Final Report

Increasing the Predictability and Success Rate of Skeletal Evidence Typing: Using Physical Characteristics of Bone as a Metric for DNA Quality and Quantity[1]

NIJ Grant# 2002-IJ-CX-K016

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1. Portions of this report were previously reported in the theses of Lisa Misner (2004), Amy Barber (2005), Virginia Clemmer (2005), Rebecca Elk (in prep), Andrea Halvorson (2005), Timothy Lange (in prep), and Lisa Ramos (in prep).

2. This project was carried out at Michigan State University. The study involved the joint effort of the following individuals: Dr. David Foran, PI, Dr. Doug Ubelaker (The Smithsonian Institution), Dr. Todd Fenton (Michigan State University, Department of Anthropology), Lisa Misner, Andrea Halvorson, Virginia Clemmer, Lisa Ramos, Amy Barber, Rebecca Elk, Timothy Lange, Michael Mutolo, and Melissa Meyers (Forensic Science Graduate Program, Michigan State University).

Abstract

Owing to their longevity, skeletal remains can supply important forensic information long after death occurs. Further, when simpler tests are not available, bone DNA can be invaluable in skeletal identification. Unfortunately, while bones are the longest lasting tissues postmortem, they degrade over time, as does the DNA within. This relationship between bone weathering and DNA degradation is poorly understood, and an analyst can only make an educated guess as to which bones might harbor useful DNA, and how degraded that DNA might be. If the appearance of bone could be related to the quantity and quality of the DNA within before testing commences, substantial time and effort would be saved, and laboratory productivity would increase.

Previous studies on DNA from aged bone have used samples of varying age and type, obtained from different environments. To avoid these confounders, a large set of discovered skeletons from a mid-1800's cemetery near Pittsburgh were tested. Long bone, flat bone, rib and tooth were collected from remains at varying stages of degradation. Quantitative PCR was used to measure the amount of mitochondrial DNA remaining, while DNA quality was assayed by amplifying progressively larger segments of DNA. Results indicate that there is little relationship between skeletal/bone weathering and DNA quantity or quality. In contrast, bone type played a significant role in obtaining DNA results. Long bones contained more DNA and were more likely to produce usable results than ribs, which were more likely to produce usable results than flat bones. The amount of DNA recovered did not play a significant role in the ability to amplify DNA, though there was a trend in that direction. Neither sex nor age of the individual were related to DNA typing success.

Several ancillary projects were undertaken related to this research. Soil samples assayed indicate pH influences bone preservation, while several other factors do not. PCR inhibition was found to greatly affect quantitative PCR results, remedied to some extent by the addition of BSA. Amplification using primers moved in towards the target sequence (Mini-STRs) made little difference on the ability to amplify nuclear DNA, while the use of nested PCR did. Whole genome amplification, while promising for high molecular weight DNA, was of little use on aged skeletal material.

Overall, the research indicates that while the outward appearance of bone is generally not related to successful DNA typing, the bone type chosen for analysis needs to be considered.

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Executive Summary

Background

Skeletal material, including bones and teeth, are the biological samples most likely to require analysis by the forensic scientist well after an individual's death. Although bone persists in general, it does degrade over time, in part due to the dissolution of the minerals, most importantly hydroxyapatite. Likewise, the organic molecules in bone, including DNA, break down over time. This breakdown can result from direct strand breakage, chemical modification, or enzymatic actions of microorganisms. It is not known however, how the breakdown of bone and DNA correlate.

For the forensic scientist a knowledge of the relationship between bone and DNA degradation would be advantageous. If an analyst could look at a skeleton or bone and, based on its appearance or level of weathering, make a reasonable prediction regarding the likelihood of successfully obtaining DNA, valuable time and resources could be saved. When a certain level of bone degradation was reached the analyst would know not to expend the effort on DNA analysis. Likewise, if the level of bone weathering related to DNA degradation levels, the analyst could more accurately predict what size fragment of DNA could successfully be analyzed. On the other hand, if no such relationship exists, this too is important, as the analyst will know that just because skeletal material appears to be poorly preserved, it does not preclude successful DNA analysis.

While there have been numerous studies on DNA and bone, most have worked with mixed skeletal samples. Some varied greatly in age, some in the environment where they existed prior to analysis, some in bone type analyzed, and some in all of these variables. Comparing results from a 20 year old bone with one 150 years old makes little

sense. Similarly, trying to draw conclusions on DNA degradation from a bone found in a woods to one found in a swamp or one found in a desert is nearly impossible. Likewise, it is not known to what extent DNA results from one bone type, for instance a long bone like a femur, can be extrapolated to a flat bone such as the pelvis.

The goal of this study was to use a more uniform set of skeletal remains to analyze the relationship between bone weathering and DNA degradation. For this, a set of burials from a cemetery used over a relatively short period of time was tested (details below). Weathering was graded at both the overall skeletal level as well as for individual bones. Similar bone types (generally femur, rib, pelvis, and tooth) were collected from skeletons over a range of weathering states. DNA quantity was assessed using quantitative (real time) polymerase chain reaction (PCR), while DNA quality was estimated by attempting to amplify progressively larger pieces of DNA. The focus of the research was on mitochondrial DNA (mtDNA) as its analysis is more likely to be successful in aged skeletal remains than is nuclear DNA, although nuclear loci were examined as well. Soil was analyzed to help determine what role it plays in degradation. Age and sex of individuals was considered. Finally, a series of experiments designed to better isolate and analyze DNA from samples with limited DNA quantity or quality was performed.

Skeletal Samples

The primary skeletal material used in this study originated from the Voegtly Cemetery, associated with the First Evangelical Church of Allegheny (The Voegtly Church), near Pittsburgh. The cemetery was used between 1833 and 1861, thus all the

remains are about 150 years old. The plot was small, and church members were buried in a uniform fashion, therefore environmental conditions should be similar for all burials except perhaps at the extremely localized level. When the church and long-forgotten graveyard were declared to be in a highway right-of-way, the church was largely disbanded. When highway construction commenced the cemetery was discovered. Because there were no records on these individuals, all skeletons were donated to the Smithsonian Institution. There, anthropologist Doug Ubelaker and co-workers estimated age and sex of each individual, and gave each skeleton an overall weathering score, based on a 0 – 5 scale, with 0 being bone showing no weathering. Sections of long bone (primarily femur), flat bone (generally pelvis), rib, and a tooth were collected from up to 20 individuals at each weathering stage (when available) for DNA analysis.

A second, separately funded study in our laboratory, involves analysis of skeletal material from a large burial mound near Kamenica, Albania. This material ranges from 2500 – 3000 years old. Sections of long bone, teeth, and the hardest bone in the human body—the petrous portion of the temporal bone—were collected.

Methods

Bones were cleaned and either drilled or ground to obtain bone powder. Bone powder was weighed and DNA isolated using a standard organic extraction method. MtDNA primers and an internal oligonucleotide probe were generated for the quantitative TaqMan assay. MtDNA was quantified on an ABI 7700. The effect of PCR inhibition on quantitation was tested by adding known amounts of target DNA to DNA samples that had shown inhibition in previous experiments, and observing how the

known DNA quantity was altered. The same experiment was conducted with the addition of substances thought to reduce PCR inhibition, a commercial PCR enhancer, as well as bovine serum albumin (BSA). MtDNA quality was assayed by attempting to amplify a 220 base pair (bp) fragment of mtDNA. If this was successful, progressively longer amplifications were attempted. If it failed, a 107 bp amplification was attempted. If this failed the sample was considered negative.

Both quantity and quality data were compared to skeletal weathering stage and bone type using single factor Analysis of Variance (ANOVA). During the study it was found that individual bones often differed in their level of weathering in comparison to the skeletal grade, thus each bone was restaged on a 4 point scale, allowing a second comparison of DNA quantity and quality to individual bone weathering using ANOVA. Quality and quantity results were compared to one another using a t-test.

Although direct comparison was difficult given the very different nature of the Albania bone samples, these samples were prepared and mtDNA amplified similarly to the Voegtly bones. Comparisons of amplification success of the different bone types were made.

A number of techniques were tested for enhancing PCR results from low copy or degraded DNA. Mini-STR sets, in which the PCR primers are moved closer to the DNA region of interest thus producing a shorter amplicon (more likely to be successfully recovered from degraded DNA) were obtained for 6 nuclear loci from scientists at the National Institute of Standards and Technology (NIST). Mini-STR primers for the sexing locus amelogenin were produced in-house. Both sets of primers were tested on a subset of the Voegtly bones that amplified using mtDNA primers. Nested PCR, in which two

rounds of amplification are conducted, uses an external set of primers for the first round of amplification, followed by a second round using primers internal to (nested within) the first set. This allows for an increased number of PCR cycles (advantageous when very little DNA is present) without causing non-target DNA to amplify—which often happens when DNA is over-amplified using a single set of primers. Nested PCR was conducted on mtDNA when necessary, on the amelogenin locus, and on a set of STR loci using a commercially available PCR kit (Identifiler™) followed by the NIST Mini-STR set.

Whole genome amplification (WGA) are methods for amplifying all the DNA in a sample prior to DNA testing. Two methods of WGA, Improved Primer Extension Preamplification (I-PEP) and Multiple Displacement Amplification (MDA), were tested on a subset of the Voegtly bones that amplified using mtDNA primers. Following WGA, mtDNA amplification was attempted.

Soil samples from a number of Voegtly remains were tested for levels of a variety of organic and inorganic compounds, to determine if any had an effect of bone decomposition. The pH of the soil was also analyzed.

Findings

MtDNA quantities showed a statistically significant difference when skeletal weathering was taken into account, although this outcome was not as expected. When all individuals were considered, the most weathered skeletons tended to produce the highest levels of mtDNA ($p=0.036$) (but see individual bones below). It is surmised that this might result from the relative ease of retrieving DNA from the more friable material, although it is also possible that factors or conditions that help preserve skeletons (those

with the least weathering) are disadvantageous for DNA. In this regard, the only soil factor that showed a correlation with overall skeletal weathering was pH; more degraded skeletons tended to have lower soil pH (R^2=0.21). It seems possible that while the lower pH tended to lead to skeletal breakdown, it was likely not low enough to damage DNA. On the other hand, the more neutral pH found with the less-weathered bones may have created conditions detrimental to DNA, perhaps by encouraging microbial activity.

DNA quality was not significantly correlated with skeletal weathering, although the most degraded stage generated the highest percentage of positive results. When the quantitative and qualitative results were broken down by bone type for each skeletal weathering stage (e.g., stage 5 femora) there was no significant differences in stages, however this practice reduced sample sizes considerably. When quantitative and qualitative results were compared against each other, there was no statistical relationship between the number of DNA copies in a sample and the maximum amplicon size produced.

The restaging of each individual bone (as opposed to overall skeletal staging, above) did not result in a significant effect of bone weathering on DNA quantity or quality, contrary to the quantitative skeletal results. On the other hand, when bone type was taken into consideration it was readily apparent that certain bones produced more amplifiable DNA than others. While this was not significant in DNA quantity, bone type and the likelihood of successful PCR amplification was very significant (p=0.006). Femora had the highest amplification success (79%) as well as the most DNA. Ribs were next in PCR success (64%) while pelves were lowest (36%). Ribs and pelves produced

similar amounts of DNA. Similar percentages were obtained for the Albanian samples, with both femora and petrous portions amplifying greater than 80% of the time.

No effect of age was observed on DNA quantity or quality, while sex did appear to have an influence, although the actual sex of many of the remains was tentative based on anthropological sex estimation of poorly persevered specimens.

PCR inhibition was measured by adding target DNA to samples that had previously shown inhibition. Target copies ranged from 600 – 6,000,000 in 10-fold dilutions. The same experiments were conducted with the addition of a commercial PCR enhancer or BSA. The untreated samples showed substantial inhibition, with estimated quantities of the target DNA reduced by 79 – 96%. Samples with the enhancer added also had high levels inhibition (41 – 99%) and were not significantly different from those without. In contrast, samples with BSA added had significantly lower levels of inhibition at all levels except at 6,000 and 60 target copies.

The enhanced techniques for use on low copy or degraded samples met with mixed success. The NIST Mini-STRs for nuclear loci did not amplify any of the Voegtly samples, nor did the amelogenin primer set produced in-house. On the other hand, nested PCR using the amelogenin primers allowed the nuclear DNA to be amplified in 12 of 65 Voegtly samples and 4 of 17 Albanian samples. Similarly, DNA from Voegtly teeth could not be reliably amplified until nested PCR was initiated.

WGA, while successful on high quality DNA, was not useful on the more degraded DNA of the Voegtly samples. Because WGA reduces DNA size it is assumed that the vast majority of DNA produced is too small to be amplified.

Conclusions

From the experiments and results presented here it seems clear that skeletal weathering/appearance cannot be used as a reliable metric of mtDNA quantity or quality, and indeed the only statistically significant result obtained was that more weathered burials resulted in higher DNA yields (other factors being equal). Likewise, the weathering stage of an individual bone is not a useful measurement of DNA quantity or quality. On the other hand, bone type plays a substantial role in DNA results, particularly the ability to successfully produce an amplicon. This should be taken into account when selecting skeletal material for processing. Likewise nested PCR can be a powerful tool when amplifying very small amounts of DNA. Owing to its extreme sensitivity precautions need to be made, particularly running reagent blanks and negative controls to test for contamination, however with these in place DNA results can be obtained from material that would otherwise generate a negative result.

Introduction and History

An important goal of any forensic investigation involving unidentified human remains is positive identification. Forensic specialists including anthropologists and odontologists may evaluate the remains, estimating the individual's sex, stature, age at death, and ancestry. Any identifiable characteristics such as fingerprints, if flesh is still intact, unique skeletal features, and dental arrangement are also noted. However, when antemortem reference records are not available for comparison or remains are fragmented or otherwise in a state in which definitive conclusions cannot be made as to the person's identity, DNA analysis may be required. Indeed, identification through analysis of DNA from human skeletal remains has been used in numerous cases, beginning in 1989 (Pääbo *et al*., 1989). Advancements in techniques and applications occurred in the 1990's (Boles *et al*., 1995; Hagelberg *et al*., 1991; Primorac *et al*., 1996; reviewed in O'Rourke *et al*., 2000), and in 1991 the Armed Forces DNA Identification Laboratory was established for the identification of the remains of U.S. military personnel (Holland and Parsons, 1999). A decade later a high-throughput method was developed for the analysis of the thousands of bones connected with the attacks on the World Trade Center (Holland *et al*., 2003).

Bones encountered by the forensic biologist often vary in their degree of degradation, and if more than one bone is available, the scientist may sensibly choose which bone to analyze based on its appearance; a bone in good condition would logically contain 'better' DNA than a more weathered sample. However, the lack of any proven association between the degradation level of a bone sample and the quality or quantity of its DNA means that it is currently difficult or impossible to reliably predict DNA typing success for aged bones. Further, because of the high levels of variability among aged

skeletal samples that have been studied to date (including bone type, age, the environment where it was found, etc.), DNA preparation and typing techniques that have been shown to work well on one sample may not be useful for another. If criteria were available for relating the appearance of aged bone to the DNA within, the efficiency, affordability, and reliability of analysis could be greatly enhanced. Given the type of skeletal material (long bone, flat bone, tooth, etc.) and its level of weathering, valuable predictions could be made about the probable amount of obtainable DNA and its level of degradation, thus leading the scientist to the best genetic loci and polymerase chain reaction (PCR) primers for analysis.

Genetic Analysis of Aged Skeletal Material

Although bone (and relatedly, dental remains) can persist for exceedingly long periods of time, this does not mean that bone, at either the gross or molecular levels, does not change with time (the so-called taphonomic process). Like any tissue, bone breaks down postmortem, and given enough weathering becomes so fragmented that it may be unidentifiable. Likewise, the organic molecules within a bone sample used for individual identification, including DNA, mutate and degrade as time passes. It is not known however, how these two species of degradation inter-relate. Bone, for instance, is often damaged mechanically, hastening its breakdown, but this may or may not have a large influence at the molecular level.

In describing bone diagenesis, Hedges (2002) summarized the histological and biochemical changes that occur as a bone degrades. The primary medium in which skeletal remains are located is soil. Analyzing the effects of soil conditions, soils of

neutral pH often have calcium and phosphate concentrations similar to those found in hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; the major bone mineral), which likely leads to a slower bone dissolution rate. In contrast, soils of low pH generally have lower calcium and phosphate concentrations and dissolution occurs faster, as the protons from the soils replaced the calcium ions in the hydroxyapatite of the bone. DNA is also negatively impacted by acidic conditions, although other variables, such as warm temperature and high moisture, accelerate DNA degradation as well, and may be as or more important than soil pH (Parsons and Weedn, 1996; Smith *et al*., 2003). DNA from bone can also be broken down enzymatically or chemically with nicks, chemical cross-bridges, or extraneous molecular attachments occurring (e.g., Rogan and Salvo, 1990; Tuross, 1994; reviewed in O'Rourke *et al*., 2000). Microorganisms also affect the stability of bone DNA, producing nucleases that directly attack it (Rogan and Salvo, 1990). Finally, as long as soft tissue persists, additional enzymatic attack can occur from autolytic enzymes.

Multiple authors (e.g., Gilbert *et al*., 2003; Gotherstrom *et al*., 2002; Kalmar *et al*., 2000; Pääbo *et al*., 1989), have noted that mitochondrial DNA (mtDNA) analysis is the primary mode of DNA typing of aged skeletal remains. Mitochondria are cytoplasmic organelles containing a small, maternally-inherited, circular piece of DNA distinct from the linear chromosomes of the nucleus. Thousands of mitochondria can exist in a single cell, and as such there are thousands of copies of mtDNA as well. This is far different then the two copies (maternal and paternal) of a DNA locus that are examined in most forensic analyses. Human mtDNA was first sequenced in its entirety in 1981 (Anderson *et al*., 1981) and is approximately 16569 bp in length. The majority of genes in mtDNA encode proteins essential to mitochondrial function, thus these areas are highly conserved

among individuals, and are generally not useful for identification. However, an approximately 1125 bp stretch of non-coding DNA known as the control region contains two segments that mutate frequently and therefore vary among unrelated individuals. The sequence variation of these two areas, known as hypervariable region I and II (HVI and HVII), are generally the focus of forensic analysis (reviewed by Holland and Parsons, 1999). Typically, this involves amplification of both hypervariable regions using PCR followed by a sequencing procedure to determine the exact DNA sequence, which is then compared to the Anderson reference sequence. The nucleotide differences between the sample and the reference sequence constitute the mtDNA profile for that sample, which can then be compared back to a known sample or a maternal relative for identification.

Project Goal

The objective of this project was to determine if the visual appearance of skeletal remains can be used as a reliable metric of the DNA (particularly mtDNA) found within, or: is there a relationship between the level of bone weathering and the quantity and/or quality of DNA that may be used for analysis? While many scientists have obtained DNA from bone, no study to date has systematically looked at bone appearance and related it to successful DNA typing. The utility of this to the forensic scientists is plain: can we examine a skeletal specimen and make a reliable estimate about how much DNA it might contain, and how large a fragment of DNA exists for analysis?

To accomplish this goal it was necessary to eliminate confounding factors that exist in most research on DNA and skeletal remains. Foremost among these is non-uniform samples. In a systematic study it is not useful to begin with samples of different

ages, that come from different peoples, have been 'stored' under different environmental conditions, and otherwise do not resemble one-another, and then try to directly compare results from sample 1 to sample 2. Therefore, a set of skeletal remains was sought that eliminated most confounders, and instead differed only by their appearance (or weathering level) to the greatest extent possible. From these we could start to address the relationship between bone appearance and DNA quality and quantity in an objective and systematic way. These results could then begin to be compared to other skeletal samples to see how generalized the findings were. Bone type (long bone, flat bone, teeth, etc.) was considered, as it is possible that different bones generate different results. Other factors that might affect bone diagenesis, most notably soil conditions, were tested as well. Finally, methods for enhancing the acquisition of DNA data from aged skeletal remains were examined.

The Samples: The Smithsonian Institution and the Voegtly Cemetery

The two key features of this study were having 'uniform' samples for the controlled, correlative portions of the work, and having large sample sizes for statistical analysis. Skeletal material for this study came through collaboration with Dr. Doug Ubelaker of The Smithsonian Institution. These samples originated from a single burial site that was utilized for a short period of time about 150 years ago, known as the Voegtly Cemetery.

The Voegtly Cemetery was associated with The First Evangelical Church of Allegheny (The Voegtly Church), located in 'Old Allegheny Town', across the Allegheny River from Pittsburgh; its history is documented in Ubelaker and Jones

(2003). The founders were Swiss-German immigrants (the Voegtly family immigrated to the area in 1822) who started Voegtly Church in 1833. The cemetery, adjacent to the church, was utilized from that point until 1861, when a new cemetery site was purchased. The Church was expanded and modified over the next century, and the historic cemetery, without permanent markers or other identifying materials, was built over.

In 1959 it was announced that the Church was located in the right-of-way for a new highway, and thereafter membership declined. In 1972 the last Voegtly associated with the church died, the parsonage was destroyed, and in 1984 the Pennsylvania Department of Transportation acquired the entire property. The Church was officially disbanded in 1985; its original cemetery was discovered two years later when highway construction unearthed the first human bones. Church records from the time, written in a rare German dialect, had deteriorated and were of little use in immediately determining which or how many persons were buried at the site. Official city records of burials were not initiated until 1882, and remaining parishioners had no knowledge of the cemetery's existence as it had been largely assumed that all bodies had been moved to different sites in the 1860s.

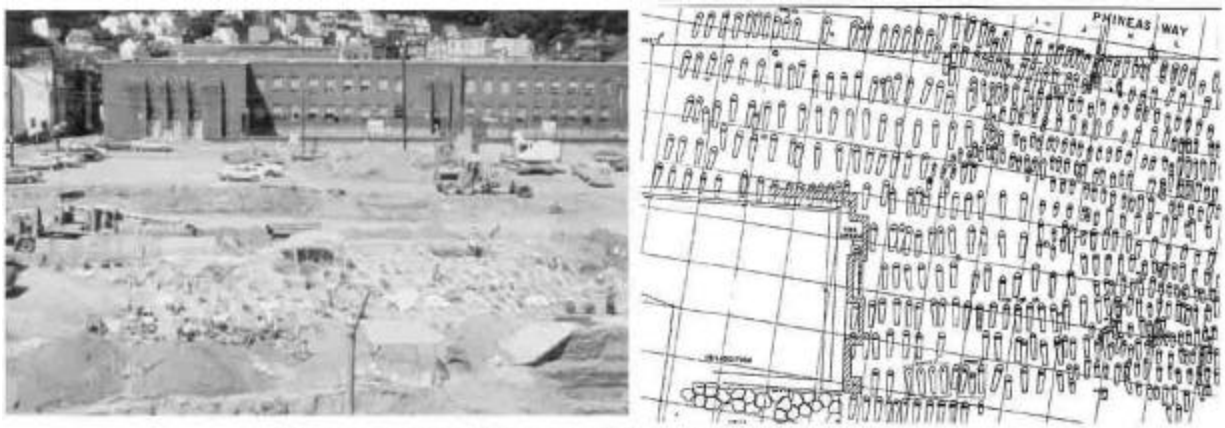
The Layout and Excavation of the Cemetery

The ¾ acre Voegtly Cemetery was excavated over a relatively short period (June – September of 1987). As no gravestones or other markers were present the site was treated as an archeological dig (Figure 1, photo and burial map). Excavations consisted of mechanical removal of topsoil followed by shoveling and hand-excavation to expose, map, and record each grave. Coffins were all similar—wooden, six sided, and wedge

shaped—with the major differences being in the degree of ornamentation. Following primary bone and artifact collection, soil was sifted through 0.25-inch hardware cloth to collect remaining artifacts and small bone fragments. All skeletal material was wrapped in aluminum foil, placed in plastic bags, and labeled.

Since 1987 the German church records have been interpreted and analyzed; these indicate approximately 823 individuals were buried, however remains from only 724 were located. Over the years church pastors kept different records; in some cases very little information about the deceased was given. This was most true of children, some labeled as "not seen", indicating miscarriage or stillbirth. For the 799 persons to which church records assigned age, 39% were less than 1 year old, 24% were between 1 and 5 years, and 8 % were between 5 and 20. The mean age at death was 14.6 years, a datum influenced by the high level of infant mortality. For known sexes (47% female) 99 females and 131 males were age 20 or over, while 266 females and 278 males were younger.

Figure 1. The excavation and layout of graves at the Voegtly Cemetery site



Excavation Site and Layout of Graves

Causes of death were varied and often unrecorded, but beyond stillbirths (the most common), illnesses such as cholera, typhoid, scarlet fever and consumption (tuberculosis) were seen, and often ran through families. Other illnesses are more poorly described, with only brief notations such as "Long Illness" or "Feverish Illness". The highest percentages of deaths occurred in July and August (13.4 % and 11.9 % respectively) generally affecting children. Seventy percent of individuals were buried 1 day after death, 23% after 2 days, and 5% on the same day.

The Skeletal Remains

All skeletal remains were transported to The Smithsonian Institution in Washington DC for physical analysis. While preliminary cataloging began several years ago, an intensive effort to finalize the visual analyses began in 2000 and is now complete. Bones were unpacked, cleaned, soil samples collected, and a variety of data recorded. The nature of the cleaning depended on the state of the bone (detailed below), with those in excellent condition washed under water while more fragile specimens were gently dry-brushed to remove dirt. Data were collected according to the Standards for Data Collection from Human Skeleton Remains (Buikstra and Ubelaker, 1994). Sex, age and stature were estimated when possible using all available material. Sex of children was not determined, and higher emphasis was placed on the pelvis, when available, for adults. Ages were placed into general ranges, these being typically more refined in children due primarily to dental development. Stature was estimated using the femur. Results relevant to this proposal are given below, while complete data can be found in Ubelaker and Jones (2003).

The overall condition (degree of weathering) of each skeleton was carefully assessed and logged. The classification scheme was taken from Behrensmeyer (1978) and is detailed in Buikstra and Ubelaker (1994). Briefly, a 0 to 5 scale is generated:

Stage 0—A bone sample whose surface shows no sign of cracking or flaking due to weathering;

Stage 1—Bone shows some cracking, usually longitudinally in long bones;

Stage 2—Cracks and some flaking of bone;

Stage 3—Bone surface has rough patches of weathered compact bone down to 1.5 mm, with extensive flaking although bone fibers are still attached to each other;

Stage 4—Bone surface is course, splinters may exist and fall out, and weathering reaches the interior portions (Figure 2);

Stage 5—Bone is easily broken and is disintegrating. Original shape may be hard to determine.

Figure 2. Example of skeletal weathering stage 4

Individual records for each of the burials are available in Ubelaker and Jones (2003) . These records encompass several hundred pages and hence cannot be reproduced here, but in brief they include as much information as available on: Each bone detected; Skeletal weathering score; Teeth present; The deceased's age range and sex if possible; Cultural information (coffin and other artifacts found); Other observations (e.g. discoloration of bone, dental decay, evidence of disease, etc.).

The vast majority of bones in the Voegtly sample are in weathering stages 2 – 5, with a smaller number classified as stage 1. It is not surprising for samples that have been buried for 150 years that virtually no bones were found at stage 0 (showing no weathering). This is not of particular concern for this study as bones at this stage are basically 'fresh' and as such are not usually challenging for identification or genetic studies. In general, confirmed male samples were in slightly better condition than females in the same age class, and younger adults were in better condition than the very old, presumably resulting from osteoporosis. Also, when compared to the church records, a large number of infant burials were not located. Because of the high number of stillbirths and similar deaths described in the records, it seems likely this deficit results from a lack of calcified bone in the very young.

Comparison to Other Skeletal Samples: The Albania Project

In addition to the skeletal remains from the Voegtly Cemetery, extensive research has now been conducted on a set of skeletal remains from a tumulus (burial mound) in Kamenica, Albania, in collaboration with Dr. Todd Fenton. The site at Kamenica is a Late Bronze Age, Early Iron Age tumulus located in eastern Albania just outside the

village of Kamenica in the southeastern corner of the fertile Korca basin. The Tumulus at Kamenica was utilized as a cemetery over many centuries, ranging from approximately the 12th to 6th century BC, introducing the variable of age to these analyses. The site was first identified in the early 1990s. Excavations began at the tumulus in 2000 as a rescue effort to preserve the burials after the site was badly damaged by looters from 1997 to 1999. There were three seasons of excavation from 2000 – 2002, which unearthed approximately 400 human skeletons.

While the burials at Kamenica appear to be in excellent condition (Figure 3), upon their recovery it was discovered that they were extremely friable, therefore only small fragments were recoverable. Because of this, the hardest bones available, the femur, teeth, and the petrous portion of the temporal bone, were collected for DNA sampling.

Figure 3. Example of (double) burials at the Tumulus at Kamenica, Albania

Experimental Studies

Determining MtDNA Quality

The degree to which DNA is degraded in a sample dictates the size of DNA fragments that can be amplified, and thus the level of genetic information that can be retrieved. If a sample is too far degraded the remaining small fragments of DNA are of no use for identification. In contrast, a sample retaining high molecular weight DNA, regardless of age, can yield a wealth of data.

DNA fragment length isolated from ancient DNA is typically small (O'Rourke *et al.*, 2000). To more exactly pinpoint useful size ranges for analysis, PCR can be used to determine the largest size class of DNA existing in each sample by targeting a series of amplification products. Progressively larger segments of DNA can be amplified until a negative result is obtained, this cutoff size can indicate how degraded a sample is. Amplicon sizes can then be compared within a weathering stage to see what generalities can be observed, within a stage and among stages, to discern any statistically meaningful differences in degradation levels.

It is important of course to distinguish between samples that do not amplify because there is no target DNA of a given size, and samples that do not amplify owing to the presence of PCR inhibitors. One way to test this is to add a small amount of high quality DNA to the PCR reaction containing a non-amplifying sample (or 'spike' it). If a positive PCR result is obtained, it indicates that the sample is clean enough for PCR to occur, and that there is not adequate product in the sample. On the other hand, if the control DNA does not amplify it indicates that inhibitors are present and that the sample requires additional purification.

Determining mtDNA Quantity

DNA quantity can also be assessed from bone samples and compared among weathering stages and bone type. In aged skeletal remains it is likely that not enough DNA exists for more traditional forensic methods of quantitation (such as Quantiblot), therefore a DNA amplification method is requisite. Quantitative PCR (QPCR, also called real time PCR) works by detecting increases in amplified DNA product (the amplicon) during the amplification process. In standard PCR, the amount of DNA produced bears little relationship to the starting amount of DNA, as a plateau exists when one or more reagents become limiting and amplification ceases. QPCR takes advantage of the fact that at lower concentrations of DNA, the rate of amplification proceeds in an exponential manner. During the exponential growth phase of amplification, samples pass a preset threshold of amplification rate. This value is taken as a sample's critical threshold (C_t) value, which is directly related to its starting DNA concentration. In order to assess the relationship between C_t and starting concentration, DNA standards—samples of known concentration—are amplified at the same time as unknown samples. A standard curve is then derived from a dilution series of these standards by plotting the concentration of the standards versus their C_t values.

Detection of the amount of amplicon in a reaction can be done in one of two ways. Double stranded DNA in the reaction can be detected with a dye, such as Syber Green, which intercalates between the strands of DNA, causing the dye to fluoresce when excited by a laser. This fluorescence will increase in a linear relationship to the concentration of double stranded DNA in a sample. The second method of detection is a probe that can hybridize to a specific sequence in the amplicon. Depending on the nature

of the probe, it may fluoresce only when hybridized to the amplicon or after degradation of the probe. In the research detailed below a TaqMan probe was used for detection, which is a short length of DNA, typically between 18–27 bp, with a fluorescent molecule at the 5' end (a "reporter") and a "quencher" molecule at the 3' end. The reporter is excited throughout the amplification process. However, when the probe is intact, the reporter is in close proximity to the quencher, which absorbs any emissions from the reporter. When the amplicon is present, the probe hybridizes to it and is digested by the 5' exonuclease activity of the polymerase, freeing both the reporter and quencher. This allows the fluorescence of the reporter to be detected by the QPCR instrument. Since the target level of fluorescence increases in a linear relationship to the concentration of the target amplicon, the starting concentration of DNA can be calculated using a standard curve.

Enhanced Techniques

When dealing with degraded skeletal samples, DNA cannot always be amplified using standard PCR. Bone diagenesis may leave little DNA to be extracted, and any DNA can be highly fragmented. One solution is to increase the chance of amplifying degraded DNA targets by moving primer sites closer to the DNA region of interest. This method can be useful on both nuclear and mtDNA. A second tactic is to increase DNA yields by increasing the number of PCR cycles attempted. A problem with this approach is that excessive PCR cycles often result in amplification of undesired, non-target DNA, that can jeopardize subsequent analyses. In addition, methods exist for pre-amplifying all the DNA that exists in a sample. One final solution may be to modify DNA isolation

procedures to increase DNA yields; for instance, most bone DNA preparation protocols leave visible bone behind, which may harbor useful DNA. These methods have the potential to generate large amounts of DNA and allow for multiple rounds of testing.

Mini-STRs Sets for DNA Analysis

Moving primers closer to the DNA region of interest can be advantageous when analyzing degraded DNA. For instance, most commercial STR kits amplify DNA segments from 100 to 450 bp. However, if a DNA sample is degraded, its length can be reduced to a size range smaller than this (Zelman and Moran, 1996), resulting in allelic or locus dropout and an incomplete genetic profile (Whitaker *et al.,* 1995). By moving primers as close as possible to the ends of the repeat region, successful amplification of smaller fragments of DNA may occur (Chung *et al.,* 2004). Butler *et al.* (2003) developed primers for amplifying CODIS STRs and additional markers. Likewise, multiple primers exist for the mtDNA control region, which can be used in various combinations to better amplify degraded material.

Nested PCR

It has been shown that DNA existing at very low copy number can be successfully amplified using nested PCR (Strom and Rechitsky, 1998), a technique in which two successive rounds of PCR are used to amplify small amounts of DNA. In the first round, DNA is amplified under standard conditions. In the second round, the reaction is supplemented with product from the first round reaction, providing it with an enriched template. This subsequent round utilizes primers that sit internal to the first round

primers, meaning that only fragments amplified in the first round that contain the second round primer sites are amplified. This allows for a greater number of PCR cycles while eliminating non-specific amplification products, and serves as a powerful technique for amplifying very small copy number samples with relatively high fidelity. A related technique is semi-nested PCR, in which the second round of PCR uses one of the primers from the first round and a second primer internal to the first set.

Whole Genome Amplification

Another potential method for dealing with limited starting DNA is to increase the overall amount of DNA prior to beginning standard laboratory procedures. Whole genome amplification (WGA) is a procedure used to duplicate DNA, but unlike PCR, which generally amplifies small sections of the genome, WGA is capable of replicating the genome in its entirety. WGA methods utilize random primers (oligonucleotides) to amplify large portions of the genome. Two WGA methods, I-PEP (Improved Primer Extension Preamplification) and MDA (Multiple Displacement Amplification) are widely used in clinical research for this purpose.

I - PEP is completed under conditions similar to that of typical PCR, except that random 15-mer oligonucleotides are utilized. I-PEP WGA reactions are, in theory, capable of replicating the genome in an unbiased manner as the random 15-mer oligonucleotides can locate a complimentary sequence anywhere in the sample DNA. I-PEP WGA has been shown to result in a 30 fold increase in product yield (Dietmaier *et al.*, 1999).

MDA was first described by Dean *et al*. (2002) and utilizes the DNA polymerase Phi 29. This polymerase is very processive and also displaces double stranded DNA in front of it. This results in longer product length and more access to the template and non-template strands so random primers can anneal. In MDA, random hexamer primers are used to amplify the genome. Hosono *et al*. (2004) determined that up to a 10,000 fold increase in DNA yield can be achieved with MDA.

Methods

Collection of Samples

A subset of 89 bones from the total set obtained from The Smithsonian Institution was analyzed in this study. Upon recovery of the skeletal remains, the bones were described in terms of sex, estimated age, and one of the six skeletal weathering stages (Stages 0 – 5). As detailed above and by Ubelaker and Jones (2003), the weathering stages originally assigned to the samples by the anthropologists at The Smithsonian considered the condition of the skeleton as a whole. In order to allow ratings on individual bones, a new staging system was developed at MSU and assigned to each bone based on visual inspection for the DNA study. This individual bone staging system was separated into four categories:

Stage 1: Bone surface shows minimal flaking. The bone piece is still whole, with no large pieces broken off.

Stage 2: Bone surface shows some flaking, pieces of bone are coming off in sheets. Small pieces of bone are breaking away from the cut piece.

Stage 3: The bone is fragmented into several pieces. At least one large piece is still present.

Stage 4: The bone is extensively fragmented. No large pieces are present.

Various bone types were collected for this study. When possible, femur sections represented long bone while pelves represented flat bones, and ribs an intermediate. If femora were not available, another long bone was sampled. If pelves were not found among the skeletal remains, crania were chosen. In addition, teeth were also collected

from the burials. A total of 2 crania, 28 femora, 1 fibula, 25 pelves, and 33 ribs were tested, coming from a total of 36 individuals. In addition, 31 teeth were examined.

The bones samples presented here spanned five of the six weathering stages assigned by Ubelaker and Jones (2003): 6 from stage 1, 23 from stage 2, 22 from stage 3, 18 from stage 4, and 20 from stage 5. The three samples from the single individual classified as stage 0 were not tested due to the insufficient sample size and nonexistence of comparison samples. Of these, 11 were female, 17 were male, 1 was labeled as "possibly male", and 7 were undetermined. This possible male had large mastoid processes and supraorbital ridges, but female sex could not be ruled out.

For the Albania bone samples, specific burials were chosen for testing based on the information mtDNA analysis could contribute to the Kamenica project. Long bones, such as femur, and teeth were collected whenever possible. In addition, the petrous portion of the temporal bone was taken if available, as it is one of the hardest bones in the body. Integrity of the bone samples was rated using a 0 through 5 system (below) based on their completeness. Teeth were rated in a similar fashion, but with some descriptive adjustment made within the scale to represent their unique endurance. Unfortunately, because the skeletons had already been removed to a new location, and because the bones were largely broken, the anthropologists on site were forced to use rating criteria modified from those used on the Voegtly material. Rating systems were as follows:

Bones (excluding teeth)

0 – bone is complete; surface of bone has a sheen

1 – proximal/distal ends beginning to break; sheen may be present

2 – 3/4 to 2/3 bone present

3 – 1/2 to 1/4 bone present

4 – small pieces (1 – 4 inches) of identifiable bone

5 – tiny (less than ½ inches), unidentifiable pieces of bone

Teeth

0 – no erosion visible

1 – little erosion

2 – moderate erosion

3 – great erosion, but crown still attached to root

4 – crown and root attached, but one or both is/are broken

5 – only crown or root present

Sample Preparation and DNA Extraction

Approximately 1 cm^3 of bone was cut from the source section using a Dremel MultiPro tool and was collected in a tube. For tooth samples, the tooth was cut in half exposing both the crown and the root. Samples were then immersed in filter-sterilized wash buffer (1% SDS, 25 mM EDTA) and 0.1 mg/ml proteinase K, and incubated for one hour at room temperature. Following the incubation, the wash buffer was poured off and each sample was washed with 1ml of sterile dH_2O six consecutive times. Samples were allowed to air dry. Bone powder from the dried bone samples was collected in one of two ways. Bone was either ground to powder using an IKA A11 Basic Grinder or drilled using a the Dremel tool or a 12 inch Craftsman drill press, both fitted with $1/16^{th}$ inch drill bits. Teeth were drilled in all cases. The resultant powder was collected in a

microfuge tube and weighed. Between samples the grinder was rinsed with digestion buffer (see below), which was carried through the entire analysis as a reagent blank.

Four hundred microliters of digestion buffer (20 mM Tris, 100 mM EDTA, 0.1% SDS) and 0.4 mg/ml proteinase K was added to each ground bone sample and incubated overnight at 56ºC. A standard phenol/chloroform organic extraction was performed on each of the samples. DNAs were precipitated using 3M sodium acetate and 95% ethanol, vacuum dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA) based on the original mass of the bone powder. For the Albania bone samples, the final aqueous layer was processed using a Microcon-30 column and retentate volume adjusted based on the original mass of the bone powder.

Several precautions were taken to prevent contamination during the experiments. Grinders and drills used to generate bone powder were washed with 70% EtOH and 10% bleach, and were UV irradiated between each sample prep. Pre-amplification and post-amplification steps were carried out in separate rooms. Finally, negative controls and reagent blanks were included in all experiments.

Optimizing DNA Amplification

Multiple steps were taken during DNA amplification procedures to decrease the effects of PCR inhibitors found in the amplification reaction. If amplification was inhibited using undiluted DNA extract, a 1:20 dilution of the DNA was used. In addition, 5X HotM Enhancer (Eppendorf) and/or bovine serum albumin (BSA) was added to the amplification reaction to decrease inhibition. A hot-start Taq polymerase (HotMaster, Eppendorf) and accompanying 10X buffer were also used. Finally, the use of the

Microcon columns was incorporated as part of Voegtly DNA preparations when a sample caused PCR inhibition. This allowed removal of both small pieces of DNA that could potentially act as random primers, and small water soluble inhibitors not removed during organic extraction.

Table 1. Mitochondrial and nuclear primers used for DNA amplification. Also included is the TaqMan® probe used in QPCR experiments.

Name	**Sequence**	**Region**
Mitochondrial Primers		
F82	5'ATAGCATTGCGAGACGCTGG3'	HVII of mtDNA
F155	5'TATTTATCGCACCTACGTTC3'	HVII of mtDNA
R285	5'GTTATGATGTCTGTGTGGAA3'	HVII of mtDNA
R484	5'TGAGATTAGTAGTATGGGAG3	HVII of mtDNA
F15989	5'CCCAAAGCTAAGATTCTAAT3'	HVI of mtDNA
F16144	5'TGACCACCTGTAGTACATAA3'	HVI of mtDNA
F16190	5'CCCCATGCTTACAAGCAAGT3'	HVI of mtDNA
R16251	5'GGAGTTGCAGTTGATGT3'	HVI of mtDNA
F16400	5'ACCATCCTCCGTGAAATCAA3'	HVI of mtDNA
R16410	5'GAGGATGGTGGTCAAGGGGAC3'	HVI of mtDNA
D-loop	5'ACCCTGAAGTAGGAACCAGA3'	D-loop of mtDNA
Nuclear Primers		
BF	5'CCCTGGGCTCTGTAAAGAATAGTG3'	Amelogenin Gene
BR	5'ATCAGAGCTTAAACTGGGAAGCTG3'	Amelogenin Gene
LF	5'AAGAATAGTGTGTGGATTCTTTATCCCA3'	Amelogenin Gene
LR	5'GGAACTGTAAAATCGGGACCACTTGAG3'	Amelogenin Gene
Forward Amel	5'CTCCCCTCCTCCCTGTAAAA3'	Amelogenin Gene
Reverse Amel 496bp	5'AGCAGAGGCAAGCAAGAGAC3'	Amelogenin Gene
Reverse Ame1 200bp	5'TAAACTGGGAAGCTGGTGGT3'	Amelogenin Gene
Probes		
TaqMan® probe	5'CCTCGCTCCGGGCCCATAAC3'	D-loop of mtDNA

DNA Quality: PCR Amplification and Sequencing

Voegtly and Albania Bone Samples

DNA quality (maximum obtainable amplicon size) from the Voegtly bone samples was assayed using several different mtDNA primers in hypervariable region I (HVI). Primer sequences used for amplification can be found in Table 1. Initially, PCR was performed using F16190 and R16410. If the sample did not produce the 220 bp amplicon, F16144 and R16251 were used to produce a 107 bp amplicon. When amplification of the 220 bp amplicon was successful, amplification of progressively larger amplicons (329 bp followed by 402 bp) was attempted. The amplification reaction consisted of denaturation at 94ºC for 2 min, followed by 35 – 40 cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 45 s, and extension at 72ºC for 45 s. The last cycle was followed by an additional extension step at 72ºC for 5 min. PCR reactions were in 20 µl volumes, containing 1U of enzyme, 1X buffer, 0.2 mM each of dNTP, and 2 µmol each of mtDNA-specific primers. Fragment size and amplification quality were visualized by running 5 µl of the PCR product on a 2% agarose gel followed by staining with ethidium bromide. If only a light PCR product was seen, an additional amplification reaction was performed to increase the amount of product to be used in sequencing reactions. In these reactions, 1 µl of the original PCR product was used as a template and the number of cycles was reduced to 20.

Voegtly Tooth Samples

Samples were amplified using a semi-nested PCR technique because single PCR did not provide enough DNA for sequencing. A portion of the HVI was amplified. For

the first round of PCR, primers F16144/R16410 were used. The primer set F16190/R16410 was used for the second round of PCR, resulting in a 220 bp amplicon.

MtDNA Sequence Determination

When amplification was successful, the remaining 15 µl of PCR product was purified using a Microcon-30 column with 300 µl of TE. The samples were washed two additional times with TE before retentate was brought back to 15 µl with TE. Up to 5 µl of template DNA was sequenced using a BeckSeq kit (Beckman-Coulter). Per the manufacturer's recommendations, 50–100 femtograms of DNA were used for sequencing reactions; estimations of concentration were made from the yield gel of the original amplification. The primers used for sequencing were the same as those used for amplification, and as a modification to the protocol provided with the kit, the total reaction volume was reduced to 10 µl. The sequencing reaction was followed as outlined in the kit manual. Following sequencing, 4 µl of stop solution (1.5M NaAc, 50 mM EDTA, 2.5 mg/ml glycogen) and 30 µl of cold 95% EtOH were added to each reaction, which was then centrifuged at 14000rpm for 15 min. The supernatant was removed and the sample was washed twice with 200 µl of cold 70% EtOH with a 2 min, 14000rpm spin between washes. After the final wash, the supernatant was removed and the sample was vacuum-dried for approximately 10 min, then resuspended in 40 µl Sample Loading Solution (SLS, Beckman-Coulter). Samples were sequenced on a CEQ 8000 Genetic Sequence Analyzer (Beckman-Coulter), using the LFR-1-60 program (capillary temperature 50ºC, denature 120 s at 90ºC, inject 15 s at 2.0kV, and separate 60 min at

4.2kV). Resulting sequences were aligned using BioEdit Sequence Alignment Editor (Hall, 2004) and compared to the Anderson *et al*. (1981) reference sequence.

MtDNA sequences for scientists conducting the DNA work were determined in all cases, to ensure DNA sequences were not derived from laboratory personnel. To be considered a 'positive' result, the same mtDNA sequence had to either be obtained from more than one bone from a skeleton, or obtained from two independent DNA preparations of a bone. Further, reagent blanks had to be negative throughout.

DNA Quantity: Quantitative PCR

A subset of 60 bones that had previously produced mtDNA PCR products was used for quantitative analyses. Also included were samples that did not produce amplicons yet did not show PCR inhibition, in order to address DNA levels that resulted in unsuccessful amplification. Samples that had PCR inhibition were not chosen for this portion of the DNA quantity experiment. The two mtDNA primers used in the QPCR reactions, F16400 and D-loop, amplified a 118 bp region in HVI. A TaqMan® probe was then designed to sit between the forward and reverse primer sites (Table 1). Primer/probe concentrations were optimized. The parameters recommended for quantitative TaqMan® assays (as described in the TaqMan® Universal PCR Master Mix protocol, 2002) were utilized, except that the reaction volume was decreased to 10 µl. Further, based on the work of Andreasson *et al.* (2003), cycle number was increased from forty to fifty. Based on optimization experiments, bone DNAs were diluted 50-fold for amplification, and BSA was added. Standards (known amounts of the same region of human mtDNA) were designed as ten-fold dilutions, from $6x10^7$ copies/µl down to 6 copies/µl. The samples

were processed in triplicate on an ABI 7700. Results were analyzed using the standard curve method.

Effects of PCR Inhibition

A set of 11 Voegtly bone samples that showed extensive/complete PCR inhibition were tested to determine what effect it had on DNA quantity estimates, and how addition of substances used to reduce PCR inhibition altered results. One microliter of DNA extract was spiked with 1µl of the mtDNA standards (in ten-fold dilutions from 6,000,000 – 60 copies/µl) and analyzed by QPCR. The 11 spiked samples were also tested in the presence of a commercial product designed to augment PCR (TaqMan® 5x PCR Enhancer (Eppendorf)) and BSA.

Enhanced Techniques

Mini-STR Experiments

Mini-STR primers were supplied by Michael Coble and John Butler of NIST. These included 6 individual primer pairs, and one mini A1 primer set that contained a combination of all primers. Table 2 lists the primers included in the set, and additional information on each. The DNAs analyzed were selected based on results from the amelogenin nested PCR amplification results and in some cases, on previous ability to obtain mtDNA sequence.

Table 2. Mini-STRs utilized in this study. Information from Butler *et al*. 2003

STR Locus (Dye Label)	Allele Range	Allele Spread	Product Size (STR Kit)	Mini-STR Size	Size Reduction
FGA (6-FAM)	12.2 – 51.2	156 bp	196 – 352 bp	125 – 281 bp	71 bp
TH01 (6-FAM)	3 – 14	44 bp	160 – 204 bp	51 – 98 bp	105 bp
D16S539 (NED)	5 – 15	40 bp	233 – 273 bp	81 – 121 bp	152 bp
D18S51 (VIC)	7 – 27	80 bp	264 – 344 bp	113 – 193 bp	151 bp
D2S1338 (PET)	15 – 28	52 bp	288 – 340 bp	90 – 142 bp	198 bp
Amelogenin (6-FAM)	X, Y		106 – 114 bp		

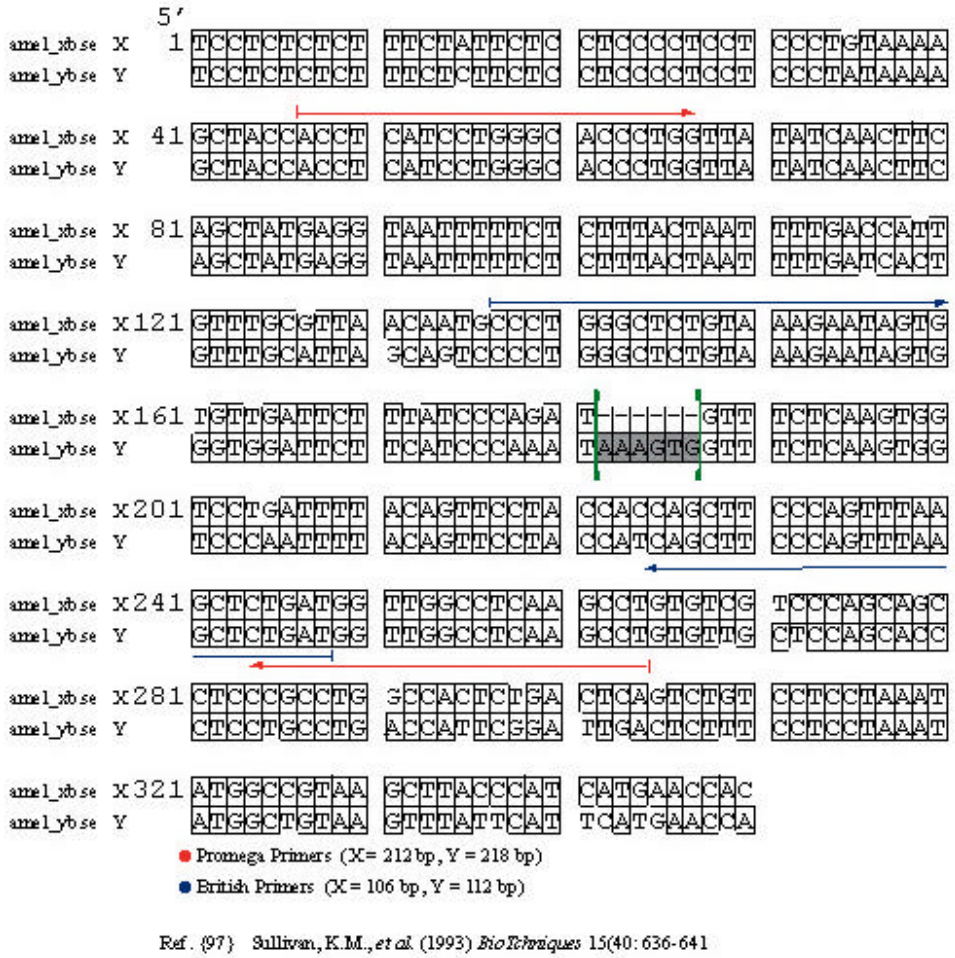
Amplification parameters were based on the authors' recommendations, although increased cycle number was also attempted. In addition, the Mini-STRs were tested using a nested technique. DNAs were first amplified for 30 cycles using an Identifiler™ kit and the manufacturer's protocol at 30 cycles in a 10 µl sample volume. One microliter of the resultant product was transferred to a reaction mix containing the Mini-STR set and PCR components listed above, and amplified for an additional 30 cycles. Half the reaction was electrophoresed on a 2% agarose gel, and examined for PCR product. Amplification products visible on the gel were electrophoresed on an ABIPRISM® 310 Genetic Analyzer.

Nested PCR of Nuclear Loci

Nested amelogenin primer sets (Sullivan *et al*., 1993) were created based on the amelogenin sequence from Genbank (Figure 4). The external primer pair amplified a 112

bp region in males and a 106 bp region in females. The internal primers, one of which was labeled for capillary electrophoreses, produced 68 bp and 62 bp amplicons.

Figure 4. Amelogenin gene sequence and primer positions



The amelogenin sequence from Genbank for both the X and Y chromosomes. The British Primer set, labeled in blue, was chosen as the external primers for nested PCR. Primers for the second round of amplification were chosen internal to the British primer set (see Table 1). Figure taken from www.cstl.nist.gov/ div831/strbase/jpg_amel.htm.

Voegtly DNAs shown to have amplified using mitochondrial primers were tested. A 1:10 dilution of the DNA was used in the first round of PCR in a 10 µl volume. Cycling parameters were as above, for 30 cycles. One microliter of product was

transferred to a second tube containing the PCR mix and the internal primers in a 20 µl volume. This too was amplified for 30 cycles. Five microliters of the reaction was electrophoresed on a 3% agarose gel and examined for the presence of bands. Samples that had an amplification product were analyzed on a Beckman-Coulter CEQ™ 8000 Genetic Analysis System

Whole Genome Amplification

Five Voegtly femur samples (burials 27F, 114F, 249F, 448F, and 704F) were tested using WGA. A 1:20 dilution of the DNAs was amplified using two techniques, I-PEP (Dietmaier *et al*., 1999) and MDA (Dean *et al*., 2002). I-PEP was performed using Expand High Fidelity PCR system (Roche Applied Sciences) and MDA was performed using a GenomiPhi DNA amplification kit (Amersham Biosciences). The whole genome amplified products were purified using a Microcon-100 column and amplified using mitochondrial DNA primers F16190/R16410, a 220 bp region of HVI. In addition, F155/R484 were used to amplify a 329 bp region of HVII. PCR products were visualized by running 5 µl of the PCR product on a 2% agarose gel. Purified mtDNA amplified DNA was sequenced on a Beckman-Coulter CEQ™ 8000 Genetic Analysis System as detailed above, and sequences compared to those previously obtained.

Soil Analyses

Samples were collected from all Voegtly burials in which soil could either be brushed off skeletal remains or collected from the box where the skeletal remains had been stored. Control soils samples were collected from the Voegtly Cemetery site in early

2004. Descriptions of the soils were generated using particle size analysis. Soils were tested for relevant inorganic compounds, including phosphorous, magnesium, potassium, calcium, beryllium, cobalt, indium, cerium, cerium oxide, bismuth, uranium, berkelium, arsenic, zinc, uranium oxide, lanthanum, and barium, as well as total organic carbon, based on X-ray fluorescence. Calcium, magnesium and potassium levels were determined using ammonium acetate extraction followed by atomic absorption. Extractable phosphorous was isolated using acidic Bray extraction and analyzed by atomic absorption. Additional elemental analysis was conducted using inductively coupled plasma in line with mass spectrometry (ICP-MS). Finally, the pH level of each soil sample was determined using a pH meter with a standard two point calibration.

Statistical Analyses

Statistical analyses were performed in Microsoft Excel. Single factor Analysis of Variance (ANOVA) was used to examine the effect of weathering stage, sex, age, and bone type on the DNA quantity and quality results from the Voegtly bone samples. ANOVA was also used to examine the effect of skeletal weathering on amplification success of multiple bones from a single individual, as well as the effect of skeletal weathering, bone weathering, and bone type on amplicon size. DNA quantities for sex and age statistics were averaged when multiple bones originated from a single individual. Amplification success vs. DNA quantity was examined using a t-test with equal variance assumed. The effect of PCR inhibition during QPCR on spiked samples with and without anti-inhibitory addition (BSA or commercial enhancer) was examined using a two-tailed paired t-test. In all cases results were considered significant at $p<0.05$.

Results

The Voegtly Samples

The Voegtly bone samples tested, bone type, mtDNA QPCR yields, maximum mtDNA amplicon size obtained, and PCR inhibition are detailed in Table 3.

Table 3. Overall DNA quantity and quality results from Voegtly samples

The Voegtly mtDNA quantity and quality results are displayed below. Headings denote burial number, bone type, mtDNA quantity (copies/µl), and the maximum obtainable mtDNA amplicon size. Samples that were not tested for mtDNA quantity are left blank in the mtDNA quantity box. Those samples in which an amplicon was not produced and there was no primer activity are labeled as "inhibited". Samples which did not produce an amplicon but showed primer activity were considered negative and all boxes were left blank. * indicates samples that were processed with a Microcon-YM 100 column.

Burial Number	Bone Type	MtDNA quantity (copies/µl)	107 bp	220 bp	329 bp	Inhibited
27	Femur	76.2		X		
27	Rib	508		X		
30	Femur	64.2		X		
34	Femur	304.3	X*			
34	Pelvis	61.9				
34	Rib	162.2		X		
47	Femur					
47	Pelvis	141.8		X		
47	Rib	122.2		X*		
47	Rib					
111	Femur	67.8	X			
111	Rib	146.5	X			
111	Pelvis		X			
114	Femur	122.9		X		
124	Femur	277.1	X			
124	Pelvis	138.6	X			
124	Rib	117.9		X*		
126	Femur	63.7				

126	Pelvis	14.9				
126	Rib	78.6				
132	Femur	49.2	X			
132	Pelvis	24.2		X*		
132	Rib	66.21	X			
164	Femur	38.9			X	
164	Pelvis	134				
164	Rib	110.9			X	
167	Femur	10.3		X		
167	Pelvis	22.4				
167	Rib	175.4				
192	Femur	101.2		X		
192	Pelvis	53.4		X		
192	Rib	9.5				
203	Cranium				X	
203	Fibula	64.9		X		
203	Rib					
256	Femur					
256	Pelvis					
256	Rib					
260	Femur	31.8	X			
260	Rib	78.8	X			
322	Femur	24	X			
322	Pelvis	69		X		
322	Rib	13.9	X			
328	Femur	34.3	X			
328	Pelvis					X*
328	Rib	71.4		X*		
331	Femur	161	X			
331	Rib	100.2	X			
345	Femur					
345	Pelvis					X*
345	Rib	27			X	
345	Rib				X	
348	Femur	74.7				
348	Pelvis	57.1				
348	Rib	92.7	X			
349	Femur			X		

355	Femur					
355	Rib					
381	Femur	107.1	X			
381	Pelvis	157.4				
381	Rib	11.2	X			
389	Pelvis					
389	Rib					
402	Pelvis					X*
409B	Femur	195.6	X			
409B	Pelvis					
409B	Rib	57.5		X*		
447	Femur			X		
447	Rib			X		
448	Femur	52.1		X		
448	Rib	7.2		X		
489	Pelvis	45.8				
489	Rib					X
529A	Femur	251.4		X		
529A	Pelvis					
529A	Rib	0.6		X		
539	Pelvis					
539	Rib					X*
540	Pelvis	76.9		X		
540	Rib					
545	Femur	55.7	X			
545	Pelvis	56.3				
545	Rib	42		X*		
546	Pelvis			X		
546	Rib					
686	Cranium			X		
704	Femur	468.3		X		
704	Pelvis	566.7	X*			
704	Rib	33.2		X*		

MtDNA Quantity

Skeletal Weathering Stage

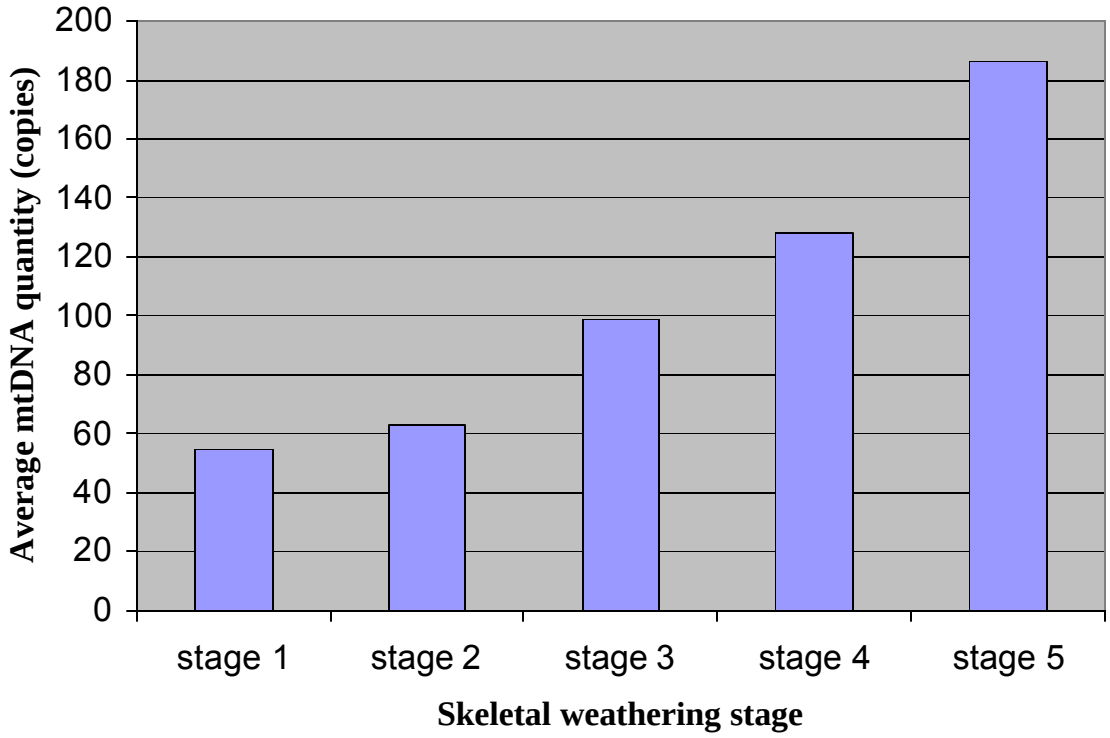
The mtDNA quantity calculated for each sample was categorized by the complete skeletal weathering stage of the Voegtly samples, where a degradation level was assigned to each skeleton. Table 4 gives the average mtDNA quantity values obtained for each skeletal weathering stage. There was a notable trend (Figure 5) between mtDNA quantity and the skeletal weathering stage, with more weathered skeletons producing more mtDNA, and differences among stages were statistically significant (p=0.036).

Table 4. Average mtDNA quantity in each skeletal weathering stage

Skeletal weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	54.7	4
Stage 2	63.0	20
Stage 3	98.6	15
Stage 4	128.3	9
Stage 5	186.4	12

The average mtDNA quantities (in copies/µl) found in each skeletal weathering stage. There were no stage zero bones available for analysis.

Figure 5. Average mtDNA quantity in each skeletal weathering stage



The average mtDNA quantity (given in copies of DNA) is on the y-axis, and the skeletal weathering skeletal stages range from 1 (least weathered) to 5 (most weathered) along the x-axis.

Skeletal Weathering Stage Categorized by Bone Type

The mtDNA quantity was further broken down by bone type (ribs, femora, and pelves) in each skeletal weathering stage (Tables 5, 6, and 7, Figure 6). For all samples, the greatest average DNA quantity was found in stage four or five, and the lowest quantity was found in either stage one or two. The average mtDNA quantity found in ribs increased from stage one to stage two, then decreased in stage three. The quantity then increased from stage three to stage five. The quantity of mtDNA in femora stayed approximately the same between stages one and two, then increased sharply from stage two to three. The quantity increased between stages three and four, then decreased in

stage five. The average mtDNA quantity in pelves decreased between stage one and two, then increased in stages two through five.

Table 5. Average mtDNA quantity in rib samples categorized by skeletal weathering stage

Rib samples categorized by skeletal weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	42.0	1
Stage 2	91.5	7
Stage 3	80.8	6
Stage 4	90.6	4
Stage 5	162.2	4

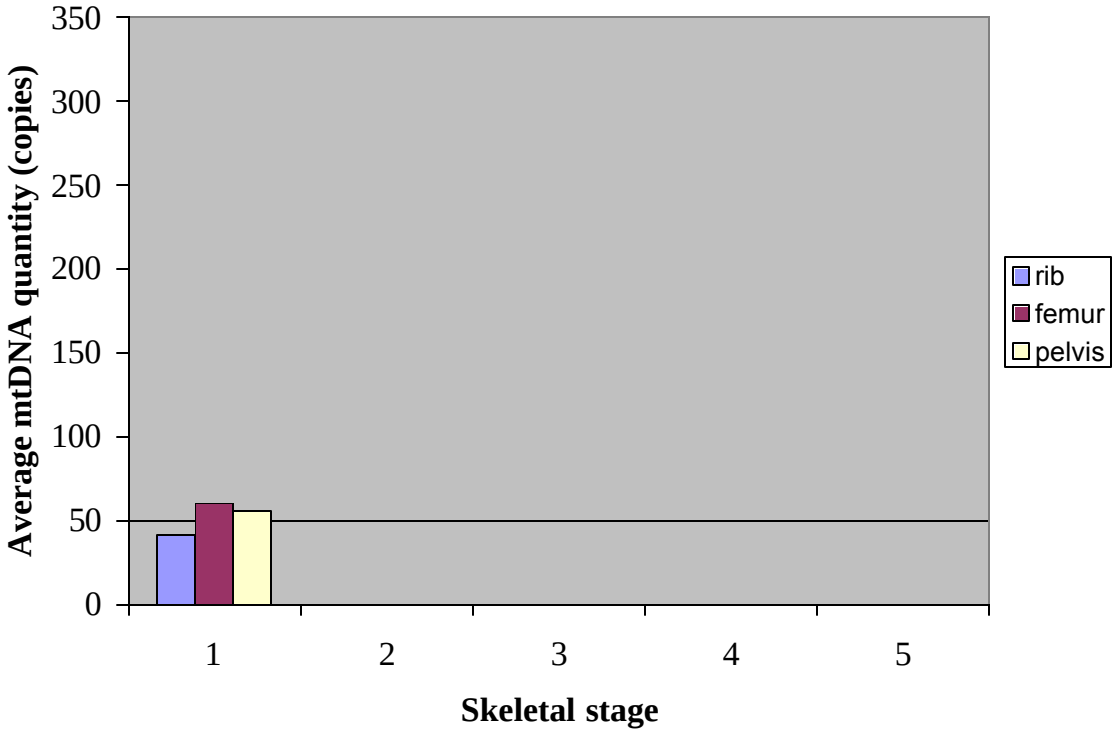
Table 6. Average mtDNA quantity in femur samples categorized by skeletal weathering stage

Femur samples categorized by skeletal weathering stages	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	60.3	2
Stage 2	56.3	7
Stage 3	130.0	5
Stage 4	195.8	3
Stage 5	157.5	6

Table 7. Average mtDNA quantity in pelvic samples categorized by skeletal weathering stage

Pelvic samples categorized by skeletal weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	56.3	1
Stage 2	37.7	6
Stage 3	86.1	4
Stage 4	101.8	2
Stage 5	321.8	2

Figure 6. Average mtDNA quantity in each skeletal weathering stage categorized by bone type



The average mtDNA quantity in each skeletal stage is given on the y-axis, and the skeletal weathering stages range from 1 (least weathered) to 5 (most weathered) along the x-axis. In each stage, the left column portrays rib samples, the middle column femur samples, and the right column pelvic samples.

As is apparent, there is an upward trend in mtDNA yields in relationship to increasing levels of skeletal degradation. However, based on ANOVA there was no significant difference among the stages divided by bone type for ribs, femora, or pelves ($p=0.78$, $p=0.31$, and $p=0.13$ respectively), in part due to the much smaller samples sizes generated by the increasing number of categories.

Individual Bone Weathering Stage

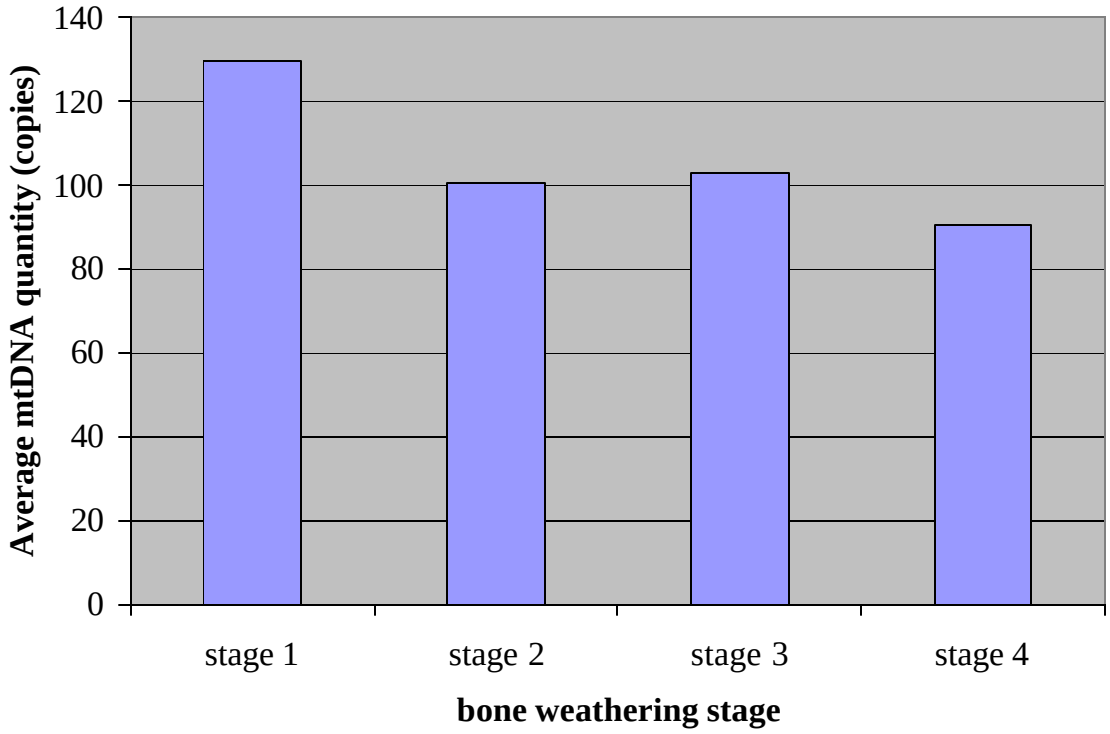
If the level of degradation is predictive of the quality of DNA within the bone, a bone rating system might reveal a correlation that was masked when the whole skeleton rating system was used. A new four stage weathering classification system was created and applied to each bone. Reclassification of individual bones confirmed that the level of degradation of a skeleton was not consistent throughout the skeleton. In general, bones within a skeleton were reclassified into different stages, and only rarely were all three bones classified to the same stage.

MtDNA quantities were calculated based on the weathering level of individual bones (Table 8 and Figure 7). In contrast to skeletal weathering, there was no significant difference in the amount of mtDNA extracted across the bone weathering stages ($p=0.91$).

Table 8. Average mtDNA quantity in each bone weathering stage

Bone weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	129.6	15
Stage 2	100.7	25
Stage 3	103.1	14
Stage 4	90.7	6

Figure 7. Average mtDNA quantity for each bone weathering stage



The average mtDNA quantity (in copies) is on the y-axis, and the bone weathering stages range from 1 (least weathered) to 4 (most weathered) along the x-axis.

Bone Weathering Stage and Bone Type

The mtDNA quantities obtained for each bone type were also categorized into bone weathering stage for analysis (Tables 9, 10, and 11). As can be seen in Figure 8, there is no common trend among the three bone types. However, there were trends within bone types. Ribs showed a general increase in mtDNA yields with advanced weathering, while the opposite was true for pelves. Femora appeared to change little. This was borne out statistically in that there was no significant difference in femora ($p=0.69$), ribs ($p=0.57$), nor pelves ($p=0.053$) although pelves approached significance. This difference was due to one pelvic sample in stage 1 with a very high mtDNA yield. Note that there were no femur or pelvic samples graded as stage 4.

Table 9. Average mtDNA quantity for rib samples categorized by bone weathering stage

Rib samples categorized by bone weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	9.5	1
Stage 2	78.9	7
Stage 3	137.6	8
Stage 4	90.7	6

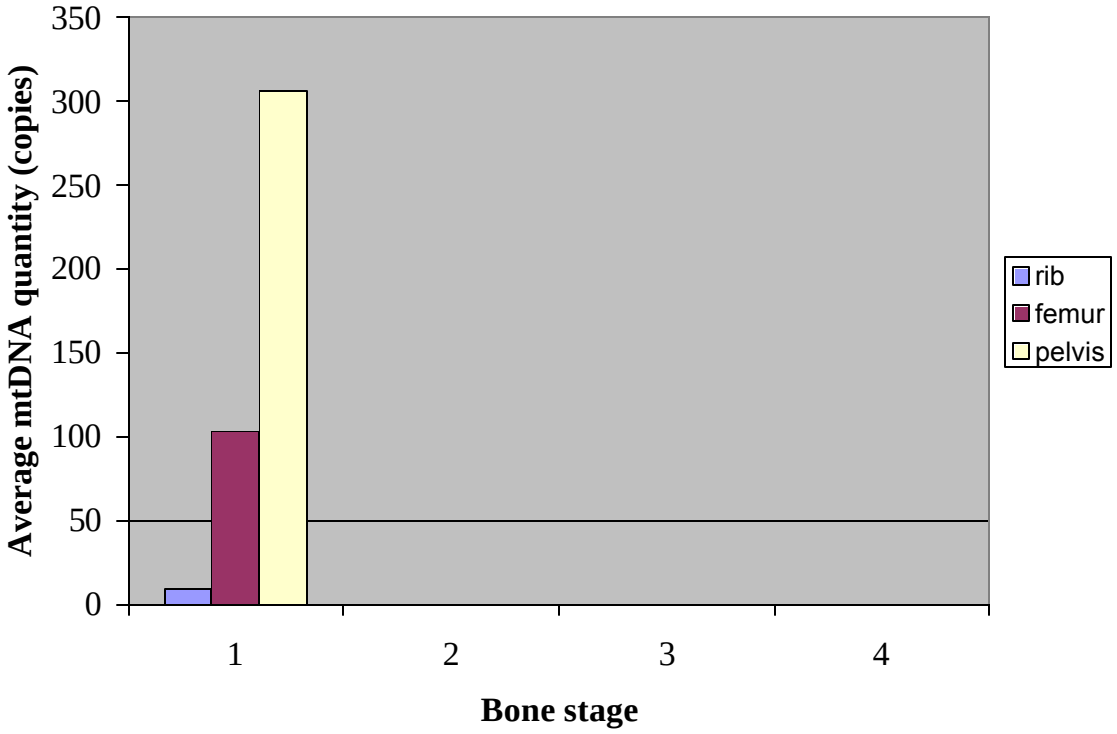
Table 10. Average mtDNA quantity for femur samples categorized by bone weathering stage

Femur samples categorized by bone weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	103.2	12
Stage 2	139.4	10
Stage 3	64.2	1

Table 11. Average mtDNA quantity for pelvic samples categorized by bone weathering stage

Pelvic samples categorized by bone weathering stage	Average mtDNA quantity (copies/µl)	Number of samples
Stage 1	306.3	2
Stage 2	72.9	8
Stage 3	55.6	5

Figure 8. Average mtDNA quantity versus bone stage categorized by bone type



The average mtDNA quantity (in copies) in each bone stage is shown. The bone stages range from 1 (least weathered) to 4 (most weathered) along the x-axis. The high stage 1 results for pelves stemmed from a single high sample. No femur or pelvic samples were rated as stage 4.

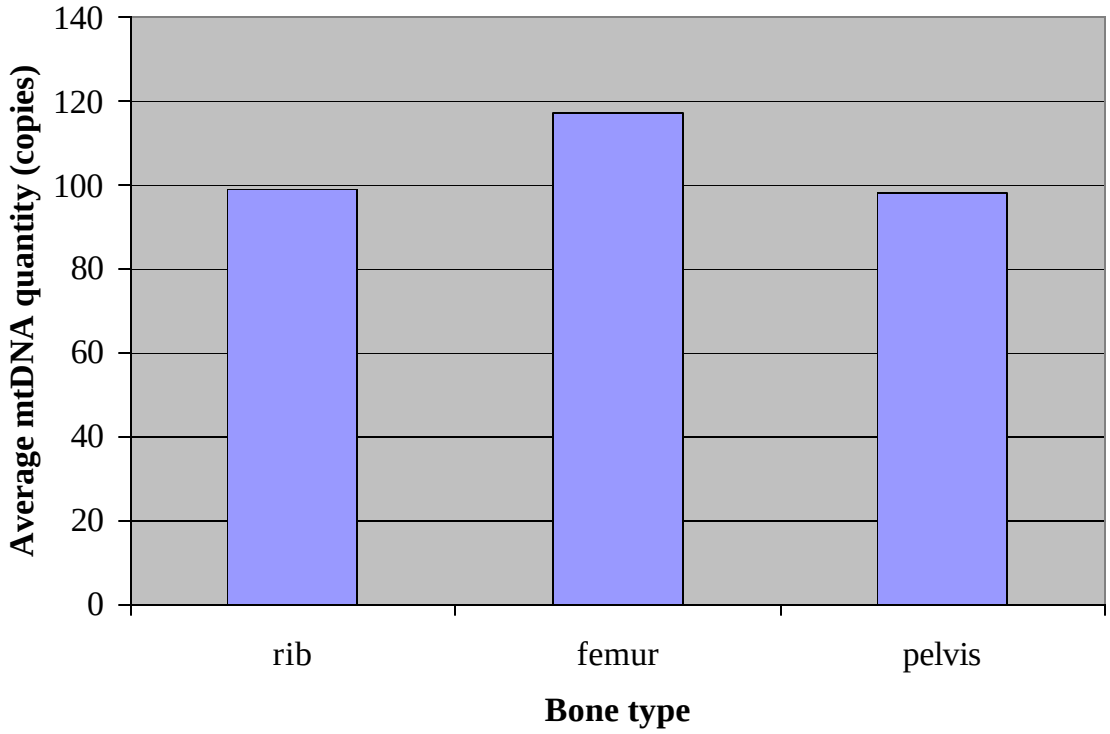
MtDNA Quantity and Bone Type

MtDNA quantity was examined based on bone type (Table 12 and Figure 9). Femora were found to generate the highest mtDNA quantity, while ribs and pelves were quite similar. Statistical analysis showed no difference in the quantity of mtDNA obtained from each bone type ($p=0.84$).

Table 12. Average mtDNA quantity in each bone type

Bone type	Average mtDNA quantity (copies/µl)	Number of samples
Ribs	99.0	22
Femora	117.2	23
Pelves	98.3	15

Figure 9. Average mtDNA quantity versus bone type



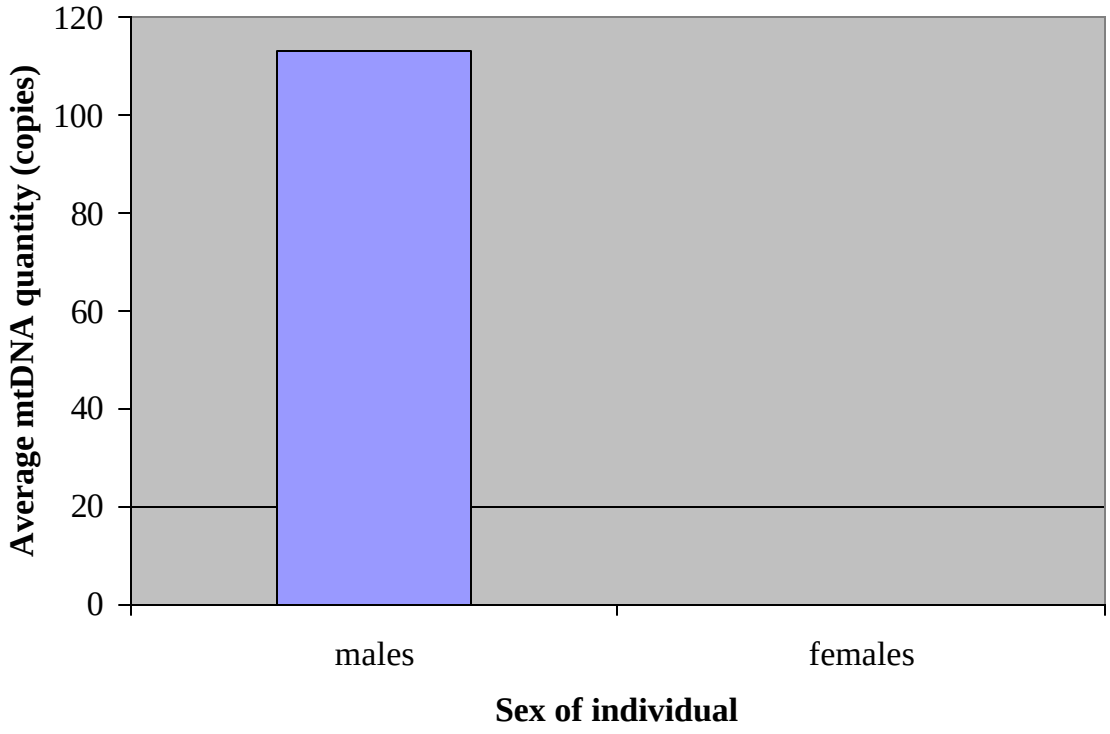
The average mtDNA quantity (in copies) in relation to bone type. MtDNA quantity is on the y-axis, and the bone type is on the x-axis.

Sex Estimates and Average mtDNA Quantities

The average bone mtDNA yield was compared to the individual's sex estimated anthropologically; samples where the sex was not estimated were excluded from these analyses. If there were multiple bone results from an individual, average mtDNA quantity was used. The mtDNA quantity extracted from males (n=17) averaged 113.2 copies/µl,

while the average from females (n=8) was 98.6 copies/µl (Figure 10). There was not a statistical difference in mtDNA quantity between the sexes (p=0.88).

Figure 10. Average mtDNA quantity for each sex



The average quantity of mtDNA for males and females. Sex of the individual is on the x-axis.

Age at Death and DNA Quantity

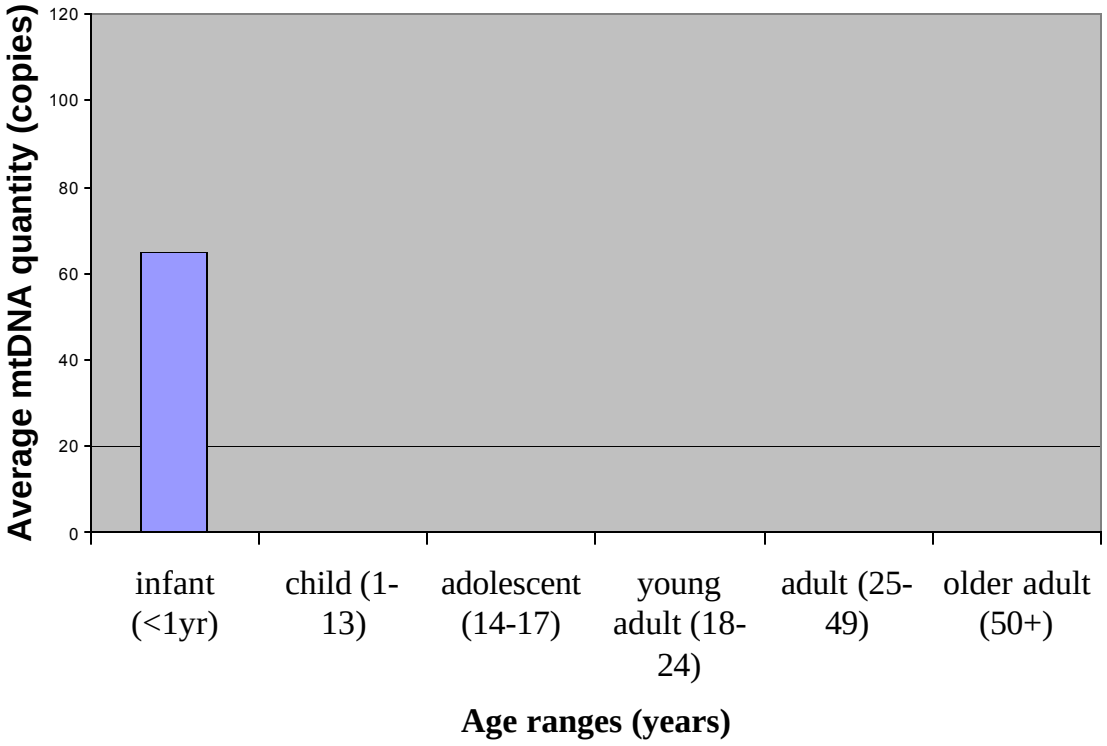
The anthropologically estimated ages at death were put into six categories for analysis: infant (less than 1 year old), child (1–13 years), adolescent (14–17 years), young adult (18–24 years), adult (25–49 years), and older adult (50+ years); mtDNA quantities by age are shown in Table 13 and Figure 11. If there were multiple bone results from an individual, average mtDNA quantity was used. There was no apparent trend in DNA quantity with age, and there was no statistical difference in mtDNA yields from each of

the age ranges (p= 0.97). It should be noted that the multiple categories made samples sizes very small in some cases.

Table 13. Average mtDNA quantity in each age range

Age ranges	Average mtDNA quantity (copies/µl)	Number of samples
Infant (<1 year)	64.9	1
Child (1–13)	108.8	5
Adolescent (14–17)	70.3	2
Young adult (18–24)	106.3	4
Adult (25–49)	103.0	15
Older adult (50+)	54.7	1

Figure 11. Average mtDNA quantity in each age range

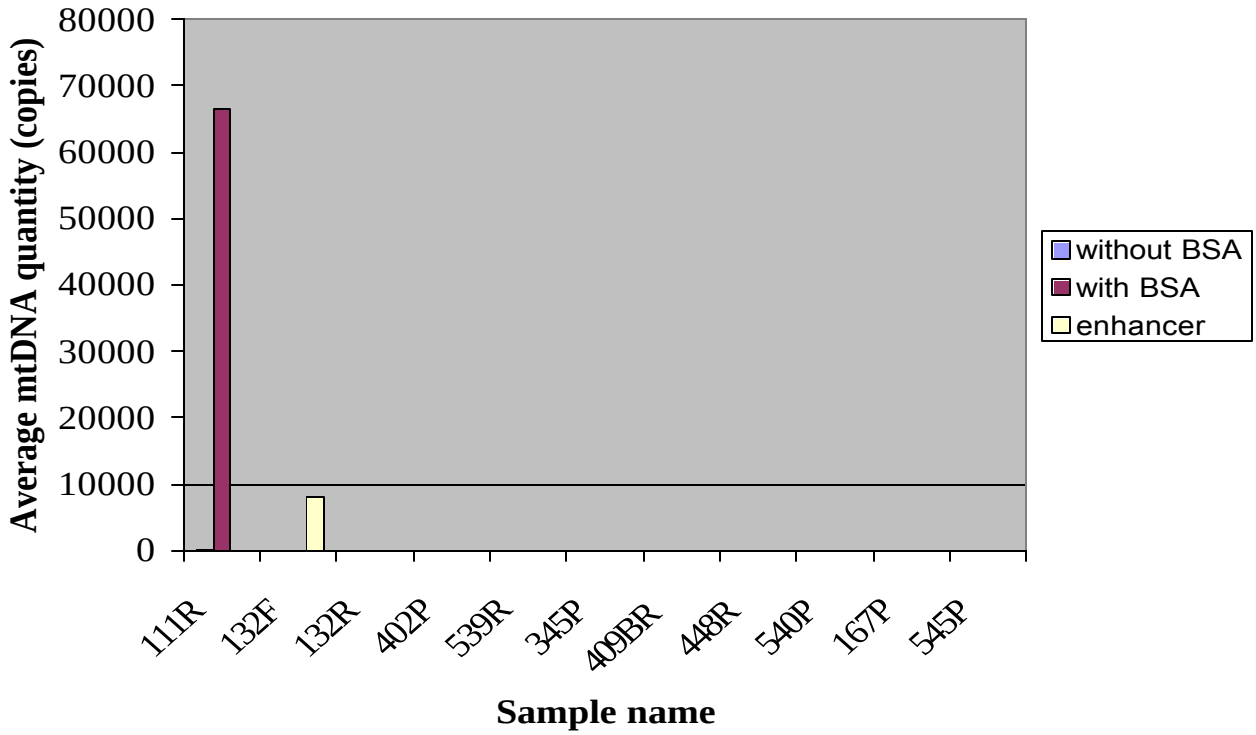


The average quantity of mtDNA for each age range is. The age ranges (in years) are along the x-axis.

The Effects of PCR Inhibition on DNA Quantification

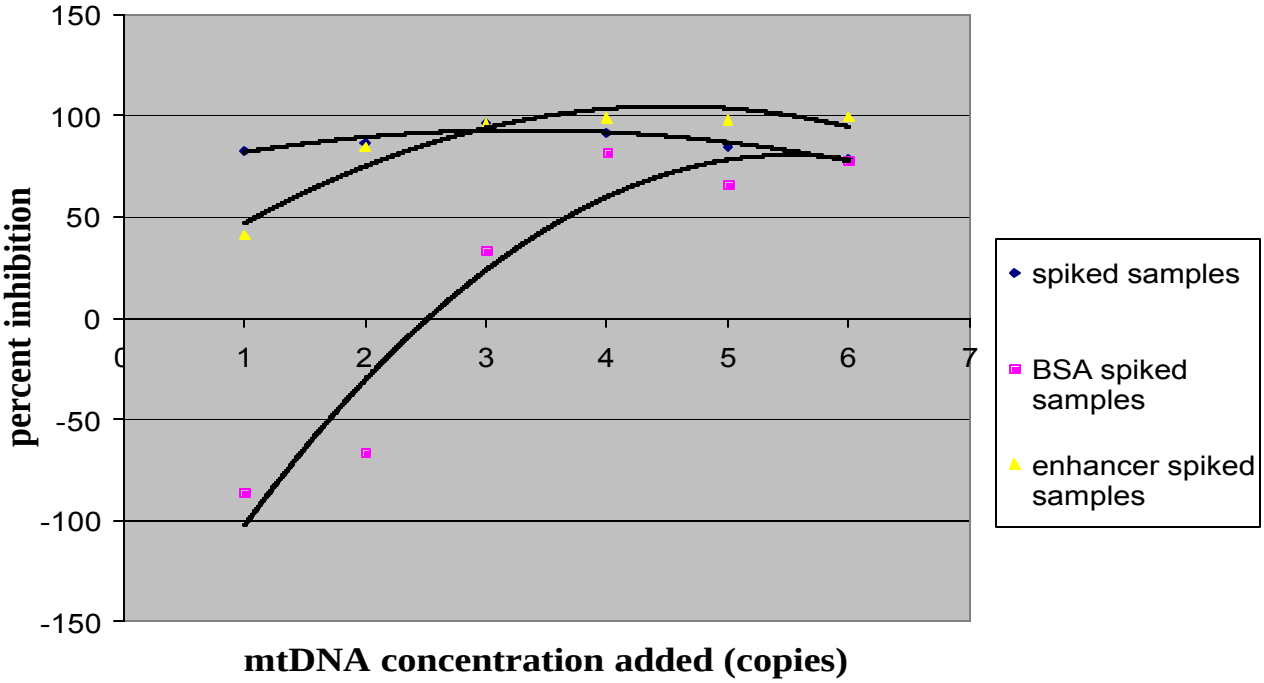
Samples that showed PCR inhibition (based on a lack of primer activity) were analyzed through addition of (spiking with) known amounts of target sequence, using 6,000,000 – 60 copies in 10-fold intervals. The same samples had either BSA or a commercial PCR enhancer added as well. Following QPCR, results among treatments were compared. An example, with 60,000 copies of target DNA added, is shown in Figure 12, with a graph of all results shown in Figure 13. The native samples all showed a high level of PCR inhibition (79% and above), as did the samples with the commercial PCR enhancer, (41% and above, although all but the sample containing 6,000,000 copies of added target were 85% inhibited and above). In contrast, samples with BSA added were not inhibited at high target DNA addition levels, and surprisingly even showed enhanced PCR. At lower target levels samples with BSA were inhibited. There were no statistical differences between native samples and those with the commercial enhancer at any added DNA concentration. Samples with BSA differed significantly from native DNA at all added DNA levels except at 6,000 and 60 copies.

Figure 12. Samples spiked with 60,000 copies of control DNA



An example of QPCR measured inhibition in Voegtly bone samples amplified with and without reagents thought to reduce PCR inhibition. 60,000 copies of the target DNA was added to each sample. Sample name indicates the burial number and bone type (R=rib, F=femur, P=pelvis).

Figure 13. Percent PCR inhibition of bone samples containing known amounts of DNA



Average QPCR results for inhibited bone DNAs spiked with known amounts of target DNA. Spiked samples are numbered 1 – 6 on the X axis, indicating 6,000,000 – 60 copies of target. Percent inhibition ranges from close to 100 at the top of the figure, to excess DNA estimated below 0 on the Y axis for some samples containing BSA.

MtDNA Quality

The Voegtly Samples

Final mtDNA PCR results for the Voegtly Cemetery samples, arranged by burial number and bone type, are shown in Table 3. If amplification was successful, it was noted by the maximum fragment length obtainable. Samples that were subject to Microcon-100 processing are denoted by a star. Thirty-four samples (10 femora, 11 ribs, 6 pelves, 1 fibula, 1 cranium, and the 5 detailed below) produced the 220 bp amplicon, all of which were additionally analyzed using the 329 bp set of primers. Five samples (203

cranium, 164 femur and rib, and 345 rib 1 and 2) produced the 329 bp amplicon. PCR of those five samples with the 402 bp primers was carried out, but negative results were obtained from all. Of the 50 samples that did not produce a 220 bp band, but did not show PCR inhibition, 21 produced a 107 bp amplicon. Samples that had a maximum amplicon of 107 bp included 11 femora, 7 ribs, and three pelves.

When amplification product was available from two or more bones from any one individual, amplicons were sequenced to ensure that the DNA extracted from the different bones originated from the same source. For all bones sequenced from a skeleton, the sequencing results were consistent within an individual. In no instance was incongruent mtDNA sequence derived from bones from a single burial (Table 14).

Table 14. Mt DNA sequence in skeletons generating PCR products from more than one bone

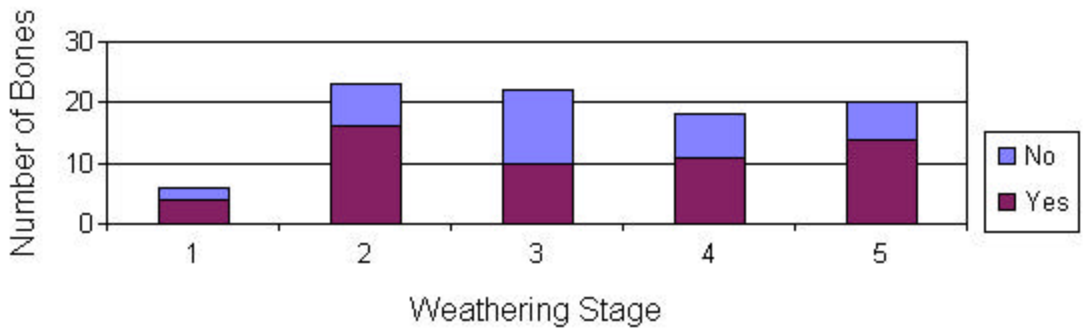
Burial Number and Bone Type	Sequence Interval	Sequence Polymorphism
27 femur F rib F/R	16121-16232 16272-16376	 16340C
164 femur F/R rib F/R	16173-16216 16247-16385	 16267T
192 femur F/R pelvis F	16167-16390	None
203 fibula F/R cranium F/R	16167-16388	16270T
447 femur F/R rib F/R	16169-16260 16299-16378	 16248T, 16340C
448 femur F rib R	16177-16382	None
529A femur F/R rib F/R	16169-16387	None
704 femur F/R rib F/R	16186-16378	None

The sequence interval available for comparison between at least two bones of an individual, with differences from the human mtDNA reference sequence noted. F=forward, R=reverse sequence.

Skeletal Weathering Stage

Amplification results broken down by skeletal weathering stage are displayed in Figure 14 and show that there was no obvious relationship between weathering stage and amplification success. Bones from stage five, the most weathered, amplified the most often at 70% (14 out of 20), followed by stage two at 69.57% (16 out of 23), stage one at 66.67% (4 out of 6), and stage four at 61.11% (11 out of 18). Samples from stage three amplified the least often, at 45.45% (10 out of 22). The difference in amplification among all weathering stage categories was not significant (p=0.460).

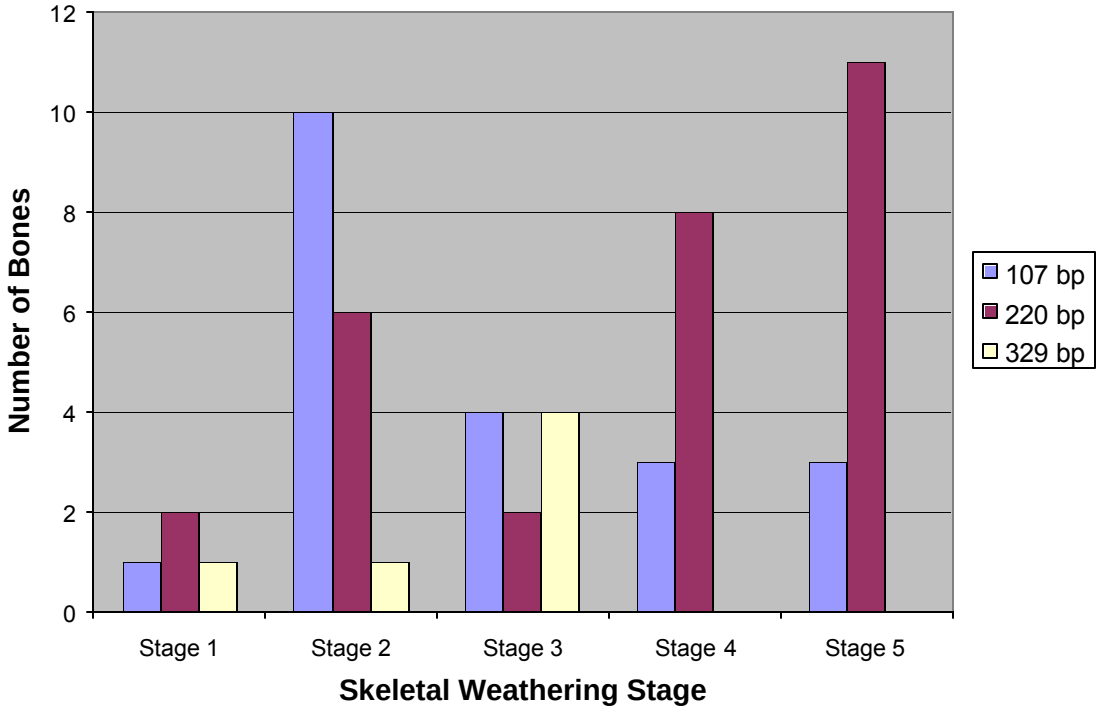
Figure 14. Amplification results in comparison to skeletal weathering stage



Graph depicting amplification success (yes) and failure (no) in relationship to the five skeletal weathering stages.

Amplification results were broken down further to examine if skeletal weathering had any influence on amplicon size. For each skeletal weathering stage, bones were categorized by the maximum amplicon length that was amplified. When compared across skeletal weathering stages, Figure 15 shows no relationship between stage and the maximum amplicon length. However, the largest amplicon produced, 329 bp, was only seen in the first three weathering stages. When compared, there was no significant difference between the size of the amplicon produced and skeletal weathering stage ($p=0.128$).

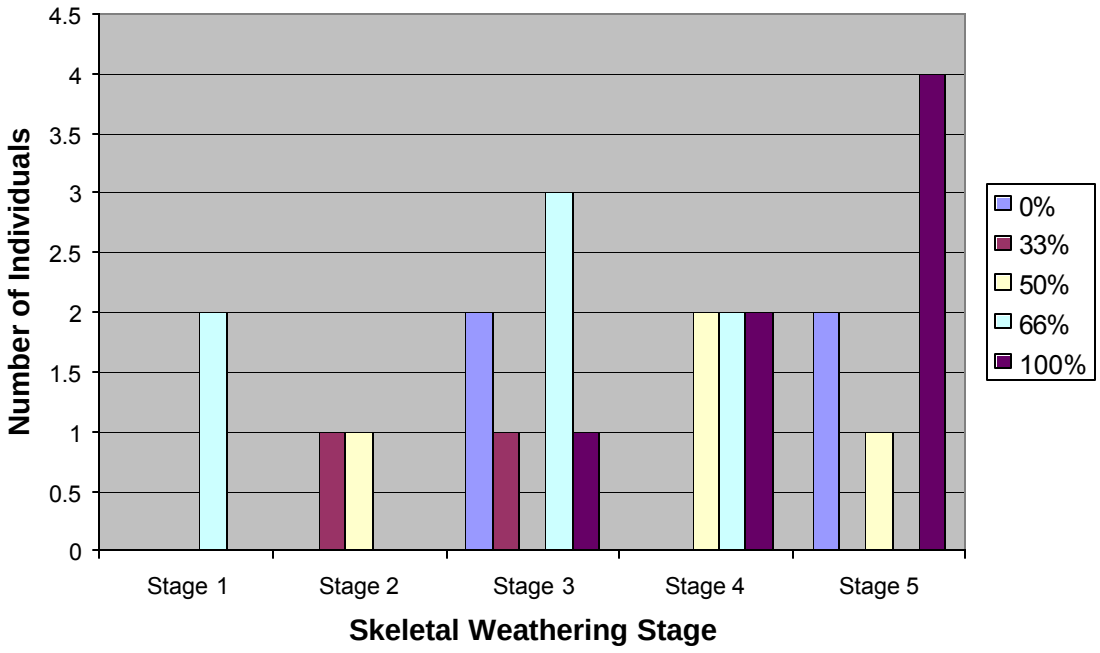
Figure 15. Maximum obtainable amplicon produced for each skeletal weathering stage



Graph depicting maximum amplicon length for bones at each skeletal weathering stage.

Most skeletons from the Voegtly cemetery had more than one bone available for analysis, thus it was interesting to note if less weathered skeletons were more likely to have DNA from multiple bones amplify. Figure 16 displays the amplification success of these at each weathering stage. Amplification was determined in terms of the percentage of bone DNAs that amplified from a single individual (0%, 33%, 50%, 66% or 100%). There was no significant difference between how often multiple bones from a single individual could be amplified and weathering stage ($p=0.679$).

Figure 16. DNA amplification rate of multiple bones from individuals at each weathering stage



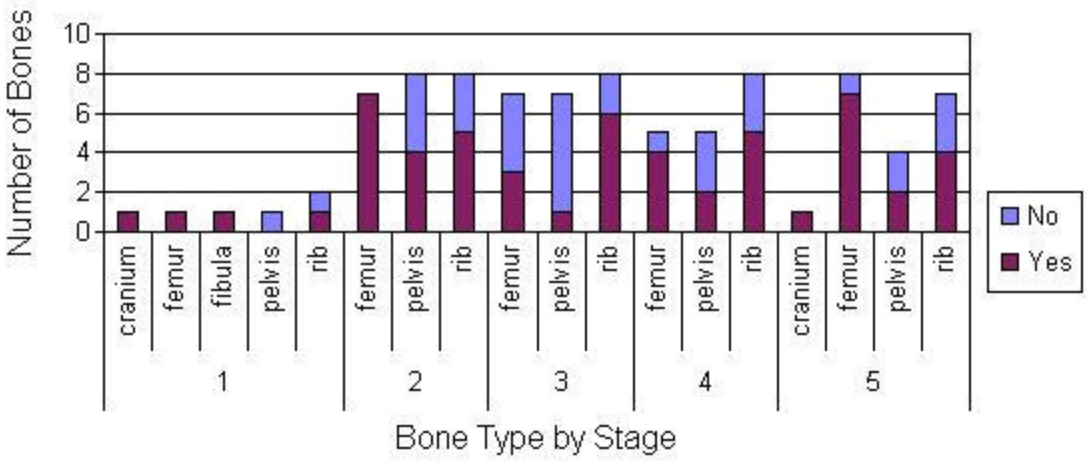
Graph depicting the success of bone DNA amplification for individuals with more than one bone available for analysis.

Skeletal Weathering Stage Categorized by Bone Type

To incorporate the potential influence of specific bone types on amplification success, each skeletal weathering stage was further divided by bone type. Amplification success is similar across the five weathering stages, with pelvis and rib samples amplifying the least often at stage one (0 out of 1 and 1 out of 2, respectively), while femur samples amplifying the least often at stage three (42.9%, 3 out of 7). There was no significant difference in the pelvis, rib, or femur data among the five stages ($p = 0.604$, $p = 0.956$, and $p = 0.076$, respectively). This further indicates that staging based on skeletal weathering does not aid in the prediction of the quality of DNA that can be extracted

from the bone, although due to the large number of categories, samples sizes became quite small.

Figure 17. Amplification results in comparison to skeletal weathering stage and bone type

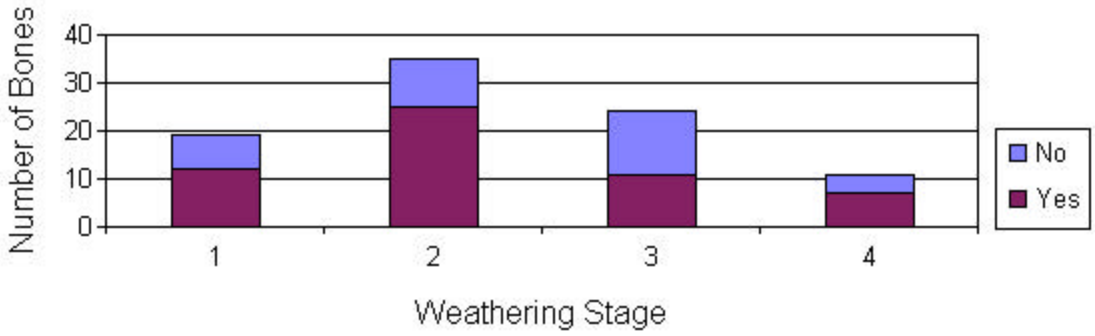


Graph depicting amplification success (yes) and failure (no) arranged by bone type within each of the five skeletal weathering stages.

Individual Bone Weathering Stage

Figure 18 shows amplification results based on the individual bone weathering criteria in which each bone was staged individually. Amplification success across the stages (without considering bone type) was similar, with the highest at stage two (71.4%, 25 out of 35) followed by stage four (63.6%, 7 out of 11), stage one (63.2%, 12 out of 19), and stage three (45.8%, 11 out of 24). Statistical analysis comparing all four weathering stages showed the variance in amplification success across the stages is not significantly different ($p=0.269$).

Figure 18. Amplification results in comparison to individual bone weathering stage

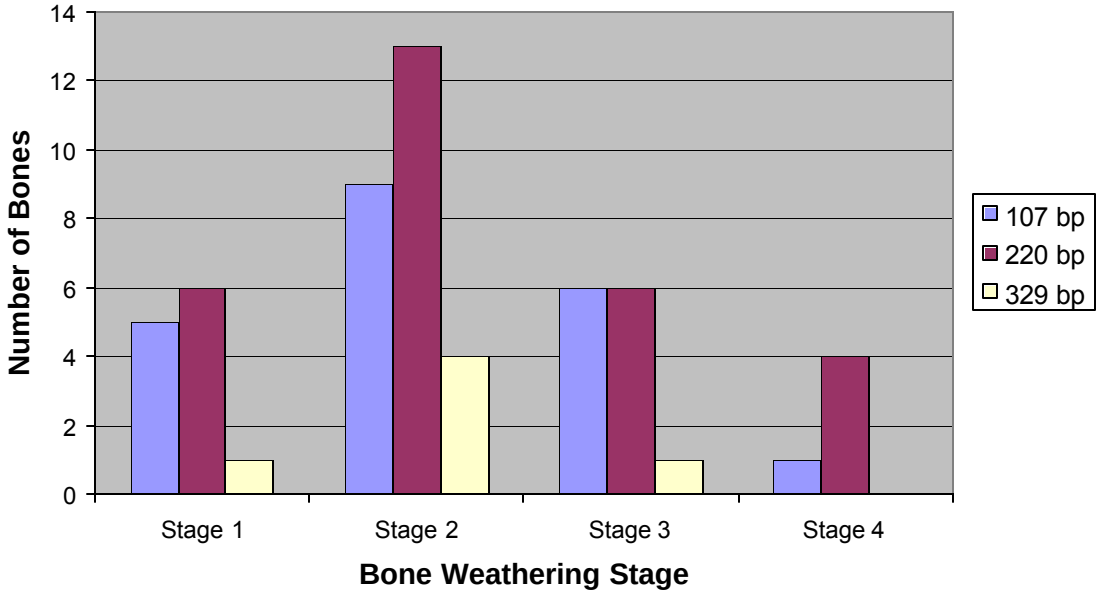


Graph depicting amplification success (yes) and failure (no) in relation to the four individual bone weathering stages.

It should be noted that as a consequence of reclassification, the sample sizes across the four weathering stages was not equal. Because of this unequal sampling, differences among the stages may be hard to uncover, particularly among the most weathered bones. Regardless, the data do not appear to follow a trend and indicate that individual bone weathering condition is not predictive of DNA quality.

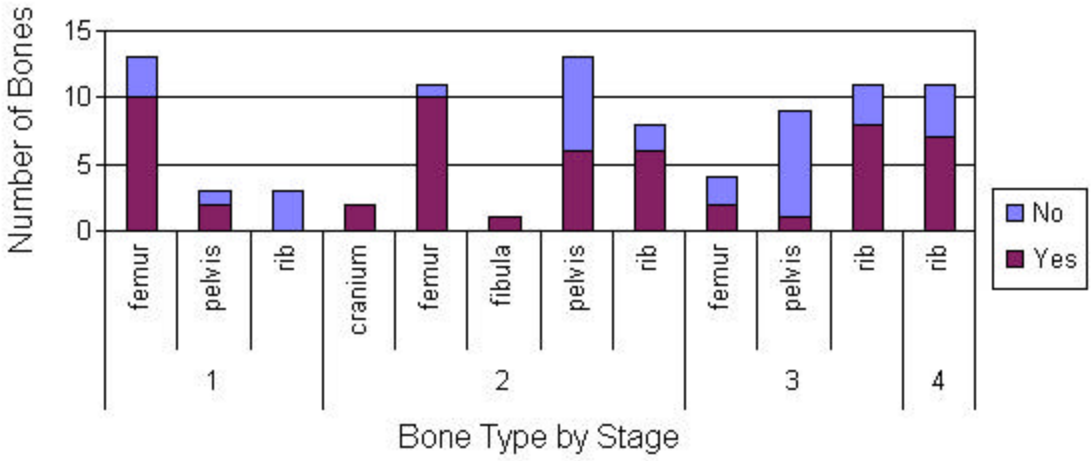
Amplification results were broken down further to examine if individual bone weathering had any influence on maximum amplicon size. Figure 19 shows no apparent relationship between bone weathering stage and the maximum amplicon length ($p=0.929$).

Figure 19. Maximum obtainable amplicon produced for each bone weathering stage



Amplification success of the different bone types varied across the four weathering stages (Figure 20), however the differences were not significant. For example, stage two femora had the highest amplification success at 90.9% (10 out of 11), followed by those at stage one at 76.9% (10 out of 13), and stage three at 50% (2 out of 4) (p=0.213). The pelvic samples showed a decrease in amplification success from stage one to stage three (as with femora, none of the pelvic samples were categorized as stage four) (66.7%, 2 out of 3; 46.2%, 6 out of 13; and 11.1%, 1 out of 9 for stages one, two, and three, respectively) (p=0.130). Rib samples had the highest amplification success at stage two (75%, 6 out of 8), followed by stage three (72.7%, 8 out of 11), stage four (63.6%, 7 out of 11), and stage one (0%, 0 out of 3) (p=0.111).

Figure 20. Amplification results in comparison to individual bone weathering stage and bone type

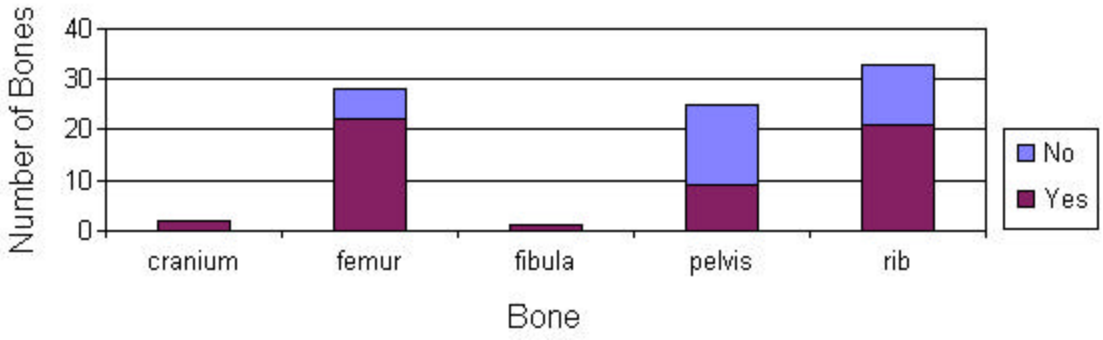


Graph depicting amplification success (yes) and failure (no) arranged by bone type within each of the four individual bone weathering stages.

MtDNA Quality and Bone Type

The strongest influence on amplification success appeared to originate from bone type. The effect of bone type was analyzed on its own, showing a strong difference in amplification success among pelves, femora, and ribs. As shown in Figure 21, amplification occurred in the 2 crania samples (100%), 79.3% (23 out of 29) of the femur/fibula samples, 36% (9 out of 25) of the pelvis samples, and 63.6% (21 out of 33) of the rib samples.

Figure 21. Amplification results in comparison to bone type

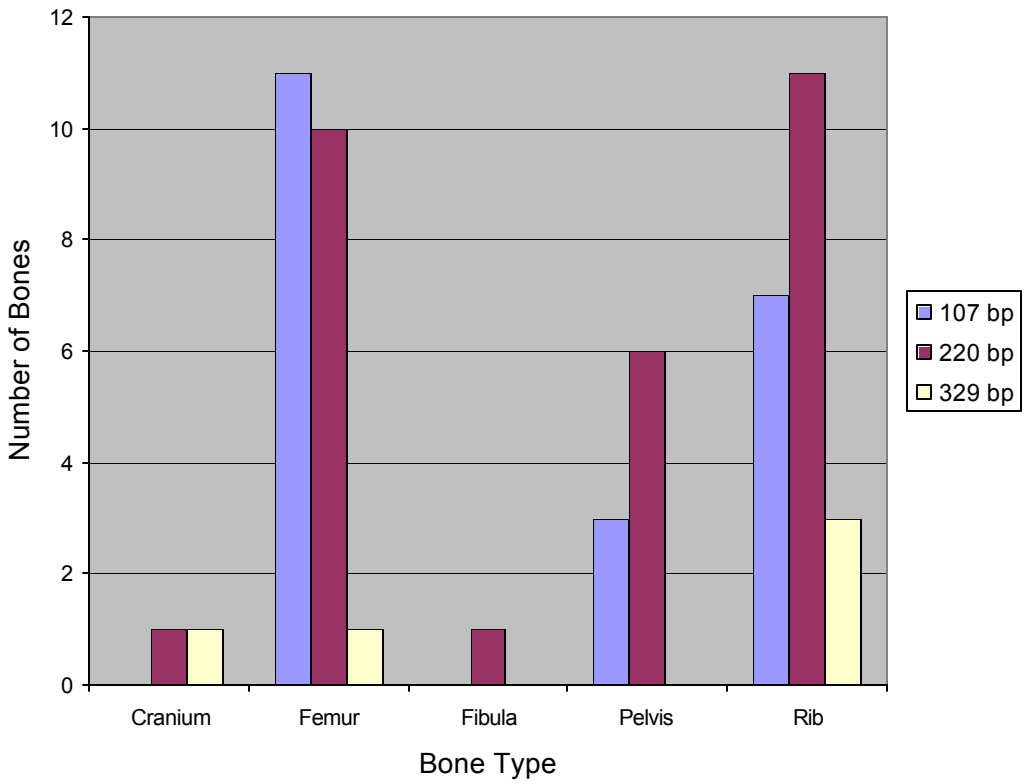


Graph depicting amplification success (yes) and failure (no) arranged by bone type.

Across bone types (excluding crania and fibula with their small sample sizes) there is strong statistical evidence ($p=0.006$) for a difference in amplification success, with the greatest difference between femora and pelves ($p=0.0009$), followed by ribs and pelves ($p=0.037$). There is no significant difference between ribs and femora ($p=0.181$).

In addition to amplification success for bones type, amplification was further broken down into largest amplicon size obtained for each bone type (Figure 22). Although femora had the overall highest amplification success rate, in many instances these were the smaller 107 bp amplicons. However, there is no significant difference between the maximum obtainable amplicon and bone type ($p=0.44$). Once again, crania and fibula were not included in the statistical analysis due to the small sample sizes.

Figure 22. Maximum obtainable amplicon in comparison to bone type



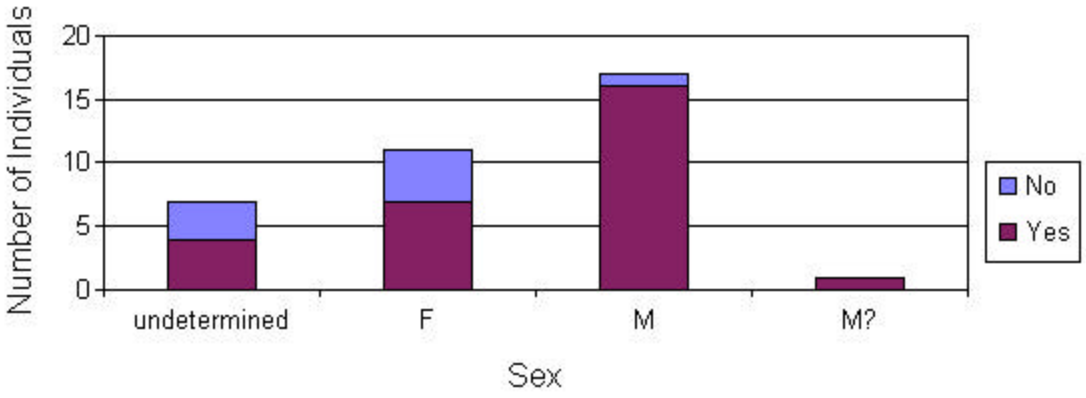
The largest amplicon size by bone is displayed. Note the far great number of femur, pelvis, and rib samples, which were the focus of this study.

Sex Estimates and mtDNA Quality

Amplification results are shown in Figure 23 by the four sex categories (female, male, "possible male", and undetermined). Amplification occurred in 57.1% (4 out of 7) of the undetermined individuals, 63.6% (7 out of 11) of the females, and 94.1% (16 out of 17) of the males. The one individual classified as "possibly male" (M?) also amplified. There was no significant difference in amplification success among the four categories ($p=0.120$). However, removing the undetermined individuals from the analysis revealed a significant difference in amplification success between males and females ($p=0.041$),

with male samples amplifying more often. Adding the "possible male" to the male category dropped the p-value to 0.034.

Figure 23. Amplification results in comparison to sex of the individual

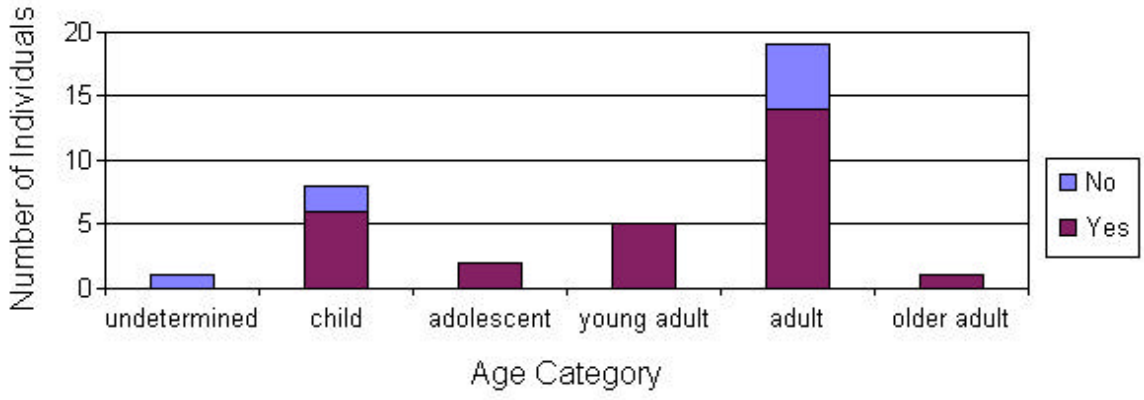


Graph depicting amplification success (yes) and failure (no) in relation to the anthropologically estimated sex of the individual. Four categories were used to describe the individuals sampled in this study: undetermined (sex of the individual could not be determined), female (F), male (M), and possible male (M?).

Age at Death and DNA Quality

The effect of estimated age of the individual on amplification success is displayed in Figure 24. There was no significant difference in the amplification success across the five age categories (p=0.748). The amplification results of each age category are as follows: 6 out of 8 (75%) children, 2 out of 2 (100%) adolescents, 5 out of 5 (100%) young adults, 14 out of 19 (73.7%) adults, and 1 out of 1 (100%) older adult amplified. The age of one individual (from burial number 389) was not estimated at the time of recovery and therefore is not included in the statistical analysis.

Figure 24. Amplification results in comparison to estimated age



Graph depicts amplification success (yes) and failure (no) of each bone sampled in relation to the five age categories. The age of one individual was not estimated.

The Relationship Between DNA Quantity vs. DNA Quality

The ability/inability to amplify mtDNA (regardless of size) and DNA quantity from the Voegtly samples was examined. The mean quantity of mtDNA obtained from samples that were successfully amplified for sequencing was 114.9 copies/µl while the mean quantity from samples not amplified was 73.2 copies/µl. These did not differ statistically ($p = 0.125$) although this was likely due to the large variance in the quantitative data.

Voegtly Teeth Samples

Amplification and sequencing success was compared between the root and crown portions of tooth samples. Success rate using standard PCR was extremely low, thus a nested PCR procedure was used. When crown samples were amplified using semi-nested PCR, 30 out of the 31 were successfully amplified. Root samples showed the same results for amplification success, with 30 out of the 31 samples being amplified. For the crown samples, 26 of the thirty samples were successfully sequenced, while 24 out of 30 root

samples could be sequenced. There was no relationship between skeletal weathering stage and tooth amplification success rates. It must be remembered that tooth appearance bore little relationship to overall skeletal appearance.

Albania Bone Samples

Bone Type and Amplification Success

Eight anthropologically sexed females, seven males, and one child whose sex could not be determined were analyzed. Among these 16 skeletons, DNA isolation was attempted from 62 different bones. These included 1 clavicle, 15 femora, 6 humeri, 11 petrous portions, 1 mandible, 1 metacarpal, 4 radii, 2 scapulae, 1 skull portion (non-petrous), 5 tibiae, 13 teeth, and 2 ulnae. Femur samples were divided equally between females and males, the mandible belonged to a male skeleton, the metacarpal to a female skeleton, the radii were all from males, while the ulnae were from female skeletons.

A total of 52 bones (84%) produced DNA product that was successfully sequenced, while 10 (16%) did not. Of the 12 bone types, 6 produced sequences from all individuals, including clavicle, humerus, mandible, metacarpal, radius and scapula. The clavicle, mandible, metacarpal, radius, and scapula all had sample sizes of 4 or less. The femur, petrous, and tooth bones had sample sizes of 15, 11, and 13 respectively. The femur was next most successful at 87%; the petrous at 82% and the tibia at 80%. Seventy-eight percent of the teeth and 50% of the ulnae produced sequence, while the non-petrous skull portion resulted in none. Taking sample size into consideration, femora, petrous portions, and teeth were all bones that could reliably be amplified and sequenced.

Bone Integrity and Amplification Success

Albania bones were given an integrity rating based on appearance. Bones rated 3 produced the highest amplification rate; out of the 27 analyzed, 85% were successfully amplified and sequenced. This success was followed closely by a bone rating of 1 (84%), 2 (82%), and 4 (80%). However, the sample size of bones rated 4 was quite small with only five bones being analyzed. As with the Voegtly samples, there are no visible trends between the different bone integrity ratings and amplification/sequencing success.

Sex Estimates and Amplification Success

Sexual disparity between sequence production in HVI were investigated. Femur, humerus, petrous, tibia, and tooth were collected from both anthropologically estimated male and female skeletons. No trends were seen, in that femur and tibia both produced sequences slightly more often from female skeletons, while humerus and petrous produced sequence more often from males. Teeth produced similar results between the sexes.

Enhanced Techniques

Mini-STR Sets

The Mini-STR sets used for nuclear DNA analysis, both for CODIS loci obtained from NIST and the amelogenin primers designed in house, did not produce profiles for any loci tested (but see nested results below).

Nested PCR

Nested PCR of mtDNA was successful on the Voegtly tooth samples (see above). For nuclear DNA, 65 Voegtly samples that produced mtDNA results were analyzed using the newly designed primers for nested amelogenin amplification. Twelve produced an amelogenin profile. Likewise, 4 of 17 Albania bone samples produced profiles.

Two of the Voegtly bones that were successfully amplified using the amelogenin nested PCR technique were then tested with the Mini-STR sets. These samples were initially amplified for 40 cycles using the individual primer sets, along with mini A1, but no bands were present on a 2% agarose gel. Nested PCR was then attempted beginning with the Identifiler™ primer set followed by the Mini-STR sets. No amplification products were observed on a 2% gel. For more information on these experiments see Ramos, 2005.

Whole Genome Amplification

The 220 bp mtDNA amplification that was successfully conducted on Voegtly bones acted as the basis for assaying PCR following WGA. Five samples were tested (Table 15). Of the five I-PEP samples, one was successfully PCR amplified based on agarose gel electrophoresis. All PCR products following MDA WGA were unusable due to a high number of non-target bands on the gel. These results pointed to the general reduction in DNA size following WGA (see Discussion). For more information on these procedures see Barber, 2005.

Table 15: Amplification of mtDNA from whole genome amplified samples

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

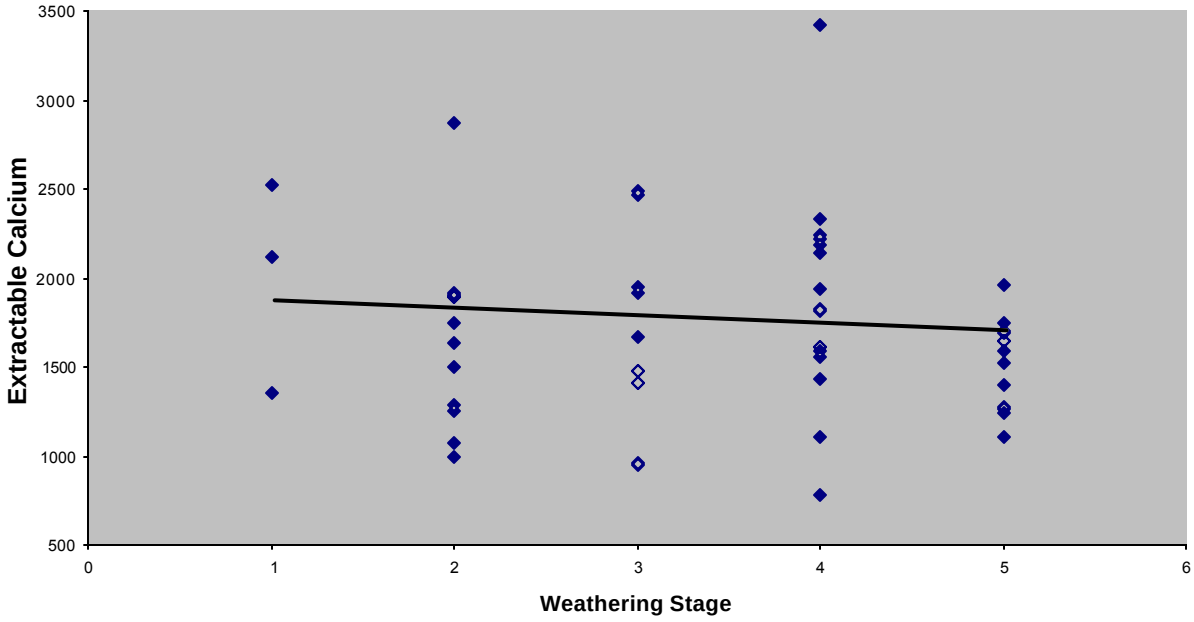
Treatment type is displayed on the top of each column. * indicates data were obtained from Misner (2004), + indicates successful amplification, - indicates amplification failure, [+] indicates sample amplification was successful, but multiple nonspecific bands were present.

Soil Analysis from the Voegtly Cemetery

The soil from all Voegtly burials were from the clay loam textural class, or were a combination of clay loam and sandy dry loam. The soil samples from the burials contained nearly equal amounts of sand, clay, and silt; specifically the soils were composed of approximately 30 – 40 % sand, 30 – 35% clay and 25 – 35% silt.

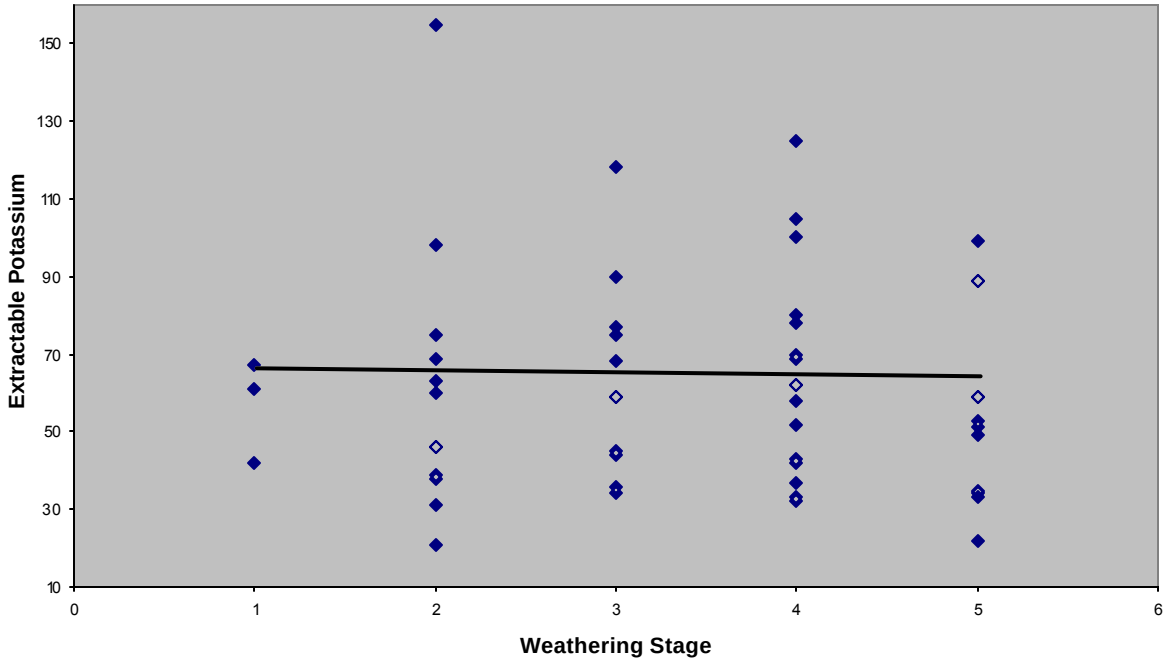
The soil collected from each burial was tested for levels of several inorganic and organic compounds, as well as soil pH (see Methods). Each soil element was compared to the weathering stage assigned by Ubelaker and Jones (2003). The only notable correlation between skeletal weathering and any of the factors tested was with the pH of the soil. In this case, skeletal weathering was more extensive at lower pHs, with an R^2 value of 0.2064.

Figure 25. Skeletal weathering stage and the amount of extractable calcium from soil from Voegtly burials



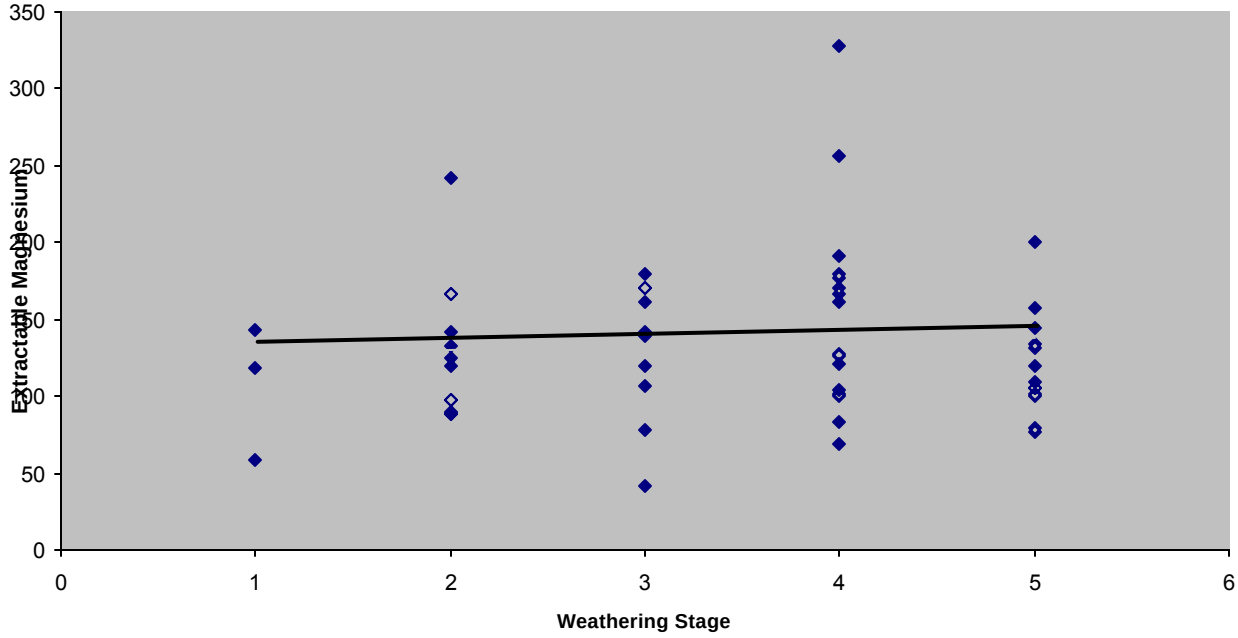
Graph depicting the relationship between skeletal weathering stage and the amount of calcium found in soil from the Voegtly burials. The two factors have an R^2 value of 0.0108.

Figure 26. Skeletal weathering stage and the amount of extractable potassium from soil from Voegtly burials



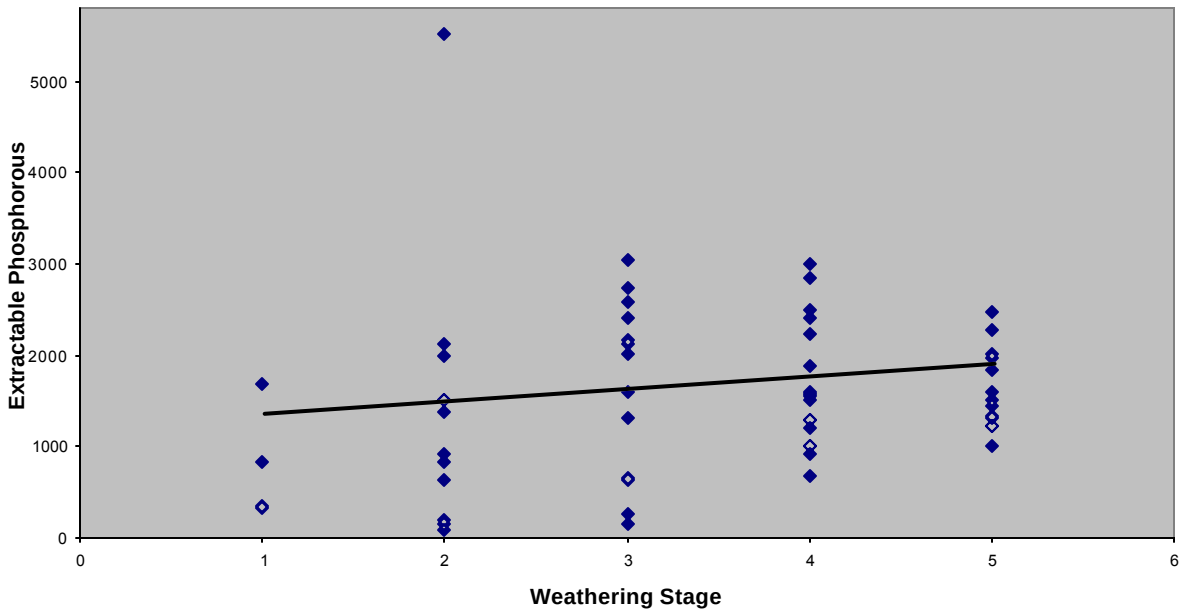
Graph depicting the relationship between skeletal weathering stage and the amount of potassium found in soil from the Voegtly burials. The two factors have an R^2 value of 0.0007.

Figure 27. Skeletal weathering stage and the amount of extractable magnesium from soil from Voegtly burials



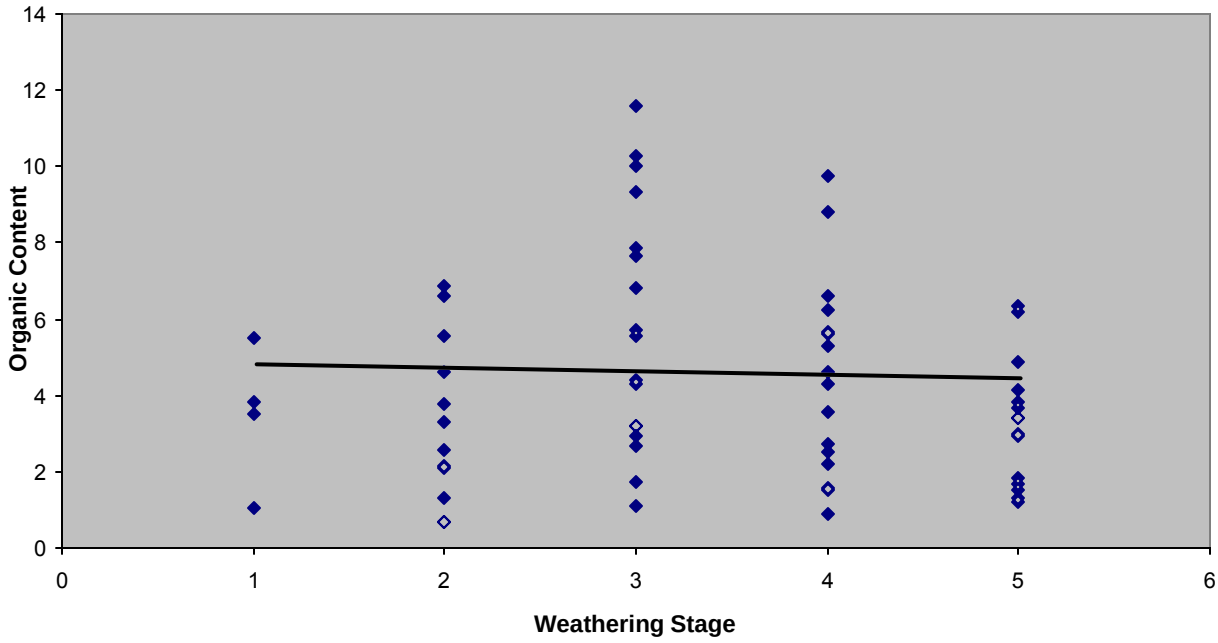
Graph depicting the relationship between skeletal weathering stage and the amount of magnesium found in soil from Voegtly burials. The two factors have an R^2 value of 0.0033.

Figure 28. Skeletal weathering stage and the amount of extractable phosphorus from soil from Voegtly burials



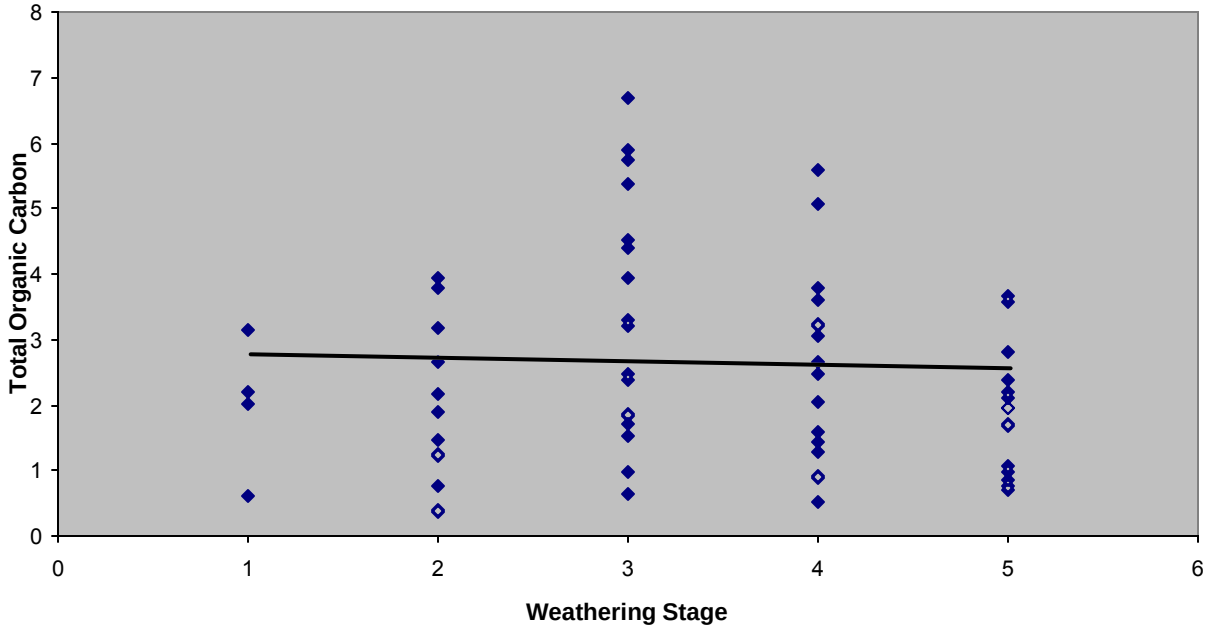
Graph depicting the relationship between skeletal weathering stage and the amount of phosphorus found in soil from Voegtly burials. The two factors have an R^2 value of 0.0335.

Figure 29. Skeletal weathering stage and the organic content from soil from Voegtly burials



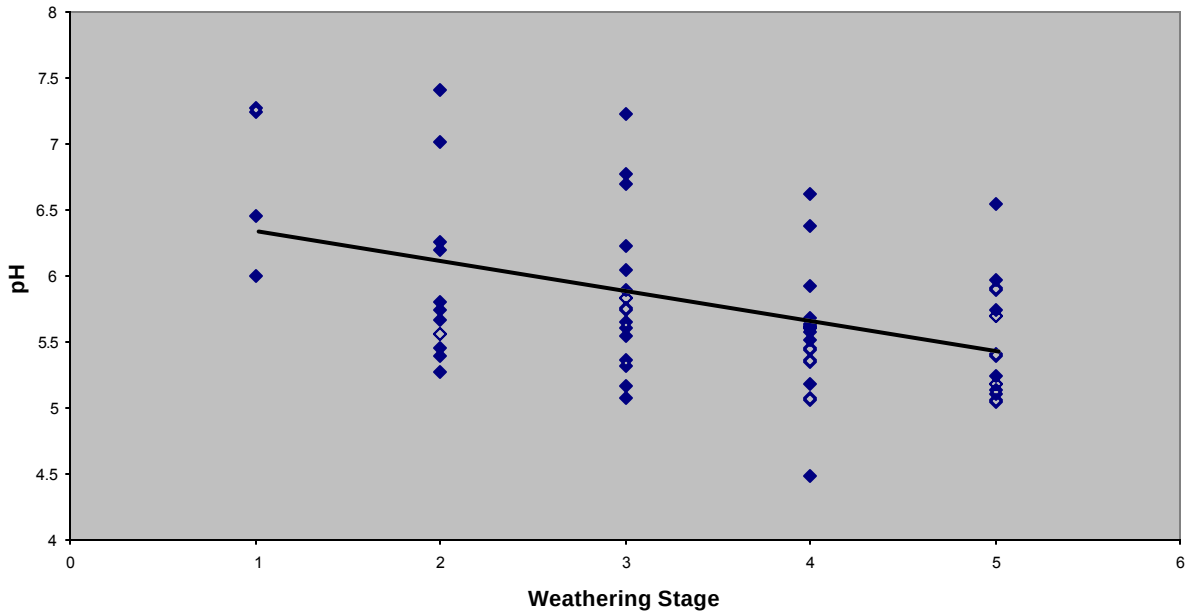
Graph depicting the relationship between skeletal weathering stage and the organic content found in soil from Voegtly burials. The two factors have an R^2 value of 0.0018.

Figure 30. Skeletal weathering stage and the amount of extractable organic carbon from soil from Voegtly burials



Graph depicting the relationship between skeletal weathering stage and the amount of organic carbon found in soil from Voegtly burials. The two factors have an R^2 value of 0.0018.

Figure 31. Skeletal weathering stage and pH level of soil from Voegtly burials



Graph depicting the relationship between skeletal weathering stage and the pH level of the soil from Voegtly burials. The two factors have an R^2 value of 0.2064.

Discussion

The goal of this study was to determine if there is a relationship between the outward appearance of skeletal material (considering bone type and level of weathering) and the quantity and quality of the DNA within. Being able to predict the amount of obtainable DNA and its level of degradation has the potential to assist the forensic scientist in providing a more efficient and productive analysis of skeletal material. If the scientist can examine skeletal material visually and make a reasonable assessment of what sized fragment of DNA is likely to be successfully amplified, as well as how many PCR cycles may be required to generate analyzable product, both time and resources will be conserved. Likewise, if given an assortment of skeletal material, to know which is most likely to harbor analyzable DNA would also be useful. Further, if other variables, such as the anthropologically estimated age or sex of the remains have an influence on DNA results, these too would be worth knowing, as could being aware of soil characteristics from which the remains were recovered. Finally, if knowledge about skeletal remains can help predict when standard forensic DNA testing is likely to fail, while advanced techniques may prove successful, then identifications might be made that would otherwise be impossible.

The research detailed above ties together a large number of separate studies, perhaps eight in all, although even this number may expand with work being done on cellular features of the skeletal material. Key to this was having a standard set of skeletal remains to work with. In this regard the Voegtly Cemetery material was ideal: it provided a large number of skeletons from which different bones could be examined, that were located at a common site for a similar amount of time, thus removing environmental

variables except at the micro-habitat level. Yet the Voegtly material displayed a remarkable variety of weathering, ranging from complete and rather sturdy skeletons to small bone fragments that could easily be crumbled by hand (indeed many burials in the Voegtly Church were never found). Access to such a large number of skeletons of common burial age and environment generated a robust dataset and a number of interesting findings.

Skeletal Weathering and DNA Quantity and Quality

First among these findings was the relationship between the weathering stage of a skeleton and the DNA found within. Intuitively one would expect good bone to yield good DNA, and highly degraded bone to little or no DNA. In contrast not only was such a relationship not found over the course of this research, the complete skeleton data point towards the opposite—more weathered samples generated more DNA ($p=0.036$). Likewise the most weathered skeletons had the highest PCR success rate (DNA quality), although these differences were not as strong as DNA quantity. In contrast, there was no correlation between skeletal weathering and the largest amplicon that could be generated, nor with the number of bones from an individual that produced amplifiable DNA.

The reason for the increase in DNA yield with increased skeletal weathering is not clear (and deserves further study) but several possibilities exist. The first is that DNA is simply easier to recover from degraded bone. There is little known on the cellular or sub-cellular level about how DNA, and particularly mtDNA, is stored or degrades in bone. A very reasonable hypothesis is that bone that is quick to crumble is also quick to release any DNA it contains, while bone that is firm will hold that DNA.

A second possibility is that whatever process acted to keep some bones from the Voegtly cemetery in relatively good shape (less weathered) was detrimental to DNA. Among the many chemicals that were tested from the soil, none were found to correlate greatly with skeletal preservation. Calcium levels were slightly higher among more preserved samples, although it does not seem likely that this would negatively impact DNA. Soil acidity did show a correlation with skeletal preservation, with more degraded remains found at lower pHs. Low pH is known to create nicks in DNA, however given the lowest ph found here (above 5) this may not have an influence on DNA while still affecting bone. In turn, at the higher pHs more conducive to bone preservations it is possible that microbial activity or other factors that degrade DNA could be more active. In that sense two related but different factors might be acting on the skeletons and their DNA, with low pH aiding in the degradation of bone, while more neutral pH allowing factors to degrade DNA.

The overall condition of a skeleton was the primary grading criterion used on the Voegtly remains, as it was conducted first by the anthropologists who characterized the remains. However, during the course of this study it became apparent that skeletal staging and the condition of an individual femur, rib, pelvis, etc., did not go hand-in-hand. This might be due to sampling bias, as workers at a site are likely to (and perhaps logically forced to) collect bones that are in the best condition. If all the ribs but one are in very poor shape and disintegrate upon handling, the single good rib will be collected, leading perhaps to a difference between skeletal staging and bone staging. (This should not affect forensic analyses tremendously, as the same would be true of any discovered remains, however it might bias the results obtained here.) Rarely in this study were individual

restaged bones given the same classification as whole skeletons, and often different bones from a single skeleton did not fall into the same weathering category. In the long run this did not seem to matter however; when the DNA yields results were compared to restaged bones, no significant correlation was seen. Further, there was no correlation between bone staging and the maximum amplicon size produced. Once again the appearance of a bone was not a good indicator of the DNA within.

The Influence of Bone Type

In contrast, the type of bone tested did seem to have an influence on both mtDNA quantity and DNA typing success. Amplification attempts among long bones, flat bones, and ribs, showed that long bones (femora and fibula) produced both the most DNA and the highest successful PCR rate (teeth are not considered here, as they required nested PCR for typing, a technique not widely used in crime labs currently and not directly comparable to the other bone preparations). These were followed by rib samples, and lastly by pelves. Pelves contained similar quantities of DNA as ribs, however their PCR success rate was much lower (36 vs. 64%). This PCR success differences among bone type was highly significant ($p=0.006$). Note that amplicon size was not influenced by bone type, in that femoral DNA often amplified at the smallest size class. Regardless, from these results it becomes clear that bone type is an important factor when considering samples to analyze.

Because bone type had a significant influence on successful mtDNA amplification as well as the amount of DNA present, it was interesting to note the correlation between the two of these. Indeed the average copy number for samples that amplified was 115

while for those that did not amplify it was 73 (this result was not significant due largely to the high level of variance in the quantitative data). However, there was no 'cutoff' for successful amplification; samples with the lowest copy number could be amplified, meaning that quantitative PCR results will likely not be a good indicator of downstream PCR success.

The Influence of Sex and Age at Death

Age of an individual at death did not seem to influence the quantity or quality of DNA recovered. One might expect that the very young, with poorly developed bones, would have increased bone/DNA degradation. This may be true, as few infant remains were recovered (see Introduction and History). Older individuals might also be expected to have poorer bone strength, but here again very few elderly individuals were recovered. Among other age groups no relationship was seen.

Sex did have an effect on DNA quality (though not quantity). While this result was significant, it should be viewed with come caution. The skeletal remains were often poorly preserved, and the pelvis, which is the best tool for sexing a skeleton, was frequently degraded. Sex was then estimated based on overall skeletal robusticity, a far weaker criterion. Also, a disproportionate number of estimated males was recovered. It is possible that this group of people, of southern German/Swiss descent, was particularly large, and that female skeletons were miscalled. It is also possible that males did preserve better, causing their bones to be recovered more often. However, experiments involving the amelogenin locus (see below) indicate some called male skeletons are likely female.

PCR Inhibition

Another factor that could readily influence both quantitative experiments and PCR amplification is inhibition. PCR inhibition was seen in several of the bone samples amplified for sequencing. In many instance it could be overcome with the addition of BSA or a commercial PCR enhancer, or through the use of a Microcon column. However, because standard PCR is not quantitative, it was impossible to tell if these positive samples still had some level of PCR inhibition, or if they were amplifying like pure DNA.

It then became of interest to understand how PCR inhibition might be influencing the results obtained. A series of eleven bone samples known to be inhibited from earlier experiments were tested using QPCR, with the addition of known amounts of the same DNA used for the quantitative standards (from 6 million to 60 copies as 10-fold dilution). If inhibition occurred in a 'standard way' (e.g., there was 20% inhibition) one would expect the spiked bone samples to all generate quantitative values approximately 20% lower than anticipated, with some correction for the bone DNA present. On the other hand, it is possible inhibition might be 'overcome' with the addition of large levels of DNA while having its greatest influence at the very dilute DNA levels expected in skeletal remains. The same experiment was then conducted in the presence of BSA or the enhancer, and the average percent inhibition for all eleven samples was determined for each quantity of spiked standard.

The results from these experiments were quite surprising (Figure 13). The samples without BSA or enhancer were inhibited at high levels at all spiked concentrations, from 79 – 96%. Addition of the enhancer did little to decrease inhibition,

except at the most concentrated DNA level (6,000,000 copies added), where inhibition dropped to 41%. In contrast, BSA addition dramatically affected amplification, again most notable at the highest concentrations. At the two highest concentrations the quantification results were actually substantially higher than what was added, at 186% and 166%. At lower concentrations inhibition occurred and reached a level similar to untreated samples. The reason for this phenomenon is not directly apparent, and it is possible multiple phenomena are occurring. Certainly the samples are inhibited and this inhibition occurs regardless of spiked DNA quantity added. If addition of BSA simply binds up factors that would otherwise bind to and inhibit Taq, it seems it would work at all DNA concentrations, not only at the high concentrations seen in Figure 13.

In contrast, if an inhibitor binds to DNA it may be possible to out compete it with additional DNA. For instance, the hydroxyapatite found in bone binds DNA and could inhibit PCR. Adding thousands or hundreds of thousands of copies of target DNA could make numerous copies available for PCR. However, if this was solely the case, all samples with high amounts of DNA, not just those with BSA, should amplify. Thus, it seems as if a combination of factors are causing PCR inhibition in these samples, and that both BSA and high DNA quantity are required to overcome it. We are still left to wonder why the high DNA/BSA samples produced quantitative results surpassing the amount the samples were spiked with. Certainly the QPCR inhibition results open up new paths for research.

Other Skeletal Material Analyzed

The other skeletal samples utilized in this research, including teeth from the Voegtly Cemetery and skeletal materials from the Tumulus at Kamenica, Albania, were used to provide supporting information for the Voegtly burials. Teeth, generally molars, from the Voegtly graves did not follow the weathering scheme used for whole skeletons, as most were solid without caries. These were divided into root and crown to test if there were differences in PCR results between the two. It was not possible to obtain PCR products from these samples without using nested PCR, making them difficult to compare to the other skeletal remains discussed above, where this procedure was not used. No difference was found between root and crown amplification success.

The Albania tumulus bones were far older than the Voegtly material (2500 – 3000 year old vs. ca 150 years old). While many of the skeletons appeared to be in excellent condition (Figure 3), most of the bones fell apart during collection. In many instances pelves of the Albania skeletons had disintegrated, meaning this bone could not be compared to the Voegtly results. However, long bones and teeth were collected, as was the petrous portion of the temporal bone, the hardest bone in the human skeleton. Each of these bone types produced DNA results at a frequency similar to their counterparts in the Voegtly Cemetery. For instance, femora from Voegtly successfully produced PCR product 79% of the time, while the Albania samples resulted in product 87% of the time. The one important bone that was impossible to compare was the petrous, as these were not collected from the Voegtly remains. For the Albania samples the petrous led to PCR product 82% of the time, and should be considered when skeletal remains undergo DNA testing. The disadvantage of the petrous is that it is located on the inside of the skull and

for practical purposes is impossible to reach if the skull is not open. Obviously in a forensic case it would be undesirable to damage an intact skull. On the other hand if skull sections are one of or the only bones available, the petrous may be the best target available. It would be interesting to conduct a complete study on skull bones and DNA typing success.

Enhanced Techniques

Three enhanced techniques were conducted during the study of the Voegtly material. While these were not part of the original proposal, because the Voegtly bones already had DNA extracted it seemed worthwhile to examine the effectiveness of each method on DNA know to be in limited quantity and/or degraded. The first of these, using nuclear DNA primer pairs that reduced amplicon size (both the Mini-STRs from NIST and amelogenin primers designed in house), had little effect on amplification success. Naturally this may simply have been due to a lack of DNA. When the amelogenin primers were used in nested experiments, with the first round of amplification producing amplicons just over 100 bp and the second round producing products approximately 70 bp, over 20% of the ancient remains produced a profile, showing the potential of this technique. However, when an Identifiler™ kit was used in combination with the NIST Mini-STRs, no products were produced. This is most likely due to the larger starting size of the Identifiler™ amplicons.

Finally, two methods of WGA were tested on the Voegtly samples. This work was part of a larger study on WGA, thus only a small sample of bones was tested. The findings, which are concordant with other degraded samples we have tested, is that WGA

is not a functional technique when DNA is highly fragmented. This finding makes sense in that all methods of WGA will inherently shorten DNA as it is amplified, as the primers are unlikely to sit at the very end of the fragment they will replicate. Therefore, with each round of WGA the targets will become shorter and shorter. For instance, if a target strand of DNA is 400 bp in length, a primer may anneal at any point in the sequence. While some products may be larger and some may be smaller, the average amplified DNA will be approximately 200 bp. This 200 bp product will likely be shortened in the next round, etc. Methods of WGA will plainly need to be redesigned if they are to be used on degraded DNA.

Conclusions and Recommendations

The research presented here leads to a number of conclusions. 1) The overall appearance (level of weathering) of a skeleton is not likely to be useful in estimating if DNA testing will be possible. Likewise, the appearance of an individual bone does not make a worthwhile metric for subsequent DNA testing success. In this sense attempting DNA typing on skeletons/bones that appear to be in very poor shape may be prudent. 2) Bone type can offer substantial guidance as to the likelihood of successfully obtaining analyzable DNA. Long bone, teeth, and petrous portions, and to a lesser extent ribs, make good targets, while spongy bone does not. 3) DNA quantity, while related to PCR results, cannot act as a hard and fast assay for downstream PCR success; bones with the lowest level of DNA were capable of generating results. 4) DNA quality does not relate to bone/skeletal appearance; mtDNA at all stages was generally degraded down to sizes of about 200 bp. Attempts to produce amplicons larger than this were rarely successful,

and if the forensic scientist is attempting to amplify larger regions, consideration should be given to assaying smaller segments of DNA. 5) Nested PCR can be a powerful tool when dealing with samples that contain very little DNA. Naturally, extreme caution needs to be used given the sensitivity of the technique, with appropriate blanks and negative controls run each time, and results replicated at a separate time. However it was only with this technique that we were able to obtain results from a number of samples.

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Acknowledgements

This project was supported under award 2002-IJ-CX-K016 from the National Institute of Justice. Points of view in the document are those of the authors and do not necessarily represent the official view of the U.S. Department of Justice. We would like to gratefully acknowledge the Office of the Vice President for Research and Graduate Studies, the Graduate School, and the School of Criminal Justice at Michigan State University for support. We would also like to gratefully acknowledge collaborators Dr. Doug Ubelaker of The Smithsonian Institution and Dr. Todd Fenton from Michigan State University. The study involved the joint effort of the following individuals: Dr. David Foran, PI, Dr. Doug Ubelaker (The Smithsonian Institution), Dr. Todd Fenton (Michigan State University, Department of Anthropology), Lisa Misner, Andrea Halvorson, Virginia Clemmer, Lisa Ramos, Amy Barber, Rebecca Elk, Timothy Lange, Michael Mutolo, and Melissa Meyers (Forensic Science Graduate Program, Michigan State University).