

The Effect of Common Imaging and Maceration Techniques on DNA Recovery from Skeletal Remains

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DEDICATION

To Drs. Janice and Kevin Frank (aka. Mom and Dad) for your continued love and support, as well as helping me bury the occasional road-kill specimen in the backyard.

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ABSTRACT

DNA testing is an essential tool for human identification, particularly from skeletal remains. Therefore it is important to understand how different processing methods used to clean and prepare bones for research and analysis influence the recoverability of CODIS marker DNA from skeletal remains. Thirteen elements from three sets of recently skeletonized remains were macerated with hot water, scanned by computed tomography (CT), X-rayed, or exposed to a combination of the three methods. The number of CODIS marker loci, relative fluorescent units (RFU) values and DNA yields were compared to an unprocessed control group to determine if there was a significant difference in STR-DNA recoverability between processed and unprocessed remains.

The RFU values differed significantly between the processed and unprocessed groups ($p=0.000$) and among samples from each separate processing treatment. CODIS-DNA yields showed a different pattern. None of the processing methods significantly affected yield. There was a significant difference in the profile completeness (number of loci) between the overall group of processed and unprocessed remains ($p=0.007$). However, results indicate no significant difference when comparing each method individually. Due to the inconsistencies between the different measures of DNA quality and quantity, it is still unclear how these three processing methods affect DNA recovery from skeletal remains.

Identifications are established by amplifying a standard set of CODIS marker

loci comprising a “complete profile,” but complete profiles do not indicate an equal recovery of CODIS marker DNA between samples. It is only necessary for the quantity of CODIS marker DNA to exceed a minimum RFU threshold for a locus to be identified. Since processing skeletal remains appears to reduce RFU values, maintaining an unprocessed element from every skeleton would maximize CODIS marker preservation for future testing without significantly changing current processing procedures.

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CHAPTER I

INTRODUCTION

After an organism dies, decomposition processes reduce the remains to its skeletal components. Various insects and microorganisms consume the soft tissue, breaking it down and releasing organic molecules for use by future organisms. The organic collagen components of bone and teeth also decompose over time; however, this breakdown occurs more slowly than soft tissue decay due to the mineral component of these tissues. Therefore, bone typically persists well after the loss of other tissues. Furthermore, the matrices of bone and dentin retain the organism's deoxyribose nucleic acid (DNA) and protect it from breaking down (Gotherstrom et al. 2002; Foran 2005). Since bone and teeth are often the last remaining tissues of an organism, they are important resources for identifying deceased individuals through DNA comparison. Traditionally, skeletonized remains are identified based primarily on characteristics of the biological profile along with dental records (Ciaffi et al. 2011). Recently, the increases in proficiency of CODIS DNA recovery techniques have enabled researchers and crime labs to more easily extract and analyze DNA profiles from bone.

CODIS loci DNA has been used extensively in disaster victim identification (DVI) projects and human rights investigations in which unidentified remains are often fragmentary, burned or commingled (Butler 2006). DNA is particularly valuable when dealing with extensive fragmentation of individuals from a large, population in which the identity and the number of victims are unknown (Blau et al. 2008). When fragmentation is significant, remains may not possess characteristics traditionally used to identify a

body, such as fingerprints or teeth (Mundorff et al. 2008). Dispersal of remains throughout the recovery site and commingling of multiple individuals frequently makes individual identification more difficult and prevents re-association of body parts. In these situations CODIS loci DNA is the primary means of identification (Mundorff et al. 2009)

While bone and teeth provide the only avenue for obtaining a DNA profile after the soft tissues are gone, they are nevertheless vulnerable to extrinsic factors. If DNA can be recovered from the bone, it is often degraded or of insufficient quantity to be detected, even after amplification (Foran 2006; Minser et al. 2009). Introduction of DNA from extrinsic sources further complicates the problem, since the contaminating DNA is can overshadow the signal of the target DNA. Therefore, maximizing the potential for DNA extraction is critical.

In the context of this study, processing refers collectively to techniques used to prepare the remains for study including removal of soft tissue, and to creation of a record of data or images of the remains. Heat processing, or heat maceration, specifically refers to the use of hot water to remove soft tissue from skeletal remains. Processing is vital to the identification process since it allow pathologists to examine in the remains thoroughly via visual inspection of the features present on the cleaned outer surface of the bones as well as images of the internal structure. However, some processing techniques may comprise the integrity of remains. Steadman et al. (2006) assessed the quality of maceration techniques based on the appearance and texture of the skeletal material before and after processing in terms of preservation of morphological characteristics. Preserving bony features is important due to the information they can yield about the individual's activities during life. Additionally, recent advancements in CODIS DNA recovery and

analysis allows for the collection of additional information from skeletal remains. Thus, the evaluation of maceration techniques was extended to favor processes that preserve the quality of DNA as well as the bone itself (Steadman et al. 2006; Lee et al. 2010).

Reconsideration of processing procedures is now essential to ensure that critical information is not inadvertently lost.

Purpose/ Objectives

The purpose of this study is to assess the potential effects different processing techniques may have on the recoverability of CODIS DNA from bone. Specifically, this study focuses on three common techniques used by the Forensic Anthropology Center (FAC) at the University of Tennessee, Knoxville (UTK). The scope of this study is limited to assessing procedures used by the FAC, which may differ from methods used by other facilities.

Prior to inclusion in the William M. Bass Skeletal Collection, all individuals' remains are allowed to decay in an outdoor environment at the FAC's Anthropological Research Facility (ARF). Therefore, all skeletons in the collection are exposed to a similar environment during decomposition. In general, the bodies are placed on the ground surface prone and unclothed but loosely covered with a black tarp. Most bodies are not buried. Depending on the season, decomposition rates could vary from a few weeks in the hot summer months to over a year, but most bodies are at the facility about two years. This ensures sufficient time for microorganisms to break down the bulk of the soft tissue.

After the remains are mostly skeletonized, they are collected and stored in biohazard bags to await processing: once in the laboratory facility, the bones are washed to remove dirt and any adherent tissue. If tissue is still present (such as in instances of mummification), the affected elements are placed in heated water for intervals of 45 minutes until the tissue can be removed easily (Jantz and Taylor 2009). The remains are then allowed to air-dry before curation into the Bass Collection. This skeletal collection is used for different types of osteological research. Typically, the research is nondestructive to bone, subjecting the bones to measurement and visual assessment. The bones may also be exposed to electromagnetic radiation via X-ray and Computed Tomography (CT) scanners. For example, many of the skeletons in the Bass Collection have been CT scanned to produce a digital record that can be used by researchers to take measurements and model the shape and movement of various skeletal elements (Moore and Schaffer 2011).

No previous study has examined the individual or combined effects of the UTK FAC processing methods, yet it is becoming more common for these skeletons to be used for DNA research (Mundorff et al. 2011). Additionally the UTK processing methods are different from other methods previously tested (Arismendi et al. 2004; Steadman et al. 2006; Lee et al. 2010). The bodies donated to UTK require minimal maceration because they are allowed to decompose naturally until the majority of soft tissue is gone. Previous studies have started with completely fleshed bone samples and then removed the soft tissue via chemical and heat maceration, except for the controls in which the tissue was removed with a scalpel (Steadman et al. 2006; Lee et al. 2010).

The objectives of this study are to assess the potential effects of maceration, X-ray, and CT scans on the recovery of DNA from skeletal remains. Furthermore, the combination of all three processes will also be analyzed. DNA sequences from processed elements will be compared to the sequences previously generated from the same individual's antimeric bony elements (Mundorff et al. 2011). Because the comparative (baseline) profiles were generated prior to any processing, differences between the two sets of results can be used to determine if processing techniques affect the recoverability of DNA from bones.

Some previous studies have been limited by small sample sizes or access to only nonhuman bones. The dataset for the current study was comprised of a relatively large, human sample (N=72) from three individuals. The 72 samples included 24 previously tested, unprocessed samples, which constitute the control group. Additionally, 48 samples from the same three individuals comprising the control group were tested after processing. Comparing results collected from the same individual before processing and after processing helped to reduce distortion from unknown environmental factors acting on the bone's nuclear DNA (Graw et al. 2000). Comparing the opposite side of the same element (right vs. left), from the same individual, helped to ensure disparities in results are not caused by differences arising from comparisons between different skeletal elements or between different individuals. Previous studies have shown that different skeletal elements and bones from different individuals yield nuclear DNA at different success rates (Milos et al. 2007; Mundorff et al. 2009). Therefore, results from previous studies showing disparity in DNA yield between processed and unprocessed bones may simply be due to comparing two different elements or comparisons between different

individuals (Arismendi et al. 2004; Lee et al. 2010). This study is unique in that it compared the same element type from the same individual, before and after processing.

Significance

The results of this study will build upon a growing body of literature in the fields of biological anthropology, forensic biology and genetics. The results will be applicable to future studies extracting CODIS DNA from bones that have been processed, radiographed, or CT scanned. Most of the skeletons in the WM Bass Donated Skeletal Collection have been processed using the previously discussed methods. Therefore, DNA based research using these skeletons will benefit from understanding the potential effects of FAC processing methods on the ability to recover CODIS loci DNA. Findings will also be useful to researchers developing procedures for handling human remains, whether in a curatorial or a medico-legal context.

While the heat maceration procedures are specific to the FAC, similar dosages of radiation are supplied to bones subjected to X-ray and CT scanning procedures during research and forensic analysis. Removing a bone sample for DNA testing is often the final step in a forensic examination, with X-ray and CT scans occurring earlier in the examination (Blau et al. 2007). If the recoverability of DNA is compromised by radiation, it may be important to change the examination sequence and collect DNA samples prior to X-ray and CT scans. CODIS DNA analysis may provide the only means to establish a positive identification; therefore it is important to understand the possible effects of these processes on skeletal remains.

This study will also directly inform another study assessing DNA yield from bone (Mundorff et al. 2011). Mundorff et al.'s study compares DNA yields between recently skeletonized elements and the corresponding elements from individuals of increasing post mortem intervals (PMI). The most recent skeletonized remains in this study were collected from the ARF and sent directly for testing prior to undergoing any FAC curatorial procedures. The skeletons from longer PMIs came from the WMB Donated Skeletal Collection, having undergone various combinations of heat maceration, X-ray, and CT scanning. Understanding potential effects from these procedures on DNA yield will assist in the comparison between recent unprocessed skeletons and older, processed ones. If no difference is found between the pre- and post- processed elements, the effects of hot water maceration, X-ray, and CT scanning can be excluded when assessing differences between the recently skeletonized remains and those for increasing post mortem intervals. Instead the differences in DNA profile recovery are more likely attributable to the increasing PMI. If, on the other hand, significant differences are found in the quantity or quality of CODIS DNA between the processed and unprocessed elements, differences in DNA yield could be an artifact of processing. Therefore the effect of processing would need to be taken into account when comparing the impact of increasing PMIs.

CHAPTER II

LITERATURE REVIEW

The preservation and curation of skeletal materials has always played a significant role in research conducted by anthropologists, as well as zoologists and other scientists in many different fields (Simonsen et al. 2011). Comparative collections are important because they provide a way to assess variation among individuals within a population. Collections also offer a means of assessing change in population features over time since the stored samples may be accumulated over a lengthy time period. To ensure that useful information is preserved, the specimens must be preserved and stored properly. Therefore, specific processes have been established to clean specimens while preserving their structural integrity (Steadman et al. 2006).

Prior to being accessioned into a comparative collection, skeletal remains must be cleaned in such a way as to prevent decomposition from occurring in storage facilities. In cases of recently deceased individuals, extensive maceration efforts are often required to remove the soft tissue. Various methods have been used to remove soft tissue from bone, including chemical treatments, heat treatments, and controlled insect activity (Steadman et al. 2006). Enzyme activity has also been tested on soft tissue as an alternative maceration method. Results indicate that enzymes efficiently remove soft tissue with minimal odor (Simonsen et al. 2011). However, techniques that are effective for removing tissue can have long-term negative consequences. Heat and soaking methods, for example, can damage the internal structure of bone causing it to become friable. The task of removing soft tissue quickly and efficiently must be balanced against the goal of preserving the specimens for future study (Steadman et al. 2006). With the advancement

of DNA research and an increase in its use as an identification tool, preservation of the bone and the DNA contained within are now important considerations when selecting a maceration method.

Studies on the Efficacy of “Processing”

Advances in radiographic technology have allowed radiologists and pathologists to create three-dimensional visualizations of human remains without removing the soft tissue. While radiographic images allow anthropologists to efficiently visualize bone, subtleties may be missed that would otherwise be seen on a clean skeleton. Therefore, clean bone is still important for a forensic anthropologist to conduct a thorough exam.

One study examined the effects of processing human remains using a variety of maceration techniques (Arismendi et al. 2004). The samples to be tested were taken from two individuals from UTK’s ARF. The sample comprised six ribs from each individual (N=12). The ribs were subjected to five different processing treatments including: 1) simmer one and a half hours, 2) simmer one and a half hours with detergent, 3) simmer one and a half hours with Arm & Hammer[®] washing soda, 4) boiling/drying/simmering process over the course of seven days, and 5) day one: simmer one and a half hours with detergent, day two: simmer one and a half hours with degreaser. Two ribs, one from each individual, were subjected to each process. The sixth ribs were not treated and served as the control.

Following maceration, DNA was extracted and tested to determine if the sample would produce a genetic profile for two loci used in the CODIS system (CSF1PO and AMEL). Out of the 12 samples, one control rib and one boiled rib failed to produce

results for either loci. All other samples produced adequate profiles. The authors suggest that excessive heat could be responsible for the failure of the boiled sample, even though the boiled rib from the second individual yielded a successful profile. The failure of the control sample is attributed to the presence of “inhibitors” within the bone after decomposition. Various substances, including blood, can prevent CODIS DNA recovery, by interfering with the enzymes used to replicate DNA sequences. These are PCR inhibitors. When DNA amplification is prevented, the quantity of DNA in the sample is insufficient to be detected. If inhibitors were responsible for the failure of the control sample, it would suggest that some degree of processing might be beneficial for generating DNA profiles because it removes chemicals or human fluids that might interfere with DNA amplification (Arismendi et al. 2004).

Rennick and colleagues (2005) compared different techniques to prepare skeletons (Rennick et al. 2005). Nuclear DNA was isolated from 36 samples of the femora and ribs from sheep, cow, and pig. A small sample of human bone was included for comparison, comprising three one centimeter (cm) wedges of femur, two metatarsals, and a foot phalanx. The study compared the effects of boiling elements in 1) 25% bleach solution, 2) purified water, and 3) sodium carbonate. DNA yields varied greatly across the specimens. These inconsistencies were attributed to differing species and element types. There was no statistically significant pattern illustrating one species consistently producing better DNA profiles than another. Although DNA could be extracted from bones following all treatments, a statistical reduction in DNA was only noted from the bones treated with bleach. Bones subjected to the bleach treatment produced significantly less amplifiable DNA. On the other hand, carbonate treated bones

performed better than those boiled in water alone, though these results were not statistically significant.

Steadman et al. (2006) compared different maceration methods using pig ribs to determine which techniques allow for the easiest soft tissue removal while maintaining DNA yields. The sample comprised fresh ribs from twelve pigs, which were divided into sets of three contiguous ribs. The rib sets were weighed and placed in one of each different treatment. Many treatments to remove soft tissue were evaluated, including: 1) manual removal with scalpel (control), 2) soak (22°C room temp), 3) hot water (90°C), 4) boiling (100°C), 5) microwave at one minute intervals, 6) 10% bleach soak (22°C), 7) hydrogen peroxide soak (22°), 8) EDTA and papain soak (45°), 9) meat tenderizer and Palmolive © soak (90°C), 10) detergent and sodium carbonate (90°C), and 11) detergent and sodium carbonate followed by degreasing in ammonia. The process continued until the bones were stripped of soft tissue.

Steadman et al. (2006) then scored samples for odor, soft tissue texture, ease of flesh removal and bone quality. These assessments were used to quantify the effects of maceration on the bone. The assessment was then compared with the quantity and quality of nuclear DNA recovered from the bones. Results indicate that the length of processing had a greater effect on DNA recovery than the solution used. Long periods of processing increased DNA degradation regardless of the solution used. These findings suggest that exposing bones to short periods of high temperature, instead of long periods at lower temperatures, maintains DNA while effectively removing soft tissue.

Lee et al. (2010) retested Steadman et al.'s (2006) maceration methods (except soaking at room temperature) using six amputated human lower limb bones rather than

pig ribs. The tibia and the fibula were sectioned to provide a larger sample size. The distal femur was used as a control and not subjected to any maceration procedures except for the removal of tissue with a scalpel. The effectiveness of each method was assessed for odor, ease of soft tissue removal, and bone quality, as per Steadman et al. (2006). Treatments continued until the bones were stripped of soft tissue and duration for each method was recorded. Nuclear DNA was sequenced using two different kits for comparison of fragment size amplification. The microwave and the detergent treatments allowed for complete DNA profiles and removed the soft tissue most rapidly. Overall, the results were consistent with those of Steadman et al. (2006), with the exception of the degreasing process (treatment 10), which took significantly longer for human bones. The authors attributed the divergence to differences in the structure of the marrow cavity and the bone's fat content. The authors suggest that along with chemical and heat methods, the processing duration can be damaging to the bone's integrity. Methods causing more damage also remove soft tissue more rapidly than gentler maceration techniques. Since the bone is exposed to the more damaging treatments for less time, overall the cumulative damage is less than that of the gentler treatments that take a greater time to complete. Thus, DNA degradation may actually be minimized by exposing bone to harsher maceration techniques because the bones are cleaned more rapidly, reducing the total exposure. One issue the authors did not address is the effect of sectioning the bones to increase the sample size. As opposed to intact bone, sectioning exposed the bones internal aspects to the maceration treatment, potentially affecting the results. Since more surface area is exposed to processing, the overall effect of processing may be amplified leading to a greater loss of genetic information.

Computed Tomography (CT)

CT scanning is a radiographic technique used to visualize three-dimensional structures. CT scans are capable of capturing and preserving information from a wide variety of body features, including shape and dimension of bone and soft tissue structure (Rutty et al. 2007; Sidler et al. 2007; Blau et al. 2008). The quality of CT images is dependent on the parameters used during the scan. These parameters include slice length, time of scan, number of slices per scan, length of table, and intensity of radiation. The amount of radiation imparted to the skeletal remains can be adjusted by altering the parameters. CT scans are composed of many images taken along the length of the object and compiled to produce a 3D representation. Slice length refers to distance between the images and ranges from 0.625mm to 10mm. Decreasing slice length and increasing the total number of slices produces higher quality images, but also increases radiation exposure.

Pathologists use CT scans to visualize potentially dangerous items in a body bags since metallic and other types of materials appear on the scan differently than bone tissue. CT scans can also be used to find individualizing characteristics (such as prostheses) that can help establish personal identification. Though CT scanning is often performed on complete bodies, it is also a useful tool when assessing fragmentary or commingled remains. The CT scans can be performed before opening the body bag, limiting disruptions to the friable remains inside. In cases with extensive damage from burning or other skeletal trauma, individual elements can be documented from a CT scan. This type of pre-physical examination can later be used to help reassociate fragmentary remains and sort out commingled remains.

CT scans offer many advantages extending beyond traditional photography or X-ray. They provide three-dimensional images of the remains that can be manipulated into any position, eliminating the need to take X-rays from multiple angles. CT scans of skeletal elements are also useful for research purposes because detailed digital measurements can be collected by positioning the images in different orientations. The images can be saved as a digital file and later referred back to when descriptions of feature shape and size do not correspond to ante-mortem descriptions. These images can also be shared with experts around the world when incidents occur in small jurisdictions with limited experts and resources.

Imaging and documentation often occur prior to the collection of DNA samples (Rutty et al. 2007; Blau et al. 2008). Although the scans offer the practitioner the ability to collect important information about the appearance of the remains, CODIS DNA is more often the tool used to establish a positive identification. Therefore it is important to consider the possible affects of CT scanning on the recoverability of CODIS DNA.

X-ray

Prior to the development of CT scanning, X-ray images provided the most effective means to visualize internal body structures without opening up the body cavity (Ciaffi et al. 2011). X-ray machines produce images by exposing an object (e.g., body, suitcase) to penetrating electromagnetic radiation, and capturing the negative image of where the particles are prevented from passing through the material. The radiation passes easily through less dense mediums, such as skin or clothes, but is impeded by materials of greater density such bones, teeth or metal.

Radiographs are commonly used to diagnose skeletal injuries, thus providing a record of bone trauma. These radiographs can be compared to post-mortem X-rays for personal identification. For example, frontal sinuses take on a unique appearance during development. Therefore, if ante-mortem radiographs of the frontal sinuses are available they can be compared with post-mortem images to establish an individual identification (Christensen 2005). Dental X-rays are frequently used for forensic identification. Dental fillings and appliances provide distinctive features to match ante-mortem to post-mortem records (Campobasso et al. 2007).

CT Scan and X-ray Studies

In a study by Grieshaber et al. (2008), researchers tested the idea that X-ray and CT analyses were not destructive to the nuclear DNA within skeletal material. To measure the potential loss of genetic information from bone the authors compared the lengths of the amplified fragment as well as the number of cycles needed to attain a sufficient amount of amplified nuclear DNA. Fragments were assessed based on the number of base pairs (bp) that were quantifiable. Each complementary set of nucleotides (A, T, C, G) in a double strand of DNA represents a base pair, thus longer or less degraded double stranded DNA segments would have more base pairs. Fragments of 100 bp, 200 bp and 400 bp were compared. The study sample comprised 124 foot and ankle bones from nine domestic pigs (*Sus scrofa*). Prior to testing, the bones were defleshed with separate instruments to prevent cross contamination. Four bones from each foot were used in the different trials. The four trials measured single or multiple exposures to X-ray or CT scan. A control set for each pig was retained (N=36).

During the X-ray trial one set of bones (n=36) was exposed to a single x ray of 60kVp, 200 milliamps (mA) for 27 m seconds at a distance of 101 centimeters (cm). The second set (n=4) was exposed to the same levels three times. CT scan exposure parameters were 120 kilovolts (KV), 140 milliamps (mA) s in 2mm slices. A single CT scan was preformed on one set of bones (n=36). Three exposures using the same parameters were preformed on the second set of bones (n=12).

Results indicated that the nuclear DNA was most difficult to amplify in the phalanges that had been exposed to three CT scans. No sample produced 400 bp sequences but 200 and 100 bp sequences were amplified from all samples. The amount of amplified DNA did not significantly differ across the treatment and control groups. However, the control groups required shorter durations to amplify the DNA than their comparable test groups. This suggests the possibility that radiation increases nuclear DNA fragmentation.

Bone Histology

Osseous tissue is a combination of collagen and hydroxyapatite. This unique combination of fibrous and crystalline tissues forms a connective matrix that is simultaneously strong and flexible (White et al. 2011). Bone is composed of individual cells, called osteocytes, which mature from osteoblasts following the cells' entrapment in the bone's tissue matrix. Although all bone is formed from osteocytes, the cell's organization affects the overall bone structure.

Bone is typically categorized into two types based on the underlying structure of the cells. The first type, referred to as compact or cortical bone, forms from a series of

structural units called osteons. Each osteon has a central canal surrounded by densely packed osteocytes organized into a series of concentric layers. The blood and nerve supply for the bone travel through the central canal, providing innervation and nutrients to maintain the bone cells. The tight packing of osteons provides the rigid structure of long bone shafts. The second type of bone, cancellous or spongy bone, has a much greater surface area with greater space between bone cells, giving the appearance of a sponge. Spongy bone is distributed throughout the skeleton and makes up the epiphyseal ends of most long bones. The loosely packed organization allows stresses to be distributed over a wider surface area and reduce concentrated forces that might damage the bone.

Although it is unclear exactly where DNA is preserved within bony tissue, recent studies suggest the preservation of DNA is contingent on the preservation of both the collagen and hydroxyapatite components (Gotherstrom et al. 2002; Kemp and Smith 2005; Compos et al. 2012). Originally researchers expected the collagen portion of the bone to provide a greater amount of DNA since it housed the organic component of the cell. However, the hydroxyapatite component appears to play an important role in protecting the DNA and may bind to it. Furthermore, degradation of the hydroxyapatite component of bone has a negative impact on the recoverability of DNA (Gotherstrom et al. 2002). The overall number of osteocytes present in a compact bone sample has been compared to the amount of DNA recovered. The researchers found a correlation between the amount of DNA recovered and the osteocyte quantity in the bone sample (Soler et al. 2011).

Nuclear DNA recovery is also mediated by the skeletal element tested (Milos et al. 2007; Mundorff et al. 2009; Mundorff et al. 2011). The study by Milos et al. compared differences in DNA yield across skeletal elements. Results indicate that load-bearing lower limb long bones (i.e., femur and tibia) provide significantly better DNA samples than their upper limb counterparts (Milos et al. 2007). This finding was thought to be the result of the densely packed cell structure of cortical bone. Authors suggest that the densely packed cortical bone cells provide better protection for the DNA from degradation than the more dispersed cancellous bone cells (Milos et al. 2007). Although areas of cancellous bone may start out with more available DNA due to increased vascularization, these unmineralized cells are not well preserved during decomposition and skeletonization.

A more recent study performed by Mundorff et al. produced similar results with the lower limb long bones continuing to out-perform the upper limbs (2009). Mundorff et al. also found that small ankle, foot, and sesmoid bones, not included in the Milos et al. 2007 study, produced even better DNA profiles. These smaller elements have a much thinner layer of cortical bone over the cancellous structure and therefore the samples consisted of a mixture of bone types. These results question the original assessment that dense cortical long bones provide the best source of DNA in recently deceased individuals. The results also exposed a need to reevaluate the conventional expectation that weight-bearing cortical bone provides the best samples for DNA testing (Mundorff et al. 2009).

Both the Milos et al. (2007) and the Mundorff et al. (2009) studies were retrospective with little control over taphonomic influences. A follow-up study by

Mundorff et al. (2011) systematically evaluated each bone type for the quality and quantity of recoverable DNA. Attempting to control for confounding factors, the sample was limited to bones from only three individuals, and the same bones were tested from each individual. All three individuals decomposed in the same environment, reducing the likelihood of differential taphonomy influencing the results. Mundorff et al. (2011) found that upper limb and skull bones continued to perform poorly, but the tarsals, hand and foot phalanges, and metacarpals / metatarsals outperformed the femur and the tibia of the same individual.

Misner and colleagues (2009) assessed the differences in mitochondrial DNA degradation by skeletal element for an archeological sample. They found that element type had a significant impact on the quantity of DNA recovered from the bone. Although the likelihood of femora and ribs to produce a successful DNA amplification were not statistically different, both were statistically more likely to produce a profile than the pelvis. The successful amplification of DNA profiles varied by element type, resulting in a 79% (23/29) success rate for femora, a 64% (21/23) success rate for ribs, and a 36% (9/25) success rate for pelvis. Furthermore, the authors noted the breakdown of the outer layer of cortical bone was usually more pronounced on sample pelvis, offering a possible histological correlate with DNA degradation (Misner et al. 2009).

DNA Degradation

Differentiating between nuclear and mitochondrial DNA is an important factor to consider when assessing DNA degradation. Offspring inherit nuclear DNA from both parents. As the name indicates, nuclear DNA resides in the nucleus of the cell. It is the

underlying template for the production of proteins and enzymes used to build structures in the body. Unlike nuclear DNA, mitochondrial DNA resides in the mitochondria of the cell and is matrilineally inherited. Furthermore, mitochondrial DNA is viable in cells for a significantly longer period of time than nuclear DNA so it is commonly used to examine questions of human evolution or past human migration patterns (Schultz and Smith 2008).

Taphonomy: Moisture

Environmental conditions associated with decomposition appear to have a significant impact on the recovery of DNA from bone and tissue. Varying environmental conditions can be both general, such as geographic region, and specific, such as soil chemistry. Skeletal remains are also exposed to varying taphonomic factors including weathering and sun bleaching from surface exposure, varying degrees of moisture above and below ground, and different conditions within a burial such as pH levels (Pfeiffer et al. 1999; Graw et al. 2000; Minser et al. 2009; Soler et al. 2011).

Iwamura and colleagues (2005) compared DNA degradation in bones exposed to several different environmental conditions. The results suggest that different environments cause varying degrees of DNA degradation. Bones exposed to soil performed better than those immersed in fresh water. Degradation occurred more rapidly in aquatic conditions than arid ones, and humid conditions also accelerated DNA degradation (Iwamura et al. 2005). Bone samples exposed to tropical rainforest conditions showed a decrease in osteocyte counts, which was correlated with a decrease in the availability of quantifiable DNA. Since there are relatively few osteocytes per unit

of bone, the authors suggest that more sensitive DNA amplification techniques are necessary to extract a complete profile from a degraded sample (Soler et al. 2011).

A study by Graw et al. (2000) examined nuclear DNA degradation in four skeletonized forensic cases recovered from damp environments. The skeletons were from various postmortem intervals ranging from 6-45 years (or unknown). Although the remains were recovered from “damp environments” the condition of the remains, as well as the remains themselves, varied significantly from case to case. Different elements were recovered for each case. DNA from the mastoid, calotte, and teeth were sampled from all four cases, though none yielded a DNA profile. Phalanges from one case and humeral fragments from a different case were also tested. DNA was successfully extracted from the humerus but not from the phalanges. Decomposed brain matter yielded full profiles in cases where bone had failed, leading the authors to conclude that damp environments degrade skeletal DNA more quickly than DNA in other tissues.

Taphonomy: Burial and Surface Exposure

Burial conditions provide another set of variables to be considered. Pfeiffer et al. (1999) tested the effects of soil on nuclear DNA degradation. They sectioned 24 teeth in half. One half of each tooth was buried in garden soil and dug up for testing at intervals of 6, 12, and 18 weeks. The other tooth halves were frozen and sampled at the same time intervals to provide a control. Results suggest that DNA degradation occurs rapidly following burial. The DNA concentration from the buried teeth was reduced by 90% after six weeks when compared to the control teeth.

Misner et al. (2009) examined the effects of weathering on the recoverability of

mitochondrial DNA from different skeletal elements. The skeletons came from an archaeological collection excavated from Voegtly cemetery in Pennsylvania. Individuals were interred in the cemetery from 1833 and 1861, thus the skeletal sample had a known post mortem interval and relatively consistent burial conditions. This information limited the number of confounding variables so that differences in DNA degradation could be attributed to the extent of weathering. A total of 36 skeletons were ranked on a zero to five scale, based on the degree of weathering, before DNA tests were conducted. The specific elements to be DNA tested were ranked separately from their entire skeletal rank because the degree of overall weathering was often different to weathering on specific elements. DNA samples were mostly taken from the femur, pelvis, and rib. If these three bones were not present then different elements were sampled. The largest base pair sequence targeted in this study could not be produced from any sample but shorter sequences were obtained from some of the remains. The authors did not find a statistically significant correlation between the degree of weathering and the quantity or quality of DNA. The results suggest that skeletal weathering did not influence the amplification of mitochondrial DNA.

Misner et al. (2009) concluded that visual signs of weathering and break down of bone was not necessarily indicative of the degree of mitochondrial DNA degradation. Thus the preservation of a bone's surface morphology is not representative of DNA preservation. Furthermore, different processes might have a greater or lesser affect on different skeletal elements and DNA in cortical and cancellous bone might degrade via different mechanisms.

Taphonomy: DNA Extraction and Contamination

While environmental conditions associated with the recovery of remains have a significant impact on the recovery of DNA, other factors associated with the sampling and extraction procedures can produce a negative impact on DNA yields (Kemp and Smith 2005; Adler et al. 2011). Prior to DNA extraction, bone samples must be reduced to a powdered state so that the DNA in the cells is accessible. Although there are several techniques used to produce bone powder, the most common is sampling with a drill. This technique is generally considered preferable to crushing a window section removed from the bone, because a single drill hole is less destructive to the whole bone.

Recently a study by Adler et al. (2011) found that samples collected with drilling produced poorer mitochondrial DNA profiles than those powdered using a bone mill. Samples produced by drilling yielded up to thirty times less DNA than those pulverized by a bone mill. The researchers attribute the decrease in DNA collected from drilled samples to heat damage produced by the friction created by rapidly spinning drill bits during drilling. The decrease in mitochondrial DNA recovery disappeared when using slow drill speeds; corroborating the conclusion that high speed drilling negatively affected the recovery of DNA from bone samples. While the process of drilling is destructive to the bone, it also provides an avenue for contamination of the sample with extraneous DNA from the surface of the bone.

Contamination is serious problem when working with ancient DNA since the ancient DNA is already degraded and the modern DNA may mask the signal since it is also replicated during the amplification process (Kemp and Smith 2005). In order to prevent such contamination for occurring, bleach is used on the surface of the bones and

teeth prior to sampling. The process of bleaching the bones is effective because it removes the surface contaminate without damaging the internal mitochondrial DNA of the sample. Even the most extreme treatment, submersion of samples for twenty-one hours at concentrations of 6.0 % sodium hypochlorite, did not prevent the amplification of DNA from the ancient bone samples. These techniques are also applicable to forensic cases as samples may be contaminated during collection and the DNA from the sample might be degraded from taphanomic processes.

Postmortem Interval

Perry et al. (1998) designed a study to estimate the postmortem interval based on DNA degradation in bone. The authors observed similar rates of DNA degradation, across multiple individuals when bones were maintained in sealed plastic containers and incubated at a similar temperature and humidity. They hypothesized that if environmental conditions were known for specific remains, the time of death could be reconstructed. However, difficulties determining the skeleton's exact exposure condition complicated their ability to establish postmortem interval from DNA degradation. The authors concede that DNA degradation varies based on temperature and humidity more than it does between individuals, and that different skeletal elements from the same individual may decay at different rates.

DNA

DNA can be divided into coding and non-coding regions. Coding regions are those that supply the information for the creation of all the proteins needed to build and maintain body structures (Hartl 2009). These portions of the DNA are usually highly

regulated, since minor changes or substitutions often have a significant, usually deleterious effect on the organism. Since the proteins created by these regions are necessary for proper function of various body systems, even small alterations can threaten the viability of the zygote.

On the other hand, non-coding regions do not directly affect the physiological function of the organism (Hartl 2009). These portions are removed from the DNA prior to transcription and translation into proteins so mutations often go completely unnoticed. One type of coding error seen in non-coding portions of DNA is called a short tandem repeat or STR. An STR is a short sequence of nucleotides or base pairs (A, G, C, T) that repeats one or more times, frequently following the original sequence. For example, if the sequence appeared as AGTC[CTCCGCTCCGCTCCG]TTGGA, the bracketed segment would represent a short tandem repeat of the nucleotides CTCCG.

Polymerase Chain Reaction (PCR)

One technique used to visualize DNA is to amplify the sequence of a specific region in order to analyze the series of base pairs coding for that portion of DNA. Amplification is used to increase the number of copies of a DNA sequence. One specific process used to amplify DNA in a forensic context is polymerase chain reaction (PCR). During this reaction, double stranded DNA is heated, separating the two strands of DNA to expose the bases. Primers are short sequences of DNA that are artificially synthesized, and that are complementary to the sequence to the ends of the locus to be amplified. The primers bind to the desired section of the DNA, which allows polymerase enzymes to continue adding complementary base pairs to sequence until the entire length of the DNA

strand is completed. Thus the net result of the reaction is the production of two complete double stranded DNA sequences.

Repeating this process many times produces an exponential effect since each segment of DNA present from the previous cycle of PCR produces two segments in the subsequent round. Thus the process of PCR amplification produces many identical copies of the desired sequence, thereby amplifying the frequency of that sequence in the solution (Saiki et al. 1986; Saiki et al. 1988). Following amplification, the sequence can be visualized by running it through a gel electrophoresis which separates out the molecules based on fragment size and density, because light particles can travel farther than heavier ones.

Recently, new DNA extraction kits have been developed and optimized to overcome the damaging effects of chemical treatments to bone thus increasing DNA recoverability. However, the new techniques for degraded DNA have not been thoroughly validated for use in forensic contexts. Therefore researchers such as Franchi and colleagues (2011) still advocate preservation of a bone fragment not subjected to any maceration techniques in order to maintain a DNA sample.

Inhibitors

Inhibitors pose a significant challenge to the recovery of DNA. Many different substances can act as inhibitors. Inorganic ions such as calcium, indigo dyes, organic acids, as well as human tissues (collagen) or pigment (melanin) have all been documented as PCR inhibitors (Opal et al. 2009). Inhibition occurs during the amplification process, but different inhibitors affect different aspects of the amplification

reaction. Inhibitors can prevent amplification by binding to the polymerase enzyme, interacting with or binding to the DNA, preventing the polymerase from functioning correctly during primer extension, or depleting necessary cofactors. Moreover, inhibitors can act in one or more of these fashions, and multiple different inhibitors can be present in a single sample.

Methods to combat inhibitors fall into two different categories, those that reduce the effect of inhibitors by altering PCR reaction ratios or cycling conditions, and those that remove the inhibitor through purification processes prior to amplification (Kemp et al. 2006). Often water is added to inhibited samples to reduce the ratio of inhibitors to PCR reagents. Although the ratio of PCR reagents to DNA is also reduced, without the effect of the inhibitors, the PCR amplification process increases the amount of DNA to quantifiable levels. Altering DNA melting temperature or size of amplification products can also reduce the impact of some inhibitors, but these methods are not effective for all inhibitors. Therefore, these techniques would be of limited use unless the specific inhibitor could be identified (Opel et al. 2009).

CODIS

While DNA can provide information about ancestry and sex via genetic markers at specific loci, it is of limited use for identifying an individual when it cannot be compared to a known sample. CODIS DNA is most effective when a genetic profile collected from a known individual can be matched to an unknown sample. A repository of known CODIS DNA profiles provides an avenue to compare unknown profiles against in order to identify the unknown sample. Therefore, the establishment and curation of a

database to store CODIS DNA profiles is necessary for DNA to be an effective identification tool.

The development of the Combined DNA Index System (CODIS) has provided a way to standardize and compare CODIS DNA profiles to aid in the identification of victims and criminals (Butler 2006). One subsection of CODIS is the National DNA Index System (NDIS), which contains the DNA profiles contributed by local, state, and federal forensic laboratories. When a sample is collected during an investigation the profile is uploaded and compared to those already in the database (CODIS fbi.gov). A “hit” occurs when a new profile under investigation matches one already in the database. A CODIS profile includes thirteen STR markers and amelogenin. The standard markers include: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 (Budowle et al. 1998). The combination of these markers has proven to be a reliable tool for establishing a unique identity. These specific markers were selected based on a comparison of multiple human STR loci because they showed a high variability and produced minimal stutter product (Butler 2006). Since the loci are highly variable among individuals, the chance that any two people would match at all 13 autosomal STR loci is unlikely. Stutter product occurs when the DNA is not replicated in its entirety due to slippage of the polymerase enzyme. Samples with stutter product have extra, reduced peaks adjacent to the actual STR peak, which can be confused for a second degraded DNA profile mixed with the primary profile. Therefore, minimizing stutter product is essential for accurately identifying a profile. Since the CODIS loci meet these criteria they have become a reliable and respected tool for establishing the identity of an individual.

CHAPTER III

MATERIALS AND METHODS

Sample Background

The research sample comprises skeletal elements from three individuals who self-donated to the FAC. To minimize confounding factors, only unautopsied individuals with similar demographics were considered. The individual subjects used in this study were males ranging from 45-75 years old at death, and self-identified as white.

Shortly after death, the bodies were placed unclothed on the ground surface and covered with a black tarp (loose enough to not inhibit insect activity) for the purposes of natural decomposition at the ARF located in Knoxville, Tennessee. The bodies remained on the surface throughout the duration of soft tissue decay for approximately one year. All individuals were exposed to similar environmental conditions during decomposition.

The skeletons were assigned the following FAC accession codes: UT07-09D, UT45-09D, and UT116-09D (Table 1) and are same skeletons used in a previous study by Mundorff et al. (2011). Overlap in the elements tested in the two studies was avoided by processing elements from the opposite side of the skeleton, or antimere elements. The elements tested in Mundorff's study were used as an unprocessed control group to provide a comparative baseline of the potential DNA from comparative elements of the three individuals.

Table 1. Demographic and Placement Data

Year	UT ID #	Race	Sex	Age	Date of Placement	Date of Collection
2009	UT07-09D	W	M	50	1/21/09	2/17/10
2009	UT116-09D	W	M	47	11/20/09	3/18/11
2009	UT45-09D	W	M	69	4/24/09	3/18/11

Sample Selection

Procedural Considerations

To minimize destruction, DNA samples were limited to 0.2 grams of bone powder removed by drilling a 3/8" circular hole. The sample was taken from a pre-determined site on each element to avoid destroying significant morphological features. Thus, every effort was made to minimize the impact of this research on use of the skeleton for future projects.

Although the original sampling of left elements by Mundorff et al. (2011) was limited to a 3/8" hole, the internal structure of the bone was exposed. Since it is unclear how processing with hot water affects the internal aspects of bone and subsequent DNA yields, only right elements were tested for this study. The left elements served as controls because they were tested prior to any processing. Element side does not appear to affect DNA yield, thus comparing right and left elements from a single individual maintains consistency.

Bone Type Considerations

Bone type was also considered during the selection of elements for this study. Bone can be classified into two different types based on their structure components: cortical bone and cancellous bone (White 2012). Therefore, the elements of both cancellous and cortical bone were chosen for this study to access possible differences in the effects of processing on bone's structural differences. A long bone shaft offers a good example of cortical bone while tarsals, ribs, and phalanges offer a mixture of both cancellous and cortical bone.

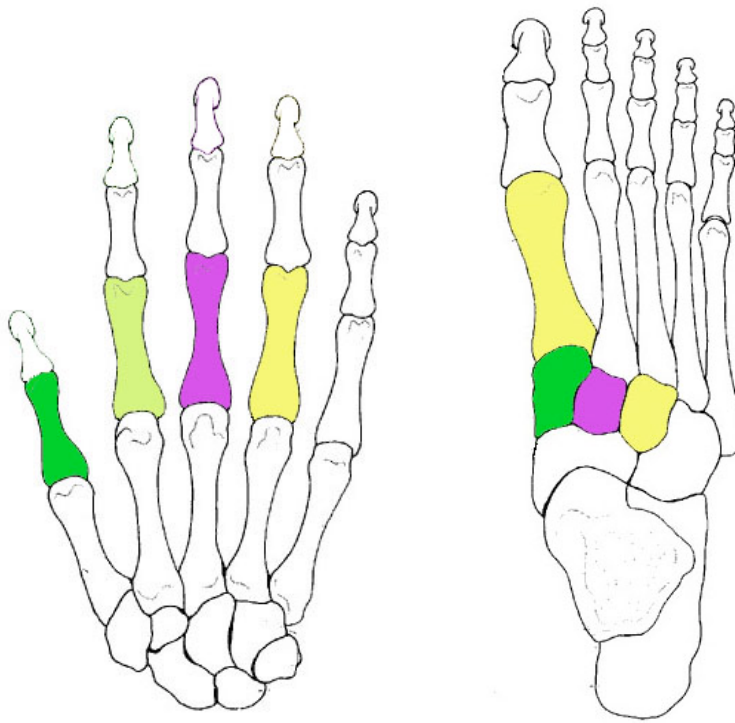
Data Set

Control Dataset

The dataset serving as the control is a subset of previous work provided by Mundorff et al. (2011). This study sampled STR DNA from all element types in the human skeleton (n= 55) from the same three individuals as this study to establish a systematic ranking of DNA yield. The skeletons were not processed prior to DNA testing. Following natural decomposition, the skeletons were picked up from the facility and brought directly to the laboratory. The bones were rinsed with a new toothbrush and water. A new toothbrush was used for each individual to prevent cross contamination. The skeletons were washed briefly to remove the surface coating of dirt, but no other processing procedures were applied to these skeletons.

Project Dataset

The elements tested are the left femur, tibia, 1st metatarsal, middle rib (6 or 7), 1st, 2nd, and 3rd cuneiforms, and the first proximal hand phalanx. These specific elements were chosen because they were found to consistently produce complete profiles by Mundorff et al. (2011). With the exception of the long bones (femur, tibia, and 1st metatarsal) the other elements selected for this study ranked among the top ten elements for profile quality. Although the long bones did not perform as well, they were chosen for comparison to include elements consisting mainly of cortical bone. The structural composition of the other elements consists mainly of cancellous bone, thus the inclusion of cortical bone elements offers a useful counterpoint.



Elements Sampled for Processing Comparison

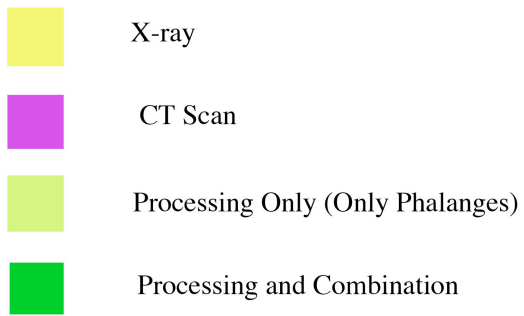


Figure 1. Specific bones sampled from the hand and foot

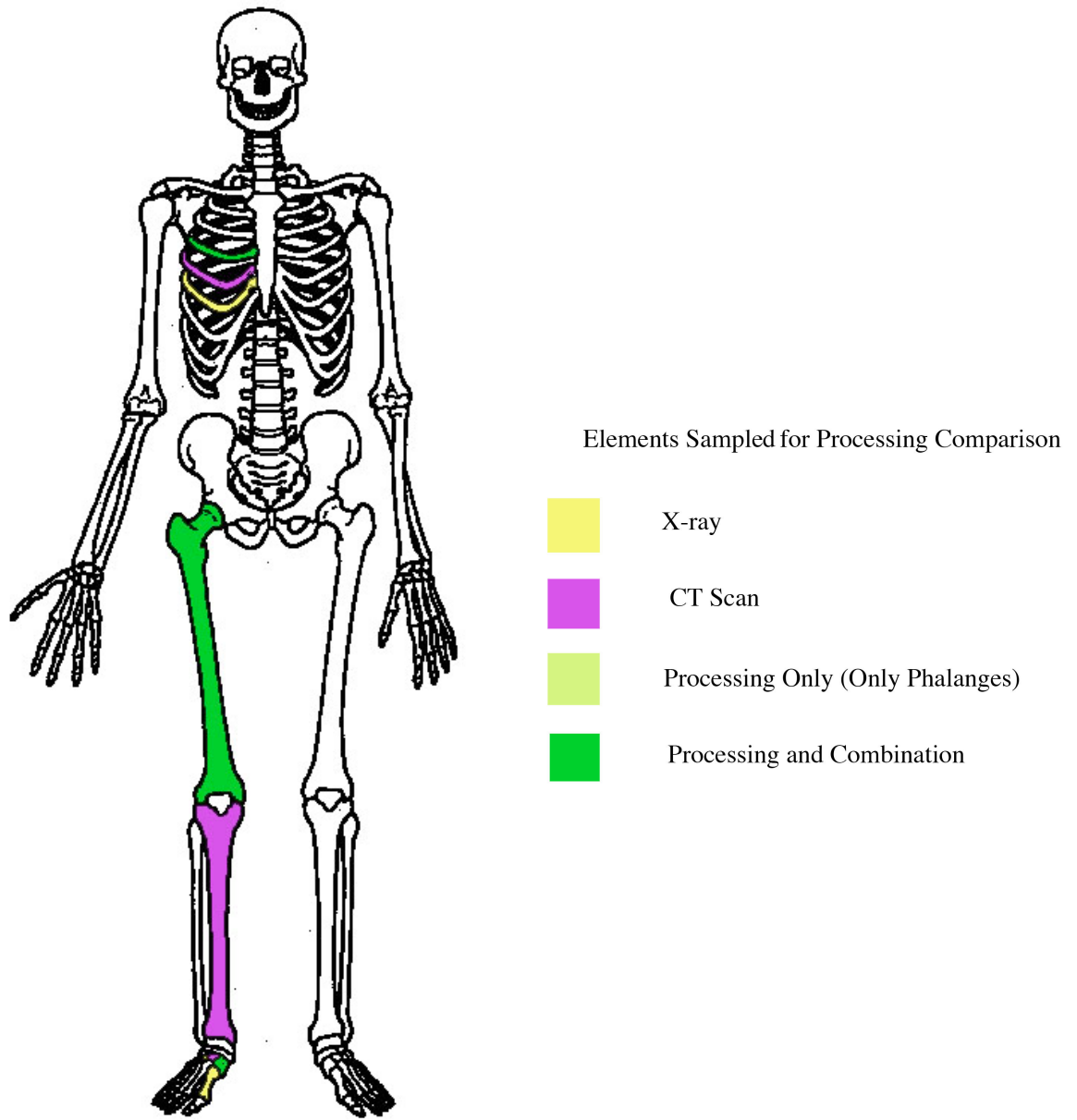


Figure 2. Long Bone and Rib Sampling

Processed Sample

The specific right bone elements include: three middle ribs (6,7,8), the femur, the tibia, the 1st metatarsal, the 1st, 2nd, and 3rd cuneiform, and four proximal hand phalanges (Figures 1&2). The effect of heat maceration was tested with the femur, a rib, the 1st cuneiform, and a proximal hand phalanx from each of the three individuals. The 1st metatarsal, a rib, the 3rd cuneiform, and a proximal hand phalanx were utilized to test the effects of X-ray. The CT-scanned trial tested the tibia, a rib, the 2nd cuneiform, and a proximal hand phalanx.

The exact bones used to test the effect of heat maceration were also the bones used to test the three methods combined. This was in part due to the limited number of long bones that performed well in the Mundorff et al. (2011) study.

Reusing bones for the combined trial also limits the number of elements exposed to destructive analysis, and provides the opportunity to directly compare the effects of multiple treatments on the same skeletal elements. The proximal hand phalanx was the only exception. This bone was not tested again because it was unclear whether its small size would provide adequate bone powder during a second sampling event. Instead, another phalanx was used during the combination trial. In total, this research tested 39 elements comprising 48 STR DNA samples.

Processing Methods

Methods 1: Hot Water Maceration

At the ARF, human remains are placed outdoors and allowed to skeletonize through natural decomposition processes. Mummification may occur on occasion, or

small quantities of soft-tissue may remain adhered to bones. In these cases prolonged outdoor decomposition is insufficient to remove the tissue, so remains are collected and cleaned using the established FAC processing procedures (Jantz and Taylor 2009). Following the FAC guidelines, crock-pots were used for heat maceration. Bones were fully submerged in the crock-pots to allow uniform heating. The femur was too large to fit into the crock-pots and was therefore placed individually in a large metal pot directly on the stovetop (in accordance with FAC protocols). Bones from different individuals were macerated separately to prevent DNA contamination. Bone elements from the same individual were heated in the same pot.

Following the FAC guidelines, bones in this study were submerged in the heated water for 45 minutes to soften the tissue for easy removal with a toothbrush. The crock-pots and stove pot were carefully monitored for water depth and the temperature was recorded every 15 minutes to make sure that the water did not reach a boil (Tables 2-4). The contents of the pots were periodically agitated to prevent a bone from resting against the bottom of the pot for the entire heating process. This allows for even heating and prevents a single element from overcooking.

After 45 minutes, the bones were removed and allowed to cool for several minutes. Next, they were rinsed under cool tap water and cleaned with a new toothbrush to remove any adherent soft tissue. The pots were cleaned with soap and hot water then sprayed with enviroid in between skeletons to reduce the likelihood of DNA contamination.

The bones did not require a second round of heating since most had little to no adherent tissue. After maceration the bones were placed on clean, clearly labeled trays

Table 2. Heat Maceration UT45-09D

UT45-09D				
Bone	Time Interval	Time Actual	Temp (F*)	Depth (mm)
Femur	0	2:45	138	140
	15	3:00	140	
	30	3:15	146	
	45	3:30	150	
Rib/ Tarsal/ Phalanx	0	2:46	127	137
	15	3:01	135	
	30	3:16	142	
	45	3:31	144	

Table 3. Heat Maceration UT07-09D

UT07-09D				
Bone	Time Interval	Time Actual	Temp (F*)	Depth (mm)
Femur	0	10:06	150	138
	15	10:21	150	138
	30	10:36	150	137
	45	10:51	148	137
Rib/ Tarsal/ Phalanx	0	10:09	136	136
	15	10:24	140	135
	30	10:39	145	135
	45	10:54	147	135

Table 4. Heat Maceration UT116-09D

UT116-09D				
Bone	Time Interval	Time Actual	Temp (F*)	Depth (mm)
Femur	0	3:10	140	138
	15	3:25	150	137
	30	3:40	152	136
	45	3:55	152	136
Rib/ Tarsal/ Phalanx	0	3:15	105	144
	15	3:30	113	143
	30	3:45	118	143
	45	4:00	124	143

covered with multiple layers of brown paper bags (to absorb wetness) and allowed air dry completely.

Method 2: CT Scan

Numerous skeletons from the Bass collection were CT scanned to build a three-dimensional inventory by Moore and Schaefer (2011). These authors used a GE Lightspeed 16 Slice computed tomography scanner with a 1.5-meter long table. Bones were placed in polyurethane foam lined cardboard boxes. Elements were positioned to prevent contact with each other. The following parameters were used:

1. Mass – 150 at .8 seconds
2. Field of View– 32 cm
3. Resolution - .625 mm slices
4. 100 KiloVolts (KV)
5. Use Bone Algorithm 512/512 mm to display three-dimensional image matrix of scanned element

The same CT scanner used by Moore and Schaefer (2011) was not available for this study; therefore scans were produced using a Siemens mCT 64 slice at the UT Medical Center. The three skeletons were scanned separately to prevent contamination and commingling. Each set of bones was exposed to a single scan. The four elements were placed directly on the CT scanning bed positioned close enough to each other to be captured by a single scan while still preventing contact. The scan had the following parameters.

1. Resolution - .600 mm slices

2. 120 KV
3. Scan time: 10.4 seconds

The scans were processed and rendered via the CT scanner bone algorithm to visualize the element as a three-dimensional image. Although the actual images were not part of this study, the individual slices have been stored on a CD for future use.

Method 3: X-ray

The bones were X-rayed to assess whether or not X-ray radiation affected DNA recovery from bone. X-rays are commonly used in forensic casework and expose the samples to less radiation than CT scans. Following the protocol established by the UT Health Center, which has radiographed been used on skeletons from the Bass collection previously, the bones were exposed to the amount of radiation required to produce an adequate image.

Exposure was a single X-ray of 50-kilovolt peaks (kVp), 150 milliamps (mA), for 0.030 seconds at a distance of 40 inches. Bones from same individual were X-rayed together and the three skeletons were X-rayed separately from each other. For each X-ray the bones were arranged on a digital X-ray board adjusted to properly fit the entire group in a single image. Although the images were not part of this study, they were checked to ensure a good quality X-ray was produced and the images were saved on a CD for future study.

Method 4: Combination of the four methods

A trial combining the three procedures together offered the opportunity to access multiplicative affects on DNA yield. Baseline effects for individual methods were set by

trials one to three. The combined trial assessed the interaction of multiple sequential methods on DNA yield. The same bones used during the maceration trial were again used to assess the combination of methods. Having already undergone DNA sampling, each element had a 3/8” drill hole at the sampling site. Thus, a new sampling site, adjacent to the original, was selected to prevent re-use of the original site while still sampling a similar area of the bone.

The bones used to test the effects of heat maceration were reused following the first round of DNA tests. These same bones (and a different phalanx) were then subjected to X-ray and CT scanning. This additional phalanx was previously macerated with the other elements (Table 5). The same CT and X-ray parameters were repeated for the combined trial to ensure consistency.

Table 5. Heat Maceration Phalange for Combined Trial

Bone	Time Interval	Time Actual	Temp (F*)	Depth (mm)
07-09 phalanx (combined)	0	10:10	142	121
	15	10:22	148	121
	30	10:40	152	121
	45	10:55	155	121
45-09 phalanx (combined)	0	10:12	141	114
	15	10:27	148	114
	30	10:42	152	114
	45	10:57	156	114
116-09 phalanx (combined)	0	10:14	143	79
	15	10:29	150	79
	30	10:44	153	78
	45	10:59	157	78

DNA Analysis

For comparison purposes, the DNA sampling procedures were the same as those used by Mundorff et al. (2011). Profiles extracted from the samples were assessed for both quality, based on the completeness of the profile, and quantity, based on the RFU value and total DNA yield. Bode Technology, an ASCLD/LAB and FQS ISO certified laboratory, preformed the CODIS loci DNA extraction and amplification.

Decontamination and Sampling

Prior to DNA extraction, the surface of each bone was cleaned with a 10% bleach solution. The bones were then rinsed with water purified via the Millipore Water Purification System, followed by a 70% ethanol solution. Bone sampling was conducted using a 3/8" circular drill to remove 0.2-grams of bone powder from each element. Although greater amounts are often used, the samples were limited to 0.2-grams of bone powder to ensure equivalent samples across bones. Each sample was weighed prior to DNA extraction to ensure consistency between samples. The bone powder was collected in a 50-milliliter (ml) conical tube.

DNA Extraction

The powdered bone was maintained at 56°C in a solution of EDTA for 18 hours to facilitate demineralization. Next, DNA was extracted using the Qiagen QIAamp extraction method. The EDTA was conserved, and the entire solution was eluted into 50 µl of TE⁻⁴. Extractions were preformed in groups of eleven samples with an additional reagent blank maintained for control purposes.

DNA Quantification

The Applied Biosystems (ABI) Quantifiler was used to quantify the extracted DNA. A ratio of 6.25 µl: 5.25 µl Quantifiler Buffer to Quantifiler Primer was used for each reaction. For each reaction, 2 µl of DNA was added to 11.5 µl of the buffer/primer solution. The samples were analyzed with an ABI 7500 SDS instrument using compatible version 1.2.3 software. STR DNA yields were calculated by multiplying the quantification value in ng/µl by the elution volume (50 µl) and then dividing by the mass of sampled bone. Thus DNA yields are given in ng DNA/ gram of bone.

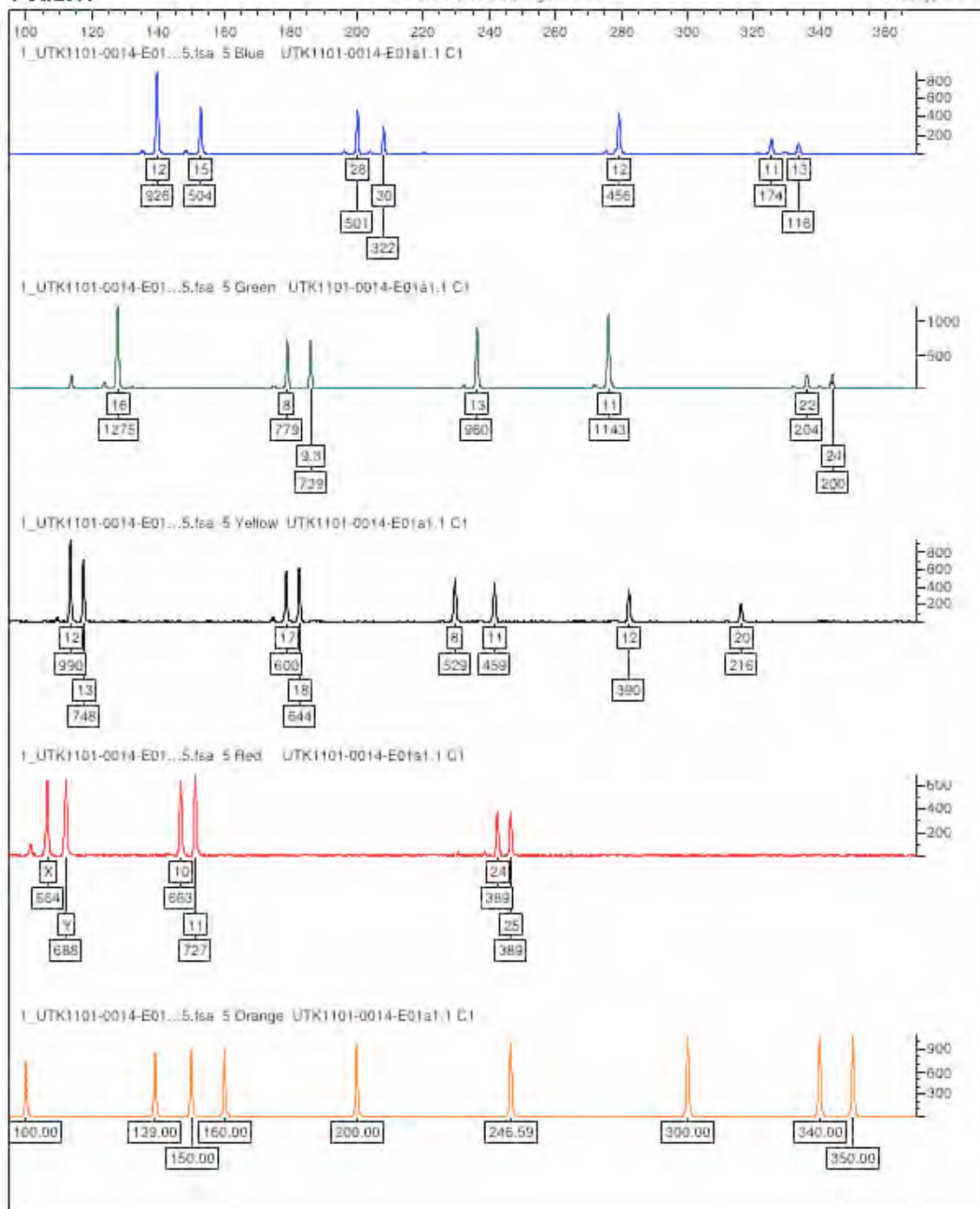
DNA Amplification

Polymerase chain reaction (PCR) was used to facilitate amplification of the designated STRs. For this study, DNA extracts were amplified using the AMPfISTR Identifiler™ system. The proportions of amplification components were maintained for each reaction at a ratio of 10.5 µl amplification buffer: 5.5 µl Identifiler Primer: 1.0 µl Taq Gold. A total of 15.5 µl of the mix was added to 9.5 µl (2 ng) of DNA extract for each reaction. Each sample was run on an ABI 9700 thermocycler through 28 cycles to allow sufficient amplification.

Although a single amplification was usually sufficient to produce a DNA profile, several samples showed inhibition. Inhibition was detected by including a non-human internal positive control (IPC) within the PCR reaction. A separate reaction targets the non-human DNA, which has a known amplification duration of 27 cycles. In the presence of inhibitors the IPC reaction is delayed or in some cases fails to amplify at all. In these cases a second round of amplification was performed to overcome the inhibition.

For re-amplification, less DNA extract was added to the amplification reaction solution to reduce the interference of the inhibitor. Once the sample had been amplified, the STR products were separated using an ABI 3130xl fragment analyzer. For fragment analysis, 0.7 µl of amplified STR product was added to a mix of 10 µl formamide and 0.12 µl (ILS) GS500 Liz.

The ABI GeneMapperID software version 3.2.1 was used to measure the signal strength (peak heights) at the specific loci analyzed in this study (Figure 3). Peak height was measured in relative fluorescent units (RFU), since STR loci with greater quantities of amplified DNA produce a stronger RFU signal. To be considered viable, a peak had to measure at least 75 RFU for a locus at which the individual was heterozygous, and at least 200 RFU for the homozygous cut-off. Furthermore, the allelic balance had to be at least 50% for the allele to be considered quantifiable. For each allele, RFU value was calculated as the average of the two peak heights (heterozygous). When the individual was homozygous at a locus, the single peak was divided in half to generate the average. The loci examined for this project include the thirteen standard CODIS STRs: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11, plus Amelogenin (AMEL). The Qiagen QIAamp micro kit also includes two additional loci: D19S433 and D2S1338. The location and magnitude of the 15 autosomal STR loci and the amelogenin gene can be seen in figure three. Quality of the STR DNA profile was assessed based on the number of loci recovered out of the 16 total loci. RFU values and DNA yield values were used to measure the quantity of STR DNA present in a sample.



For Forensic and Research Use Only

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Not for use in Diagnostic Procedures.

Figure 3. STR results for the control femur from skeleton UT 07-09D.

CHAPTER IV

RESULTS

Results will be presented in two sections. The first section presents the data used to assess the quality and completeness of the DNA profile for each sample. The second section includes the values indicative of the quantity of DNA recovered from each of the samples. Test results from assessing the quality of DNA profiles, for each method, are presented in Tables 6 to 10, respectively. A “full profile” or “complete profile” consists of 16 total loci, which includes 15 autosomal STRs and the amelogenin gene. The specific numbers of STR and amelogenin loci are presented for the samples that did not yield full profiles. Results are highlighted in either yellow or orange based on their relationship to the control values. Samples highlighted in yellow yielded a less complete profile than the comparative control value while samples highlighted in orange yielded a more complete profile than the comparative control. Samples not highlighted produced an identical profile to their respective control.

Since the control sample was taken from Mundorff et al. (2011), only a single middle rib and proximal hand phalanx were used from each individual. This single element was then compared to multiple elements of the same type (ie. rib) that underwent processing. Therefore it is significant to note that there is not a one-to-one relationship between the control and processed elements, as the control results are reused. For example, the single control middle rib from each individual is used for comparison against the heat-macerated rib, the X-rayed rib, the CT scanned rib, and the rib that underwent a combination of treatments. If a unique control rib was tested for comparison

Table 6. Control Profile Results

	No Processing					
Element Type	UT07-09D		UT45-09D		UT116-09D	
	Sample #	Result	Sample #	Result	Sample #	Result
Middle rib	5	Full Profile	58	Full Profile	111	Full Profile
Femur	14	Full Profile	67	14 + amelogenin	120	13 + amelogenin
Tibia	15	7 + amelogenin	68	14 + amelogenin	121	14 + amelogenin
Proximal phalanx (hand)	26	Full Profile	79	Full Profile	132	Full Profile
1 st metatarsal	28	Full Profile	81	11 + amelogenin	134	Full Profile
1 st cuneiform	39	Full Profile	92	Full Profile	145	Full Profile
2 nd cuneiform	40	Full Profile	93	Full Profile	146	Full Profile
3 rd cuneiform	41	Full Profile	94	Full Profile	147	Full Profile

Table 7. Profile Results: Hot Water Maceration

	Heat Maceration					
Element Type	UT07-09D		UT45-09D		UT116-09D	
	Sample #	Result	Sample #	Result	Sample #	Result
Middle rib	600	Full Profile	604	Full Profile	608	Full Profile
Femur	601	9 + amelogenin	605	Full Profile	609	13 + amelogenin
1 st cuneiform	602	6 + amelogenin	606	Full Profile	610	Full Profile
Proximal phalanx (hand)	603	Full Profile	607	Full Profile	611	Full Profile

Table 8. Profile Results: CT Scan

	CT Scan					
Element Type	UT07-09D		UT45-09D		UT116-09D	
	Sample #	Result	Sample #	Result	Sample #	Result
Middle rib	800	Full Profile	804	Full Profile	808	Full Profile
Tibia	801	4 + amelogenin	805	Full Profile	809	9 + amelogenin
2 nd cuneiform	802	Full Profile	806	Full Profile	810	Full Profile
Proximal phalanx (hand)	803	Full Profile	807	Full Profile	811	Full Profile

Table 9. Profile Results: X-ray

	X-ray					
Element Type	UT07-09D		UT45-09D		UT116-09D	
	Sample #	Result	Sample #	Result	Sample #	Result
Middle rib	700	Full Profile	704	Full Profile	708	Full Profile
1 st metatarsal	701	11 + amelogenin	705	14 + amelogenin	709	Full Profile
3 rd cuneiform	702	Full Profile	706	Full Profile	710	Full Profile
Proximal phalanx (hand)	703	Full Profile	707	14 + amelogenin	711	11 + amelogenin

Table 10. Profile Results: Combined Treatments

	Combined					
Element Type	UT07-09D		UT45-09D		UT116-09D	
	Sample #	Result	Sample #	Result	Sample #	Result
Middle rib	900	Full Profile	904	Full Profile	908	Full Profile
Femur	901	5 + amelogenin	905	12 + amelogenin	909	7 + amelogenin
1 st cuneiform	902	No Results	906	Full Profile	910	Full Profile
Proximal phalanx (hand)	903	Full Profile	907	Full Profile	911	Full Profile

against each sample separately, more variation in recovered STR loci might be noticeable due to normal fluctuations in the samples. The repeated use of a single control could provide a false impression of consistency within the control data set, because it might appear that many control elements produced identical profiles.

All 24 unprocessed bones produced at least a partial autosomal STR profile and amelogenin and 18 produced full profiles (Table 6). The middle rib, proximal hand phalanx, 1st cuneiform, 2nd cuneiform, and 3rd cuneiform produced a full profile for all three individuals. The femur, tibia, and 1st metatarsal failed to produce complete profiles for at least one skeleton. Two 1st metatarsals produced complete profiles while the third produced a partial profile of 11 autosomal STR loci (Table 6). The femur produced a full profile for one skeleton, and partial profiles, 14 autosomal STR loci and 13 autosomal STR loci, for the other two. Tibial samples did not produce any full profiles (Table 6). Two tibial samples yielded nearly complete profiles, 14 autosomal STR loci each, and 7 autosomal STR loci were produced from the third.

Hot Water Maceration Results

The middle rib and the proximal phalanx produced full profiles for each of the three skeletons (Table 7). Two out of the three 1st cuneiforms produced complete profiles while 6 autosomal STR loci were amplified from the third. One femur produced a full profile, another produced a partial profile (13 autosomal STR loci) and the third produced 9 autosomal STR loci. A single bone outperformed the control by a single loci and another bone yielded fewer loci compared to the control (9 autosomal STR loci/ full profile). All other results were consistent with the comparative controls (Table 6).

CT Scan Results

Most bone sample exposed to CT scans produced identical results compared to the controls. All middle ribs, 2nd cuneiforms, and proximal phalanges yielded full profiles, consistent with the control sample results (table 8). The tibia is the only element with different results than the control. Two were less successful than the control with one dropping from 7 autosomal STR loci to 4, and the other dropping from 14 autosomal STR loci to 9. The third tibia produced a slightly better profile after CT scanning increasing from 14 autosomal STR loci to a full 15 autosomal STR loci profile (Table 8).

Amelogenin was successfully amplified for all samples.

X-ray Results

All middle ribs and 3rd cuneiforms maintained full profiles after X-ray (Table 9). X-ray radiation had a variable effect on the 1st metatarsal's results with no consistent trend between X-ray exposure and profile completeness. Compared to the control samples, one metatarsal dropped from a full profile to 11 autosomal STR loci, a second increased from 11 autosomal STR loci to 14 and the third maintained a full profile (Table 9). Results from the proximal hand phalanges show a decrease in loci for two of the three samples. Both dropped from complete profiles to 14 and 11 autosomal STR loci, respectively. The third phalanx maintained a complete profile. The amelogenin gene was recovered for all control and processed samples.

Combination Results

Results from the group of bones subjected to all three methods (maceration, X-ray, and CT scan) are comparable to both the control group and the heat-macerated

group. The middle rib and proximal phalanx maintained the same profiles as their respective control samples as well as when the bones were simply macerated (Table 10). Two 1st cuneiforms maintained full profiles across the control, heat maceration, and combined methods. The third 1st cuneiform, UT07-09D, shows a decrease in loci between the trials (Table 10). The control sample produced a complete profile, six autosomal STR loci plus amelogenin were amplified after heat maceration, and no results were detectable following the combined trial. PCR inhibitors were identified in both post-processing samples when the quantification value was measured. Since the control sample was from the unprocessed antimer element it is unclear if the loss of genetic information is due to processing or if other factors may have been involved.

None of the femurs were as successful following the combination of methods as either the control samples or the samples exposed to heat maceration alone. None produced a full profile of autosomal STR loci, though amelogenin was identified for all three (Table 10). Nine autosomal STR loci were recovered from one femur following heat maceration and the same bone yielded five autosomal STR loci following the combined method, both a drop from the control's full profile. The other two femoral bones showed reduced profiles. One dropped from the control's 14 autosomal STR loci to 12 following the combination of methods and the other dropped from 13 autosomal STR loci to 7.

RFU Values and DNA Yields

While profile completeness can be an indicator of DNA quality in a sample, RFU values and DNA yield provide information about the quantity of DNA available

following the amplification process. RFU and DNA yield results highlighted in yellow indicate that the value is greater than the comparative control (Table 11), while results highlighted in orange are less than their comparative control. RFU results that are not highlighted are nearly identical (Tables 12-15). Quant values for inhibited samples are highlighted in blue. In some cases, bones were retested because inhibitors were present or the original results were unreliable. The results from the original sample are included in the tables, but they were not used for analysis. They appear in gray text to distinguish them from the final results.

Control Samples RFU Results

The RFU values for the control samples are taken from Mundorff et al. (2011). RFU values range from 301 to 4841 (Table 11). The lowest RFU value from a sample that also yielded a full profile came from a 1st metatarsal (UT116-09) with a value of 550. The greatest RFU value from a sample that did not yield a complete profile came from a tibia (UT116-09) with a value of 558.

The DNA yields per gram of bone range from 0 to 926 nanograms (ng) for the control samples. Identical elements did not consistently yield DNA in similar quantities. For example, the 2nd cuneiform produced a DNA yield of 1 ng/g, 415 ng/g, and 926 ng/g for the three samples. Since a single unprocessed element contained the entire variation of the data set, it difficult to determine what is causing the variation between samples.

Hot Water Maceration RFU and DNA Yield Results

All RFU values from bones that underwent hot water maceration were lower than their respective controls. The values ranged from 130 to 3152 (Table 12). Two samples

Table 11. Control RFU Results

Individual	Sample	Quant ng/ul	ng DNA / g of bone	RFU/ Sample
UT07-09	Middle rib	2.04	49	3225
	Femur	0.89	19	977
	Tibia	0.77	17	301
	Metatarsal 1	1.17	34	951
	Cuneiform 1	0.86^	23	1471
	Cuneiform 2	0.04^	1	2879
	Cuneiform 3	0.52^	36	966
	1st proximal phalange of hand	1.75	46	1430
UT45-09	Middle rib	1.97	29	3206
	Femur	0.85	36	491
	Tibia	0.70	106	547
	Metatarsal 1	0.45	38	385
	Cuneiform 1	1.35	51	2341
	Cuneiform 2	1.99	926	2704
	Cuneiform 3	unknown (maxed out)^	664	3411
	1st proximal phalange of hand	0.43	73	617
UT116-09	Middle rib	1.16	52	1984
	Femur	1.64	19	456
	Tibia	2.09	18	558
	Metatarsal 1	1.44	11	550
	Cuneiform 1	0.47^	281	4841
	Cuneiform 2	1.85	415	1596
	Cuneiform 3	2.09^	0	2845
	1st proximal phalange of hand	2.29	11	3173

Table 12. RFU Results: Hot Water Maceration

Individual	Sample	Quant ng/ul	ng DNA / g of bone	RFU/ Sample
UT07-09	Middle Rib	0.205	44.6	401
	Femur	0.146	31.7	136
	1st cuneiform	<0.001	0	0
	1st cuneiform	<0.001	0	130
	Proximal phalanx (hand)	0.189	47.3	526
UT 45-09	Middle Rib	0.399	95	690
	Femur	0.129	25.8	300
	1st cuneiform	1.38	246.4	718
	Proximal phalanx (hand)	0.203	42.3	449
UT 116-09	Middle Rib	0.113	23.5	323
	Femur	0.0953	18.3	194
	1st cuneiform	<0.001	0	4090
	1st cuneiform	<0.001	0	3152
	Proximal phalanx (hand)	0.247	61.8	535

Table 13. RFU Results: CT Scan

Individual	Sample	Quant ng/ul	ng DNA / g of bone	RFU/ Sample
UT07-09	Middle Rib	0.0811	17.6	710
	Tibia	0.015	3.3	59
	2nd cuneiform	0.247	61.8	1275
	Proximal phalanx (hand)	0.0881	22	472
UT45-09	Middle Rib	0.172	45.3	1569
	Tibia	<0.001	0	545
	2nd cuneiform	0.84	175	1415
	Proximal phalanx (hand)	0.0588	14	587
UT116-09	Middle Rib	0.0835	16.7	781
	Tibia	0.0298	6.5	148
	2nd cuneiform	1.52	330.4	2423
	Proximal phalanx (hand)	0.105	25	825

Table 14. RFU Results: X-ray

Individual	Sample	Quant ng/ul	ng DNA / g of bone	RFU/ Sample
UT07-09	Middle Rib	0.386	107.2	496
	MT1	0.127	33.4	170
	3rd cuneiform	0.695	173.75	584
	Proximal phalanx (hand)	0.157	43.6	426
UT45-09	Middle Rib	0.186	51.7	503
	MT1	0.0587	12.8	361
	3rd cuneiform	1.32	287	752
	Proximal phalanx (hand)	0.195	42.4	357
UT116-09	Middle Rib	0.107	26.8	376
	MT1	0.622	124.4	549
	3rd cuneiform	3.26	776.2	500
	3rd cuneiform	3.26	776.2	995
	3rd cuneiform	3.26	776.2	1205
	Proximal phalanx (hand)	0.0242	5.5	147

Table 15. RFU Results: Combined Treatments

Individual	Sample	Quant ng/ul	ng DNA / g of bone	RFU/ Sample
UT07-09	Middle Rib	0.165	39.3	833
	Femur	0.0627	14.3	180
	Femur	0.0734	16.7	114
	1st cuneiform	<0.001	0	0
	1st cuneiform	<0.001	0	0
	Proximal phalanx (hand)	0.216	51.4	1016
UT45-09	Middle Rib	0.256	58.2	879
	Femur	0.0452	9.8	223
	1st cuneiform	1.07	243.2	921
	Proximal phalanx (hand)	0.0997	23.7	583
UT116-09	Middle Rib	0.111	27.75	561
	Femur	0.042	10.5	128
	1st cuneiform	<0.001	0	1537
	1st cuneiform	<0.001	0	1978
	Proximal phalanx (hand)	0.414	103.5	668

were retested due to the presence of inhibitors in the original sample. The original results were not used to evaluate the quality or quantity of the sample, but are included in Table 12. The DNA yield from heat-macerated bones was highly variable. All four elements produced DNA yields that were increased compared to the control as well as yields lower than the respective control.

CT Scan RFU and DNA Yield Results

Although most of the RFU values from the CT scanned samples were lower than their respective control values, one sample was higher than the control. The 2nd cuneiform (UT116-09) had RFU values of 2423, which is greater than the 1596 RFU value from the control sample (Table 13). A tibia had the greatest RFU value, 148, without yielding a complete profile. The proximal hand phalanx showed the lowest RFU value (472) associated with a complete profile.

Although most of the samples showed a decrease in DNA yield after CT scanning, three of the samples produced a greater DNA yield. One middle rib, one 2nd cuneiform and one proximal phalanx performed better after CT scanning, indicating that there was not a clear pattern in the effect of CT scanning on a specific element.

X-ray RFU and DNA Yield Results

All the RFU values for X-rayed elements were less than their respective controls. The values ranged from 147 to 1205 (Table 14). The 3rd cuneiform from UT116-09 was retested twice. The first two tests were not used in the analysis of the quality or quantity of the sample, but they are included in Table 14. Reviewing results that did not yield complete profiles, the 1st metatarsal corresponded to the highest RFU value (361). The

proximal hand phalanx produced the lowest RFU value (426) associated with a complete profile.

Only the proximal hand phalanx showed a consistent reduction in the DNA yield between the control and the X-rayed elements. The other three elements alternated between increasing and decreasing DNA yields. Moreover, there was no trend between the loss of DNA yield and the individual skeletons, as none of the three skeletons had all elements affected the same way.

Combination of Methods RFU and DNA yield Results

All elements that underwent the combination of all three methods had RFU values less than the comparative controls. The values ranged from 114 to 1978 (Table 15). Three samples were retested. Two of the retested samples were inhibited, which prevented proper amplification of the original sample. The results from the inhibited samples were not used in analysis since no profile was generated. A femur yielded the greatest RFU value (223) from a sample that failed to produce a complete profile, while the lowest RFU value that successfully yielded a complete profile was 561 and came from a middle rib.

The DNA yield values did not follow a consistent trend in the relationship between the control and processed values. Two of the proximal phalanges produced greater DNA yields following the combination of processing procedures. A middle rib and a 1st cuneiform also yielded more DNA after processing. While the other samples yielded less DNA per gram of bone after processing, there is not a clear pattern in which individual skeletons or elements were more likely to be impacted by processing.

CHAPTER V

DISCUSSION

Statistical comparisons were run using data on profile completeness, RFU values, and DNA yield per gram of bone to assess whether statistical differences exist between processed and unprocessed samples. These data were measured to compare the quality and quantity of DNA recovered from the processed and control samples. The values were assessed independently as well as in relation to one and other in order to tease apart individual significance.

Profile Comparison

DNA from sixteen loci were extracted from each skeletal element, and quantified if they met the threshold peak height (heterozygous: 75/ homozygous: 200). Loci producing RFU values that surpassed the threshold were counted as part of the profile, thus profile completeness ranged accordingly from 0 to 16 loci. The majority of profiles were skewed toward 16 loci, which represents a complete profile (Figure 4). Since the data was not distributed normally and failed to have homogeneity of variance, parametric statistics were not applicable. Instead, a Wilcoxon Signed Rank test was most appropriate to compare the pairs of control and processed samples. For all statistical tests in this experiment, the threshold of significance was set at a p value of 0.05.

Comparison of the complete data set, which includes results from pre and post processing, indicates a significant difference between the unprocessed and processed samples ($p=0.007$). Since there was a significant overall difference between the control

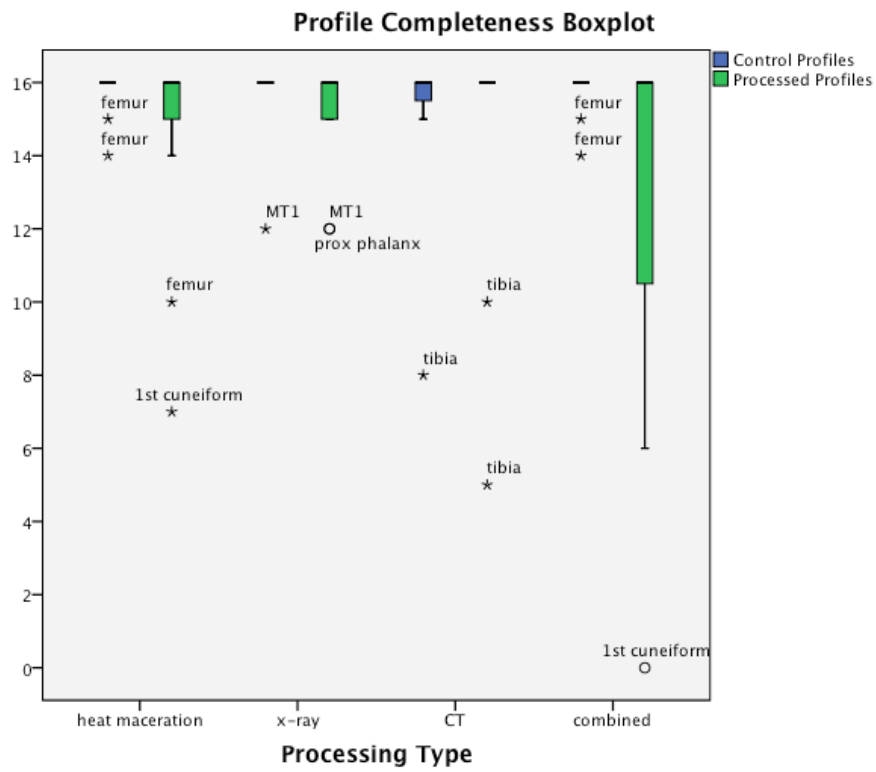


Figure 4. Boxplot of Profile Completeness

group and the processed group, additional statistical tests were run for each individual method. Isolating and comparing each process individually, hot water maceration ($p=0.285$), CT scan ($p=0.260$), X-ray ($p=0.285$), and a combination of all three ($p=0.068$) show no significant differences when compared to the respective control samples. The combination trial appears to diverge from the respective control sample more than the other trials, however the value was not significant. While the results between the control samples and the processed samples were not significant, this may be misleading because all of the element types were being compared together.

Mundorff et al. (2011) demonstrated that different elements from the same individual (exposed to the same taphonomic conditions and PMI) did not perform equally well. There was a large enough difference between the element types that they could be ranked on the overall quality of the element as a source of DNA for a forensic investigation. Although it is unknown exactly how DNA is distributed within a bone, their results imply that different skeletal elements do not maintain the same amount of DNA for equal periods of time after death. Therefore, the baseline amount of DNA in one element could be higher or lower than the baseline of other elements. This factor must be taken into account when comparing a sample set composed of different element types.

While the goal of forensic genetic testing is often the ability to recover enough DNA to generate a complete profile, profile completeness may not be the best measure of total DNA in a sample. A full profile from two different samples does not mean equal amounts of DNA are present in both samples. Since a full profile only indicates that enough DNA is present to exceed the threshold, the quantity of DNA available in each

sample could actually be any value beyond this point. Once a full profile is obtained, testing is complete and the availability of DNA is not measured beyond this point. Incomplete profiles can be assessed on the loss of genetic material compared to a complete profile, but it is more difficult to determine if one complete profile is of equal quality to another.

For example, the middle rib consistently produced a complete profile for the three control samples and for all of the processed samples (Figure 5). On the other hand, the femora produced incomplete profiles for some of the control samples. This shortcoming was also evident in the processed femur samples, which often showed an even greater reduction in the number of recovered loci. It is possible that equal degradation from processing might have occurred in both elements (rib and femur), but was not detected in the elements that started out with a greater amount of DNA. Those elements still had enough DNA available to produce a full profile even if the underlying quantity was reduced. Elements, such as the femur, appear to start out with less DNA than more successful elements such as the ribs. These elements continue to perform worse after processing giving the appearance that they suffered more degradation because the DNA loss directly affects the profile results. These differences are more noticeable because some control samples barely met or failed to meet the DNA threshold to produce a complete profile. Therefore, even if processing only caused a small amount of genetic material to be lost, it was detectable in a reduction of recovered loci since the RFU values were diminished below the threshold.

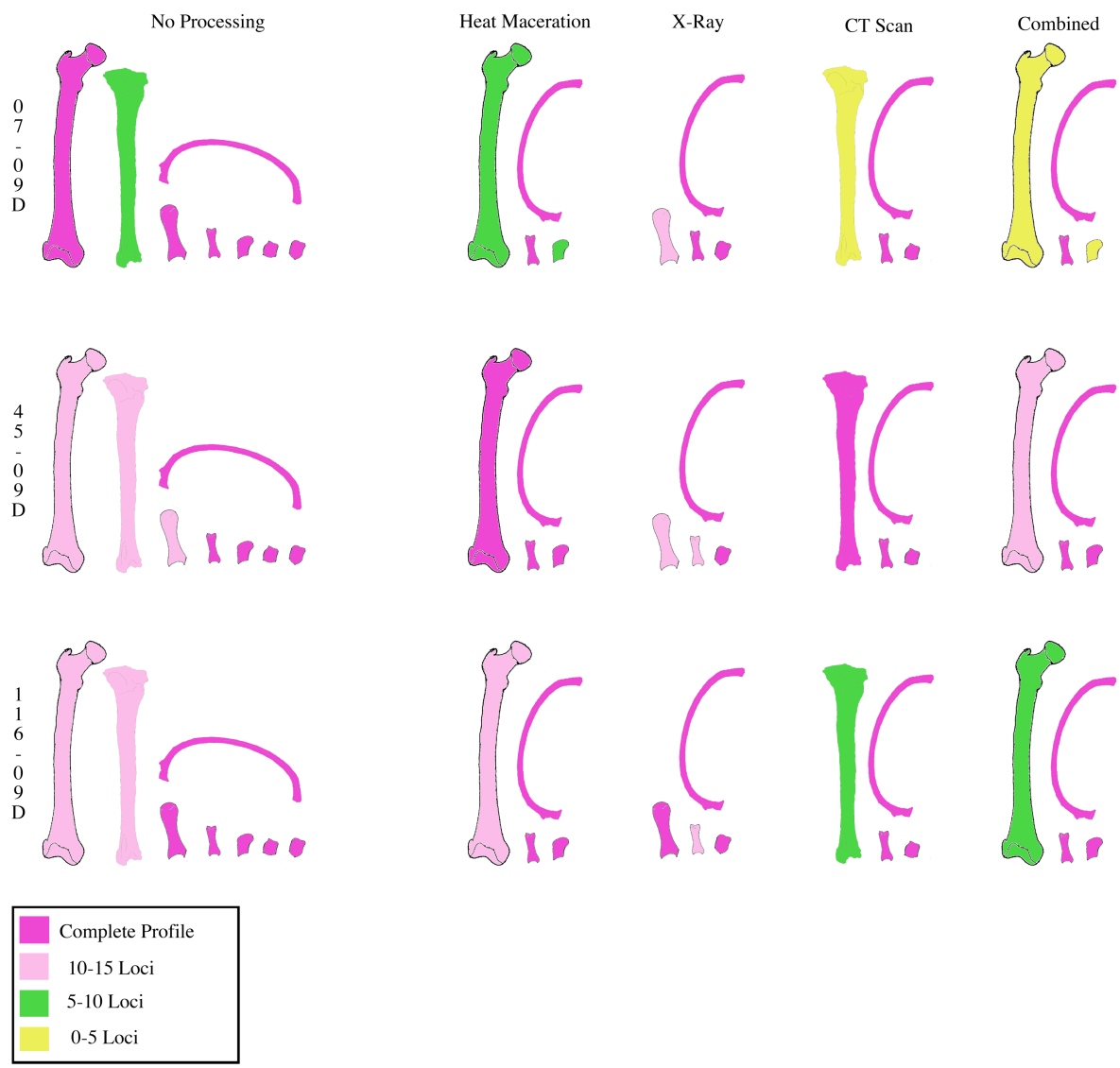


Figure 5. Comparison of Profile Completeness

RFU Value Comparison

Unlike the profile data, the RFU values were normally distributed. However, due to the broad range of values and the difference in the ranges between the control and processed samples, the data did not exhibit homogeneity of variance. To account for this a Wilcoxon Signed Rank test was used to assess the difference between the control and processed samples. A statistically significant difference was detected between the pre and post processing RFU values for the entire data set ($p=0.000$). The pair-wise comparisons for hot water maceration ($p=0.002$), CT scan ($p=0.002$), X-ray ($p=0.008$), and the combined treatment ($p=0.002$) confirmed that the RFU values were significantly different for each method.

While the significance values only reveal that there is a difference between the control and processed samples, comparison of the data indicates that the unprocessed control samples had greater RFU values than their processed counterparts (Figure 6). In fact, all control samples except one produced a greater RFU value than the comparative processed samples (Tables 11-15). Since RFU values can be used as a general proxy for the quantity of amplified DNA, these values may be a better indicator of genetic loss caused by processing, than comparing profile completeness.

RFU values are important because they are used to set the threshold for the amount of DNA necessary for a loci peak to be identifiable and considered part of the profile. RFU values must be greater than the threshold of 75 to be counted as peaks rather than background noise. Each locus in a sample has two RFU values, one for each allele. The RFU value for a specific locus is the average of these values, while the final

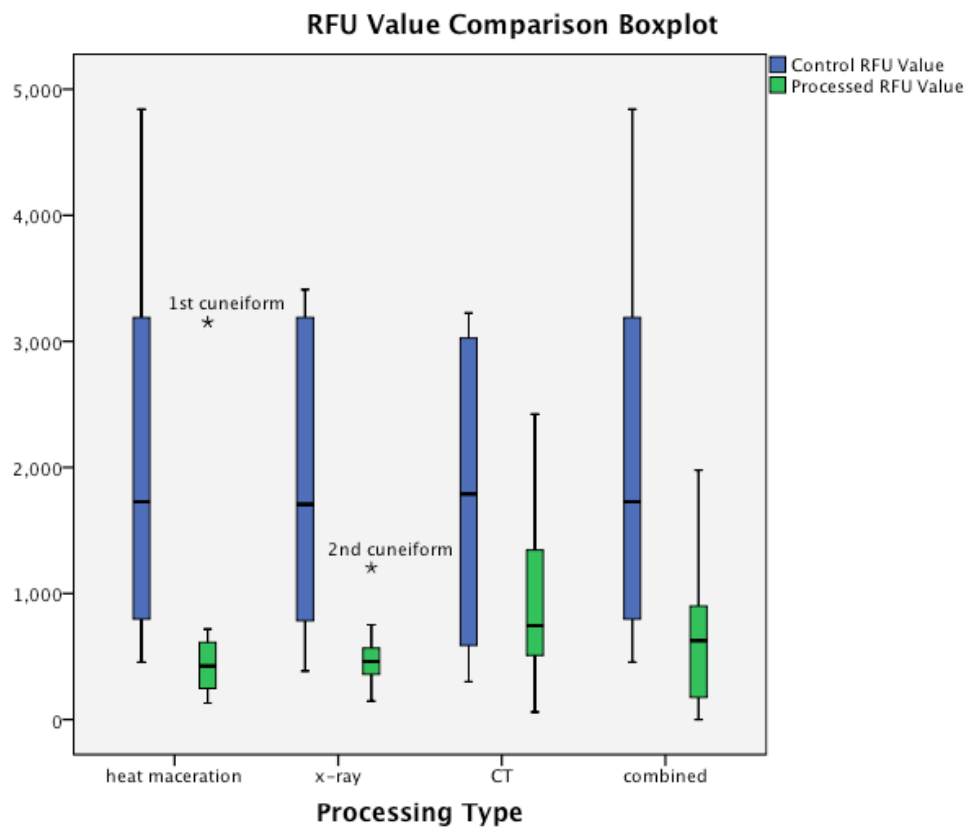


Figure 6. Boxplot of RFU Values

RFU value for the sample is an average of the RFU values from all 16 loci. Although there could be variation within the sample due to some loci producing higher or lower RFU peaks, the average gives an overall indication of the amount of genetic information available for the entire complement of 16 loci. The processed samples tend to have lower RFU values suggesting that the genetic information is diminished. Also, it is more common that samples with low average RFU values have at least one locus that did not meet the threshold, leading to an incomplete profile.

Once again it also follows that samples starting out with more genetic information would continue to produce greater RFU values after processing than those that started with less available DNA. Although the reduction might not translate into a loss in profile quality, the overall reduction in recoverable DNA would be picked up in the RFU value because it measures a greater range of variation. The more genetic information available to begin with, the longer those elements would continue to outperform elements with inherently less DNA. Even if there were a significant loss of DNA, these results would not be detected from profile completeness alone until the RFU values were reduced below the threshold. The results from this study follow this pattern because almost all of the processed samples had reduced RFU values relative to the unprocessed samples, yet only a few actually had a reduction in the number of loci detected.

DNA Yield Per Gram of Bone

The sampling procedure aimed to consistently extract ~0.2 grams of bone powder although there was some variation in the actual amount collected. Generally, collecting more bone powder increases the amount of DNA available in the sample, so DNA yields

from each sample are standardized by the amount of bone powder collected. This minimizes the effect of over or under sampling so that the DNA yields from all bone samples are comparable. The values for DNA yield represent a ratio of the amount of DNA relative to the amount of powder sampled, so parametric statistics were not applicable. A Wilcoxon Ranked Sign was used to compare the DNA yields from the control samples to those from the antimere elements that underwent processing. Five separate comparisons were run. The first test compared all control samples to all processed samples ($p=0.291$), while the other four examined each method and its respective control individually. None of the results revealed a significant difference between any process and their control values (heat maceration $p=0.875$, X-ray $p=0.754$, CT scan $p=0.071$, combined $p=0.530$).

DNA yields did not differ significantly between the processed and unprocessed samples as they did with RFU values. In theory, the DNA yields and RFU values should both be measuring the quantity of DNA available in the sample, but unlike the RFU values, there is no clear trend between processed and unprocessed samples in terms of DNA yield (Figure 7). Furthermore, the DNA yield and RFU values only show a weak positive correlation with each other ($r=0.362$). While slight variation in the quantity of bone powder collected for each sample might have affected the RFU results, overall quantity of bone used for each sample was carefully maintained around 0.2 grams. It is unclear why the two measures are not more closely correlated. Fragment length was not specifically assessed in this comparison, but it should be noted that the quantifier quantification reactions carried out in this experiment target fixed size amplification products of 62 base pairs. Generally, the STR loci range in length from 100 to 400 base

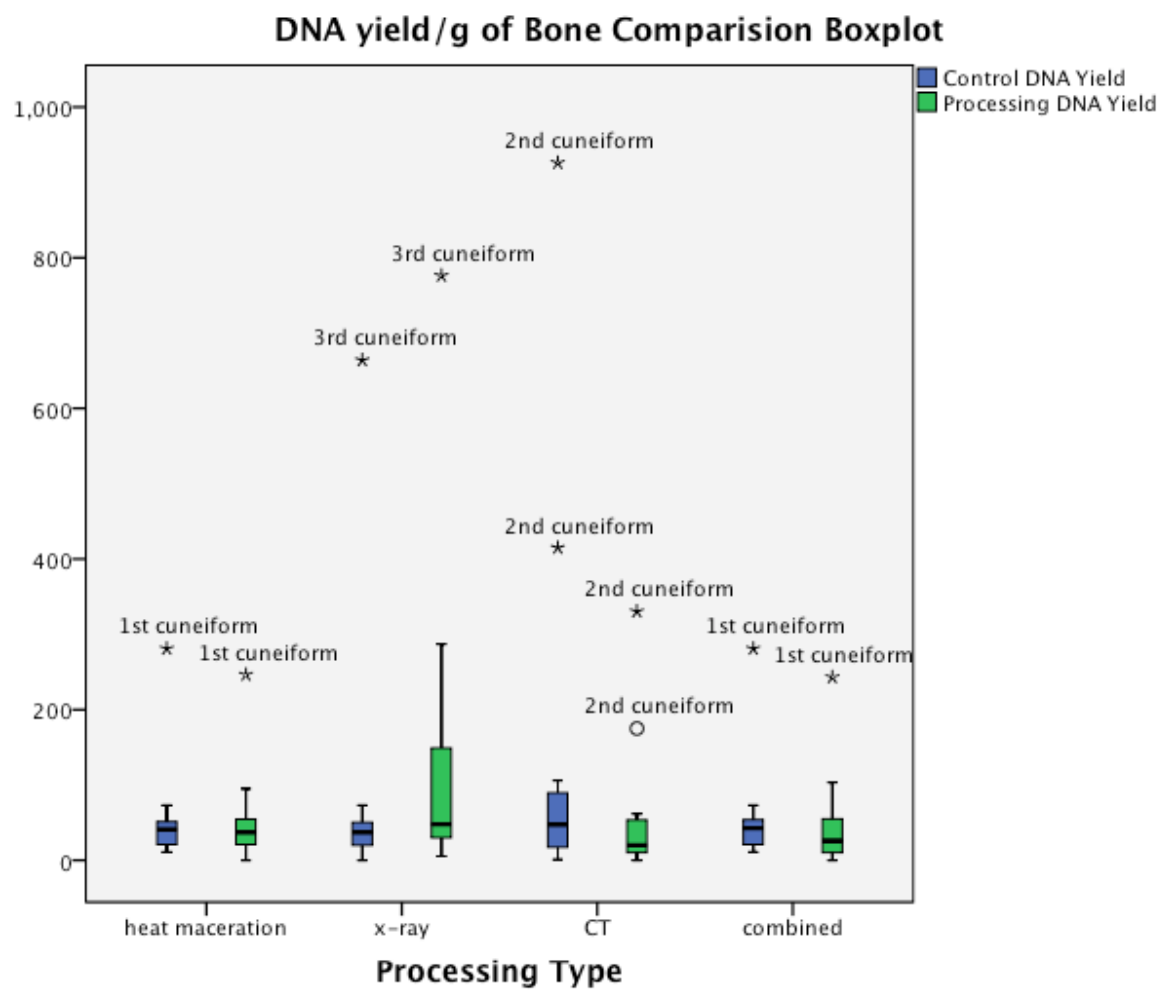


Figure 7. Boxplot of DNA yield/ g of Bone

pairs. Thus smaller, possibly more degraded, STR DNA fragments may be included in the DNA quantification value, but not in the RFU value.

RFU and DNA Yield Ranking

Differences in the average RFU values show that both element type and processing activity appear to play a role in RFU value distribution (Figure 8). The top five average RFU values all belong to unprocessed samples. They are also the elements that ranked highest in Mundorff et al.'s study (2011). The other three unprocessed elements were long bones, and in all these cases at least one of the unprocessed samples did not produce a full profile. While the average RFU values from the unprocessed elements were greater than the average RFU values from comparative processed elements, they were not greater than all processed elements. In other words, element type appears to have a greater affect on the total RFU value than processing effects. Elements that produced higher RFU values prior to processing continued to produce relatively higher RFU values after processing.

Though the average values for DNA yield/ g of bone powder do not correspond well with the RFU value ranking, there are some similarities (Figure 9). All three cuneiforms performed well for both RFU and DNA yield/ g of bone powder. However, when comparing DNA yield between elements, the cuneiforms performed better than any other bone type for both processed and unprocessed samples. The long bones (femur and tibia) produced the poorest yields, with little differentiation between their processed and unprocessed samples. While the ranking RFU values show a distribution that supports the hypothesis that both element type and processing method affect the DNA quantity,

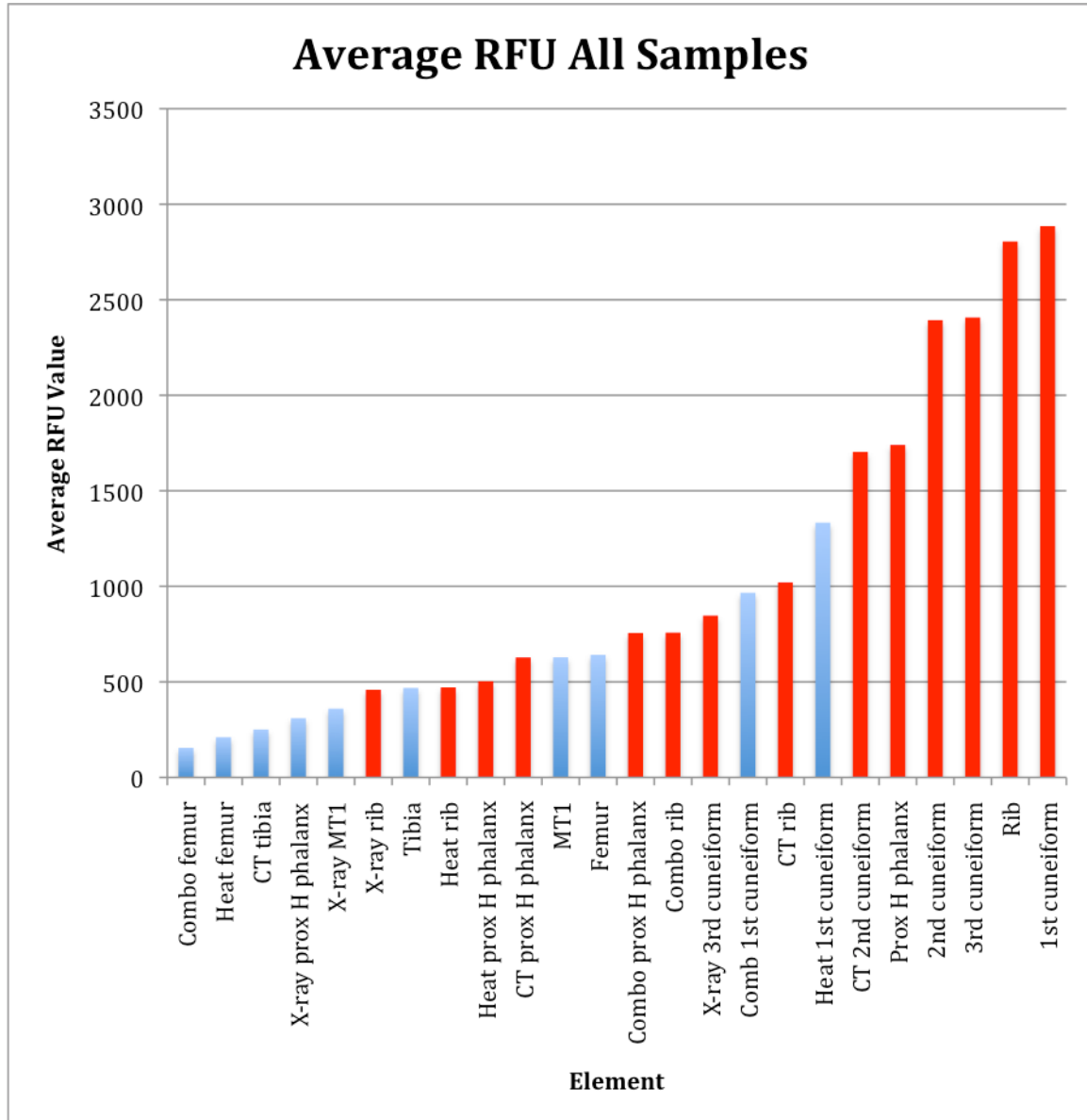


Figure 8. Ranking of average RFU value by element with the treatment noted for each. Boxes highlighted in red are samples that produced a full profile for all 3 individuals.

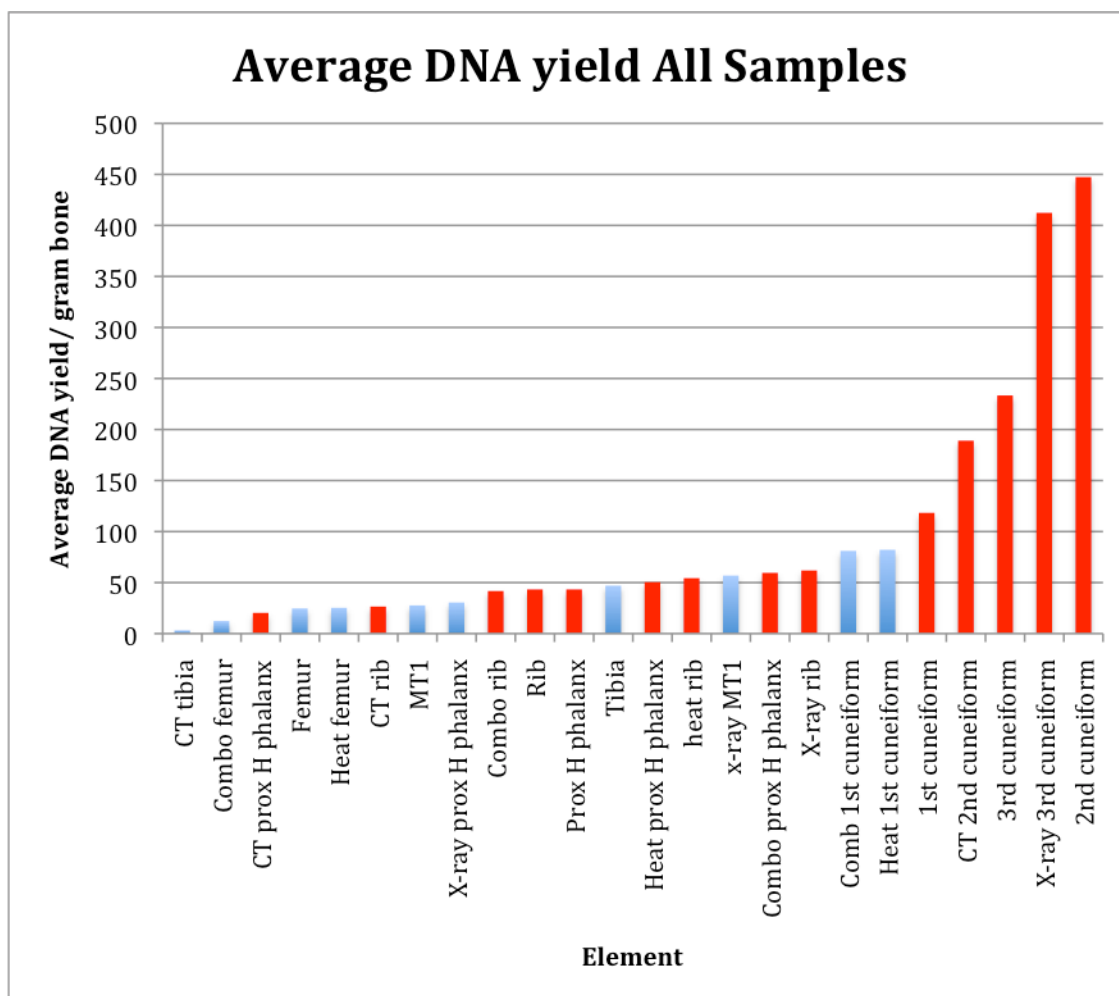


Figure 9. Ranking of average DNA yield/ g of bone powder by element with the treatment noted for each. Boxes highlighted in red are samples that produced a full profile for all 3 individuals.

the ordering based on DNA yield appears to be more influenced by the element type alone.

Comparing Specific Elements

Comparisons between the long bones (femur, tibia, and MT1) offer an opportunity to examine the loss of profile information. These bones did not meet the threshold set for a full profile resulting in variation in the number of recovered loci. Since the long bone controls produced incomplete profiles without the addition of any processing, it appeared that this type of element started with poorer quality DNA. Due to a lower initial quantity of DNA, when the long bones underwent processing they were more likely to have the amount of DNA further reduced below the threshold limit, causing their profiles to show a reduction in completeness. Overall, when comparing profiles, a drop in the number of loci is evident when long bones are exposed to numerous processing methods. This is especially noticeable with the femur since the exact same bone was sampled following hot water maceration and then the combined trial. In all three femora, the number of loci decreased with the addition of CT and X-ray imaging beyond the decreased results from heat maceration alone.

While most of the long bones showed a loss in the number of loci recovered following processing, a few showed an increase in the number of loci following processing. Since the unprocessed element was the antimere bone to the processed one the differences in DNA recovery might be an artifact of variation between the left and right elements.

One bone, the 1st cuneiform, provides a series of interesting comparisons. Based on Mundorff et al.'s (2011) study the 1st cuneiform ranked among the top ten bones consistently producing a complete profile and high RFU value. For the most part, this trend continued, as two of the three bones produced complete profiles following each processing trial. However, the third 1st cuneiform showed a different result. While the control sample produced a complete profile, the heat macerated sample dropped to seven loci and the sample from the combined processes is the only one in the entire study to not produce any loci at all. The RFU values also showed a significant drop, with the control at 1471, the heat macerated sample at 130, and the combined sample at zero

The loss of loci between trials is significant for these samples, because the 1st cuneiform along with the 2nd and 3rd cuneiforms generally produced complete profiles. Also, in this case, the heat maceration and combined trial samples were taken from the exact same bone. It is possible that the initial decrease was due to problems with the specific bone, but the second decrease cannot be attributed to the bone's original condition. The only variable that changed between the first and second decrease was the addition of X-ray and CT scanning. This series offers more support for the theory that all elements, not just long bones, experience a decrease in genetic information with additional processes. Unlike the other cuneiforms sampled, this particular cuneiform did not initially meet the DNA threshold, so the effects of additional processing could more easily be measured.

On the other hand, this specific 1st cuneiform showed the presence of inhibitors in the sample. Since inhibitors prevent the normal amplification of DNA, they might be responsible for obscuring the profile information. While the exact relationship between

inhibitors and loss of genetic information is unclear, it should be noted that inhibitors were detected in one of the other skeleton's 1st cuneiform, but those samples all produced complete profiles. While inhibitors are a significant problem in the DNA amplification process, they were not a major consideration in this project, since they affected few samples. Inhibition was limited to samples taken from the cuneiforms, and was present in both processed and unprocessed cuneiform samples. Furthermore, all but two of the inhibited samples produced a complete STR profile, which suggests that the inhibitors did not have a significant impact on the results of this study. It is unclear which specific inhibitor prevented normal amplification in this study. Therefore it is difficult to parse out which aspects of the amplification reaction processes were affected or what steps could be taken to minimize the inhibitory affect in the future.

Limitations: Normal Variation

Although some of the data appears to show a reduction of genetic information with the application of processing treatments, overall the differences between methods are inconsistent. Furthermore, there is significant variation in the quality of the DNA yields within the samples that underwent the same processing methods. For example the DNA yields for the control samples of 2nd cuneiforms produced values of 1 ng/g, 926 ng/g, and 415 ng/g (Table 11). In all three cases the element produced a complete genetic profile with all 16 loci represented. Yet there were samples with yields greater than 100 ng/g of DNA that did not produce a complete profile. Since this variation is seen in the control samples it is difficult to determine the amount of the variation due to normal fluctuations between samples of the same element type and variation due to specific

processing methods (heat maceration, X-ray, CT-scan) that induce degradation. When the data are broken down by individual element type the sample sizes are much smaller, with some elements only represented by three control and three processed samples. Therefore, it is possible that inconsistency in profile completeness represents normal variation within samples of the same element type, and only appears to be more significant due to small sample sizes.

The inconsistent measure of DNA quantities between the RFU values and DNA yields, along with variation in profile completeness do not provide a conclusive measure of the effect of processing on the recoverability of DNA in bone. While it is plausible that the processing methods are having a negative effect, it is equally plausible that DNA quantity is variable among samples and that a greater sample size would show a broader range of profile completeness for the element types, independent of processing.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Based on a comparison of unprocessed and processed skeletal elements, it appears that processing does affect the recovery of STR loci information, but the extent of its effect is unclear. RFU values were significantly reduced following all processing techniques ($p=0.000$). Since RFU values measure the amount of genetic information amplified for an STR loci segment, a reduction in this value suggests that genetic information was not recovered as effectively after an element underwent processing. Since the targeted STR loci range from 100-400 base pairs in length, the reduction in RFU value may indicate that the STR loci DNA was damaged during processing. Although DNA yields were not significantly reduced by any of the processing methods, this quantification measure targets shorter 62 base pair sequences. Therefore, the discrepancy in quantification results may reflect some degradation of the CODIS loci DNA, but insufficient damage to prevent quantification of shortened fragments.

Although it is unclear if there is a quantifiable amount of degradation that can be attributed to any of the specific processing techniques, a small amount of degradation might not affect the recovery of a complete DNA profile in a forensic context. While RFU values in processed samples showed a significant decrease compared to the unprocessed controls, none of the separate processing techniques significantly affected the number of loci recovered. Therefore, processing techniques did not impede the likelihood of an element producing a complete profile beyond the initial unprocessed state of that element.

In short, the results indicate that processing decreases the quantifiable amount of STR loci DNA. Loss of genetic information does not directly translate into loss of profile completeness, since some STR loci greatly exceed the RFU threshold quantities. Therefore, even if STR loci information is reduced, the quantity of STR loci DNA continues to exceed the RFU threshold and the loci is counted as part of the profile.

Implications: Forensic Context

In a forensic context, comparisons of DNA are made exclusively on the completeness of the profile. Therefore, for an element such as a rib, the chance of the processed and unprocessed samples producing a full profile would be equally likely since ribs from all treatment methods produced full profiles. Furthermore, for forensic samples, CODIS accepts incomplete profiles if at least 10 of the 13 required loci and amelogenin are represented (Codis.gov). Fewer loci are required for samples from missing persons and unidentified human remains. Only eight loci and amelogenin must be successfully amplified for submission into the NDIS database. In this study, only 1 of the 24 control samples and 7 of the 48 processed samples had fewer than ten loci and amelogenin. Therefore, even with processing the majority of the samples produced a sufficiently usable DNA profile.

Another practical consideration in a forensic context is element selection. In the case of unidentified remains a single element would likely be taken to test for DNA. Among the elements tested in this sample, only the rib produced a complete profile for all samples. Therefore, if elements such as the rib are targeted for collection and DNA testing, the effect of processing on profile recovery can be minimized.

Implications: Future Research

One of the aims of this research was to determine how the Forensic Anthropology Center's processing procedures may or may not affect the recoverability of DNA from bones. This was done to assess the impact on future DNA studies using the WM Bass Donated Skeletal collection. Studies comparing DNA yields frequently compare the completeness of the DNA profile since the profile is used to match unidentified skeletal remains to a known individual. Therefore, the completeness of the profiles produced from skeletons in this collection should be considered with an understanding of the possible processing variables that might have affected the bones. Since it is still unclear exactly how much of an impact each processing technique has on the total DNA remaining in the skeletal element care must be taken when comparing DNA profiles from skeletons in the WM Bass Donated Skeletal Collection with unprocessed skeletons or with skeletons that have been subjected to different maceration and imaging processes.

The results of this study offer insight in two ways. First, the results establish a baseline for the potential impact that processing could have on bones ability to yield enough DNA for a complete profile from the skeletons in the WM Bass Donated Collection. In this case it is important for researchers to realize and consider that skeletons in the collection might have a reduced DNA yield from the onset, as the result of processing rather than any other variable.

Secondly, the results may indicate that the effects of processing or other procedures that damage DNA in bone are additive. In this case the loss of loci more rapidly from samples that already showed a reduction in profile completeness may illustrate that while most bones start out with significantly more DNA than needed to

yield a full profile. It is possible that processing the bones may reduce this reservoir. As more procedures are performed on the bones, the pool of available genetic information might be lost. If that were the case, the bone samples would eventually stop producing a complete profile due to the cumulative loss of DNA.

One way to prevent different processing methods from effecting DNA yield would be to maintain a skeletal sample that is not subjected to any processing procedures. A designated element would be stored with the remains but not processed, limiting the degradative variables to increasing post mortem interval. Based on the study results, a middle rib would be the best candidate to be maintained as an untested control element. Not only do people have 24 ribs, but also the rib performed well throughout this project. Furthermore, the middle rib is not commonly used during the biological profile assessment. Plus, a rib can be easily separated from the other skeletal elements unlike small hand and foot bones, which may become mummified together. Designating a specific element to be withheld from processing also prevents variation due to element type if future researchers want to compare the unprocessed elements to each other or to their respective skeletons in future research projects.

For modern samples the best way to maximize STR DNA recovery from skeletal elements is to limit exposure to radiation and heat or chemical maceration. With increasing post mortem interval, mitigating these factors would be less effective since DNA naturally degrades over time. Loss of vascularization and increased mineralization of skeletal tissue following an individual's death increases the difficulty of generating a complete CODIS profile since the organic component of the cell is reduced. Thus, more

protected forms of DNA, such as mitochondrial DNA, may provide a better venue to assess questions of genetic variability in the distant past.

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