Final Technical Report

Document Title:	Developing an Empirically Based Ranking Order for Bone Sampling: Examining the Differential DNA Yield Rates Between Human Skeletal Elements Over Increasing Post Mortem Intervals
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Abstract

This study established an empirically based ranking of skeletal elements according to each bone's capacity to provide usable genetic material for a DNA identification and assessed how these results varied over longer post mortem intervals (PMI). Currently, selecting skeletal elements for DNA testing is based on the collective wisdom of practitioners who suggest weight-bearing long bones over all other elements. Recent evidence questions the accuracy of this collective wisdom.

This study evaluated the differential yield and preservation of DNA by skeletal element in a single individual, replacing intuition with empirically based data to increase the likelihood of successfully generating a DNA profile from skeletal material. A two-phase study was conducted to accomplish these goals. Phase 1 entailed analyzing the DNA yield rates of different skeletal elements, from 3 recently skeletonized individuals, to generate an overall rank order from most successful to least successful. Phase 2 was designed to determined if the same elements from Phase 1 were equally successful at longer post mortem intervals (0-3, 4-10, 11-20, and 20+ years) and to give an indication how DNA degradation occurs over time.

Using a single sampling (0.2 g) and a single amplification targeting 2.0 ng, both the quantity and quality of DNA were analyzed to determine which bones most successfully yielded full profiles (15 loci + amelogenin). Phase 1 results indicated that the quantity and quality of DNA obtained from different skeletal elements is highly variable. Small, predominantly cancellous bones such as a phalanx, patella, and tarsal's, out performed the femur, tibia, and other long bones, which are comprised mostly of the 'preferred' dense cortical bone. The bones that ranked at the top (full profiles) consistently for all three individuals were considered for Phase 2. Only ten

bones per individual would be tested in Phase 2, however, 23 elements and 4 teeth qualified by achieving the 'most successful' rank in Phase 1. Therefore elements were selected by yield per mass of sample, average RFU value for each element, and skeletal representation. A cervical vertebra, middle rib, patella, 4th metacarpal, 1st distal hand phalanx, 4th metatarsal, talus, 1st cuneiform, tibia, and femur were selected for Phase 2. Phase 1 results for the tibia and femur did not reach the specifications to qualify for Phase 2 however, they are elements practitioners typically prefer to sample and therefore were included for comparison.

Three skeletons from each of the four post mortem intervals (12 total) were selected. Only skeletons with similar demographics (age, sex, etc.) to the initial three individuals from Phase 1 were considered for Phase 2. Phase 2 results show that most elements produced less complete DNA profiles as the PMI increased. However, the elements that produced the best results in Phase 1 continued to perform well in later post mortem intervals, retaining their positional rank in relation to each other. This suggests that DNA degrades consistently over time. Interestingly, the distal hand phalanx and the 4th metatarsal, bones typically not sampled, were the only elements to produce full profiles in the final PMI of 20+ years.

This research project determined which skeletal elements, within a single individual, were most likely to provide both the quantity and quality of DNA needed to produce DNA profiles from increasing post mortem intervals. The results provide much needed guidance on which elements are best suited for DNA testing. Cancellous bones, typically dismissed as a potential DNA source in favor of dense cortical bones, should be reconsidered when sampling. By determining which skeletal element is most

likely to yield an STR profile allowing identification, expensive and time-consuming retesting can be avoided.

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EXECUTIVE SUMMARY

Description of the problem

Selecting the best bone to sample in order to maximize the chances of acquiring sufficient DNA for an identification can be challenging. Recent advances in genetic sequencing techniques have been driven by large-scale identification efforts focused primarily on nuclear DNA from osseous material (Whitaker et al. 1995; Hsu et al. 1999; Hoff-Olsen et al. 2003; Piccinini et al. 2004; Davoren et al. 2007). Few laboratories can conduct this type of analysis and for many jurisdictions these tests remain prohibitively expensive. Yet, as awareness of DNA capabilities continues to grow with investigators and in the popular culture, the number of unidentified remains requiring DNA identification is also growing. However, bones degrade over time, which increases the difficulty of securing an identification through DNA.

Previous research has established a practice of preferentially sampling dense, cortical, weight-bearing long bones, such as the femur. While this research has been important, it has focused only on selected elements of the skeleton. New evidence indicates that the practice of preferentially sampling cortical bone may be suboptimal. A retrospective study examining a subset of the World Trade Center remains indicated that bones such as the phalanx and the patella yield DNA as well or better than dense cortical bone such as the femur (Mundorff et al. 2009). Additional research is necessary to establish a comprehensive and empirically based sampling strategy by ranking elements according to their potential to yield usable genetic material for DNA identification and to assess how these results may vary over longer post mortem intervals (PMI). By selecting the most appropriate bone during sampling, the efficiency

(both time and cost) of DNA identification from bone will increase maximizing the success rate of DNA identifications from bone of varying post mortem intervals.

Internationally, DNA is increasingly relied upon to identify victims of mass disasters, victims excavated from mass graves, and the missing (Boles et al. 1995; Hagelberg et al. 1991; Holland et al. 2003; Biesecker et al. 2005; Marjanovic et al. 2007; Parson et al. 2007). Therefore, determining which element most likely will allow for an identification, is paramount. Thus, research is needed to establish a comprehensive, empirically based, rank order of success rates by evaluating the quality and quantity of DNA yields from all of the different skeletal elements.

Purpose, Goals, and Objectives

The purpose of this research was to address the knowledge gap outlined above by establishing the order in which all skeletal elements yield both quantity and quality of DNA from recently skeletonized remains and skeletons from longer post mortem intervals. The project's first goal was to analyze the DNA yield rates of different skeletal elements within three recently skeletonized individuals. This allowed for the ranking of elements, within an individual, from most successful to least successful. The second goal was to test a subset of the highest ranking elements, from skeletons at increasing post mortem intervals, to assess whether the same bones yield sufficient DNA for identification 0-3, 4-10, 11-20, and 20+ years post mortem.

This research project had two primary objectives. First, testing all element types from three different skeletons to develop a complete ranking, by DNA yield rate, of all the bones within an individual. Then, by using these results, establish skeletal sampling guidelines for decomposed, mummified, or relatively recent skeletal remains. These

guidelines will also be applicable identifying mass fatality victims, when bone is often sampled instead of muscle tissue (Mundorff et al. 2008; Cockle et al. 2005; Meyer 2003; Leclair et al. 2004; Olaisen 1997) or remains are fragmentary, limiting the choice of elements that can be sampled (e.g., a traumatically amputated arm). The second primary objective was to evaluate whether these results applied to older skeletal remains. Twelve individuals from increasing postmortem intervals were selected. Using the previously established rank order, the ten most successful elements were tested using the same methods. These results were then used to develop sampling guidelines for older skeletonized remains including those interred in graves for an extended period of time. By comparing the results of recent versus older remains, this study also evaluated how DNA degrades over time.

Key objectives can be summarized as:

1) Evaluating the quantity and quality of DNA from a subset of bones, which represent all of the element types in a human skeleton, to determine how different bones differentially yield DNA.
2) Establishing a comprehensive rank order, from most successful to least successful, of all the element types in the human skeleton.
3) Evaluating whether this order holds true for skeletons from longer post mortem intervals.
4) Evaluating whether DNA in different bone elements degrade differently over time.
5) Providing a comprehensive and empirically based bone sampling strategy to maximize efficiency (both time and cost) and success rates of DNA

identifications from osseous material from remains of varying post mortem intervals.

The results were presented at the 2012 American Academy of Forensic Sciences annual meeting in Atlanta. Results were also presented at the 2012 Annual International Forensic Research Institute's Forensic Science Symposium and are now in preparation to be submitted for publication to Forensic Science International: Genetics. There is no other study in the forensic literature that provides a comprehensive, empirically based rank order of DNA yield success rates from each of the different element types in the human skeleton.

Research design and methods

In order to conduct this research, the authors decided to split the project into two phases. The first process was to select three recently skeletonized individuals for Phase 1 testing. To minimize confounding variables, the three donor individuals had similar demographics: adult males between 40-70 years at the time of death; self reported as white; died within the same year. They also decomposed on the same plot of land, in the same position (surface and prone) and in the same gross environmental conditions. In order to evaluate the differential DNA yields, each skeletal element type and tooth type was tested (n=55). It was not necessary, or practical, to test all 206 bones in the adult human skeleton. Many bones are duplicated (left and right sides) or redundant (5 lumbar vertebrae), so representatives of each type (all from the left side) were selected by the authors. The same bones were tested from the three different skeletons to ensure that results from one individual were not anomalous (n=165).

Before testing, each bone was assigned a unique number, the sampling spot was marked, and photographs were taken with and without an arrow pointing to the designated sampling spot. The position of the designated sampling spot on each bone was chosen to minimize interference with morphological traits of the skeletal elements. The spot was cleaned with a 10% bleach solution followed by sterile water and 70% ethanol. To minimize destruction during sampling, a 3/8" circular hole was drilled in the bone rather than cutting out a window or wedge. This sampling approach allowed the collection of ~0.20 g of bone powder from both small bones and larger long bones. No bone was completely consumed in testing and they were relatively unchanged, allowing for their use in future research projects.

The samples were extracted using a modified QIAamp micro procedure (Qiagen, Hilden, Germany). Extracted DNA was amplified using a 25 µl AmpFlSTR Identifiler® reaction at manufacturer's conditions with double the amount of AmpliTaq Gold Polymerase. Samples were injected on an ABI PRISM 3100 or 3130xl Genetic Analyzer and interpreted using a 75 RFU reporting threshold. Results are based on a single sampling (~0.2g) and a single amplification targeting 2.0 ng. Both the quantity and quality of the DNA from each sample were analyzed to determine which bones most successfully yielded full DNA profiles (15 loci + amelogenin). Based on the results, the bones were then ranked in order from the most successful to the least successful at yielding usable genetic material for DNA identification.

Phase 2 was designed to determine if the same skeletal element types from Phase 1 are equally successful at longer post mortem intervals and to give an indication how DNA degradation occurs over time. Therefore, results from Phase 1 were used to select the specific bones to be tested during Phase 2. Only ten bones per individual

were going to be tested in Phase 2, and teeth were not available because of their scarcity in the skeletal collection. Initial selection was limited to bones that ranked as 'most successful', which meant that the element type yielded full profiles, consistently, for all three individuals. More than 10 element types qualified for Phase 2, specifically, 23 bones and 4 teeth met the requirements. Therefore, elements were selected by yield per mass of sample, average RFU value for each element, and skeletal representation. For example, six of the seven tarsals met the requirements, so two were chosen as representatives.

The ten elements chosen for Phase 2 analysis were: a cervical vertebra, a middle rib, a patella, the 4th metacarpal, the 1st distal hand phalanx, the 4th metatarsal, the talus, the 1st cuneiform, the tibia, and the femur. Although results from the tibia and femur did not actually reach the threshold to qualify for Phase 2, practitioners typically prefer sampling these elements to the bones that actually qualified. Therefore femur and tibia were included for comparison, to demonstrate their success rates against the other, 'most successful' bones from Phase 1. Once the 10 Phase 2 element types were chosen, 3 skeletons from each of the 4 predetermined post mortem intervals (0-3 years, 4-10 years, 11-20 years and 20+ years) were selected (n=12). Phase 2 skeletons came from the curated collection at the University of Tennessee. In an effort to limit as many of the confounding variables as possible, only skeletons that decomposed on the same plot of land, in the same gross environmental conditions and with similar demographics (age at the time of death, sex, etc.) to the initial three individuals tested during Phase 1, were considered for Phase 2. Ultimately, 10 elements from 12 skeletons at 4 different PMIs were tested (n=120). The testing procedures for Phase 2 generally mirrored those in Phase 1. Particular attention was paid to whether the elements retained their

positional rank in relation to each other since this data may indicate if DNA degrades consistently over time.

Results

Phase 1

The average yields per mass of sample for skeletons 07-09D, 116-09D and 45-09D are 114 ng/g standard deviation (*sd*) =204, 136 ng/g *sd* =171, and 90 ng/g *sd* =118, respectively. The DNA quantity obtained from different skeletal elements is highly variable. The average yield by sample type, as determined by Applied Biosystem's Quantifiler™ Human DNA Quantification kit (Foster City, CA), ranged from ~6 ng of DNA per gram of bone with a standard deviation of 4 for the ulna, up to ~448 ng of DNA per gram of bone with a standard deviation of 53 for the 1st distal hand phalanx. A few samples, such as the 1st distal hand phalanx, gave relatively consistent yields with a standard deviation of approximately 10% between individuals. Most samples had significant differences in yield between the 3 skeletons. Tooth samples were highly variable between individuals with the left 1st molar having 1036, 152, and 73 ng per gram of tooth for an average of 435 ng per gram of tooth and a standard deviation of 535.

Although only a few loci could be recovered from some of the samples, many others produced full profiles. Overall, there was a trend toward cancellous bone yielding more viable DNA than cortical bones. Bones typically sampled for DNA, such as the femur and tibia, did not perform as well as bones that are generally overlooked in DNA sampling. The average yield for 18 long bones not showing PCR inhibition during quantification is 26.3 ng of DNA per gram of bone *sd*= 30.1 while the 28 foot bones not

showing PCR inhibition during quantification averaged 184.1 ng of DNA per gram of bone *sd*=199.8. Even with the large variation t-test results show that the foot bones performed significantly better *p*=0.0003. The 1st distal hand phalanx, the patella, and all seven tarsals outperformed the long bones of the arm and leg, including the femur. In fact, contrary to conventional wisdom, most predominantly cancellous bones, including small elements, yielded more DNA and better STR profiles than the predominantly cortical bones.

Phase 2

During Phase 2, 120 bones (12 individuals, 10 bones each) from increased post mortem intervals were assessed. Overall, a similar trend evident in the Phase 1 results was also evident in the Phase 2 results. Predominantly cancellous bones outperformed the predominantly cortical bones in terms of DNA quantity and STR profiles obtained. The data from Phase 2 was quite variable possibly due to the increased number of individuals being tested.

Generally the DNA recovered from samples was lower as the PMI increased. Of the 10 element types tested in Phase 2, only the patella had the highest average yield at the shortest PMI and then had progressively decreasing yields with increasing PMI. The other 9 elements were more variable, with some having unusually high quantities of DNA from the longer PMIs. The 1st distal hand phalanx at more than 20 years PMI averaged more than 4000 ng/g of bone approximately 10 times higher than it averaged in Phase 1. The average yield from the 1st cuneiform was highest at the 10-20 year post mortem interval, averaging more than 1050 ng/g of bone.

When comparing each of the 10 elements from the 12 skeletons tested during Phase 2, only one tibia and one femur yielded full profiles, and both were from the

earliest PMI (0-3 years). The other 8 elements each yielded 5-8 full profiles throughout the increased PMIs. Specifically, the patella and cervical vertebra each yielded 5 full profiles; the middle rib yielded 6 full profiles; the talus and 1st cuneiform each yielded 7 full profiles; the 4th metacarpal, 4th metatarsal, and the 1st distal hand phalanx each yielded 8 full profiles.

There is a distinct difference when considering DNA yield from older bones. Most elements produced less complete DNA profiles as the post mortem interval increased. However, the elements that produced the best results in Phase 1 testing continued to perform well from later post mortem intervals. Interestingly, the 1st distal hand phalanx and the 4th metatarsal were the only elements to produce a full profile in the final PMI of 20+ years.

Conclusions

Phase 1

When comparing DNA yield per gram of bone (Ng/g) from all of the element types within a single skeleton, bones such as the 1st distal phalanx, tarsals, and metatarsals outperformed any of the long bones, whether from the arm or the leg. This pattern was replicated in all three skeletons. These more successful elements are typically dismissed as potential DNA sources in favor of weight bearing cortical long bones. While relatively larger cortical bones are regularly considered for a DNA sample, it is the smaller cancellous bones that offer a better success rate. They are also easier to sample than a long bone. A distal phalanx or metatarsal can be removed, in its entirety, with a disposable scalpel, minimizing destruction to the body. Since these smaller elements can be removed intact, opportunity for contamination within the bone

is reduced. Furthermore, removing intact small elements with a disposable scalpel reduces the chance of contamination between sampling events, which can occur when the bone-saw blade is not sufficiently decontaminated between cases. Using scalpels is physically easier for the practitioner, who may spend long days conducting bone sampling and also eliminates expensive equipment requiring electricity (Mundorff et al. 2009). On the other hand, cutting out a wedge or a window from a long bone's midshaft has to be done with a saw, typically an electric saw, which increases destruction to the body, requires electricity, is more labor intensive, and is less efficient because of the time spent decontaminating the saw blade between cases.

Using the order of successful elements generated from Phase 1, investigators can make more informed choices during bone sampling. While there are many options when sampling a complete skeleton, forensic practitioners are also faced with partial or severely fragmentary remains. For example, the 260 passengers from the American Airlines 587 crash in 2001 comprised 2,058 body fragments, a victim-to-remains ratio of approximately 7.75 remains from every victim (Mundorff 2008). Knowing which element to sample from a partial or fragmentary set of remains reduces time-consuming and costly retesting which can in turn reduce the time needed to establish identifications.

Phase 2

Phase 2 establishes sampling guidelines to use when skeletal remains are recovered from longer post mortem intervals (up to 20 years PMI). Again, conventional wisdom did not hold true when comparing multiple elements from a single individual. While most elements produced less complete DNA profiles as the PMI increased, the distal hand phalanx, metatarsal, and metacarpal, continued to out perform the femur and tibia. Therefore, when recovering or exhuming older skeletonized remains,

forsaking traditional sampling strategies in favor of the preferential ranking established here may increase DNA identification success rates. Additional research into the differences between cortical and cancellous areas within a single bone is needed. This may help to narrow down not only which specific element to sample, but where on the specific element to sample.

Implications for policy and practice

This study's results will be useful for forensic scientists and law enforcement agents in the U.S. criminal justice system investigating missing persons or unidentified remains. These data will also be useful for international investigators attempting to identify mass fatality victims or remains recovered from mass graves. The ability of DNA testing to aid in establishing identifications is directly related to the ability to obtain sufficient DNA. While previous research has documented the potential for some skeletal elements to yield better than others, those studies analyzed retrospective data and were either limited to mitochondrial DNA or did not include all skeletal element types within a single skeleton.

The current selection of skeletal elements for DNA testing is not empirically based; instead it is founded on the collective wisdom of practitioners. The experimental outline of this research project provided empirical data indicating which skeletal samples are most likely to provide both the quantity and quality of DNA needed to produce viable DNA profiles from skeletal remains at varying post mortem intervals. The results described here offer much needed guidance on sample selection. Sampling strategies vary significantly accordingly to whether the target material is from recently decomposed individuals, highly fragmented victims from mass fatality events or acts of

terrorism, or involve remains from longer post mortem intervals, such as unidentified cold cases or remains excavated from clandestine graves. Tailoring sampling strategies for a particular scenario allows investigators to focus only on those bones most likely to yield an identification through DNA analysis. This will reduce expensive and time consuming retesting, and will also speed identifications, which is beneficial to investigators as well as to the decedent's family.

I. INTRODUCTION

Identifying skeletal remains often challenges forensic investigators because ante mortem records are not always available. This is particularly true during identification efforts tied to mass grave excavations, disaster projects where entire families or communities have been killed or simply a cold case. In such instances, the only means of identification may lie in the bones' DNA. Experience teaches that, as remains decompose, skeletal elements including bones and teeth yield higher levels of DNA than muscle. Additionally, skeletal material often survives long after muscle tissue has decomposed. While bone protects DNA better than muscle, all bones are not equal and some yield DNA at higher rates than others. The ability of DNA testing to aid in identification is directly related to the ability to obtain sufficient DNA. Investigators are increasingly relying on DNA testing to identify remains. Those tests are expensive and time-consuming; therefore, by determining which skeletal element is most likely to yield an STR profile, allowing identification, expensive and time-consuming retesting can be avoided. This has tremendous implications for current policy and practice.

Statement of the Problem

Current DNA sampling practice is based on the collective wisdom of practitioners. Weight bearing long bones, such as the tibia and femur, are the elements most typically sampled over all other bones. In fact, the most recent Interpol DVI Guide (disaster victim identification) recommendations, used by their 188 member nations, suggest sampling "long compact bones (4-6 cm sections…)" "(~10g, if possible; preferably cortical bones with dense tissue" (DVI Guide: Interpol 2009:40). In the United States, the National Association of Medical Examiners has written a mass fatality plan

with DNA specimen collection guidelines. When sampling severely decomposed remains, the first request is for "long bones (either intact or 6 inches of hemi-shaft)" followed by teeth (NAME 2010:24). Yet, recent evidence questions the accuracy of this collective wisdom, and points to smaller and cancellous elements, such as the patella and metatarsals, yielding better quality and quantity of genetic material. However, there has never been a comprehensive study evaluating the differential preservation of DNA by skeletal element type. Establishing a clear DNA sampling strategy, based on a rank order of skeletal elements according to each bone's potential to provide usable genetic material for a DNA identification, offers much needed guidance on which elements are best suited for DNA testing. Thus, replacing intuition with empirically based data can maximize the success rate of identifications and avoid expensive and time-consuming retesting. It will also speed identifications, which is beneficial to investigators as well as to the decedent's family.

Literature review

DNA from Degraded Bones

The ability to extract usable DNA from bone is generally presented in terms of success or failure, where failure is commonly attributed to "degraded samples." Environments that promote human decomposition also contribute to DNA degradation. Iwamura et al. state that little is known about "the extent to which nuclear DNA remains inside the osteocyte lacuna of mineralized matrix and to what extent degradation of nuclear DNA occurs during post-mortem" (2005:33). While the relationship between DNA degradation rates and adverse environmental exposure is not fully understood, the literature points to some common themes. Various environmental and taphonomic

factors influence DNA degradation, including temperature, humidity, ultraviolet light, postmortem interval, soil microbes, fire, water, mold, and storage conditions (Arismendi et al. 2004; Burger et al. 1999; Collins et al. 2002; Edson et al. 2004; Graw et al. 2000; Grupe et al. 1993; Hochmeister et al. 1991; Imaizumi et al. 2004; Iwamura et al. 2005; Kaestle and Horsburgh 2002; Paabo et al. 2004; Pfeiffer et al. 1999; Steadman et al. 2006; Ye et al. 2004). Some studies have ranked these environmental influences according to severity, with high temperatures and water submersion listed among the most destructive (Collins et al. 2002; Gotherstrom et al. 2002; Hochmeister et al. 1991; Iwamura et al. 2005; Steadman et al. 2006).

The relationship between post mortem interval (PMI) and DNA preservation is controversial. In one study, Bar et al. determined that "generally the amount of degraded DNA correlated directly with the duration of the postmortem period" (1988:59). However, that study was limited to DNA retrieved from soft tissue, revealing that liver and kidney tissues degrade rapidly while brain tissue degrades more slowly. Further studies examining the relationship between postmortem interval and DNA preservation in bone specimens have found no correlation (Burger et al. 1999; Evison et al. 1997; Leney 2006; Parsons and Weedn 1997).

DNA research has demonstrated that bone preserves DNA better than soft tissue. Researchers believe that the structure of bone acts as a physical barrier to external influences (Graw et al. 2000; Hochmeister et al. 1991; Imaizumi et al. 2004; Ye et al. 2004). Because bone preserves DNA better than soft tissue, the next logical step in the development of DNA sampling guidelines is to rank specific bones according to their capacity to preserve DNA, and then to measure this. Little research has been conducted on this issue, but a few researchers have mentioned that DNA from different

elements may deteriorate at different rates (Imaizumi et al. 2004; Perry et al. 1988). Perry et al. examined DNA degradation in bone, finding that "when degradation of DNA in clavicle bone was compared with the DNA degradation in a rib bone from the same individual, the clavicle bone DNA seemed to be degraded more slowly" (1988:152). Alonso et al. examined the influence of microbial DNA on human DNA extracted from 8- to 50-year-old bone and tooth samples and found that "the quality of DNA obtained from long bones is higher than that extracted from skull or ribs" (Alonso et al. 2001:265). Parsons and Weedn also agree that DNA is much more reliably extracted from compact (cortical) bone than spongy (trabecular) bone, such as rib. They believe the more rapid degradation in spongy bone is due to its higher moisture content as compared to cortical bone (Parsons and Weedn 1997).

Recent Studies Examining DNA Yield by Skeletal Element

A recent study by Mundorff et al. (2009) used a subset of remains from the World Trade Center Human Identification Project to measure differential DNA preservation by skeletal element. They found that the recovery location of the remains (Ground Zero versus the Staten Island Landfill), the sex of the victim, and the victim type (civilian - usually above the level of plane impact, firefighter – usually below the level of impact, or plane passenger) did not appear to influence DNA preservation. As with previous studies, they also found that denser, weight-bearing elements yielded DNA better than non-weight-bearing elements (Figure 1).

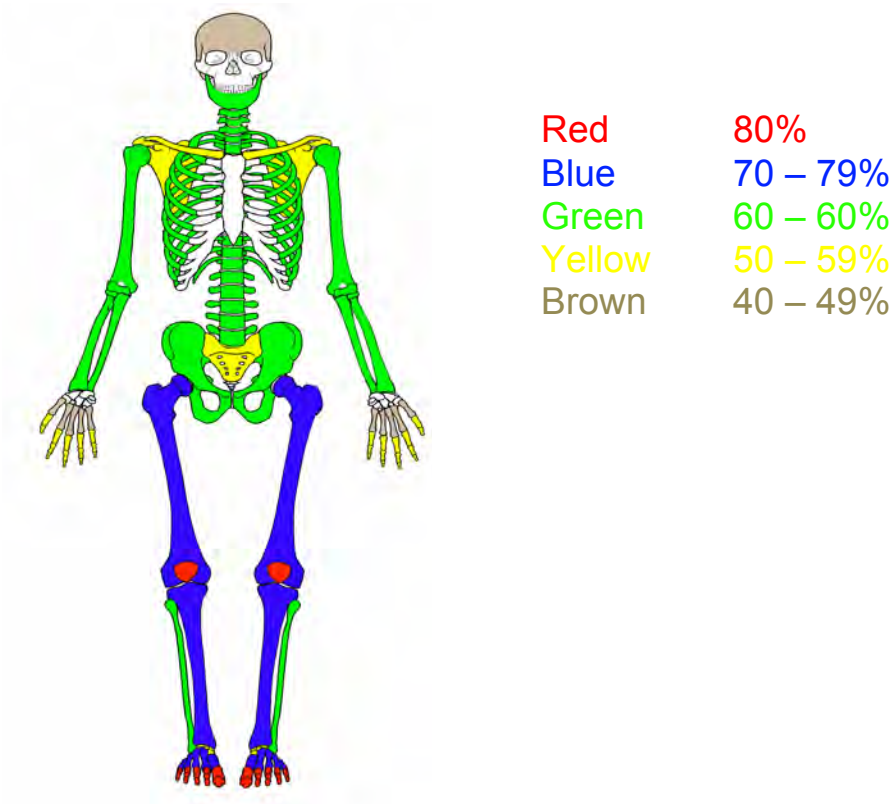

Figure 1. Success rates for different bones tested as part of the World Trade Center Identification effort (Mundorff et al. 2009).

More interestingly, they found that bones generally bypassed in DNA sampling yielded at surprisingly high rates. The better yielding leg bones included several smaller elements; patellae, metatarsals, and foot phalanges. These bones yielded DNA at a rate similar to both the femur and the tibia. Mundorff et al. argue that these results should be considered when developing DNA sampling protocols for mass fatality events. According to these researchers, small bones, such as metatarsals, can be removed easily with a disposable scalpel while sectioning midshaft femur is difficult, time consuming, and requires a bone saw. Not only are disposable scalpels less labor-intensive, they are also cheaper than bone saws and do not require electricity or decontamination between sampling (Mundorff et al. 2009). Additionally, these

researchers argue that removing intact bone specimens reduces the potential for contamination, which is higher when a section of long bone is sampled. Finally, by initially sampling those elements most reliably yielding DNA, costly and time-consuming retesting can be avoided.

In 2007, Milos et al. published a study that is similar in many respects. Their study relied on data from remains excavated from mass graves in the former Yugoslavia and focused on nuclear DNA success rates of different skeletal elements, comparing these at various postmortem intervals. The skeletal samples used in their study originated from different geographical locations between 1992 and 1999, and the DNA tests were performed between 8 and 15 years postmortem; therefore, DNA preservation was highly variable (Milos et al. 2007). Their results indicate again that dense cortical weight bearing long bones yield the best (Milos et al. 2007). Notably, this study did not test all skeletal elements; for example, patellae were not tested, and metatarsals were only sampled from two of the three gravesites. Additionally, these authors show a clear correlation between postmortem interval and DNA success rates. For example, success rates from the femur varied from 92% to 83% when comparing the 1999 samples to the 1992 samples (Milos et al. 2007).

The authors suggest that the densest compact bones, specifically midshaft femur and teeth are the best choices for DNA sampling protocols. These recommendations differ from those suggested by Mundorff et al. 2009. The differing recommendations probably result from differences between the two studies. The Milos et al. study tested skeletal remains that had been buried and subject to longer post mortem intervals while the Mundorff et al. study focused on relatively recent, yet fragmentary, remains from the WTC disaster. Additionally, the two studies considered a few different elements, such

as the patella. Because the samples in the Milos et al. study show a correlation between postmortem interval and yield rate, remains from different post mortem intervals should be examined to determine if temporal factors differentially affect element yield rates.

A similar study by Edson and colleagues examined the rate of successful mitochondrial DNA (mtDNA) extraction from different degraded skeletal elements (2004). During a three-year period, more than 1,000 samples were examined to "determine if there was a general trend among success rates versus specimen type" (Edson et al. 2004:76). These samples were remains of U.S. service members and civilians missing in past military conflicts around the world and were recovered from a variety of conditions including buried, frozen, or submerged in salt water. As with the Milos et al. study, small sample sizes precluded analysis of certain bones such as metacarpals and patellae. The findings from this study both confirm and challenge some of the anecdotal statements found in earlier studies. They argue, "Of the long bones, the weight-bearing bones, such as femora and tibiae, were the best specimen types. Metatarsals are also weight-bearing bones, but at initial glance they appear to be inadequate specimens by size alone. However, approximately 80% of the metatarsals tested produced reportable results. Ribs were also highly successful, although a larger number of specimens is needed…Cranial fragments are the most difficult of the samples tested from which to obtain quality sequence data" (Edson et al. 2004:76).

The conclusions these authors draw are similar to those of Mundorff et al., including the finding that metatarsals have a surprisingly high success rate. The authors suggest that greater efficiency is possible through careful selection of elements

used in DNA sampling, which minimizes the time and resources expended on resampling and retesting.

A study by Leney (2006), designed to provide guidance in choosing the best sample for mtDNA testing of archaeological remains, concluded that sample weight along with skeletal element were the most important factors in maximizing successful outcomes. He concluded that postmortem interval was not an important determinant of success or failure, although the climate from where the remains were found was strongly correlated. Samples recovered from temperate environments yielded better results than samples from tropical environments, even when the temperate samples were much older than the tropical samples. While heat and moisture are detrimental to DNA preservation, arid environments promote DNA preservation by slowing the biological activity that degrades DNA (Leney 2006).

Sample mass was another important factor in producing successful yields. Leney determined that "the larger the sample, the greater the probability that it will be successful, particularly up to around 7g" (2006:40). However, this result was mitigated by element choice. For example, when comparing the same sample weights from femora and humeri, the femora were successful 92.5% of the time while humeri were only successful 75% of the time. Taking into account mass and element choice, the femur and tibia were the most successful, followed closely by the os coxa, first metatarsal, and mandible. Excluding the mandible, the four most successful elements were in the lower limb or the os coxa. Leg bones were most successful, followed by arm bones and then by bones of the axial skeleton. As with the Edson et al. study, the cranial bones showed the lowest yield rates.

Leney believes that leg bones are most successful for mtDNA extraction and hypothesizes that this is because leg bones are made of dense cortical bone that bears the forces of locomotion and the stresses of carrying static body weight (Leney 2006). He proposed two explanations for higher mtDNA identification success rates of non-weight-bearing bones such as the mandible. First, bone density increases as an adaptation to the stresses of mastication. This response is critical to maintaining cortical mass and density and may be responsible for the mandibles successful DNA yield rates similar to weight-bearing bones. Second, areas where muscle attachments have reworked bone may be particularly good sources of DNA. This second hypothesis helps explain the high success rate of the os coxae, since the iliac crest (where the samples were taken) is an area where the muscle attachments are constantly reworking and remodeling the bone. Unfortunately, other bones which are also constantly reworked and remodeled from muscle attachments, such as the patella, were not included in his sample; therefore the hypothesis that reworked cortical bone presents a good source of DNA could not be comprehensively evaluated. Although the last two studies examined the mtDNA success rates, Leney argues that these results can be generalized to include nuclear DNA testing (2006).

More recently, Misner and colleagues examined the correlation between skeletal weathering and mtDNA quality and quantity (2009). This study was unique because of their ability to limit major confounding variables such as “burial age, internment style, and gross environmental conditions” (Misner 2009:822). The skeletal material was derived from excavations conducted at the Voegtly Cemetery in Pittsburgh, Pennsylvania, which was in use between 1833 and 1861 (Misner et al. 2009). Beginning in 1993, more than 700 burials were removed and taken to the Smithsonian

for examination. Each skeleton and each individual bone sampled were given a weathering score based on the condition of the remains. A total of 86 bones (femur n= 28, pelvis n= 25, and rib n= 33) were tested from 36 different skeletons. The study concluded that there was no correlation between skeletal weathering and mtDNA quality and quantity. However, a correlation between bone type and their ability to amplify mtDNA was noted (Misner et al. 2009). Like the studies by Mundorff et al., Leney, and Edson et al. (2009; 2006; 2004), these authors found that dense compact bone (e.g.,femur) performed better than the more cancellous bones such as rib and pelvis (Misner et al. 2009).

Notably, all of the above studies were retrospective analyses, and none tested all of the element types within a single skeleton. Moreover, as stated by Misner et al., and with the exception of the Misner et al. study, research involving bone DNA often " suffer from small sample sizes, compare remains from diverse geographic locations or variable habitats, have highly variable times since death" (2009:822). These and other confounders "make drawing conclusions, particularly statistically significant ones, difficult or impossible (2009:822).

A current study, by Latham and Baker, has taken on the challenge of trying to determine "the elements of the skeleton most likely to yield subsequent DNA profiles" (2012:423). However, like many of the retrospective examples, a wide array of taphonomic influences could not be controlled for and are likely to influence the results. These studies have also limited the skeletal elements being tested instead of testing the entire skeleton of one individual. To provide investigators with usable information, a study would need to measure DNA yield rates from all the elements within the same

skeleton. To date, there has never been a prospective study under controlled conditions to evaluate the differential preservation of DNA by skeletal element.

Sampling Guidelines

Despite growing reliance on DNA-based identifications, a thorough review of the literature reveals few detailed guidelines for DNA sampling of osseous remains. For instance, DNA sampling protocols used in recent mass fatality incidents varied and were often tailored to the disasters unique circumstances (Mundorff et al. 2009). Muscle tissue and rib bones were sampled from the 1995 Branch Davidian victims in Waco, Texas (Butler 2005); femur was sampled from victims of the 2002 Bali nightclub bombings (Briggs and Buck 2009); ribs and teeth were initially sampled from victims of the 2004 Boxing Day tsunami in Phuket, Thailand (Cockle et al. 2005; Lessig et al. 2006); and the anterior tibial midshaft was sampled from the victims of Hurricane Katrina in 2005 (Boyer P.C. 2006).

DNA based identifications are primarily used with fragmentary or skeletonized remains, which do not always possess more easily identifying characteristics such as fingerprints or dental. However, it is also clear, from incidents such as Hurricane Katrina and the Boxing Day tsunami, that DNA is now used to identify nearly complete yet decomposing bodies. DNA based identifications are also the primary means for identification where ante mortem data may not be available. A number of factors can make ante mortem information difficult to access. These factors may include limited health services, lack of dental care, and general disruption in a post disaster (or post conflict) society (e.g., New Orleans following Hurricane Katrina).

To address disparities in sampling strategies, particularly from mass fatality victim identification projects, agencies have begun issuing DNA sampling guidelines

and recommendations. The National Institute of Justice (NIJ) addresses sampling methods in its publication, *Mass Fatality Incidents: A Guide for Forensic Human Identification* (NIJ 2005). This guide provides general sampling guidelines, stating "the sampler obtains one of the following, listed in order of preference": deep skeletal muscle, cortical bone, canine tooth, or other portion of soft or hard tissue (NIJ 2005:61). In 2007, the DNA Commission of the International Society of Forensic Genetics (ISFG) published "Recommendations Regarding the Role of Forensic Genetics for Disaster Victims Identification (DVI)" (Prinz et al. 2007). One of the twelve recommendations addressed postmortem sampling, again confirming a preference for dense cortical bone, particularly from weight bearing leg bones (Prinz et al. 2007). In fact, the most recent Interpol DVI Guide (disaster victim identification), used by their 188 member nations, recommends sampling "long compact bones (4-6 cm sections…)" "(~10g, if possible; preferably cortical bones with dense tissue" (DVI Guide: Interpol 2009:40). In the United States, the National Association of Medical Examiners has written a mass fatality plan with DNA specimen collection guidelines. When sampling severely decomposed remains, their first request is for "long bones (either intact or 6 inches of hemi-shaft)" followed by teeth (NAME 2010:24). These existing guidelines offer very broad recommendations and do not specify which elements are most likely to produce DNA profiles under adverse taphonomic conditions or over longer post mortem intervals, particularly if the remains are fragmentary and a weight bearing leg bone is unavailable (Mundorff et al. 2009).

This research project tests the hypothesis that dense, cortical, weight bearing long bones are the best source of DNA from osseous material.

II. MATERIALS AND METHODS

Skeletal Selection

To examine the differential DNA yield rates between skeletal elements a two-phase study was conducted. First, three recently skeletonized skeletons, which had been donated for research to the University of Tennessee Forensic Anthropology Center (FAC), were selected. A specific selection criterion was established between the researchers and the FAC coordinator. The three donor skeletons were all males who died during the same year, decomposed on the same plot of land and in the same position (prone on the ground surface), were between 40-70 years at the time of death, and self-identified as white. By restricting the criteria as such, major confounders, such as post mortem interval and gross environmental conditions, are eliminated. All skeletons used in this research project were required to be older white males because the UT collection is predominantly comprised of individuals in this demographic. Young individuals, females, and minorities are not well represented in the collection; therefore destructive analysis to skeletons outside the dominant demographics is limited.

The three Phase 1 skeletons were never curated; they went from decomposing outside at the Archaeological Research Facility (ARF) at the University of Tennessee directly into this study. The preassigned donor case numbers we maintained for each skeleton through out the study: 07-09D, 45-09D, and 116-09D. All three individuals were placed at the ARF during 2009. Individual 07-09D was a 50-year-old white male. Placement lasted 12 months, 27 days. Individual 45-09D was a 69-year-old white male. Placement lasted 22 month, 22 days. Individual 116-09D was a 47-year-old white male. Placement lasted 15 months, 26 days. Placement ranges varied according to the month the individual was placed, and the availability of staff for recovery. Two of the

three skeletons (45-09D and 116-09D) were collected from the ARF on the same day, 18 March 2011. Recovery and field inventory was performed by one co-PI (AZM), the project's graduate research assistant (EF), and the FAC assistant coordinator. Recovery included picking up the bones from the ground surface and screening the dirt below the skeleton to ensure all small bones had been recovered. FAC graduate student volunteers had previously collected the third skeleton, 07-09D, employing the same recovery procedures. The skeletons were transported in large plastic bags to the FAC processing building. There, the minimal amount dirt adherent to the bones was cleaned off with room-temperature water and a toothbrush (a different, new toothbrush was used for each skeleton). The skeletons were then separately placed on decontaminated metal trays lined with brown paper bags to absorb dripping water and positioned on a rack to dry. Drying took 6 days, and then they were individually bagged in standard brown paper bags. The hands and feet were each placed in their own paper bags to minimize resorting later. The dry skeletons were then transported to the Department of Anthropology to be re-inventoried, measured, assigned individual research sample numbers, marked for sampling, and photographed.

The re-inventory list was compared to the inventory documented in the field during recovery. On site, bones can be covered with dirt masking identifying characteristics. This can lead to mistaken element identification particularly with smaller bones such as wrist and finger bones. Therefore, the skeletons are always inventoried a second time after they have been cleaned. Measurements were collected from each skeleton according to the University of Tennessee Skeletal Inventory Worksheet.

Every skeletal element and tooth type was to be DNA tested except for the carpals. The proposal initially excluded carpals because of their small size and the risk

of consuming the entire bone during testing. This decision was revisited when it became obvious that laboratory sampling procedures were so minimally destructive that a carpal could be easily sampled without being consumed. Therefore, the capitate (1 of the 8 carpals in the wrist) from each of the 3 skeletons, was included in Phase 1 testing.

The same 55 bones and teeth were tested from each skeleton (Table 1). Although adults have 206 bones, it was not necessary to test them all. Many bone types are duplicated (e.g., rights and lefts) and there is no evidence that the side the element comes from will affect its DNA yield rate. Additionally, some skeletal elements have redundancies (e.g., 24 ribs, 12 thoracic vertebrae). Therefore, 'representatives' from each element type were selected, and for consistency only bones from the left side were tested. These same 55 element types were tested from the three Phase 1 skeletons to ensure that results from one individual were not anomalous. Using the preassigned donor numbers to discriminate the skeletons from each other, each of the 55 elements was assigned a specific sample number. For example, sample "07-09D-01" was a cervical vertebra from donor skeleton 07-09D and "45-09D-54" was the cervical from 45-09D.

Table 1. Complete list of the 55 elements tested in Phase 1.

Skull	Frontal	1
	Temporal	1
	Parietal	1
	Occipital	1
	Maxilla	1
	Mandible	1
Teeth	Maxillary Lateral Incisor	1
	Maxillary Canine	1
	Maxillary 1st Premolar	1
	Maxillary Molar	1
	Mandibular Lateral incisor	1
	Mandibular Canine	1
	Mandibular 1st Premolar	1
Trunk	Cervical Vertebra	1
	Thoracic Vertebra	1
	Lumbar Vertebra	1
	1st Rib	1
	Middle Rib	1
	12th Rib	1
	Sternum	1
	Sacrum	1
	Clavicle	1
	Scapula	1
	Ilium	1
	Ischium	1
	Pubis	1
Leg	Femur	1
	Tibia	1
	Fibula	1
	Patella	1
Arm	Humerus	1
	Radius	1
	Ulna	1
Hand	Metacarpals - 1-5	5
	1st Proximal Phalanx	1
	1st Distal Phalanx	1
	Capitate	1
Foot	Metatarsals - 1-5	5
	1st Proximal Phalanx	1
	1st Distal Phalanx	1
	Calcaneus	1
	Talus	1
	Navicular	1
	Cuboid	1
	1st Cuneiform	1
	2nd Cuneiform	1
	3rd Cuneiform	1
	Total Samples	55

Once the elements were selected, a specific sampling spot was located on each bone and marked with X in pencil (Figure 2). The skeletons used for this study are part of a larger research collection, housed in the Department of Anthropology at the University of Tennessee, and frequented by researchers from around the world. Therefore, the position of the sampling spot on each bone was mainly chosen to avoid common points of measurement and in order to minimize interference with other morphological traits necessary for conducting future research. The project PIs and the FAC coordinator conducted the selection of sampling sites jointly. Tubular bones (e.g., long bones of the arms and legs, hand bones, clavicle) were all marked in the same way, along the shaft of the bone just above or below midshaft (at about 2/3rd the length). This specific site was selected to avoid midshaft, a commonly measured spot on long bones. Sampling sites on small and irregularly shaped bones (e.g., tarsals, sternum, os coxa) were placed in spots with enough surface area for the drill bit while still avoiding important morphological features. Once a spot was selected on a particular element, that exact same spot was marked on the same element from the other two skeletons.

Once all of the bones were marked, photographs were taken. Each bone was photographed with a scale, the donor case number and an arrow pointing to the marked sampling spot. Each bone was also photographed without the arrow. Additionally, the 6 standard planes of the skulls were photographed. Following photography, the specific teeth to be tested were manually extracted from the maxilla and mandible and also photographed. The teeth were then molded and cast in order to have a replica of each tooth in case of its destruction (although only a portion of the root was ground during sampling). All photographs were uploaded to a computer and labeled with their specific sample number. The bones were then individually placed in paper bags labeled with the

sample number and bone type. The labeled photographs were copied onto CDs, twice. One set of copies is maintained along with the camera's memory card, for perpetuity. The other CD, a box manifest, and chain of custody letter were included in the bone's shipping boxes as a reference and sent to Bode.

During Phase 1 there were two minor deviations from the original proposal's sampling strategy. The first, already discussed above, was the addition of a carpal to the list of elements to be tested. The second deviation was a decision taken to not test the sternum from 45-09D. This bone was not tested due to its extensive remodeling and the presence of surgical wire from previous open-heart surgery. The presence of surgical wire from surgery was not known prior to selecting the initial three skeletons.

While Phase 1 processes were ongoing, skeletons for Phase 2 were selected. Three skeletons from each of the four predetermined post mortem intervals (0-3 years, 4-10 years, 11-20 years and 20+ years) were identified (n=12). In order to test bones from longer post mortem intervals, Phase 2 skeletons had to come from the curated collection, which has maintained skeletons for over three decades. The storage conditions under which the curated skeletons are housed are not comparable to skeletons recovered directly from the field. Instead, they have been maintained in acid free boxes on shelves in a storeroom of the anthropology department. Again, in an effort to control as many confounding variables as possible, only skeletons with similar demographics (age at the time of death, sex, etc.) to those from Phase 1 were considered for Phase 2. Phase 2 skeletons also decomposed on the same plot of land and in the same gross environmental conditions as those from Phase 1. Since Phase 2 was designed to determine if the same skeletal element types from Phase 1 are equally successful at longer post mortem intervals and to give an indication how DNA

degradation occurs over time, results from Phase 1 were used to establish the list of elements for Phase 2 testing.

Following Phase 1 data analysis, ten elements (from 12 skeletons, 4 increasing PMI ranges) were selected for Phase 2 testing (n=120 bones). The 'most successful' elements from Phase 1 were those that consistently yielded full profiles for all three individuals, and a rank order was established based on these criteria. Twenty-seven elements were considered for Phase 2 testing (12 from hands or feet). Only ten bones per individual were to be tested in Phase 2, necessitating further narrowing of the selection process. Therefore, elements were selected by yield per mass of sample, average RFU value for each element, and skeletal representation. For example, six of the seven tarsals met the requirements, so two were chosen as representatives.

Although 4 of the 27 potential elements were teeth, teeth were not available for destructive analysis in Phase 2 because of their scarcity in the skeletal collection; many donors do not have teeth when they die. The ten elements chosen for Phase 2 testing are: cervical vertebra, middle rib, patella, 4th metacarpal, 1st distal hand phalanx, 4th metatarsal, talus, 1st cuneiform, tibia, and femur. Although results from the tibia and femur did not reach the threshold to qualify for Phase 2, practitioners typically prefer sampling these elements to the bones that actually qualified. Therefore femur and tibia were included for comparison, to demonstrate their success rates against the other, most successful' bones from Phase 1.

Once the ten elements were chosen, those specific bones were removed from each skeletal box. An X was marked indicating the sampling spot on each bone in the same anatomical position as marked on the Phase 1 elements. Using the same procedures as Phase 1, each bone was then photographed (with and without an arrow),

assigned a unique sample number, individually placed in paper bags, the bags were labeled with the sample number and bone type, and shipped FEDEX with a manifest, CD of photographs, and a chain of custody letter, to Bode.

During Phase 2, there were a few deviations from the original proposal. The maxilla was included in the initial ten bones selected for Phase 2 testing. However, after the first maxilla was drilled, it became clear this bone would not be suitable. The sampling site identified during Phase 1 was the posterior aspect of the alveolar bone. Most skeletons selected for Phase 2 testing were edentulous, with significant alveolar bone remodeling and resorption. This resorption left the maxillary bone fragile and more susceptible to damage during drilling, thus it's discontinued use in Phase 2. The second deviation concerned three specific elements. Skeleton 05-98D did not have a left patella; therefore the right patella was sampled. The left femur from skeleton 100-08D is currently on display in the Smithsonian museum so the right femur was sampled instead. Finally, skeleton 03-90 did not have a 1st distal hand phalanx, so a distal phalanx from another finger was sampled.

Sampling

Bone samples were cleaned by first wiping the surface with 10% bleach, followed by sterile water, and 70% ethanol. A 3/8 inch hole was drilled at the pre-designated site marked with an X (Figure 2) until approximately 0.2 grams of power was recovered. The bone powder removed from a long bone would largely include cortical bone and some smaller amounts of cancellous bone. The bone powder removed from small and irregular bones would contain a mix of cortical bone and cancellous bone although the exact volume of each has not yet been determined (see Implication for Further Research). The sampled bone powder was placed into a 50 ml conical tube and the

mass was recorded. In most cases a single hole was sufficient to collect 0.20 g of powder. A few samples, such as the middle rib, were too fragile to be drilled and therefore a cutting was taken and ground into a fine powder using a blender cup.

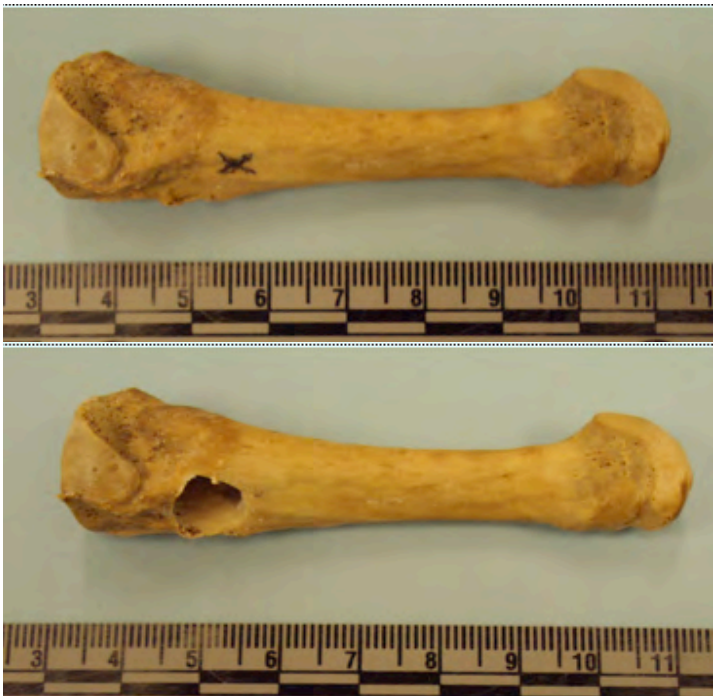

Figure 2. A marked sampling site prior to drilling (top) and after drilling (bottom).

Teeth were sampled by cutting off a portion of the root weighing approximately 0.20 g, then grinding it into a fine powder in the blender cup (Figure 3).

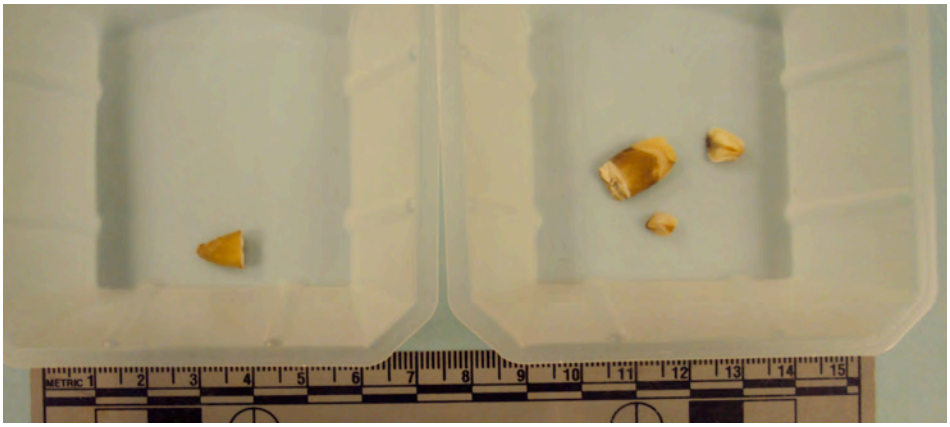

Figure 3. Sampling of 0.2 grams from mandibular canine root (left) and the remaining tooth material (right).

DNA Extraction

All skeletal samples were extracted using the same procedure. The powdered bone was demineralized with EDTA for approximately 18 hours at 56^{O}C. Following demineralization the DNA was extracted, without discarding the EDTA, using the Qiagen QIAamp extraction system and eluted into 50 μl of TE^{-4}. Samples were extracted in sets of 11 including 1 reagent blank.

DNA Quantification

DNA extracts from Phases 1 and 2 were quantified using the Applied Biosystems (ABI) Quantifiler system, using ½ reaction volumes, on an ABI 7500 SDS instrument and analyzed using the SDS version 1.2.3 software.

Amplification Component	Volume (μl) for a 1x reaction
Quantifiler Buffer	6.25
Quantifiler Primer	5.25

11.5 μl of the mix was added to each reaction
2 μl of DNA was added to each reaction

Quantification Data Analysis

The DNA quantity was assessed by taking the quantification information (ng/μl), multiplying by the elution volume of 50 μl and dividing by the mass of the sampled bone, to give the ng of DNA per gram of bone. The Quantifiler results had 18 samples that showed inhibition in the cycle threshold (ct) of the Internal Positive Control (IPC).

DNA Amplification

DNA extracts up to 2 ng or 9.5 μl were amplified using the AMPflSTR Identifiler^{TM} system and run on an ABI 9700 thermocycler at 28 cycles. The Identifiler reactions were setup as follows:

Amplification Component	Volume (μl) for a 1x reaction
Amplification Buffer	10.5
Identifiler Primer	5.5
Taq Gold	1.0
Total of mix	17

15.5 μl of the mix was added to each amplification reaction leaving up to 9.5 μl available for addition of DNA.

Re-Amplification

Most samples were subjected to a single amplification targeting 2 ng or including up to 9.5 μl of DNA extract. Several samples showed signs of inhibition and therefore a second round of amplification was attempted to overcome the inhibition. In most cases inhibition was noted during quantification and the samples displayed an undetectable quant value. The inhibited samples were initially amplified at the maximum volume and if the results were of a low quality then a second round of amplification was performed with less DNA to see if the results would improve. The best amplification was used for comparison purposes.

Fragment Analysis

Amplified DNA was prepared for fragment analysis by taking 0.7 μl of amplified product and adding it to a mix of 10 μl formamide and 0.12 μl of the internal lane standard (ILS) GS500 Liz. The amplified STR products were separated on an ABI 3130xl fragment analyzer with data collection version 3.0.

Genotyping

Separated sample data was analyzed using the ABI GeneMapperID software version 3.2.1. The minimum signal strength analyzed was 75 relative fluorescent units (RFU) and the homozygous cutoff was set to 200 RFU. For alleles to be called the allelic balance had to be at least 50%.

Data Analysis

The RFU per allele was calculated as the average of the heterozygous peak heights or the homozygous peak height divided by two. The max:min ratio was calculated by taking the value for the highest average RFU per allele at any locus in an electropherogram and dividing by the lowest average RFU per allele at any locus in the same electropherogram. The DNA yields were normalized for the minor sampling differences by taking the yield and dividing by the mass sampled. The t-tests were calculated using Excel 2010 data analysis package.

III. RESULTS

Phase I Quantification Results

Results from Phase 1 indicate that the quantity of DNA obtained from different skeletal elements of the same individual is highly variable (Table 2). Additionally the same element from different individuals generally displayed significant variation. The average yield by sample type, as determined by Quantifiler, ranged from ~6 ng of DNA per gram of bone *sd*= 4.3 for the ulna up, to ~448 ng of DNA per gram of bone *sd*= 52.6 for the 1st distal hand phalanx (Table 2, Figure 4). The femur and tibia had relatively low yields of DNA when compared to other samples, but their results were consistent with a recent study by Amory et al., where femora from a larger PMI and different environmental conditions yielded from 0.25 – 50 ng of DNA per gram of bone (2012). Some of the highest yields were obtained from foot bones and tooth samples as expected from previous studies. Consistent with previous studies the lowest yielding samples were the radius and ulna.

The average yield per mass of sample for the 28 foot bones that do not show inhibition in the Quantification results is 184.1 ng of DNA per gram of bone *sd*=199.8 and the long bones have a yield of 26.3 ng of DNA per gram of bone *sd*= 30.1.

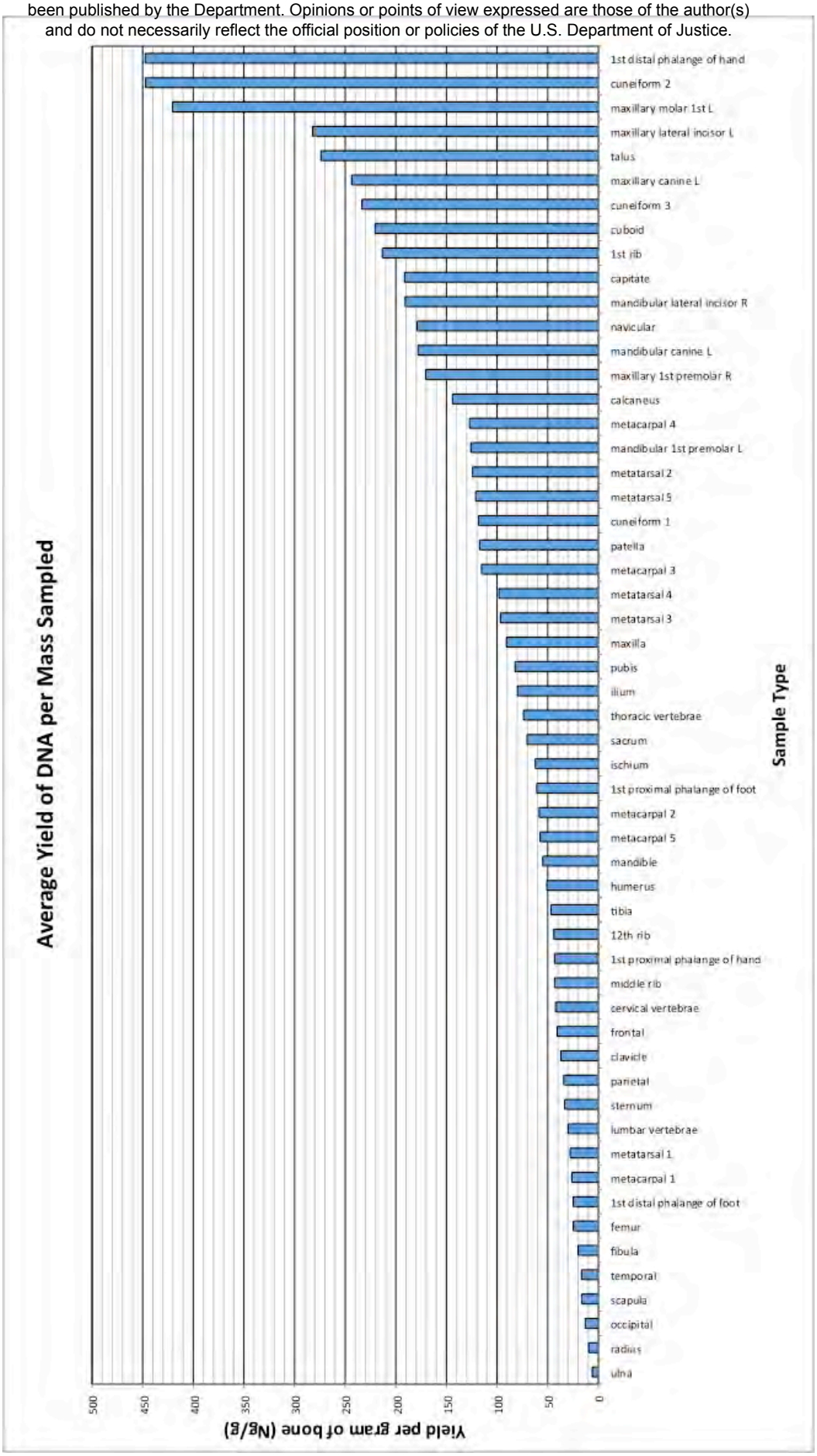

Figure 4. Average DNA quantification values per mass of sample by bone sample type including all samples from Phase I.

Table 2. Quantity of DNA per gram of bone by sample type for all samples tested in Phase 1.

				07-09D				116-09D				45-09D			
Overall Ranking	BONE TYPE	Average Yield by Bone	St.Dev	Mass Sampled	Quant Qf (Ng/ul)	Yield / Mass Sampled	07-09D Ranking	Mass Sampled	Quant Qf (Ng/ul)	Yield / Mass Sampled	116-09D Ranking	Mass Sampled	Quant Qf (Ng/ul)	Yield / Mass Sampled	45-09D Ranking
1	1st distal phalange of hand	448	53	0.20	1.91	478	5	0.19	1.47	387	6	0.23	2.20	478	2
2	cuneiform 2	447	463	0.21	0.00	1	52	0.25	4.63	926	1	0.24	1.99	415	3
3	maxillary molar 1st L	420	535	0.22	4.56	1036	1	0.21	0.64	152	14	0.23	0.34	73	20
4	maxillary lateral incisor L	282	319	0.25	3.25	650	2	0.22	0.40	92	26	0.25	0.52	104	14
5	talus	274	182	0.36	0.52	72	16	0.21	1.78	424	4	0.36	2.35	326	4
6	maxillary canine L	244	327	0.21	2.61	621	3	0.22	0.29	66	32	0.30	0.26	43	30
7	cuneiform 3	233	374	0.24	0.17	36	28	0.21	2.79	664	2	0.23	0.00	0	54
8	cuboid	221	205	0.18	0.00	0	54	0.24	1.94	404	5	0.27	1.39	257	6
9	1st rib	213	236	0.17	0.07	22	35	0.20	1.91	478	3	0.20	0.56	141	11
10	capitate	191	19	0.22	0.79	179	9	0.21	0.76	182	12	0.21	0.90	213	7
11	mandibular lateral incisor R	191	303	0.21	2.27	540	4	0.21	0.07	17	52	0.23	0.07	14	42
12	navicular	179	273	0.20	0.00	0	53	0.23	0.20	44	39	0.23	2.27	493	1
13	mandibular canine L	178	217	0.25	2.14	428	6	0.21	0.29	70	29	0.22	0.16	37	31
14	maxillary 1st premolar R	170	217	0.23	1.93	420	7	0.21	0.29	70	30	0.23	0.10	22	37
15	calcaneus	144	131	0.23	0.36	78	15	0.20	1.18	295	7	0.19	0.22	59	22
16	metacarpal 4	127	67	0.20	0.29	72	17	0.26	1.05	202	10	0.21	0.46	108	13
17	mandibular 1st premolar L	126	119	0.22	1.13	257	8	0.20	0.39	98	24	0.23	0.11	23	36
18	metatarsal 2	124	105	0.21	0.03	8	46	0.21	0.89	212	9	0.28	0.86	153	10
19	metatarsal 5	121	105	0.20	0.02	6	48	0.20	0.59	146	16	0.22	0.93	211	8
20	cuneiform 1	118	142	0.20	0.09	23	33	0.23	0.23	51	34	0.24	1.35	281	5
21	patella	117	10	0.24	0.56	116	11	0.25	0.54	108	20	0.25	0.64	128	12
22	metacarpal 3	115	123	0.20	0.22	55	20	0.21	1.08	257	8	0.22	0.15	35	32
23	metatarsal 4	98	59	0.21	0.16	38	27	0.20	0.62	156	13	0.21	0.42	101	16
24	metatarsal 3	97	73	0.20	0.07	17	39	0.21	0.47	112	18	0.21	0.68	161	9
25	maxilla	91	96	0.20	0.01	2	51	0.22	0.85	193	11	0.20	0.31	77	19
26	pubis	82	28	0.18	0.33	92	13	0.20	0.42	105	22	0.18	0.18	50	25
27	ilium	80	40	0.26	0.27	52	23	0.20	0.50	125	17	0.27	0.34	62	21
28	thoracic vertebrae	74	48	0.20	0.47	118	10	0.21	0.10	23	49	0.25	0.40	81	18
29	sacrum	70	37	0.20	0.31	79	14	0.29	0.59	102	23	0.22	0.13	30	33
30	ischium	62	7	0.31	0.39	63	18	0.23	0.32	68	31	0.25	0.28	55	23
31	1st proximal phalange of foot	61	39	0.20	0.10	25	32	0.22	0.24	55	33	0.30	0.62	103	15
32	metacarpal 2	58	40	0.20	0.41	103	12	0.28	0.26	46	37	0.23	0.12	26	35
33	metacarpal 5	57	51	0.22	0.05	11	43	0.21	0.47	112	19	0.22	0.22	49	26
34	mandible	55	83	0.18	0.04	11	44	0.24	0.72	151	15	0.25	0.02	3	51
35	humerus	51	45	0.26	0.28	54	21	0.22	0.41	94	25	0.19	0.02	5	49
36	tibia	47	51	0.24	0.08	17	40	0.22	0.47	106	21	0.21	0.07	18	40
37	12th rib	44	33	0.20	0.09	22	34	0.22	0.13	28	48	0.20	0.33	82	17
38	1st proximal phalange of hand	43	31	0.20	0.18	46	26	0.21	0.31	73	27	0.20	0.05	11	44
39	middle rib	43	12	0.22	0.22	49	25	0.21	0.12	29	47	0.20	0.21	52	24
40	cervical vertebrae	42	32	0.21	0.21	50	24	0.20	0.28	70	28	0.22	0.03	7	46
41	frontal	41	17	0.22	0.23	53	22	0.23	0.10	21	51	0.25	0.24	48	27
42	clavicle	37	14	0.20	0.08	21	36	0.26	0.24	46	38	0.18	0.16	44	29
43	parietal	34	26	0.22	0.26	58	19	0.21	0.16	38	42	0.23	0.03	6	47
44	sternum	33	1	0.26	0.17	32	30	0.24	0.16	33	45				
45	lumbar vertebrae	30	16	0.21	0.12	29	31	0.26	0.24	47	35	0.21	0.06	15	41
46	metatarsal 1	28	14	0.18	0.12	34	29	0.20	0.15	38	41	0.21	0.05	11	43
47	metacarpal 1	26	21	0.20	0.02	5	49	0.24	0.22	46	36	0.23	0.13	27	34
48	1st distal phalange of foot	25	23	0.21	0.00	0	55	0.21	0.13	30	46	0.20	0.18	45	28
49	femur	25	10	0.25	0.09	19	38	0.24	0.17	36	43	0.23	0.09	19	39
50	fibula	20	20	0.30	0.05	8	45	0.26	0.22	43	40	0.21	0.04	9	45
51	temporal	17	17	0.21	0.05	12	42	0.21	0.15	36	44	0.24	0.01	3	52
52	scapula	17	8	0.25	0.04	7	47	0.28	0.12	22	50	0.22	0.09	21	38
53	occipital	13	10	0.24	0.09	20	37	0.26	0.09	17	53	0.21	0.01	2	53
54	radius	10	3	0.21	0.05	12	41	0.20	0.04	11	55	0.22	0.03	6	48
55	ulna	6	4	0.24	0.02	3	50	0.22	0.05	11	54	0.21	0.02	4	50

A few samples, such as the 1st distal hand phalanx, capitate, and patella gave relatively consistent yields with a standard deviation of approximately 10% between individuals (Table 2). Most samples had significant differences in yield between the three skeletons. Tooth samples were highly variable between individuals with the left 1st molar having 1036, 152, and 73 ng per gram of tooth for an average of 435 ng per gram of bone and a standard deviation of 535 (Figure 5).

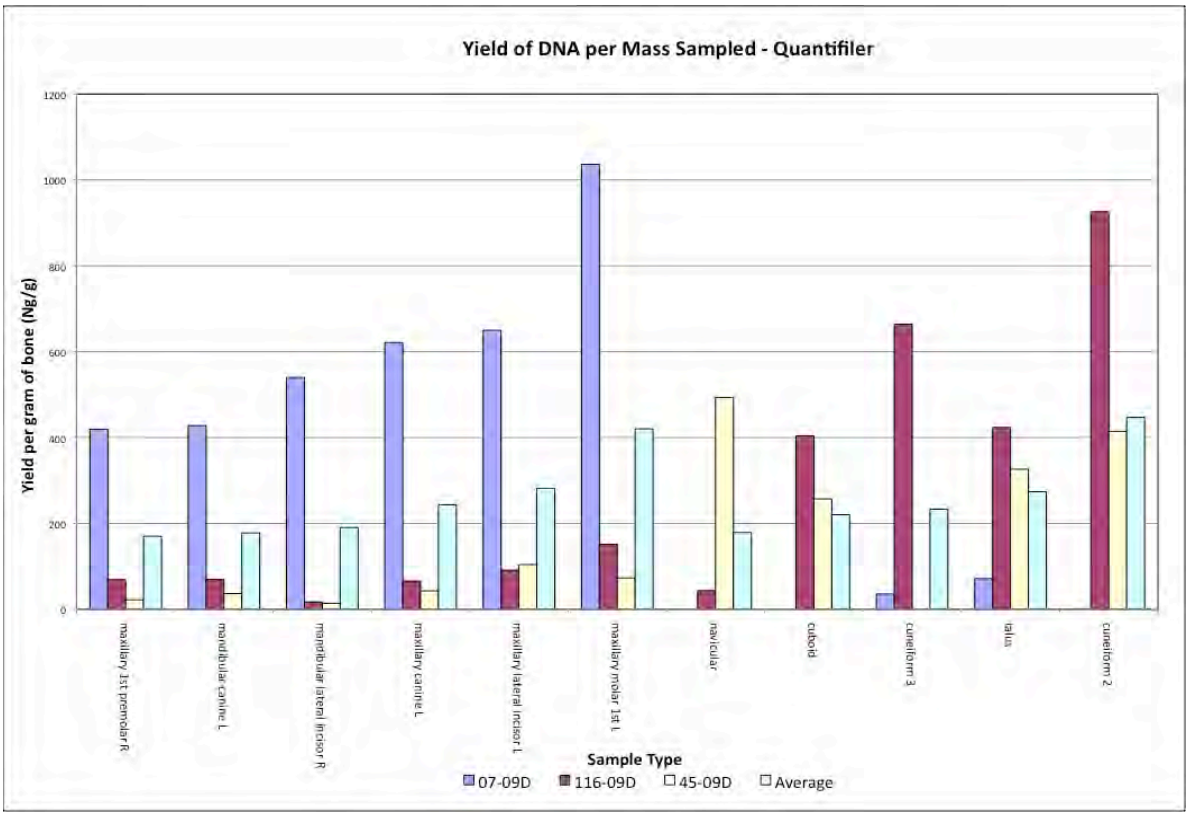

Figure 5: Comparison of yield from a sub set of skeletal samples exhibiting high variability between individuals.

Detection of PCR inhibition in the Quantifiler Results

The average cycle threshold for the internal positive control (IPC) was 27.4 for the controls. There were 29 samples that had an IPC ct value of more than 30 cycles with 12 having a ct of 30-32.99, 7 having a ct of 33-38.08 and 10 having a ct of more than 40 cycles (Table 3). The 10 most inhibited samples included 6 from skeleton 07-09D, 3 from skeleton 116-09D, and 1 from skeleton 45-09D with 9 of those samples being from foot bones. The presence of inhibition in the quantification results indicates that the quantity of DNA is likely underestimated. In nearly every case the DNA extracts that showed inhibition at the quantification stage had the lower yield than extracts from the same bone type from a different individual. The DNA extract from the 2nd cuneiform of skeleton 07-09D showed a lot of PCR inhibition (IPC > 40 cycles) and showed only

~1 ng per gram of bone while the other two skeletons had 926 and 425 ng per gram of bone.

Table 3. Quantification details for samples exhibiting inhibition in the Internal Positive Control of the quantification reaction.

Sample	Quant_Qf	IPC Ct_Qf	Individual	Bone
UTK1101-0096	0.0136	30.12	45-09D	Temporal
UTK1101-0020	0.0156	30.15	07-09D	Ulna
UTK1101-0027	1.91	30.19	07-09D	1st distal phalange of hand
UTK1101-0114	0.594	30.19	116-09D	Sacrum
UTK1101-0007	0.165	30.49	07-09D	Sternum
UTK1101-0061	0.134	30.5	45-09D	Sacrum
UTK1101-0023	0.218	31.46	07-09D	Metacarpal 3
UTK1101-0012	0.39	31.5	07-09D	Ischium
UTK1101-0057	0.563	32.27	45-09D	1st Rib
UTK1101-0064	0.337	32.29	45-09D	Ilium
UTK1101-0033	0.0994	32.52	07-09D	1st proximal phalange of foot
UTK1101-0010	0.0372	32.94	07-09D	Scapula
UTK1101-0035	0.36	33.61	07-09D	Calcaneous
UTK1101-0099	0.309	33.79	45-09D	Maxilla
UTK1101-0039	0.0908	34.95	07-09D	Cuneiform 1
UTK1101-0140	0.125	35.84	116-09D	1st distal phalange of foot
UTK1101-0054	0.0318	36.2	45-09D	Cervical Vertebrae
UTK1101-0024	0.286	37.54	07-09D	Metacarpal 4
UTK1101-0147	2.79	38.08	116-09D	Cuneiform 3
UTK1101-0034	0	Undetermined	07-09D	1st distal phalange of foot
UTK1101-0037	0	Undetermined	07-09D	Navicular
UTK1101-0038	0	Undetermined	07-09D	Cuboid
UTK1101-0040	0.00374	Undetermined	07-09D	Cuneiform 2
UTK1101-0041	0.173	Undetermined	07-09D	Cuneiform 3
UTK1101-0046	0.00998	Undetermined	07-09D	Maxilla
UTK1101-0094	0	Undetermined	45-09D	Cuneiform 3
UTK1101-0143	0.201	Undetermined	116-09D	Navicular
UTK1101-0144	1.94	Undetermined	116-09D	Cuboid
UTK1101-0145	0.234	Undetermined	116-09D	Cuneiform 1

Phase I STR results

Of the 164 Phase 1 samples, 148 (90%) gave at least 10 STR loci and 135 (82%) were typed at all 16 loci. Analyzing the different bones by element type revealed 27 sets where all 3 gave full STR profiles, 17 sets where 2 of the 3 skeletons yielded a full profile, 5 sets where 1 of the 3 skeletons yielded a full profile, 5 sets where none yielded full profiles and 1 set where 1 generated a full profile and the other did not. Only

1 sample did not generate a single callable allele and that was a radius bone from skeleton 116-09D.

In most cases the STR results were as would be expected for the quantity of DNA in the sample. The samples that were typed with the fewest STR loci were generally the ones that had the lowest quantities of DNA (Table 4). The 1st distal hand phalanx had relatively high yields from all 3 individuals and gave full 16 loci profiles from all 3 skeletons (Figure 6). DNA extracts from the femur had on average 24 times less DNA and only one gave a full STR profile (Figure 7).

There was little correlation, however, between the quantity of DNA and the STR results for samples showing inhibition in the quantification results. For example, the 2nd cuneiform from 07-09D had ~1 ng of DNA per gram of bone but when ~0.035 ng of DNA was amplified (Figure 8) the STR results averaged more than 1000 RFU per allele suggesting that the quantification results were underestimated by at least 100 times. From the ten samples that had complete inhibition of the IPC, in the quantifiler reactions, nine gave full 16 Locus STR profiles and 1 gave 15 loci. Most of the extracts showing inhibition in the quantification reaction had quite low quantities of DNA however the STR amplification results suggested that those samples all had a lot of DNA. For example the DNA extract of the cuboid bone from 07-09D showed a quantity of 0 ng/μl however amplification of 3 μl of extract gave a strong profile that is indicative of at least 3 ng of DNA (Figure 9).

Table 4. Phase 1 bone samples yielding less than full profiles.

Sample Name	Skeleton	Sample Type	Number of Loci with Data
UTK1101-0125	116-09D	radius	0
UTK1101-0098	45-09D	occipital	1
UTK1101-0020	07-09D	ulna	2
UTK1101-0096	45-09D	temporal	3
UTK1101-0018	07-09D	humerus	4
UTK1101-0019	07-09D	radius	4
UTK1101-0071	45-09D	humerus	4
UTK1101-0124	116-09D	humerus	4
UTK1101-0126	116-09D	ulna	4
UTK1101-0032	07-09D	metatarsal 5	6
UTK1101-0073	45-09D	ulna	6
UTK1101-0043	07-09D	temporal	7
UTK1101-0097	45-09D	parietal	7
UTK1101-0122	116-09D	fibula	8
UTK1101-0025	07-09D	metacarpal 5	9
UTK1101-0151	116-09D	occipital	9
UTK1101-0016	07-09D	fibula	10
UTK1101-0148	116-09D	frontal	10
UTK1101-0072	45-09D	radius	11
UTK1101-0021	07-09D	metacarpal 1	12
UTK1101-0069	45-09D	fibula	12
UTK1101-0045	07-09D	occipital	12
UTK1101-0100	45-09D	mandible	13
UTK1101-0007	07-09D	sternum	13
UTK1101-0116	116-09D	scapula	13
UTK1101-0015	07-09D	tibia	14
UTK1101-0081	45-09D	metatarsal 1	14
UTK1101-0103	45-09D	maxillary 1st premolar L	14
UTK1101-0150	116-09D	parietal	14
UTK1101-0004	07-09D	1st rib	14
UTK1101-0033	07-09D	1st proximal phalange of foot	14
UTK1101-0068	45-09D	tibia	14
UTK1101-0102	45-09D	maxillary canine L	14
UTK1101-0120	116-09D	femur	14
UTK1101-0034	07-09D	1st distal phalange of foot	15
UTK1101-0047	07-09D	mandible	15
UTK1101-0161	116-09D	mandibular lateral incisor R	15
UTK1101-0030	07-09D	metatarsal 3	15
UTK1101-0046	07-09D	maxilla	15
UTK1101-0067	45-09D	femur	15
UTK1101-0075	45-09D	metacarpal 2	15
UTK1101-0076	45-09D	metacarpal 3	15
UTK1101-0091	45-09D	cuboid	15
UTK1101-0121	116-09D	tibia	15
UTK1101-0160	116-09D	mandibular lateral incisor R	15

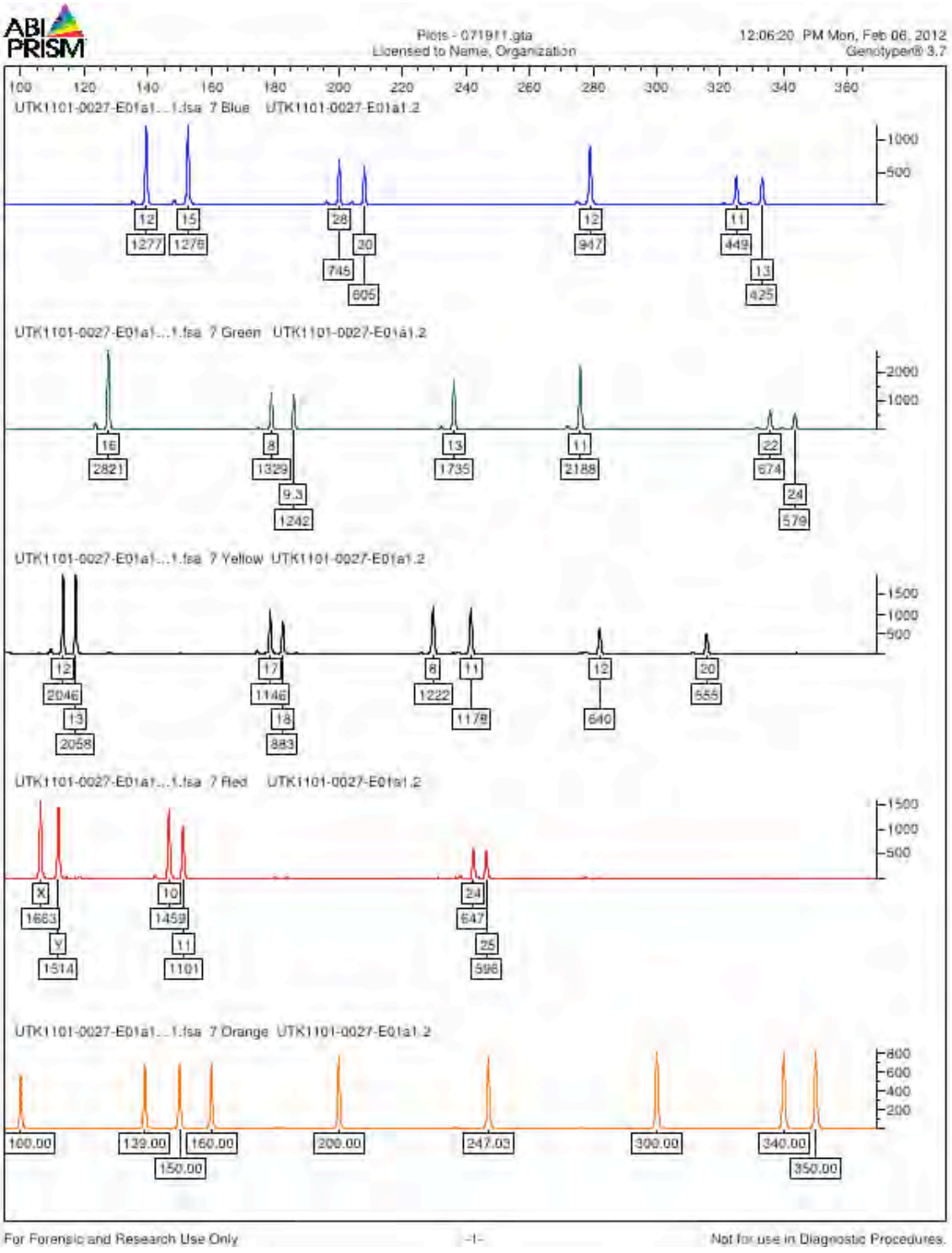

Figure 6. STR results for the 1st distal hand phalanx from skeleton 07-09D where 1.9 ng of DNA was amplified with the Identifiler system at 28 amplification cycles.

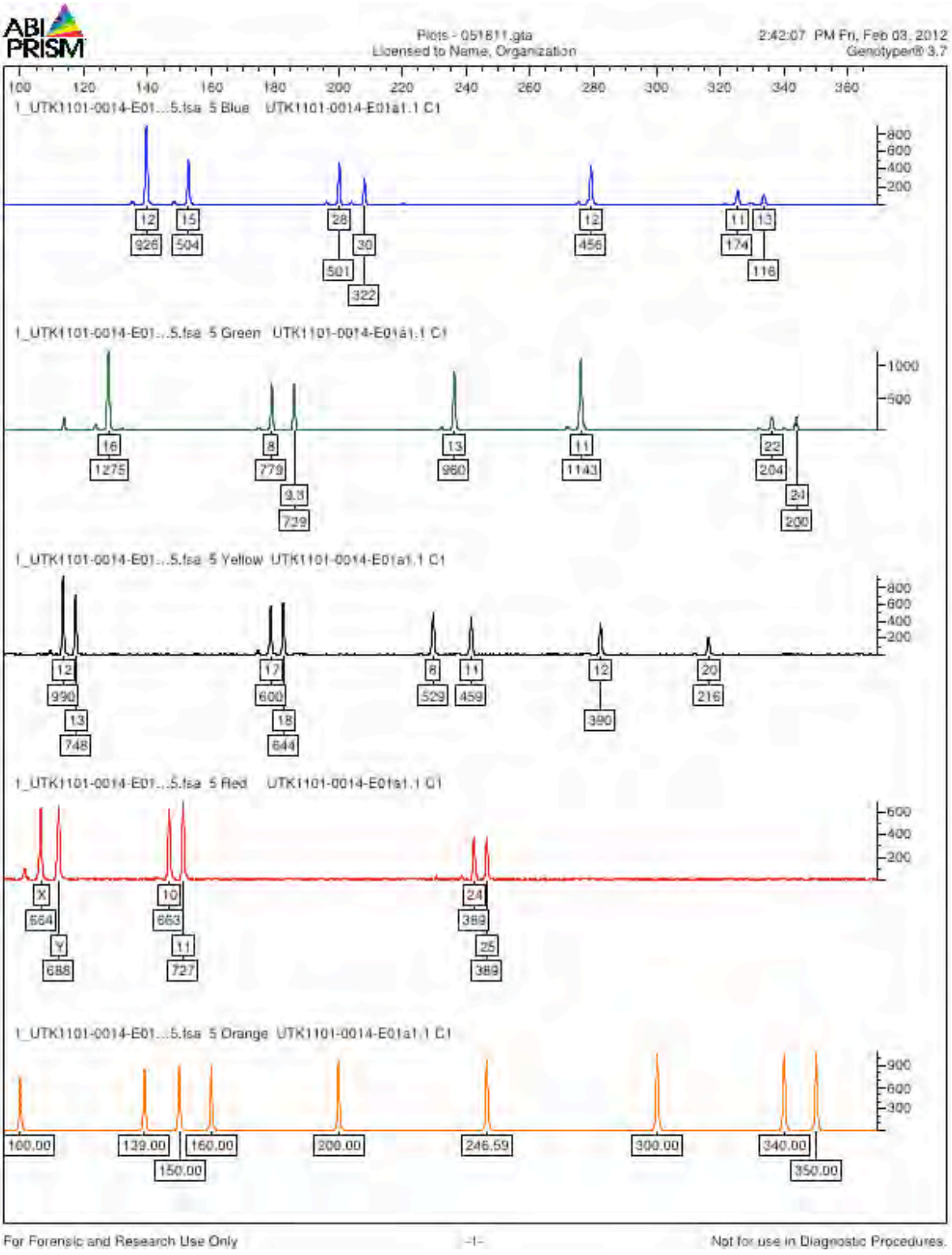

Figure 7. STR results for the femur from skeleton 07-09D where 0.9 ng of DNA was amplified with the Identifiler system at 28 amplification cycles.

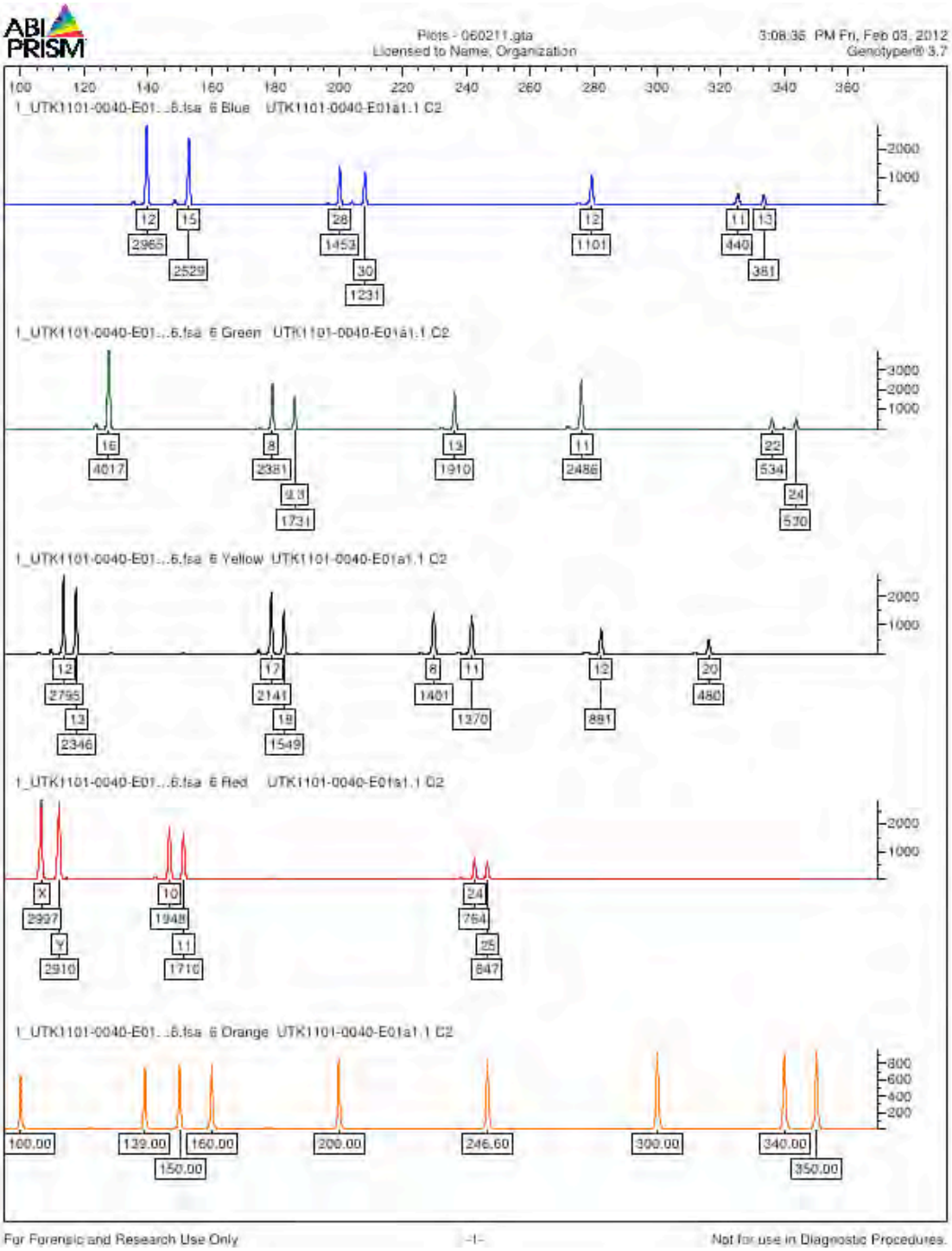

Figure 8. STR results from Phase 1 for the 2nd cuneiform from skeleton 07-09D where 0.04 ng of DNA was amplified with the Identifiler system at 28 amplification cycles. The quantification results did indicate the presence of PCR inhibitors.

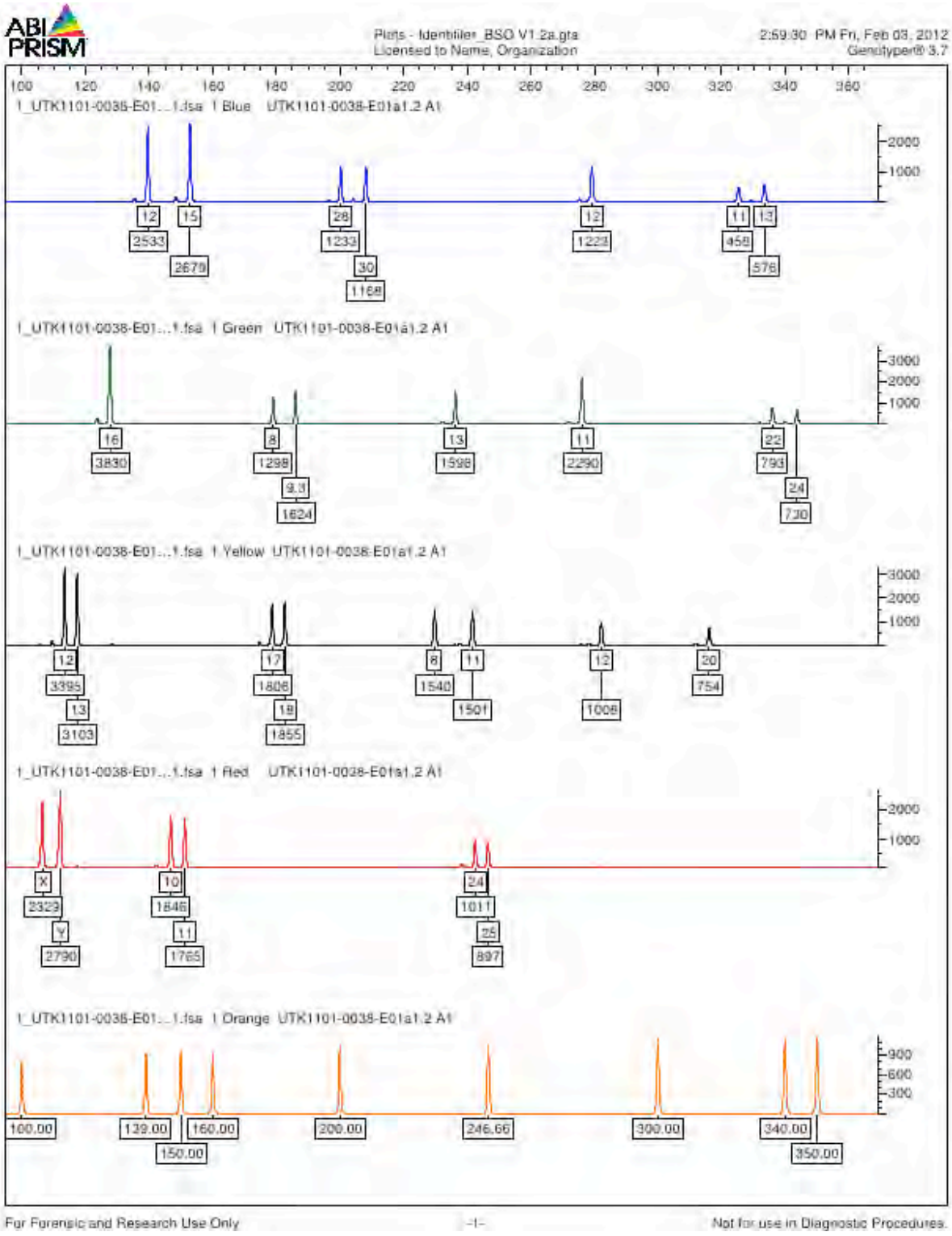

Figure 9. STR results from Phase 1 for the cuboid from skeleton 07-09D where 0.00 ng of DNA was amplified with the Identifiler system at 28 amplification cycles. The quantification results did indicate the presence of PCR inhibitors.

Phase II Quantification Results

The quantification results from Phase 2 were quite variable, possibly due to the increased number of individuals being tested. Overall the 1st distal hand phalanx gave relatively high yields of DNA while the tibia and femur had the lowest yields (Tables 5 and 6 and Figure 10). The 1st distal hand phalanx had the highest yields of DNA for six different individuals at three different PMI ranges. The 4th metacarpal was the highest yielding sample in three individuals and in two individuals the talus was the highest yielding sample. One individual had the 1st cuneiform as the highest yielding sample. Elements predominantly comprised of cortical bone were generally the lowest yielding, with the femur having the lowest yields from three different individuals at three different PMI ranges, while the tibia had the lowest yields for four different individuals at three different PMI ranges.

Overall, the samples generally maintained the rank order from Phase 1 as the PMI increased, although a few bones did not always conform to this pattern. The talus, for example, at 0-3 years PMI had 50% more DNA than the 1st distal hand phalanx. The 1st cuneiform at the 0-3 years PMI had the lowest average yield likely due to all 3 DNA extracts having high levels of inhibition in the quantification reactions. The 4-10 year PMI group generally followed the Phase 1 results for quantity of DNA recovered while the 10-20 year PMI group had significantly higher yields than the shorter PMI ranges for most samples. The tibia's ranking increased to 4th in the 10-20 year PMI and to 3rd in the 20-30 PMI. The increase in the ranking of the tibia in the 10-20 year PMI was largely due to the sample from 21-99D which yielded ~50x more DNA than the tibias from the other two skeletons at the same PMI range. The increase in the ranking of the tibiae in the 20-30 year PMI was largely due to the sample from 22-91D which yielded ~40x

more DNA than the tibiae from the other two skeletons in that PMI. The 1st cuneiform from 21-98D in 10-20 year PMI range had an unusually high DNA yield at ~20 times higher than it had in the other PMIs. Two of the 1st distal hand phalanges from the 20-30 year PMI also had unusually high DNA yields at ~20 times higher than at the other PMIs.

Table 5. Quantity of DNA by Skeleton for 0-3 and 4-10 years PMI.

SAMPLE	Skeleton	BONE TYPE	PMI	Mass Sampled (g)	DNA Quantity (Ng/ul)	IPC Ct	Ng DNA/ g bone	Ranking
UTK1102-0207	UT25-08D	talus	0-3 years PMI	0.28	2.67	28.34	477	1
UTK1102-0201	UT25-08D	middle rib		0.22	1.44	27.52	327	2
UTK1102-0204	UT25-08D	metacarpal 4		0.21	0.99	27.51	237	3
UTK1102-0206	UT25-08D	metatarsal 4		0.28	0.99	32.28	177	4
UTK1102-0205	UT25-08D	1st distal hand phalanx		0.2	0.48	>40	121	5
UTK1102-0203	UT25-08D	patella		0.22	0.45	28.52	103	6
UTK1102-0200	UT25-08D	cervical vertebrae		0.26	0.33	27.78	64	7
UTK1102-0202	UT25-08D	femur		0.25	0.25	28.13	49	8
UTK1102-0209	UT25-08D	tibia		0.21	0.06	27.77	14	9
UTK1102-0208	UT25-08D	cuneiform 1		0.26	0.00	>40	0	10
UTK1102-0217	UT49-08D	talus		0.21	2.51	27.34	598	1
UTK1102-0215	UT49-08D	1st distal hand phalanx		0.21	1.79	28.57	426	2
UTK1102-0214	UT49-08D	metacarpal 4		0.21	0.92	28.08	218	3
UTK1102-0213	UT49-08D	patella		0.22	0.89	27.74	201	4
UTK1102-0211	UT49-08D	middle rib		0.21	0.80	29.9	190	5
UTK1102-0216	UT49-08D	metatarsal 4		0.25	0.64	28.62	128	6
UTK1102-0210	UT49-08D	cervical vertebrae		0.22	0.46	28.14	105	7
UTK1102-0212	UT49-08D	femur		0.26	0.17	27.35	33	8
UTK1102-0219	UT49-08D	tibia		0.2	0.05	27.86	13	9
UTK1102-0218	UT49-08D	cuneiform 1		0.25	0.00	>40	0	10
UTK1102-0224	UT100-08D	metacarpal 4		0.2	0.74	38.5	185	1
UTK1102-0225	UT100-08D	1st distal hand phalanx		0.2	0.46	34.99	115	2
UTK1102-0221	UT100-08D	middle rib		0.2	0.16	28.62	41	3
UTK1102-0227	UT100-08D	talus		0.22	0.15	>40	33	4
UTK1102-0222	UT100-08D	femur		0.21	0.10	27.55	24	5
UTK1102-0229	UT100-08D	tibia		0.24	0.06	28.04	13	6
UTK1102-0220	UT100-08D	cervical vertebrae		0.22	0.01	>40	3	7
UTK1102-0223	UT100-08D	patella		0.2	0.00	>40	0	8
UTK1102-0226	UT100-08D	metatarsal 4		0.23	0.00	>40	0	9
UTK1102-0228	UT100-08D	cuneiform 1		0.24	0.00	>40	0	10
UTK1102-0305	UT20-05D	1st distal hand phalanx	4-10 years PMI	0.19	0.73	28.49	193	1
UTK1102-0308	UT20-05D	cuneiform 1		0.22	0.74	29.61	167	2
UTK1102-0306	UT20-05D	metatarsal 4		0.2	0.56	28.03	141	3
UTK1102-0304	UT20-05D	metacarpal 4		0.22	0.55	28.85	125	4
UTK1102-0303	UT20-05D	patella		0.21	0.47	28.12	111	5
UTK1102-0307	UT20-05D	talus		0.25	0.19	28.6	38	6
UTK1102-0300	UT20-05D	cervical vertebrae		0.2	0.13	28.31	32	7
UTK1102-0302	UT20-05D	femur		0.23	0.04	27.89	9	8
UTK1102-0301	UT20-05D	middle rib		0.2	0.03	28.54	8	9
UTK1102-0309	UT20-05D	tibia		0.22	0.02	27.95	4	10
UTK1102-0315	UT42-05D	1st distal hand phalanx		0.2	2.01	29.71	503	1
UTK1102-0317	UT42-05D	talus		0.24	1.30	28.47	271	2
UTK1102-0314	UT42-05D	metacarpal 4		0.21	0.48	28.5	114	3
UTK1102-0318	UT42-05D	cuneiform 1		0.21	0.28	28.52	66	4
UTK1102-0316	UT42-05D	metatarsal 4		0.25	0.32	28.2	64	5
UTK1102-0311	UT42-05D	middle rib		0.2	0.12	27.79	30	6
UTK1102-0310	UT42-05D	cervical vertebra		0.21	0.05	28.64	13	7
UTK1102-0319	UT42-05D	tibia		0.27	0.06	27.75	10	8
UTK1102-0313	UT42-05D	patella		0.23	0.03	28.6	7	9
UTK1102-0312	UT42-05D	femur		0.23	0.02	27.76	3	10
UTK1102-0324	UT93-05D	metacarpal 4		0.22	0.89	28.7	203	1
UTK1102-0325	UT93-05D	1st distal hand phalanx		0.24	0.96	28.25	200	2
UTK1102-0328	UT93-05D	cuneiform 1		0.21	0.42	29.43	99	3
UTK1102-0326	UT93-05D	metatarsal 4		0.24	0.33	27.73	68	4
UTK1102-0327	UT93-05D	talus		0.26	0.22	28.9	43	5
UTK1102-0320	UT93-05D	cervical vertebra		0.22	0.11	29.02	25	6
UTK1102-0323	UT93-05D	patella		0.25	0.13	27.9	25	7
UTK1102-0322	UT93-05D	femur		0.24	0.05	28.08	11	8
UTK1102-0321	UT93-05D	middle rib		0.22	0.05	28.22	10	9
UTK1102-0329	UT93-05D	tibia		0.23	0.03	27.88	5	10

Table 6. Quantity of DNA by Skeleton for 11-20 and 20+ years PMI.

SAMPLE	Skeleton	BONE TYPE		Mass Sampled (g)	DNA Quantity (Ng/ul)	IPC Ct	Ng DNA/ g bone	Ranking
UTK1102-0405	UT09-97D	1st distal hand phalanx	11-20 years PMI	0.19	2.32	28.13	611	1
UTK1102-0406	UT09-97D	metatarsal 4		0.2	1.20	28.57	300	2
UTK1102-0408	UT09-97D	cuneiform 1		0.22	1.30	28.63	295	3
UTK1102-0404	UT09-97D	metacarpal 4		0.2	0.85	28.31	214	4
UTK1102-0401	UT09-97D	middle rib		0.21	0.72	27.73	171	5
UTK1102-0407	UT09-97D	talus		0.21	0.61	28.56	146	6
UTK1102-0403	UT09-97D	patella		0.22	0.26	27.81	60	7
UTK1102-0409	UT09-97D	tibia		0.23	0.19	27.7	41	8
UTK1102-0400	UT09-97D	cervical vertebra		0.23	0.11	27.92	25	9
UTK1102-0402	UT09-97D	femur		0.2	0.09	27.8	24	10
UTK1102-0414	UT05-98D	metacarpal 4		0.22	1.70	28.1	386	1
UTK1102-0415	UT05-98D	1st distal hand phalanx		0.19	0.81	27.6	212	2
UTK1102-0416	UT05-98D	metatarsal 4		0.21	0.70	27.35	167	3
UTK1102-0418	UT05-98D	cuneiform 1		0.22	0.59	27.66	135	4
UTK1102-0417	UT05-98D	talus		0.22	0.43	27.27	97	5
UTK1102-0410	UT05-98D	cervical vertebra		0.23	0.32	28.54	70	6
UTK1102-0411	UT05-98D	middle rib		0.22	0.27	27.81	62	7
UTK1102-0413	UT05-98D	patella		0.22	0.22	28.42	49	8
UTK1102-0412	UT05-98D	femur		0.21	0.12	27.98	28	9
UTK1102-0419	UT05-98D	tibia		0.23	0.07	27.1	15	10
UTK1102-0428	UT21-98D	cuneiform 1		0.22	12.05	27.91	2739	1
UTK1102-0429	UT21-98D	tibia		0.22	3.41	27.46	775	2
UTK1102-0424	UT21-98D	metacarpal 4		0.2	1.65	27.12	413	3
UTK1102-0427	UT21-98D	talus		0.25	2.00	27.2	400	4
UTK1102-0425	UT21-98D	1st distal hand phalanx		0.2	0.64	27.14	160	5
UTK1102-0426	UT21-98D	metatarsal 4		0.25	0.72	27.25	143	6
UTK1102-0422	UT21-98D	femur		0.22	0.52	27.26	118	7
UTK1102-0420	UT21-98D	cervical vertebra		0.22	0.14	27.28	31	8
UTK1102-0421	UT21-98D	middle rib		0.22	0.14	27.24	31	9
UTK1102-0423	UT21-98D	patella		0.24	0.08	27.21	16	10
UTK1102-0505	UT03-90D	1st distal hand phalanx	20+ years PMI	0.24	20.26	29.63	4221	1
UTK1102-0507	UT03-90D	talus		0.22	0.63	28.24	142	2
UTK1102-0504	UT03-90D	metacarpal 4		0.2	0.44	27.87	110	3
UTK1102-0508	UT03-90D	cuneiform 1		0.23	0.41	28.03	90	4
UTK1102-0500	UT03-90D	cervical vertebra		0.24	0.24	27.95	50	5
UTK1102-0506	UT03-90D	metatarsal 4		0.25	0.16	28.41	32	6
UTK1102-0502	UT03-90D	femur		0.25	0.07	27.27	15	7
UTK1102-0501	UT03-90D	middle rib		0.24	0.06	27.27	13	8
UTK1102-0503	UT03-90D	patella		0.23	0.04	28.15	9	9
UTK1102-0509	UT03-90D	tibia		0.23	0.02	27.45	5	10
UTK1102-0515	UT12-90D	1st distal hand phalanx		0.23	6.61	27.55	1437	1
UTK1102-0516	UT12-90D	metatarsal 4		0.26	0.70	27.4	134	2
UTK1102-0518	UT12-90D	cuneiform 1		0.24	0.22	28.17	46	3
UTK1102-0517	UT12-90D	talus		0.23	0.12	28.37	25	4
UTK1102-0513	UT12-90D	patella		0.23	0.04	27.81	10	5
UTK1102-0514	UT12-90D	metacarpal 4		0.21	0.04	28.27	10	6
UTK1102-0519	UT12-90D	tibia		0.24	0.04	27.28	8	7
UTK1102-0512	UT12-90D	femur		0.24	0.03	27.23	7	8
UTK1102-0511	UT12-90D	middle rib		0.2	0.03	27.3	7	9
UTK1102-0510	UT12-90D	cervical vertebra		0.24	0.01	28.24	3	10
UTK1102-0525	UT22-91D	1st distal hand phalanx		0.2	27.22	28.28	6805	1
UTK1102-0529	UT22-91D	tibia		0.25	1.59	27.14	318	2
UTK1102-0528	UT22-91D	cuneiform 1		0.22	1.08	27.19	245	3
UTK1102-0524	UT22-91D	metacarpal 4		0.23	0.72	27.4	157	4
UTK1102-0520	UT22-91D	cervical vertebra		0.23	0.51	27.28	110	5
UTK1102-0526	UT22-91D	metatarsal 4		0.22	0.27	27.2	60	6
UTK1102-0527	UT22-91D	talus		0.21	0.20	27.89	48	7
UTK1102-0523	UT22-91D	patella		0.24	0.14	27.21	28	8
UTK1102-0521	UT22-91D	middle rib		0.23	0.10	27.28	21	9
UTK1102-0522	UT22-91D	femur		0.22	0.03	27.22	7	10

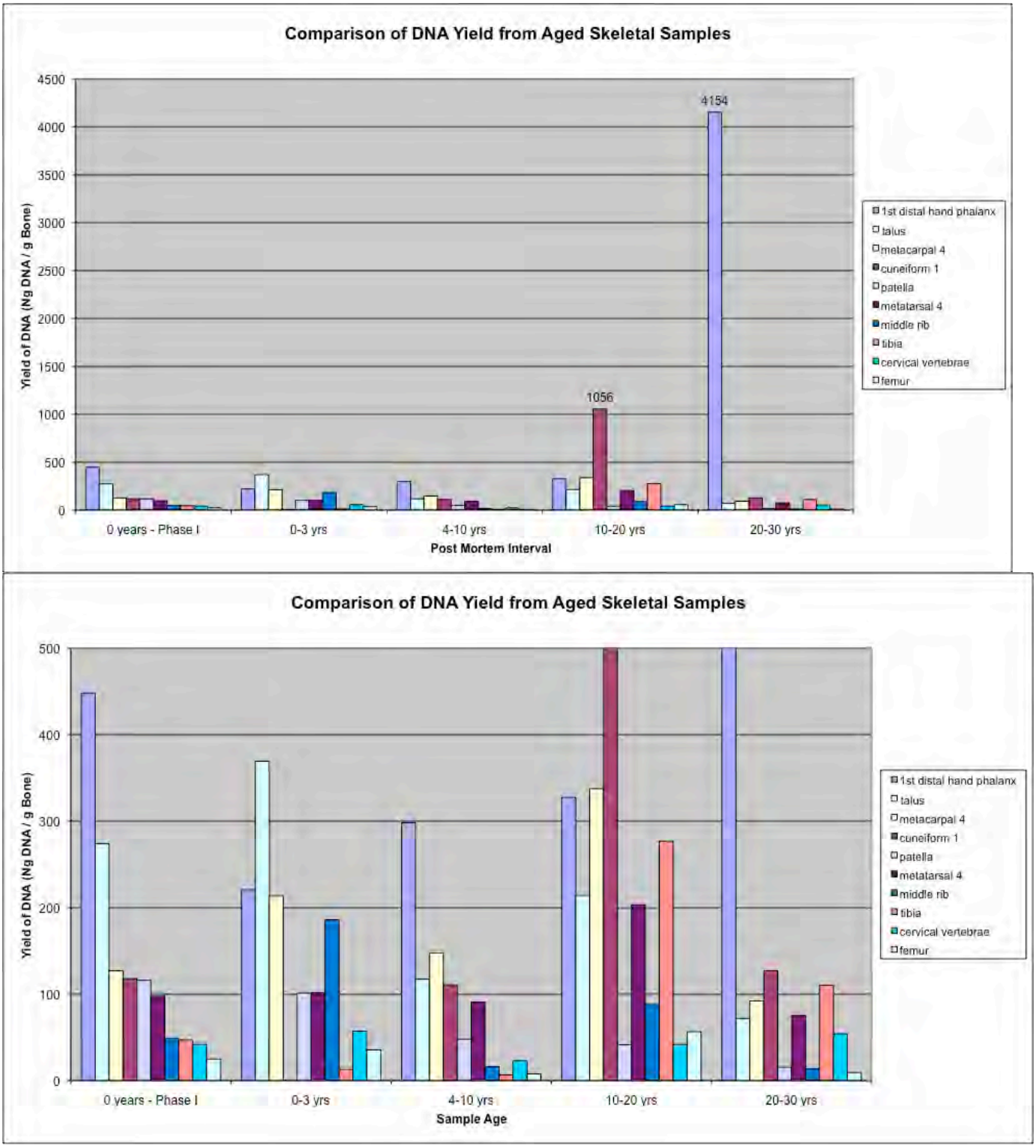

Figure 10. Yield of DNA by sample type grouped by age range. The top graph is scaled to include all data while the bottom graph is identical but scaled down to show the differences in the lower yielding samples.

Phase II STR results

Most elements produced less complete DNA profiles as the post mortem interval increased (Table 7). From the 120 different bones tested during Phase 2, full 16-locus profiles were developed for 56 of them. The full profiles were developed from 23 bones in the 0-3 year PMI, 21 from the 4-10 year PMI, 10 from the 10-20 year PMI, and 2 from the 20+ year PMI. Only two of the full profiles were from cortical long bones, one femur and one tibia, and both were from the shortest PMI, 0-3 years.

Table 7. Comparison of the number of full profiles and average number of loci by sample type at the four different post mortem interval ranges.

	0-3 years		4-10 years		11-20 years		20+ years	
Sample Type	# of full profiles	Avg # Loci	# of full profiles	Avg # Loci	# of full profiles	Avg # Loci	# of full profiles	Avg # Loci
1st distal hand phalanx	2/3	15.7	3/3	16.0	2/3	11.0	1/3	14.0
talus	3/3	16.0	3/3	16.0	1/3	14.0	0/3	9.7
metacarpal 4	3/3	16.0	3/3	16.0	2/3	11.7	0/3	8.3
cuneiform 1	3/3	16.0	3/3	16.0	1/3	15.0	0/3	11.0
patella	3/3	16.0	2/3	14.0	0/3	6.7	0/3	4.0
metatarsal 4	3/3	16.0	3/3	16.0	1/3	14.0	1/3	11.3
middle rib	2/3	14.7	2/3	14.3	2/3	14.0	0/3	7.3
tibia	1/3	14.3	0/3	9.7	0/3	10.7	0/3	3.0
cervical vertebrae	2/3	13.3	2/3	15.7	1/3	14.3	0/3	7.3
femur	1/3	11.7	0/3	9.7	0/3	10.7	0/3	2.0

Generally the signal strengths of the larger loci were reduced more than the shorter loci from the longer PMI (Figure 11). For example, a 1st distal hand phalanx from Phase 1 had approximately 4000 RFU per allele in the shortest loci and around 1300 RFU per allele in the longest loci while the same bone type at the 20+ year PMI had approximately 1000 RFU in the shortest loci and the longest loci were around 40 RFU.

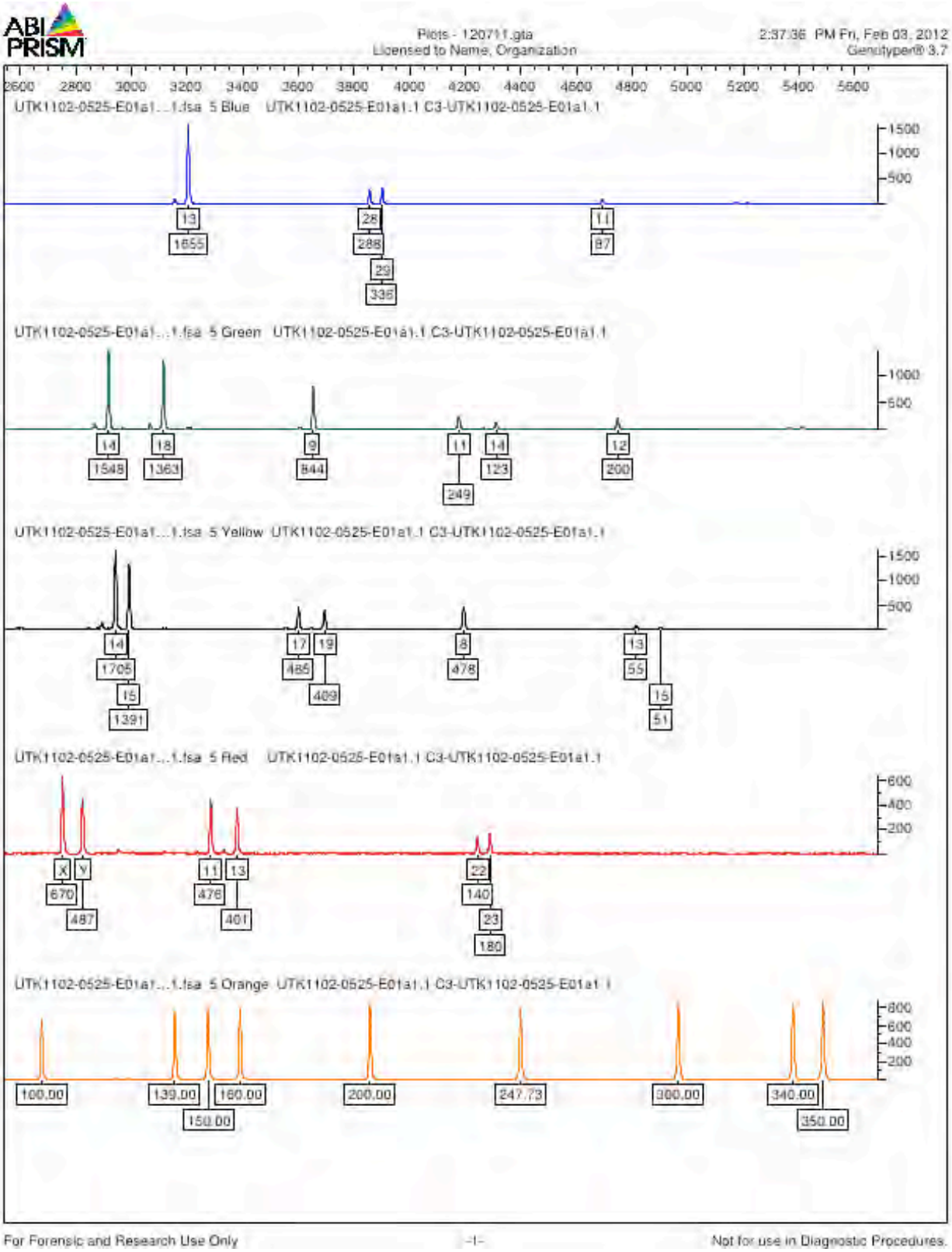

Figure 11. Electropherogram for a 1st distal hand phalanx, at the 20+ year post mortem interval, showing a reduction in signal strength from the larger loci.

Comparing the maximum average signal per locus to the minimum average signal per locus for the full profiles in Phase 2 showed an average ratio of 4.7 for the 0-3 year PMI, 6.1 for the 4-10 year PMI, 7.8 for 10-20 year PMI, and 8.6 for the 20+ year PMI (Table 8). Predictably the max:min ratio increased at the larger PMI as those samples will have increased levels of degradation.

There was no sample that was consistently better than the others in terms of the quality of the STR profile as determined by the max:min ratio. The patella had the lowest max:min ratio of 3.2 in the 0-3 and 3.1 in 4-10 years PMI ranges but in each of those ranges a patella from a different skeleton was significantly higher at 4.3 and 5.3. The 4th metatarsal has the highest max:min ratio in the 4-10, 10-20 and 20+ PMI ranges.

Table 8: Comparison of the maximum average RFU per allele at each locus to the minimum average RFU per allele at each locus by bone type, post mortem interval range and skeleton.

Sample Info	Skeleton	Max:Min Ratio	RFU / Allele	BONE TYPE	Avg Max/Min
UTK1102-0213-E01a1.1	UT49-08D	3.2	698	patella	
UTK1102-0200-E01a1.1	UT25-08D	3.2	856	cervical vertebrae	
UTK1102-0211-E01a1.1	UT49-08D	3.6	865	middle rib	
UTK1102-0216-E01a1.1	UT49-08D	3.6	880	metatarsal 4	
UTK1102-0214-E01a1.1	UT49-08D	3.6	560	metacarpal 4	
UTK1102-0203-E01a1.1	UT25-08D	3.8	805	patella	
UTK1102-0218-E01a1.2	UT49-08D	3.9	2979	cuneiform 1	
UTK1102-0210-E01a1.1	UT49-08D	4.3	615	cervical vertebrae	
UTK1102-0223-E01a1.2	UT100-08D	4.3	295	patella	
UTK1102-0215-E01a1.1	UT49-08D	4.6	886	1st distal hand phalanx	
UTK1102-0201-E01a1.1	UT25-08D	4.7	662	middle rib	
UTK1102-0226-E01a1.2	UT100-08D	4.7	1531	metatarsal 4	4.7
UTK1102-0226-E01a1.1	UT100-08D	4.7	1531	metatarsal 4	
UTK1102-0206-E01a1.1	UT25-08D	4.7	741	metatarsal 4	
UTK1102-0224-E01a1.1	UT100-08D	4.8	628	metacarpal 4	
UTK1102-0228-E01a1.2	UT100-08D	5.0	1258	cuneiform 1	
UTK1102-0217-E01a1.1	UT49-08D	5.2	437	talus	
UTK1102-0204-E01a1.1	UT25-08D	5.3	566	metacarpal 4	
UTK1102-0205-E01a1.1	UT25-08D	5.6	1522	1st distal hand phalanx	
UTK1102-0202-E01a1.1	UT25-08D	5.7	576	femur	
UTK1102-0208-E01a1.2	UT25-08D	5.8	1598	cuneiform 1	
UTK1102-0227-E01a1.2	UT100-08D	5.9	2093	talus	
UTK1102-0229-E02a1.1	UT100-08D	6.6	338	tibia	
UTK1102-0207-E01a1.1	UT25-08D	6.9	512	talus	
UTK1102-0303-E01a1.1	UT20-05D	3.1	2222	patella	
UTK1102-0308-E01a1.1	UT20-05D	4.0	1980	cuneiform 1	
UTK1102-0307-E01a1.1	UT20-05D	4.8	1846	talus	
UTK1102-0318-E01a1.1	UT42-05D	5.0	1427	cuneiform 1	
UTK1102-0328-E01a1.1	UT93-05D	5.1	1739	cuneiform 1	
UTK1102-0304-E01a1.1	UT20-05D	5.2	1362	metacarpal 4	
UTK1102-0327-E01a1.1	UT93-05D	5.2	2023	talus	
UTK1102-0310-E01a1.1	UT42-05D	5.3	341	cervical vertebra	
UTK1102-0323-E01a1.1	UT93-05D	5.3	1216	patella	
UTK1102-0324-E01a1.1	UT93-05D	5.5	1242	metacarpal 4	
UTK1102-0321-E01a1.1	UT93-05D	5.5	600	middle rib	6.1
UTK1102-0315-E01a1.1	UT42-05D	5.5	1818	1st distal hand phalanx	
UTK1102-0314-E01a1.1	UT42-05D	5.8	1259	metacarpal 4	
UTK1102-0306-E01a1.1	UT20-05D	5.9	2002	metatarsal 4	
UTK1102-0305-E01a1.1	UT20-05D	6.2	1630	1st distal hand phalanx	
UTK1102-0326-E01a1.1	UT93-05D	6.7	1282	metatarsal 4	
UTK1102-0311-E01a1.1	UT42-05D	6.7	692	middle rib	
UTK1102-0317-E01a1.1	UT42-05D	8.6	1327	talus	
UTK1102-0300-E01a1.1	UT20-05D	8.6	708	cervical vertebrae	
UTK1102-0325-E01a1.1	UT93-05D	9.1	1268	1st distal hand phalanx	
UTK1102-0316-E01a1.1	UT42-05D	11.7	1157	metatarsal 4	
UTK1102-0410-E01a1.1	UT05-98D	3.9	949	cervical vertebra	
UTK1102-0415-E01a1.1	UT05-98D	5.0	604	1st distal hand phalanx	
UTK1102-0401-E01a1.1	UT09-97D	6.0	540	middle rib	
UTK1102-0405-E01a1.1	UT09-97D	6.0	971	1st distal hand phalanx	
UTK1102-0414-E01a1.1	UT05-98D	6.3	758	metacarpal 4	
UTK1102-0411-E01a1.1	UT05-98D	6.8	799	middle rib	7.6
UTK1102-0404-E01a1.1	UT09-97D	8.9	525	metacarpal 4	
UTK1102-0408-E01a1.1	UT09-97D	9.5	448	cuneiform 1	
UTK1102-0407-E01a1.1	UT09-97D	10.3	560	talus	
UTK1102-0406-E01a1.1	UT09-97D	13.1	664	metatarsal 4	
UTK1102-0505-E01a1.1	UT03-90D	8.3	828	1st distal hand phalanx	8.6
UTK1102-0516-E01a1.1	UT12-90D	8.8	697	metatarsal 4	

IV. CONCLUSIONS

Discussion of Findings

The results of this study have created a rank order of bone elements by the amount of DNA contained in each. Phase 1 used a set of 55 bones, which are representative of every element type in the adult skeleton, from 3 different individuals. The DNA yields have been compared between the 55 bones from each individual as well as averaged together by element to give overall rank order to the samples. Phase 2 examined 10 elements from 3 individuals in each of 4 increasing post mortem intervals up to 20+ years and confirmed the initial ranking of Phase 1 elements.

This study is the first that the authors are aware of that qualitatively ranks essentially all element types from an adult skeleton. Other recent studies looking at specific bone sample success have largely focused on the cortical long bones and teeth. Additionally, previous studies were comparing the different skeletal elements between different individuals while this study compares the different skeletal elements within the same skeleton. Also unique to this study was the ability to limit many confounding variables by using skeletons that decomposed in the same geographic location and were exposed to the same gross environmental conditions, skeletons with similar overall demographics and skeletons with specific dates of death to control for post mortem interval. The results of this study generally confirms the relative ranking of previous studies, however the inclusion of more element types in the current study has identified bones that yield much more DNA per mass of sample.

The rank order as shown in Table 2 was generated simply by averaging the quantity of DNA found in each sample, from three different individuals, as determined by the Quantifiler real time PCR system. A closer look at the quantification results shows

that 29 samples showed inhibition in the IPC of the quantifiler reaction. For some of the samples containing inhibitors the IPC was more than 36 cycles indicating significant inhibition as well as a likely underestimate of the actual DNA present. If inhibition is factored into the ranking then many of the foot bones, such as the 3rd cuneiform that had all three DNA extracts showing inhibition, would move up the list.

Previous studies tested actual forensic cases including bones from the World Trade Center identification project, bones from mass graves throughout the former Yugoslavia, and bones from mass graves in Spain. Most of these studies found that dense cortical bone or teeth give the best chance of obtaining sufficient DNA for forensic testing. This study identifies small, predominantly cancellous bones as containing higher levels of DNA in the same sample size. It remains to be determined if the rank order established in this study will be maintained for buried skeletal remains.

Implications for Policy and Practice

The U.S is involved with identifying missing individuals, both civilian and military. Domestically, investigators attempt to identify human remains, whether the individual died as the result of criminal behavior, accident, or other circumstances. Internationally, the U.S. and many other countries are working to identify servicemen killed during current or previous wars, victims excavated from clandestine graves, and those killed during mass fatality events whether the cause was natural, accidental, or terrorism. In many of these examples, the deceased may be decomposed, skeletonized, or fragmentary necessitating identification through DNA testing from bone.

This research addressed current policy and practice shortcomings by examining the differences in DNA yield rates between skeletal elements from increasing post mortem intervals, and by determining a rank order for the skeletal elements most likely

to provide both the quantity and quality of DNA needed to produce DNA STR profiles. Having an idea of which bones are most likely to yield a DNA STR profile can increase success rates, especially for cases where limited sample is available. The results presented here can be used to establish comprehensive bone-sampling guidelines, which will facilitate speed, accuracy, and success rates, especially in large identification projects. These guidelines should also help reduce both the cost and the time needed to complete the identification work.

Therefore, the greatest potential gain from this research is the enhancement of the criminal justice system's ability to obtain positive identifications from skeletonized remains of varying post mortem intervals, whether the goal is simply personal identification or part of a criminal prosecution.

Implications for Further Research

Phase 1 skeletons were never curated, however, Phase 2 skeletons were stored in a cool dry environment following decomposition outside at the ARF. These storage conditions are likely to have preserved the bones and DNA better than if they had been buried in a grave for the same amount of time. There is a possibility that these results could be different had the bones been subjected to various levels of soil moisture and acidity in addition to microbial growth and other taphonomic conditions. Further research is needed using buried remains from different post mortem intervals.

The difference in the amount of cortical bone versus the amount of cancellous bone within each bone sample needs to be addressed. Clearly bones such as the femur will have had more cortical bone sampled while other bones, such as the tarsals, will have had more cancellous bone sampled. This was not something that could be accurately measured during this study. In response, a follow up study has been

initiated. CT scans of the bones will provide a more accessible and accurate picture of the sampling site. This will provide the resolution to distinguish between the cortical and cancellous bone and the measuring tools to calculate the volume of removed bone. Additionally, further research into the differences between cortical and cancellous areas within a single bone is needed. This may help to narrow down not only which specific element to sample, but where on the specific element to sample.

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VI. DISSEMINATION OF RESEARCH FINDINGS

No other study in the forensic literature provides a comprehensive, empirically based ranked order of all human skeletal elements by DNA yield success rates. Therefore, it is important to disseminate these results to as wide a community of forensic practitioners as possible, on both the domestic and international levels. To date, the results have been presented at the 2012 American Academy of Forensic Sciences annual meeting in Atlanta, GA and the 2012 Annual International Forensic Research Institute's Forensic Science Symposium in Florida. Manuscripts are currently in preparation for submission to Forensic Science International: Genetics and to the Journal of Forensic Sciences.