilutions, respectively; Lane 6: MDA reagent blank; Lanes 7 to 9: MDA DNA from aged blood at 1/10, 1/100, 1/1000 fold dilutions, respectively.

Quantity of I-PEP and MDA Product using DNA from Hair

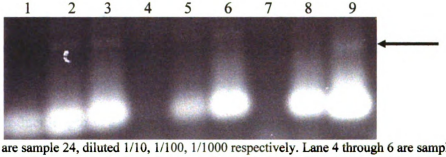
Yield experiments on DNA from hair were performed on mtDNA since it is the only type of DNA that can be regularly amplified from hair shafts. Experiments testing the quantity of I-PEP product were compared to dilutions attempted by Graffy (2003); results are displayed in Table 9. MDA quantity experiments were not attempted since multiple bands were observed in initial PCR reactions. Without WGA, PCR product was obtained from two samples diluted 100 fold, (numbers 24, and 29) the remaining three samples (number 27, 28 and 36) were amplified with a 10 fold dilution. After I-PEP, PCR product from one sample (number 29) was amplified from starting DNA 20 times more dilute than untreated DNA. PCR product from two hair samples, (numbers 24 and 36) could be obtained from starting DNA 200 times more dilute, and two samples (numbers 27 and 28) 2000 times more dilute than the same sample without WGA. As indicated in the I-PEP columns of Table 9 and in Figure 7, some PCR reactions were inhibited in the more concentrated reactions.

Table 9: Amplification of Serial Diluted mtDNA from Hair Shafts

	1/10	1/100	1/200	1/2000	1/20,000
Sample ID	No WGA*		I-PEP WGA		
24	+	+	I	+	+
27	+	-	I	I	+
28	+	-	I	I	+
29	+	+	+	+	-
36	+	-	+	+	-

The top row of each column indicates the attempted dilution. + indicates successful amplification, - indicates amplification failure. I indicates amplification was inhibited. Sample ID and data marked with a * were reproduced from Graffy (2003).

Figure 7: 203bp mtDNA PCR Product of I-PEP Diluted DNA from Hair Shafts



Lanes 1 through 3 are sample 24, diluted 1/10, 1/100, 1/1000 respectively. Lane 4 through 6 are sample 27 diluted, 1/10, 1/100, 1/1000 respectively. Lane 7 through 9 are sample 28, diluted 1/10, 1/100, 1/1000 respectively. Note the reactions which contain no product also show weak or no primer bands. The arrow points to positive bands.

STR analysis of Artificially Degraded DNA and Fresh and Aged Blood Samples

The ABI Identifiler kit is a multiplex of primers designed to amplify 16 loci (13 CODIS STR loci, 1 gender marker, and 2 additional STR loci) ranging in length from 100bp to 360bp. STR analysis was completed on artificially degraded DNA, fresh and aged blood samples using successful dilutions from initial experiments as DNA input guidelines. However, in all cases a second amplification was needed in order to add more DNA into these multiple loci amplification reactions. STR testing on artificially

degraded DNA was completed using product from the fifth trial of degradation (Table 10). Un-degraded DNA from this source was used as the reference sequence.

More loci were amplified from untreated artificially degraded fractions than whole genome amplified fractions. The untreated artificially degraded DNA from the 400 – 600, 600 – 800, and 800 – 1000 fractions matched the known profile at 14, 16 and 15 loci, respectively. Complete amplification of both alleles from the 200 – 400 size class occurred at 7 loci, partial profiles were obtained for 3 loci, and 4 loci were not typed due to multiple peaks per locus. No loci amplified from the untreated >1000 nucleotide fraction. I-PEP DNA from the >1000 fraction, matched the known profile at four loci, and an incomplete profile was generated at seven additional loci. MDA DNA from the >1000 fraction matched at nine loci, and a partial profile was obtained from 2 loci. The alleles from whole genome amplified DNA which matched the known profile were from loci of shorter product length in the multiplex; the longest loci in the multiplex are CSF1PO, D2S1338, and D18S51. No profiles were generated from artificially degraded DNA fractions below 1000 nucleotides after WGA.

Table 10: STR Profiles of Artificially Degraded DNA (Trial 5, Single Source DNA)

	Known		No WGA												I-PEP		MDA	
Locus ID	Profile		200-400		400-600		600-800		800-1000		>1000				>1000		>1000	
D8S1179	13	14	13	14	13,	14	13	14	13	14					13	14	13	14
D21S11	31	32.2	31,31.2,32.2		31	32.2	31	32.2	30, 31, 32.2								31	32.2
D7S820	10	11			10		10	11	10	11								
CSF1PO	11	12	11	12	11	12	11	12	11	12								
D3S1358	15	16	15, 15.2, 16		15	16	15	16	15	16						16	15	16
THO1	7	8	7	8	7	8	7	8	7	8					7	8	7	8
D13S317	12	12	11	12	12	12	12	12	12	12					12	12	12	12
D16S539	9	11	9, 10, 11		9	11	9	11	9	11						11		11
D2S1338	23	25	23			25	23	25	23	25							23	25
D19S433	14	15	14, 15, 15.2		14	15	14	15	14	15					13, 14	15	14	15
vWA	15	16	15	16	15	16	15	16	15	16						16		
TPOX	8	10	8	10	8	10	8	10	8	10					8, 9	10		10
D18S51	16	18		18	16	18	16	18	16	18					16			
D5S818	11	12	11	12	11	12	11	12	11	12							11	12
FGA	23	25			23	25	23	25	23	25						23	23	25
AMEL	X	Y	X	Y	X	Y	X	Y	X	Y					X	Y	X	Y

Treatment type and fraction size is indicated across the top of each column. The first column indicates the **name of each** locus. Alleles highlighted in gray matched the known profile. AMEL is an abbreviation for **amelogenin,** the sex marker.

Five of the thirteen STR loci from untreated fresh blood, diluted 10 fold, matched the known sample (Table 11). Nine additional loci displayed false homozygous peaks resulting from allele drop-out, and no results were obtained at 2 loci (Figure 8). There was no relationship between amplification success and locus length. STR tests of I-PEP DNA from fresh blood, using 1µl of WGA product, matched the known profile at all 16 loci (Figure 9). MDA product from fresh blood, diluted 1000 fold, matched at 14 of the 16 loci, (Figure 10). False homozygous peaks occurred at two loci, D3S1358 (a small locus) and TPOX (a medium sized locus).

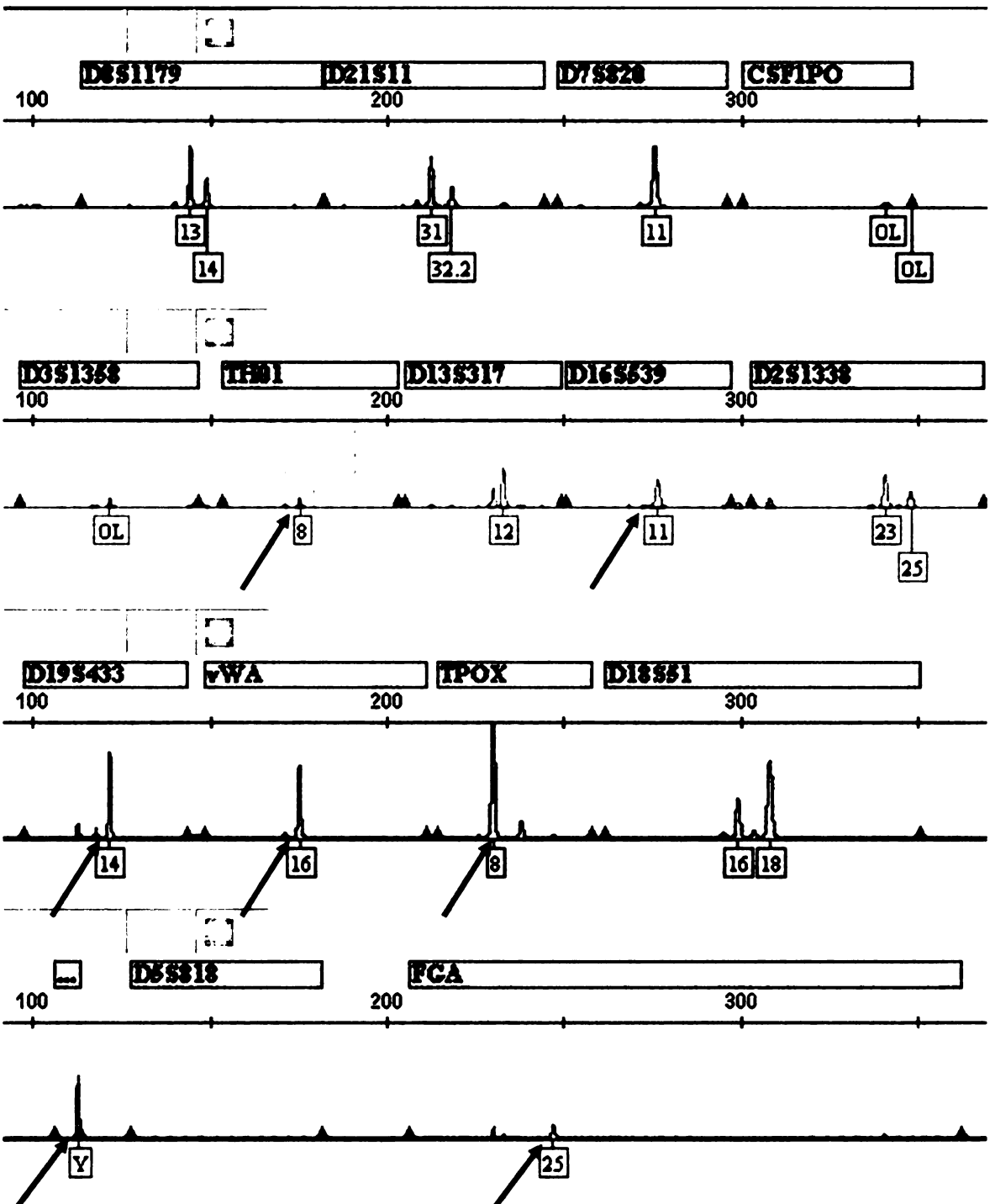
No STR profile was generated from untreated aged blood samples even when 2µl of sample (double the amount from prior experiments) was added to the amplification reaction. STR analysis of I-PEP DNA from aged blood matched at 2 loci, D19S433 and Amelogenin, which both have a short PCR product length. Partial profiles for 2 additional loci (D8S1179 and D5S818; both short loci) matched the reference profile. Aged blood DNA which underwent MDA matched the known sample profile at one allele (D8S1179).

Table 11: STR Profiles of DNA from Fresh and Aged Blood with and without WGA

	Sample		Fresh-No WGA		Fresh IPEP		Fresh MDA		Aged-No WGA		Aged I-PEP		Aged MDA	
Locus ID	Profile		1/10 dilution		1/10 dilution		1/1000 dilutio		neat		1/10 dilution		1/10 dilution	
D8S1179	13	14	13	14	13	14	13	14			13			14
D21S11	31	32	31	32.2	31	31.2	31	32.2						
D7S820	10	11		11	10	11	10	11						
CSF1PO	11	12			11	12	11	12						
D3S1358	15	16		16	15	16	15							
THO1	7	8		8	7	8	7	8						
D13S317	12	12	12	12	12	12	12	12						
D16S539	9	11		11	9	11	9	11				11		
D2S1338	23	25	23	25	23	25	23	25						
D19S433	14	15	14		14	15	14	15			14	15		
vWA	15	16		16	15	16	15	16						
TPOX	8	10	8	10	8	10		10						
D18S51	16	18	16	18	16	18	16	18						
D5S818	11	12			11	12	11	12				12		
FGA	23	25		25	23	25	23	25						
AMEL	X	Y		Y	X	Y	X	Y			X	Y		

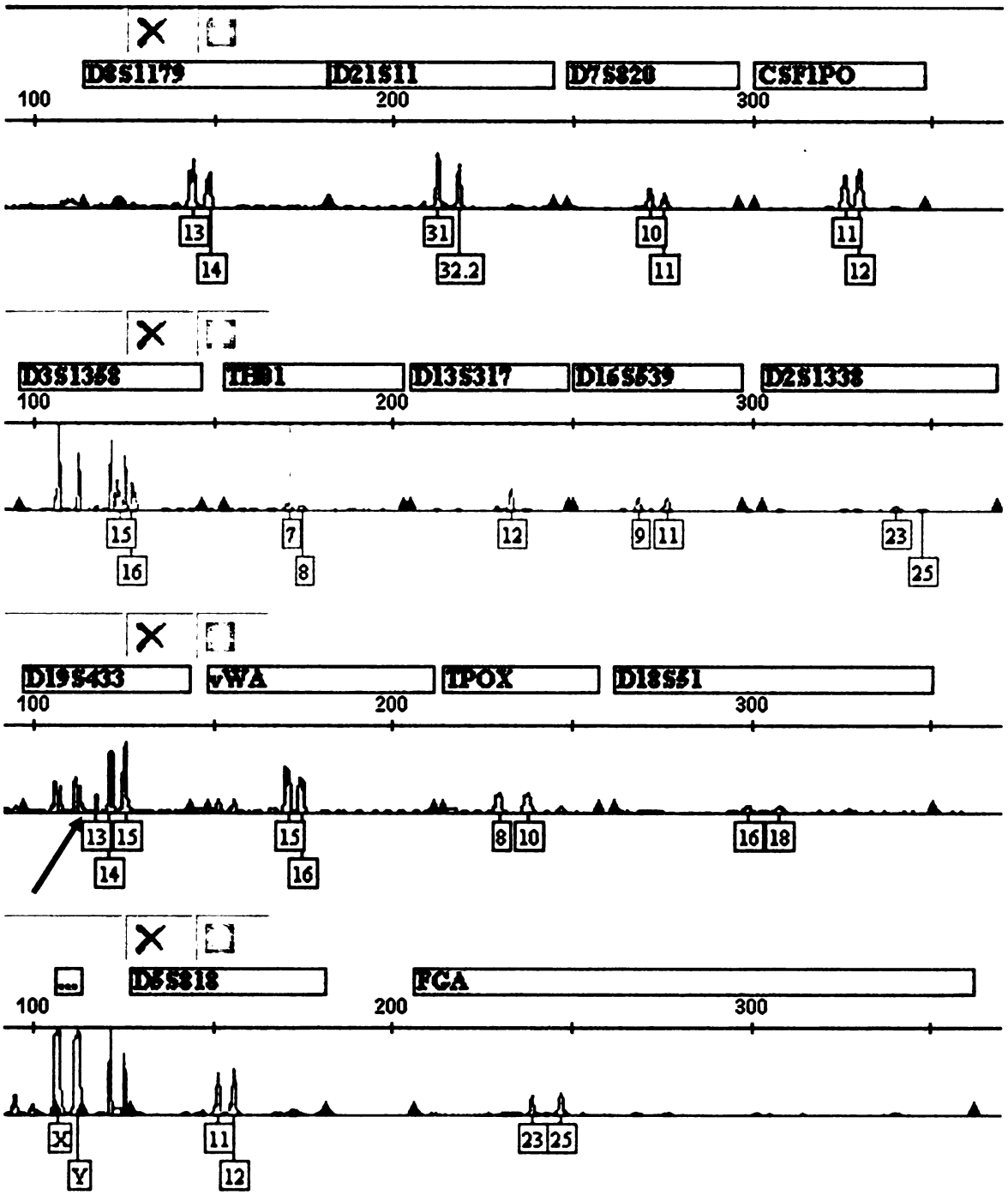
The first column indicates the name of each locus. Sample treatment is indicated across the top of each column, followed by the input dilution for amplification. Alleles highlighted in gray matched the known profile. AMEL is an abbreviation for amelogenin, the sex marker. Number not highlighted indicates a small peak was present, but no allele was called.

Figure 8: Electropherogram of Untreated DNA from Fresh Blood



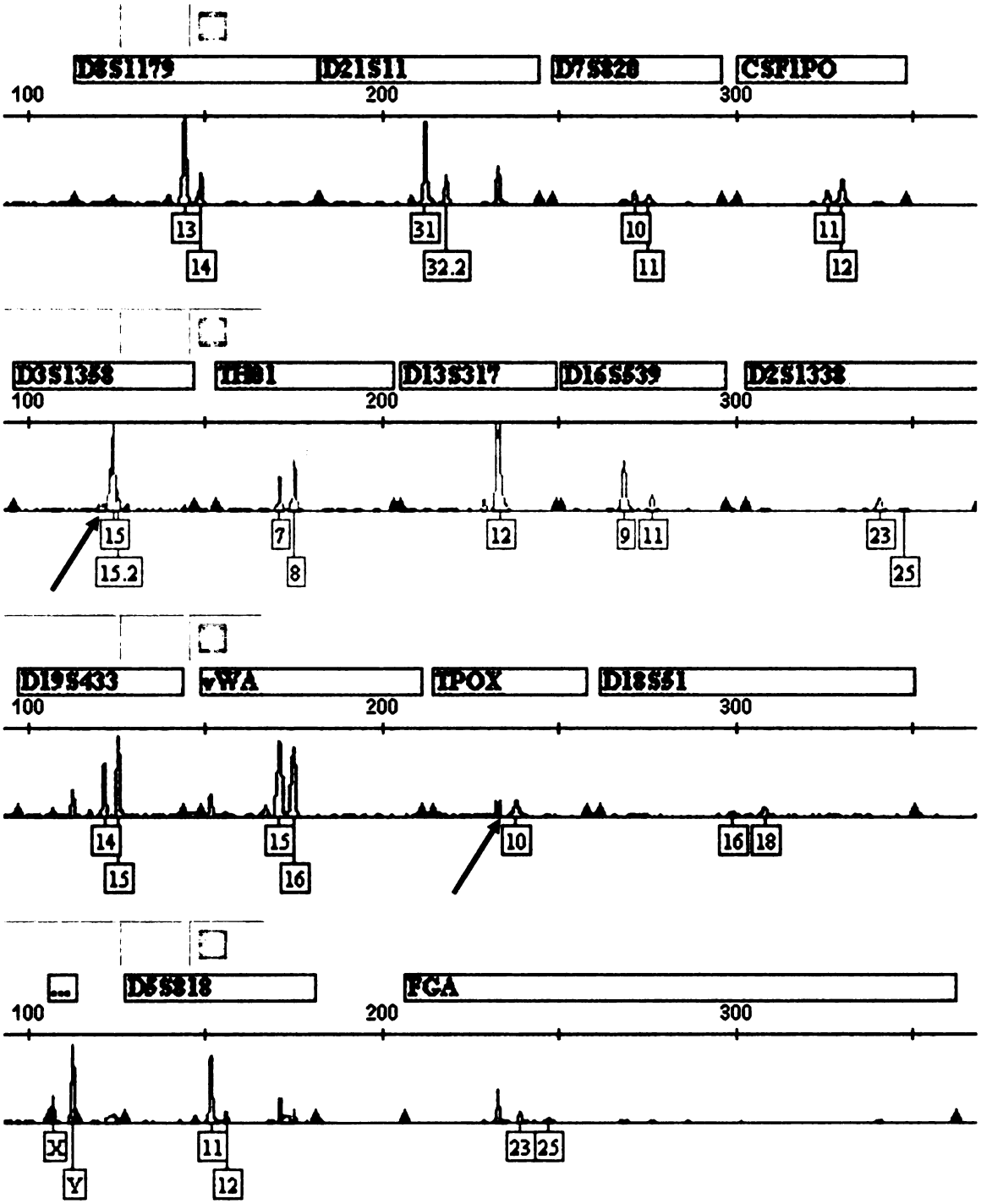
Locus name is located in the gray box above corresponding peaks. Number of repeats per allele is displayed below each peak. The amelogenin locus is indicated as '...' PCR product length increases from left to right across each row. Note the number of loci which experienced allele drop out, as indicated by arrows. Also note that product length was unrelated to PCR amplification success.

Figure 9: Electropherogram of DNA from Fresh Blood after I-PEP WGA



Locus name is located in the gray box above corresponding peaks. Number of repeats per allele is displayed below each peak. The amelogenin locus is indicated as '...' PCR product length increases from left to right across each row. Note an extra peak at D19S433, denoted by arrow.

Figure 10: Electropherogram of DNA from Fresh Blood after MDA WGA



Locus name is located in the gray box above corresponding peaks. Number of repeats per allele is displayed below each peak. The amelogenin locus is indicated as '...'. PCR product length increases from left to right across each row. Notice that at locus D3S1358, only one allele (15) matches the reference profile, the 15.2 peak is possible pull up due to spectral overlap of primer dyes. Also, notice allele dropout at TPOX. Both partial matches are denoted by arrows.

Sequencing of mtDNA from Hair Shaft and Aged Skeletal Material

Table12 displays polymorphisms in the I-PEP whole genome amplified hair samples in comparison to a reference sequence (Anderson et al., 1981) and to the sequences reported by Graffy, (2003). PCR amplification was successful for 4 of the 5 hair samples after I-PEP. DNA sequences were obtained from these 4 I-PEP hair samples. Polymorphisms in the I-PEP samples were consistent with those reported in Graffy (2003), except that at three locations the I-PEP sample showed a dual peak and Graffy (2003) observed a single peak. In one sample (sample 29), Graffy (2003) reported a possible difference; after WGA no difference was noted from the reference sequence.

Table 12: Mitochondrial Sequence Comparisons of Untreated and IPEP DNA from Hair Shafts

Sample ID	No WGA*	I-PEP
24	**97-285**	**94-279**
	185A	185A/G
	188G	188G
	288A	288A/G
	263G	263G
27	**85-284**	**141-249**
	263G	n.d.
28	**90-274**	**88-184**
	--	175C/A
	185G	
	188G	
	195N	
	228A	
	263G	
29	**84-284**	**82-286**
	146C	146C
	152C	152C
	195C	195C
	209N	--
	249G	249G
	263G	263G

Sample ID and the column marked with a * reproduced from Graffy (2003). The first line of data in bold for each sample is the range of the mtDNA genome sequenced, as numbered by Anderson et al. (1981). Differences from the reference sequence are indicated after the DNA location. 'n.d.' indicates that no polymorphisms occurred in the range sequenced, -- shows that there were no differences from the reference sequence.

Misner (2004) sequenced the five Voegtly samples, and Rennick (unpublished data) sequenced the three Albanian samples (Table 7). PCR amplification of 7 out of 8 aged bone samples were unsuccessful after I-PEP and MDA. One aged skeletal sample (Voegtly sample 704) was amplified after I-PEP. Sequencing of this sample showed no polymorphisms in this sample's sequence, which was consistent with results reported by Misner (2004).

DISCUSSION

Limited DNA can be problematic for forensic biologists. WGA, a process which randomly amplifies an entire DNA sample, has been studied in great detail on clinical biopsy material, yet there is a lack of information regarding the usefulness of WGA on forensic samples. This project characterized two methods of WGA on DNA isolated from tissues commonly seen in forensic casework. I-PEP and MDA were tested on high quality DNA from fresh blood, DNA that was artificially degraded to obtain controlled fragment lengths, and DNA from hair shafts, aged blood, and aged bone.

Advantages and Disadvantages of I-PEP and MDA

I-PEP follows a straightforward procedure which uses a reaction mixture similar in composition to PCR, and once set up, the amplification process takes no additional hands-on time. The cycling process itself is time consuming however, due to the slow 0.1°C per second temperature ramp included in each of the 50 cycles. In a field which is already backlogged, this 12 – 13 hour step seems counterproductive, but when the amplification was allowed to proceed overnight, traditional protocols could be resumed the following morning. Currently, no kits are available to simplify the I-PEP setup, but buffer, dNTPs and random primers could easily be packaged into one tube to reduce hands on time even further.

MDA is a simple reaction that requires little hands on time as kits, like GenomiPhi, have combined all necessary reaction components into 3 tubes. Although the setup process has been simplified, the 18 hour incubation was troublesome as a sample essentially had to be extracted and set up for WGA a full day in advance of any PCR

amplification. A second concern is the need to reopen sample tubes after denaturing the DNA at 95°C in order to add the Phi 29 enzyme; this could result in contamination.

Exogenous DNA is a particular concern with WGA. Any source of contaminating DNA, whether it is bacterial, fungal, or from a crime scene or laboratory technician, could be preferentially amplified, especially since the source is likely to be higher quality than that of the forensic material.

Another problem encountered with any WGA method that relies on random primers annealing throughout the genome is the shortening of product. For illustrative purposes, consider a sample of degraded DNA that is 800bp in length. This DNA has a maximum amplicon length of 800bp, assuming the random primers bind at the very ends of the template. In the vast majority of instances the random primers will anneal at other locations on the 800 base fragment, and a shortened product will be produced. In subsequent rounds, product will continue to be shortened. Similarly, consider an 800bp piece of DNA which contains primer binding sites for a 400bp PCR product located directly in the middle of the template, and 200bp of flanking sequence on either side. Only if a WGA primer anneals in the 200bp 5' of the PCR primer site will both PCR primer locations be replicated. If the WGA primer anneals within the 400bp target region, the 5' PCR primer site will be lost, and the WGA product will be useless. If the WGA primer anneals in the 3' flanking region, neither PCR primer site will be replicated. Thus in WGA, 3 out of 4 amplifications of this 800bp DNA will be useless, and as the shortening described above occurs, this ratio will only worsen. Again, as the WGA process continues and more product is amplified, trimming will worsen from one round of amplification to the next as the primers bind short of the strand ends.

General WGA Results

When WGA product was separated on an agarose gel, a vertical smear of DNA extended the length of the gel and occurred with or without input (reagent blank) DNA (Figure 2). The intensity of both positive and reagent blank smears from MDA product was greater than that from I-PEP. Further there was apparent high molecular weight DNA in the I-PEP and MDA reactions, even from reagent blanks. One possible cause of the smear, especially from samples such as aged bone which was buried in soil, was the amplification of microbial DNA. However this is not the only potential cause since the smear was observed in reagent blanks and positive reactions of all sample types, thus the smear seems to be a result of the WGA procedure in general, while the difference in smear intensity is due to a difference in the way each method amplifies product. In an I-PEP reaction, the random primers could overlap one another and act as template for the polymerases, but this product must be heat denatured in order for template to become accessible to new primers. This need to denature limits the overall yield increase since additional priming cannot occur until the next cycle. On the other hand, MDA utilizes the Phi 29 polymerase, which is capable of displacing double stranded DNA. As amplification occurs, primers anneal to template and the enzyme extends. If the template is high molecular weight DNA, the highly processive nature of the enzyme will continue to amplify sample until the DNA end is reached. In a reaction which has no input DNA, the primers (and any extraneous DNA) could overlap and act as template for the enzyme, progressively synthesizing longer product. As the enzyme extends the template strand, its displacement properties release a new strand of DNA, and amplification of this new strand can either be primed with random hexamers or the single stranded template could

potentially fold over and form a hairpin to act as its own primer. In either situation, any DNA added to the reaction will be continuously amplified by the Phi 29 enzyme.

PCR amplification of MDA treated DNA from artificially degraded sample, aged blood, hair and aged bone resulted in multiple non-target DNAs (Figure 3 and 6). The lengths of the extra products were different among samples, but if the same sample was amplified in duplicate, identical sized bands were generated. When product from an MDA reagent blank was amplified, extra bands were not produced. A simple explanation for these extra bands is that residual random primers were not washed completely from the MDA product which then acted as primers in a PCR reaction. This explanation is unlikely since the residual primers would most likely produce random products, visualized as more smearing, and not multiple bands. A second explanation is that there is random amplification of small pieces of DNA that contain the target PCR binding site, and these get incorporated into the larger pieces of DNA that result in the smears. This would account for the lack of bands in negative controls, even though these too contain smears. A final explanation involves the production of MDA artifacts related to the Phi 29 amplification process. As described above, MDA may amplify and lengthen any input DNA. If the single stranded WGA DNA were to form a hairpin loop and then extended on itself, product length could be doubled (or even tripled with a second hairpin). The resulting product would consist of tandem forward and reverse complement sequences of the original template, and could potentially contain PCR primer binding sites. Since the lengths of the non-target bands differed among samples, but were the same in serial dilutions of each WGA product, the extra PCR bands are not locus-specific (e.g., a region

of mtDNA does not generate a specific artifact). Regardless of the mechanism however, production of non-target bands will have a negative impact on subsequent analyses.

Dean et al. (2002) briefly mentioned the production of artifacts, or extra non-target DNA, from PCR based WGA methods, such as I-PEP and DOP-PCR. They suggested that the production of artifacts was due to the repeated heating of template that degraded the sample. However, they did not include any results displaying the production of non-target amplifications, did not note that the PCR process itself repeatedly heats DNA, and failed to mention similar issues with their MDA method. They did reference a paper by Cheung and Nelson, (1996) who evaluated DOP-PCR and discussed the production of non-genomic product, possibly due to non-specific priming. These authors reported that the production of non-specific DNAs influenced the success of PCR amplification in up to two-thirds of attempts. It is possible that artifacts associated with the MDA process may not have been observed by Dean et al. (2002) since they were not investigating the utility of WGA on degraded DNA.

Yet another problem encountered with I-PEP and MDA product was that PCR amplification of some whole genome amplified samples was inhibited at a dilution that was successful for untreated DNA (Table 9, Figure 7). This inhibition was generally eliminated when the WGA sample was diluted 10 to 1000 fold prior to PCR. One explanation for this PCR inhibition is that input DNA was too concentrated following WGA. It is possible that the amount of some components, such as primers and dNTPs, became limiting before the PCR process was capable of generating the desired amplicon. The amplification of hair shaft samples was a good example of this inhibition. As seen in Figure 7, hair samples diluted 10 to 100 fold (number 27 and 28) were not amplified, but

with a 1000 fold dilution, amplification was successful. A second explanation for this inhibition is that WGA artifacts were somehow acting as inhibitory agents, and as the sample is diluted so are these artifacts.

Product Quality of Untreated, I-PEP, and MDA Samples

Quality assessments of untreated and whole genome amplified DNA were completed by determining maximum PCR product lengths (nuclear or mtDNA) from artificially degraded DNA, fresh blood, aged blood, hair shafts and aged bone. These results were compared to one another to determine the usefulness of WGA on samples which have varying levels of degradation.

The effects of WGA on degraded DNA were investigated using DNA of a controlled length (Table 4). There is an apparent relationship between amplification success of I-PEP treated DNA and size class, where a 213bp nuclear product was always amplifiable from the fractions greater than 400 nucleotides, while the smaller <200 and 200 – 400 size classes had decreased PCR success, amplifying 3 and 4 times out of 5, respectively (Summary Data from Table 4). The relationship between size class and amplification success of I-PEP product is reinforced by mtDNA amplification results where larger size classes were again more successful than the small size classes. In contrast there is no apparent relationship between MDA treated size classes and amplification success of a 213bp nuclear product; the <200, 600 – 800, 800 – 1000, and >1000 MDA nucleotide fractions successfully amplified in 3 out of 5 attempts, while the 200 – 400 and 400 – 600 size classes successfully amplified 4 out of 5 times. A 270bp mtDNA amplicon amplified in 2 out of 4 attempts for all MDA size classes. The difference in amplification trends between I-PEP and MDA could be due to WGA

shortening. If this was the case, shortening of I-PEP product was only noticeable in the smaller size classes, while it appears that all MDA treated size classes were affected equally.

When comparing PCR amplification results of untreated, I-PEP and MDA treated samples from the 5 trials of degradation, 3 of the 6 I-PEP size classes had greater nuclear amplification success than untreated and 4 of the 6 had greater success than MDA treated DNA. Five out of 6 of the I-PEP size classes had a higher mtDNA amplification success than untreated size classes, and all 6 I-PEP size classes had a higher success than MDA samples. Results from untreated and MDA product indicate that PCR amplification from untreated DNA is more successful than MDA, where 4 of 6 untreated size classes had greater amplification success, 2 size classes had equal amplifications between the two treatments, and 1 untreated size class was less successful than MDA product. Clearly, I-PEP treated DNA was amplified more frequently than other treatments, across degradation trials and size classes. This is consistent with the findings noted above in which I-PEP shortening affected the larger size classes less than shorter size classes, which in turn would result in greater PCR amplification results, while shortening of MDA product affected all size classes leading to a reduction in amplification success. Further, untreated DNA was amplified more often than MDA product. The reduction of amplification success after MDA is a concern for forensic biologists and could prevent the method from being implemented in casework unless a satisfactory explanation and remedy could be obtained.

There were discrepancies in amplified PCR product lengths across trials of artificial degradation; longer product was amplified from trials 1 and 2 (496bp nuclear,

864bp mtDNA), than from trials 3 – 5 (213bp nuclear, 220 to 270bp mtDNA). This difference in product length is likely due to a change in the separation protocol, which in later trials was accomplished through the use of a longer gel. The modified procedure clearly improved separation of the digested DNA, since the amplification of an 865bp product should not be possible from a <200 nucleotide size class. Moreover, the data from the last 3 trials were more consistent with each other, indicating these data are more reliable and the modified technique was more successful at separating the DNA pieces.

Maximum amplicon length obtained after I-PEP and MDA from fresh blood, (496bp nuclear, 1064bp mtDNA) did not differ from untreated DNA (Table 5). There was also no difference in the maximum nuclear DNA product (496bp) between untreated and whole genome amplified DNA from aged blood. However, untreated aged blood had a reduced maximum mtDNA amplicon length of 865bp, while a 1064bp product was obtained from I-PEP and MDA treated DNA. Presumably the untreated DNA sample contained mtDNA of at least 1064bp, but the quantity was not great enough for successful PCR amplification. After WGA, the amount of longer DNA strands was increased beyond the minimum threshold required for PCR. Had the sample simply been PCR amplified, the evidentiary value of the DNA would have been diminished. Ironically, when tests were attempted to verify the difference in product lengths, not enough untreated DNA remained, illustrating the usefulness of WGA.

In contrast, the length of amplifiable PCR product from hair and skeletal material was clearly reduced after I-PEP. An 865bp mtDNA product was amplified from untreated DNA from hair, but after I-PEP, the maximum product length of 2 samples was reduced to 664bp and three more had a maximum amplicon length of 203bp. Without

WGA, a 220bp mtDNA product was amplified from the 5 Voegtly bone samples. Only one bone sample could be amplified after I-PEP; no product was generated from the 4 others. None of Albanian samples could be amplified following I-PEP, even though a 266bp mtDNA product was generated from them prior to WGA. The MDA method was even less successful on degraded DNA. All nuclear and mitochondrial amplification attempts from MDA treated aged bone and hair failed. In general, by comparing the PCR amplification results of all sample types, the hypothesis that the WGA process shortens product, leading to the loss of primer binding sites, is exemplified since maximum PCR product length from high quality DNA did not differ, but when testing degraded samples, there was a clear reduction in maximum amplicon length after WGA.

Product Yield from I-PEP and MDA

WGA product yield was compared to the quantity of untreated DNA by PCR amplifying nuclear (blood), or mtDNA (hair) product with serial diluted input DNA. In all cases where PCR amplification was successful, the yield of a 496bp nuclear or 203 bp mtDNA product was increased by WGA. I-PEP product yield was increased 1000 fold for fresh blood, 100 fold for aged blood and 20 to 2000 fold for DNA from hair shafts, in comparison to untreated DNA. MDA increased product yield from fresh blood 10,000 fold and aged blood 1000 fold (Table 8 and Figure 5).

The difference in product yield between fresh and aged blood is again consistent with the idea that whole genome amplified product presumably will be subjected to shortening and PCR primer binding site loss is more likely with short template. Additionally, the inconsistent yield increases among hair samples may be due to the quality of the mtDNA available in each sample.

Downstream Analysis of Whole Genome Amplified Product

STR analysis of untreated and whole genome amplified samples was performed on artificially degraded DNA from trial 5 (Table 10). The untreated 400 – 600, 600 – 800, 800 – 1000 size classes matched the reference sequence at 14, 16, and 15 loci, respectively. Of the three loci which were not typed, 2 have longer PCR products, and one locus could not be typed because 3 peaks were present. Amplification of the >1000 size class for STR analysis was unsuccessful. It is unclear why this size class did not amplify, particularly since a 213bp nuclear product was amplified from this size class in earlier experiments, but it may simply be due to PCR amplification failure unrelated to WGA.

STR amplification of the artificially degraded >1000 size class after I-PEP was less successful than untreated DNA, where 4 I-PEP loci completely matches and 7 loci were partial matches. STR amplification from the MDA treated artificially degraded >1000 fraction was also reduced in comparison to untreated sample, with complete matches at 9 loci, and partial matches at 2 loci. The unsuccessful alleles from both WGA procedures were from the longest loci in the multiplex. Therefore, it is likely that WGA product shortening is again responsible for the loss of primer binding sites from longer loci. The overall superior STR amplification results of untreated artificially degraded DNA over I-PEP treated DNA was inconsistent with results obtained from single locus testing, where I-PEP product was amplified more frequently. In earlier tests, a 213bp nuclear product was attempted; STR loci lengths are between 100 and 360bp. A reduction in the amplification success of I-PEP product may have been observed if earlier tests had been performed using primers designed to amplify a 300bp product, and it is

possible the 213bp amplicon represents the upper limit of what can be amplified from the I-PEP product.

Interestingly, STR amplification of the MDA treated fraction was more successful than I-PEP, even though amplification of the I-PEP fractions was more successful in single locus PCR reactions among all trial of degradation. This seeming inconsistency may be explained by a difference in the amount of available template. MDA is expected to increase product yield more than I-PEP, and since the same amount of sample was added to the STR amplification reaction, it is likely that MDA product had a greater amount of DNA available.

STR analysis was performed on untreated and whole genome amplified DNA from the 1uL sample of fresh blood (Table 11). STR amplification of untreated DNA (Figure 8) was successful at 5 loci, and incomplete matches, due to allele drop-out, were obtained at 9 loci. The locus length was not related to amplification success; 2 incomplete matches occurred at short length loci, and 5 incomplete matches occurred at medium length loci, while 1 long locus completely matched. These results were unexpected since the single locus amplification of a 496bp nuclear product was successful and the longest locus length in the multiplex is 360bp. In addition, equivalent amounts of DNA and similar PCR cycle numbers were used (38 for single locus, 35 for STR amplification). Therefore, the loss of heterozygosity for untreated samples may be explainable by an inadequate quantity of starting DNA.

STR amplification of the I-PEP treated fresh blood sample was successful at all 16 loci, while amplification of the MDA sample from fresh blood was successful at 14 loci, and incomplete matches were obtained from 2. There was no relationship between

locus length and amplification failure, however, more input MDA sample would likely result in a more complete profile since two peaks were observed at the TPOX locus, but one was below the minimum intensity threshold. Also, STR loci were amplified from MDA product when diluted 100 fold more than I-PEP or untreated DNA. Overall, STR analysis was more successful from whole genome amplified sample. Prior to WGA there was not enough DNA in the sample to amplify all loci; upon completion of I-PEP and MDA, more loci were amplified. These results clearly illustrate the usefulness of WGA on high quality samples that are limited in quantity.

STR testing of untreated and whole genome amplified DNA from aged blood was largely unsuccessful. No profile was generated from untreated DNA, even though additional amplifications were attempted with an increased amount of input DNA. Neither WGA method generated a complete profile, but after I-PEP 7 alleles, and after MDA 1 allele could be matched to the known profile. Alleles which matched the known profile were all from loci of short product length. In comparison to the single nuclear locus testing, where no difference in maximum product length was observed, it is surprising that there was a difference between the number of amplified STR alleles from untreated DNA and WGA product, especially since earlier PCR amplifications of a 496bp nuclear product was successful, (thus the largest STR locus, at 360bp, should be amplifiable). However, this discrepancy is consistent with the maximum length mtDNA results from the same samples, where the difference in PCR product length between untreated and treated samples was likely due to an increase in available template after WGA. In short, the ability to amplify STR alleles from untreated DNA was limited by

starting quantity, after WGA the increase in DNA quantity made STR amplification possible.

MtDNA sequencing for identification of forensic samples was also completed with WGA product. Amplification of mtDNA from five hair and eight bone samples without WGA treatment was successful. After WGA, amplification was possible from 4 of 5 hair samples and 1 bone sample. As mentioned above, differences in the amplification success of these samples before and after WGA, is likely due to the loss of primer binding sites as a result of product shortening. Sequencing of these amplicons was successful and results were consistent with known profiles, with the caveat that more heteroplasmy was observed in the WGA sequences (Table 12). It is problematic that sequence differences from two whole genome amplified hair samples resulted in dual peaks. However, as seen in sample 29, an ambiguity in untreated DNA was resolved after WGA; more experiments will be needed to fully understand the cause of these sequence differences.

Conclusions

Both methods of WGA were easy to perform, but I-PEP was preferred since non-target DNAs are not generated following PCR and valuable DNA was not exposed to potential contamination by reopening the sample tube. Further, the entire I-PEP procedure is less time consuming and thus advantageous in a field where casework is already backlogged. I-PEP and MDA are valid methods of increasing product yield for forensic samples which are brought into a crime lab in very limited quantity but not quality, such as small amounts of relatively fresh blood. Overall, MDA increased

product yield 1000 to 10,000 times, and I-PEP increased product yield between 20 and 2000 times.

Based on the variety of experiments described here, including those on controlled (artificially manipulated) DNAs, as well as forensic samples, it is unlikely that highly degraded DNA will benefit from WGA. Results of PCR amplification from aged bone and hair shaft samples were not improved after WGA; in fact, most samples had better amplification success without WGA than with. In addition, multiple non-target products were PCR amplified from highly degraded DNA which underwent MDA. Furthermore, STR analysis of untreated artificially degraded DNA generated more complete profiles than those amplified after I-PEP or MDA. The reduced amplification success is most likely due to a shortening of the whole genome amplified product, which resulted in the loss of primer binding sites. Forensic identification information could be lost if WGA were attempted on highly degraded DNA.

In conclusion, MDA was capable of increasing yield more proficiently, but PCR amplification of I-PEP product was more successful. While the principle objective of WGA is to increase product yield, the reliable amplification of forensic samples in downstream reactions is more important than large product increases. It is this regularity which makes the I-PEP method a better choice for use with forensic samples.

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