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Skeletal Weathering and mtDNA Isolation:
A Study of Dental Remains

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SKELETAL WEATHERING AND mtDNA ISOLATION: A STUDY OF DENTAL REMAINS

By

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ABSTRACT

SKELETAL WEATHERING AND mtDNA ISOLATION:
A STUDY OF DENTAL REMAINS

By

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One of the most important aspects of a forensic investigation is the identification of the deceased. Many identification techniques cannot be used to establish identity when all that remains is skeletal material. In these situations, investigators often turn to DNA evidence. This study analyzed 31 adult teeth from Voegtly Cemetery to determine which area of the tooth, crown or root, provided the best source of mtDNA. Another objective was to determine if the weathering stage of the tooth was related to the likelihood of obtaining a DNA profile. DNA was amplified using semi-nested PCR and 220bps were sequenced. There was no difference in the success of amplification or sequencing between DNA extracted from the crown or the root. Skeletal weathering was not related to mtDNA amplification or sequencing success. Sequence data can be obtained from a single tooth using powder from small, drilled holes, leaving much of the sample intact.

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Images in this thesis are presented in color.

INTRODUCTION

One of the most important aspects of a forensic case involving a deceased individual is their positive identification. First, identification allows investigators to reach a better understanding of possible causes of death based on the life history of the victim (Fisher, 2004). Second, identification is important to allow friends and family members the opportunity to make funeral arrangements and begin the grieving process. Third, many legal procedures, such as benefits from will and life insurance payments, cannot be carried out until a death certificate is obtained (Wisconsin Department of Health and Family Services, 2007, section 9). Finally, when the death is determined to have resulted from foul play, prosecution of the suspect can be very difficult if the identify of the victim has not been established.

Identification should be relatively straightforward when the deceased is discovered at home or in a hospital. When a victim is found without any identification, friends or family members are asked to visually identify the individual. If the body has been disfigured through decomposition or mutilation, it may be more difficult to discern the identity of the deceased. Fingerprints can be used for identification when visual recognition has not been successful (De Forest *et al.* 1983). In order to use this technique, antemortem fingerprints must be available; however, most people do not have a fingerprint card on file with the authorities. When the victim's body has been burned, either accidentally as in a house fire or deliberately to disguise or destroy evidence, and in situations when the hands and fingers have been removed, lost, or have decomposed, fingerprints may no longer be available. Anthropological methods of identification use estimations of sex, race, age, and stature to generate a biological profile of the deceased.

Distinguishing characteristics of the skeletal material (pathologies, anomalies, previous traumas, etc.) combined with the biological profile and the use of radiography, dental records, skull photo superimposition, and facial reconstruction allow the anthropologist to determine identification (Klepinger, 2006). However, these methods, like the use of fingerprints, can be hindered when x-rays or medical records are not available or when a single bone fragment is all that is recovered. In these instances, investigators often turn to DNA analysis to determine identification.

Nuclear DNA (nDNA) can be used to positively identify an individual because it is unique to each person (except for identical twins). A complete nDNA profile can often be recovered from blood or semen collected at a crime scene and analyzed to identify the victim and/or the perpetrator. DNA extracted from skeletal material has also been used to determine identity. In fact, the goal of the Armed Forces DNA Identification Laboratory (AFDIL) is to identify the skeletal remains of all those lost in conflict since the start of World War II, even when all that is recovered is a "handful of teeth and bone fragments" (Edson *et al.* 2004).

Researchers have shown that DNA can be extracted from ancient bone (Hagelberg and Sykes, 1989) and teeth (Woodward *et al.* 1994). Schwartz *et al.* (1991) obtained high molecular weight DNA from teeth subjected to different environmental conditions using samples obtained from oral surgeons. The researchers examined what effects sample age (time spent at room temperature: 1 to 24 weeks, 16 years, and 19 years), pH (standard calibration buffers from Fisher Scientific of pH 3.0, 7.0, and 10.0), temperature (4°, 25°, and 37°C), humidity (20% potassium acetate, 66% sodium nitrate, and 98% lead nitrate), and burial condition (sand, potting soil, garden soil) had on DNA

using Southern blots with human specific probes. For the aging study, the authors successfully extracted high molecular weight DNA from teeth stored at room temperature for all time periods studied (1, 2, 3, 4, 6, 12, 24 weeks, and 16 and 19 years). DNA was obtained from the teeth stored at 4°C for 6 weeks (longer periods of time were not analyzed) and from teeth stored for 24 weeks at 37°C. At pH 3, DNA was isolated from teeth after 1 and 3 weeks at 4°C and 37°C, but not from teeth at 25°C. DNA was extracted from teeth stored in the pH 7 solution after 3 weeks at all three temperatures and after 1 week at 4°C and 37°C. DNA was acquired from teeth stored in potting soil at 4°C, and those exposed to garden soil and sand at 4°C and 37°C after 1 and 3 weeks. DNA extraction was successful from teeth stored in 20%, 66%, and 98% humidity after 1 and 3 weeks of exposure. Despite extraction of 15 to 20μg of DNA from teeth subjected to many different environmental conditions, it was concluded that aging could cause the complete loss of nDNA under certain circumstances because DNA could not be extracted from some teeth in the study. It should be noted, however, that the authors stated that the presence of factors outside the control of the experiment (tooth type, condition of tooth, age of donor, fewer samples stored at 4°C than other temperatures, anatomical trauma, etc.) may have affected the results.

When nDNA cannot be recovered from a bone or tooth, researchers may be able to use mitochondrial DNA (mtDNA) to aid identification. In many instances, key differences between mtDNA and nDNA make mtDNA analysis advantageous. Each nucleated cell contains only a single nucleus which houses two copies of nDNA, but hundreds to thousands of mitochondria can be present in a single cell (Robin and Wong, 1988), each containing mtDNA. Mitochondria also tend to be more robust than nuclei

which allow the mitochondrion to protect mtDNA in situations where the cell nucleus cannot survive (Foran, 2006).

Not only are ancient bone and teeth a source of nDNA, but it has been established that they are a reliable source of mtDNA as well (Pääbo, 1989, Smith *et al.* 1993). Baker *et al.* (2001) extracted mtDNA from "environmentally compromised" teeth and hair. DNA was obtained from 15 teeth and 19 hair shafts, collected during autopsy, using a silica/guanidine thiocyanate method. Nine individuals had died less than 72 hours before sample collection and 5 had been dead for greater than 72 hours. Five of these had been burned and 1 body was recovered from water. Mitochondrial DNA was successfully extracted and sequenced from all teeth and hair shafts. The condition of the body at the time of sample collection, the type of tooth used, or the color of hair did not have an effect on the ability to amplify or sequence mtDNA. Additionally, AFDIL has extracted DNA from thousands of skeletal remains exposed to various environmental conditions including acidic and basic soils, jet fuel, and sea water. AFDIL has used the extracted DNA obtained from these remains to identify individuals, especially when the number of possible deceased is limited to a small population, such as the few people aboard a military plane prior to crash. In these situations, mtDNA can be used to separate the commingled remains so that each individual can be returned to their family, even years later. For example, the remains of Maj. Irwin S. Lerner and Lt. Col. Randolph A. Perry, Jr. were identified using mtDNA reference samples from maternal relatives, 30 years after the men died in Vietnam (Edson *et al.* 2004)

Many tragedies—plane crashes, natural disasters, building collapses, car bombings, and terrorist attacks—result in situations where the only biological material

recovered from the scene may be bone fragments or teeth. Misner (2004) studied what type of bone (rib, femur, pelvis) best protects mtDNA from decomposition. Amplification of mtDNA extracted from the femur samples was the most successful, followed by the pelves and then ribs. Based on Misner's (2004) work, the bone most likely to yield a DNA profile could be selected at the start of analysis, instead of testing all possible samples until a profile is generated.

If all that is recovered from a scene is a single tooth, however, it must be determined which area of the tooth, crown or root, to consume for DNA analysis so as to not use the entire sample or waste valuable time and resources on samples unlikely to yield a usable profile. Gaytmenn and Sweet (2003) collected 250 "recently extracted" teeth from oral surgeons and dentists. The samples were air dried, stored at -20°C, sectioned, and DNA extracted. The quantity of nDNA obtained was determined using the AluQuant Human DNA Quantification System (Promega Corporation). The researchers determined that more DNA could be obtained from the root of the tooth than the crown. Gaytmenn and Sweet (2003) stated that "since the root body is largely comprised of radicular dental pulp and dentin, it contains the greatest yield of DNA" and since the dental pulp contains the nerves and blood vessels of the tooth (Anderson *et al.* 1998), it makes sense that this area contains the most DNA. One limitation to Gaytmenn and Sweet's (2003) study was that all of their samples were fresh; none had undergone the DNA altering effects of decomposition. The enamel covering the crown of the tooth is one of the hardest substances within the human body (Arnheim and Prentice, 2000), and while the root may contain more DNA just after the victim's death, when DNA is

most likely not needed for identification, it is possible that after the body undergoes decomposition, the crown's enamel covering will better protect DNA from degradation.

A major limitation to much of the previous research involving ancient bone and teeth is the presence of confounding variables: variations in sample age, bone type, burial location, internment length, as well as other differences. For instance, Hagelberg *et al.* (1991) evaluated the recovery of DNA from ancient buried bone, but the locations of the burials, as well as the interment lengths, were different. One cemetery contained burials from the English Civil War period (1644 – 1663), while the other was used during earlier medieval times (exact dates not stated). Although the cemeteries were in close proximity to each other, the authors stated that "no work has been done to show how environmental conditions, such as depth of burial or soil pH, may affect DNA survival." In addition, Garcia *et al.* (1996) analyzed the effects of the environment on DNA extracted from 570 teeth subjected to different temperatures, burial conditions, and of varying ages. The authors also analyzed teeth from forensic casework where the remains had been exposed to varying environmental conditions. They determined that analysis of the samples submerged in water resulted in poorer DNA amplification than those buried in either dirt or sand.

It is hard to draw conclusions, from the studies described above, about what type of skeletal material best protects DNA from degradation since so many confounding variables were present within the studies. Many of these factors can be eliminated if a large set of samples from the same location, and interred for the same length of time, were used for analysis.

Description of the Voegtly Cemetery Teeth Used for Analysis

In 1987, the construction of a highway on the north side of Pittsburg, Pennsylvania unearthed 724 burials (Ubelaker *et al.*, 2003). The burials were discovered during the last phase of the Pennsylvania Department of Transportation's (PennDOT) Interstate 279/579 Highway Project—the erection of an interchange ramp which crossed over what was formerly the grounds of the Voegtly Church. All that was left of the Voegtly Church and Parsonage were portions of the original foundation and remnants of an asphalt paved parking lot. However, upon the discovery of buried coffins, PennDOT brought in a team of archeologists and osteologists to excavate the area in order to remove the coffins, remains, and the associated grave goods. After the area had been completely excavated, Dr. Douglas Ubelaker, from The Smithsonian Institution, was invited to Pittsburgh to discuss the possibility of analyzing the remains. The excavated material was transported to the Smithsonian in 1988 for analysis. Information describing the burials can be found in Analysis of Human Remains from Voegtly Cemetery, Pittsburgh, Pennsylvania (Smithsonian Contributions to Anthropology Series, 2003).

Landers (2003) reported that in 1787, the excavated region was located within an area known as Old Allegheny Town and until 1828, was a target of Native American attack and did not have the economic successes that Pittsburgh was experiencing. With the construction of the Pennsylvania Main Line Canal in 1828, as well as new bridges and roadways, Old Allegheny Town joined Pittsburgh in economic and social success. Early settlers to the area were Swiss-German immigrants. As the area grew, it became Allegheny City, but maintained the Swiss-German ways of the immigrants—German continued to be the language used in church services and newspapers.

The First Evangelical Church of Allegheny (Voegtly Church) was built in 1833, on land donated by the Voegtly family. The cemetery behind the church was used from 1833 to 1861. In the 1860's, land on nearby Troy Hill was acquired for a larger cemetery. After the new cemetery opened, the members buried in the Voegtly Cemetery were supposedly moved. In reality, all of the headstones and grave markings had been moved to Troy Hill, but the bodies of only two individuals, Nicholas and Elizabeth Voegtly, the church founders, were actually relocated (Landers, 2003).

As the years went on, several expansions to the Voegtly Church were built over the original cemetery. Church membership began to wane in the late 1800's, and despite resurgences, started to decline steadily in the 1950's. In 1972, the last individual with Voegtly family ties died. PennDOT acquired the property in 1984, and in 1985, the Voegtly Church was officially disbanded and destroyed. Two years later the area was excavated, every burial was assigned a number, each item unearthed from the cemetery was recorded, and the skeletons were packaged.

Ubelaker and his team analyzed the remains upon delivery to The Smithsonian Institution, where the skeletal material removed from the burial ground was unpacked and cleaned, and estimations of age, sex, and stature were made. Data collection was based on Buikstra and Ubelaker's *Standards for Data Collection from Human Skeleton Remains* (1994). In conjunction with these estimates, each burial was assigned a weathering stage using Behrensmeyer's (1978) classification system; the scale separates the degree of weathering based on a scale from 0 to 5. A summary of each stage is as follows:

Stage 0: Bone surface shows no sign of cracking or flaking due to weathering.

Stage 1: Bone shows some cracking, usually longitudinal in long bones.

Stage 2: Outermost concentric thin layers of bone show some cracking and flaking.

Stage 3: Bone surface has rough patches of compact bone. The most external layers of bone have been removed, but weathering does not extend deeper than 1.0 - 1.5mm

Stage 4: Bone surface is rough in texture. Splintering occurs and weathering extends into inner cavities.

Stage 5: Bone easily broken, large splinters present; original bone shape may be difficult to determine.

Of the 724 individuals removed from the Voegtly Cemetery, a subset was selected (approximately 20 from each weathering stage, although Stages 0 and 1 were underrepresented) for further examination. Small sections of bone and individual teeth were removed and shipped to Brazil for histological study (Braz *et al.* 2006) and to Michigan State University for DNA analysis.

The samples sent to MSU were used for several projects. As previously stated, Misner (2004) compared the influence of bone type and degree of weathering on the extraction and amplification of mtDNA. A correlation between weathering stage and amplification success was not seen, but the bone type had an impact on the ability to obtain a useable mtDNA profile. Halvorson (2005) examined the relationship between the outward appearance of a skeleton or bone and the amount of DNA that could be extracted from it, but no statistical difference was found. In the study described here, adult teeth were selected for DNA analysis. At least one tooth was sent with 44 of the 88

burials shipped to MSU, 31 of which had previously been determined to be adult dentition (Ubelaker *et al.* 2003). Nineteen teeth were estimated to be from male skeletons, 11 from female skeletons, and the sex was unknown for 1 sample. One canine, 2 incisors, 9 premolars, and 19 molars were examined.

Overview of Project Goals

One limitation to the research undertaken by Schwartz *et al.* (1991), Hagelberg and Clegg (1991), Hagelberg *et al.* (1991), Garcia *et al.* (1996), and Gaytmenn and Sweet (2003), was the lack of a sizable collection of bones and teeth that had been exposed to the same decomposition conditions. Differences in burial condition confound the data because it is impossible to determine if results obtained were from variations among the samples or disparity in the burial conditions. The Voegtly Cemetery provided an ideal collection for analysis as all of the burials were excavated from the same plot of land, eliminating large scale differences in soil, ambient temperature, humidity, and other environmental factors. The cemetery was in use for a relatively short period of time and completely excavated by 1987, meaning the skeletal material was decomposing for a similar length of time (approximately 120 to 150 years). Ethnicity, although not discussed in any of the research detailed above, could also be eliminated as a confounding variable because the members of the Voegtly Church were Swiss-German immigrants and their descendents. Additionally, the coffins from which the remains were removed were "six-sided, wedge-shaped...plain wooden coffin(s)" and differences among the coffins were due to variations in decoration associated with the growing industrialism of Allegheny and not the type of coffin used (Ubelaker *et al.* 2003). Based on this information, disparity in DNA quality from the burials was most likely to be from

differences in the teeth themselves and not variations in soil conditions, time buried, ethnicity, or other burial conditions.

Previous work has shown that teeth can be excellent sources of DNA and often survive many destructive conditions that other bones are unable to withstand (Sweet and Sweet, 1995). The primary goal of this study was to establish if there was a way to determine if useful DNA could be extracted from a particular tooth based on the physical condition or overall weathering of the skeleton. A second objective was to test which area of the tooth to use for mtDNA extraction and analysis. Finally, the results were compared to Misner's (2004) study that also used Voegtly Cemetery samples to determine if the teeth were a better source of mtDNA than rib, femur, or pelvis.

METHODS

Sample Collection

The samples used for this study were a subset of a larger collection which consisted of 724 individuals excavated from the Voegtly Cemetery (Ubelaker *et al.* 2003). A single tooth was analyzed from 31 individuals. Table 1 lists the samples by burial number, assigned weathering stage, estimated age and sex, and type of tooth. None of the skeletons from which the teeth originated were assigned to Weathering Stage 0, three were assigned to Stage 1, seven to Stage 2, nine to Stage 3, ten to Stage 4, and two were rated as Stage 5. Skeletal remains were sexed by The Smithsonian anthropologists based on skeletal morphology (Smithsonian Contributions to Anthropology Series, 2003). All four types of teeth, molars, premolars, canines, and incisors were studied, but the most common tooth used was the molar. Each of the teeth was treated as two separate samples after being sectioned—crown and root.

Table 1: Characteristics of Voegtly Cemetery Teeth

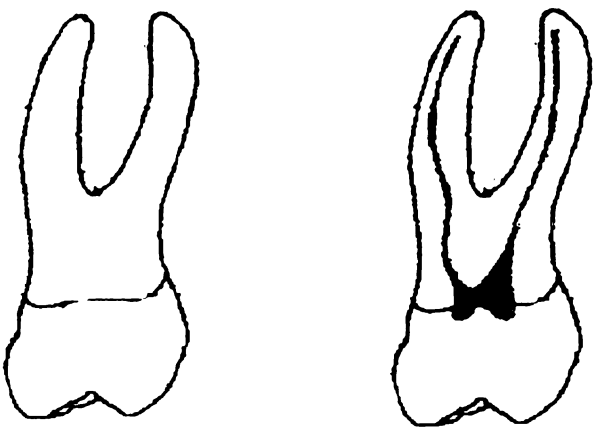
Sample	Weathering Stage	Age	Sex	Type of Tooth
3	4	30 to 45	Male	Premolar
22	4	45 to 50	Male	Incisor
23	4	22	Female	Molar
26	5	25 to 35	Female	Molar
30	5	40 to 55	Male	Molar
33	4	35 to 40	Male	Molar
34	4	35 to 45	Male	Premolar
47	4	11	Female	Molar
111	2	30 to 35	Male	Molar
126	3	25 to 30	Female	Molar
132	2	25 to 30	Male	Molar
167	2	15 to 16	Probably Male	Molar
259	2	50 to 70	Male	Canine
280	1	10	unknown	Molar
281	1	19 to 23	Female	Molar
322	2	20 to 24	Male	Molar
328	2	40 to 45	Male	Molar
345	3	adult	Male	Molar
348	3	27 to 35	Male	Molar
381	2	25 to 30	Male	Premolar
409B	3	30 to 40	Male	Premolar
449	3	27 to 33	Female	Molar
539	4	30 to 40	Female	Molar
545	1	25 to 32	Male	Molar
546	4	18 to 21	Female	Premolar
583	3	30 to 45	Male	Premolar
586	3	25 to 35	Male	Premolar
590	4	17 to 22	Female	Incisor
593	3	25 to 35	Female	Premolar
622	3	25 to 34	Male	Molar
690	4	12 to 13	Female	Premolar

Sample number was the number assigned to the skeleton at the time of excavation. The table displays the weathering stage, the estimated age, sex, and the type of tooth for each individual as determined by analysis completed at The Smithsonian (Smithsonian Contributions to Anthropology Series, 2003).

Sample Preparation

Each tooth was sectioned sagittally, as depicted in Figure 1, using a Dremel Multipro tool (model no. 395-76). One half of the tooth was returned to its original container. The second half was collected in a 17x100mm sterile tube (Fisher Scientific) and used for experimentation.

Figure 1: Sagittal Sectioning of Tooth



Tooth Prior to Sectioning Tooth After Sectioning

Each tooth was cut lengthwise down the middle, separating the tooth into two halves. One half was used for DNA isolation and the other was returned to storage.

Dirt from burial was present on many of the samples. To remove it, as well as possible exogenous sources of DNA, samples were washed for one hour at room temperature in 1 to 3 ml (enough to cover the sample) of wash buffer (1% SDS and 25mM EDTA). Samples were agitated intermittently to try to loosen some of the more persistent soil. The wash buffer was removed and each tooth was rinsed six times with sterile water (1 to 3 ml based on sample size) (Cline *et al.* 2003). Teeth were dried using compressed air passed through a 0.45μm filter (Millipore). The air was directed from the filter to the sample using a sterile Pasteur pipette.

Bone Powder Collection

All equipment—clamp and supporting pieces, drill bits, and forceps—was washed with 10% bleach and then 70% ethanol. Items were placed in an ultraviolet light box for 300 seconds on each side. The drill press itself was cleaned between samples using the same solutions. During the drilling process the researcher was careful to not breathe on the exposed samples. This prevented the powder from being blown off the platform prior to its collection and also helped to eliminate the possibility that the researcher's own DNA would be transferred to the samples.

Two collections of bone powder were made from each tooth; the first from the crown and the second from the root. The cut tooth was placed on a piece of weigh paper and positioned in a Columbian table clamp (model number 69997) with the interior surface of the tooth facing up. A 1/16" drill bit was inserted into a 12" Craftsman drill press (model number 137.219120). The clamp holding the tooth was placed under the drill and the bit was slowly lowered to the surface of the tooth. Holes were drilled into the crown area of the tooth until enough powder was produced to fill approximately 1/3 of a 1.5ml microcentrifuge tube (2 – 3 holes were needed). The tooth was removed from the weigh paper leaving the drilled powder behind. The paper was used to funnel the powder into a sterile, labeled microcentrifuge tube. This procedure was repeated to collect powder from the root of the tooth, using a new piece of weigh paper and a clean drill bit.

DNA Extraction

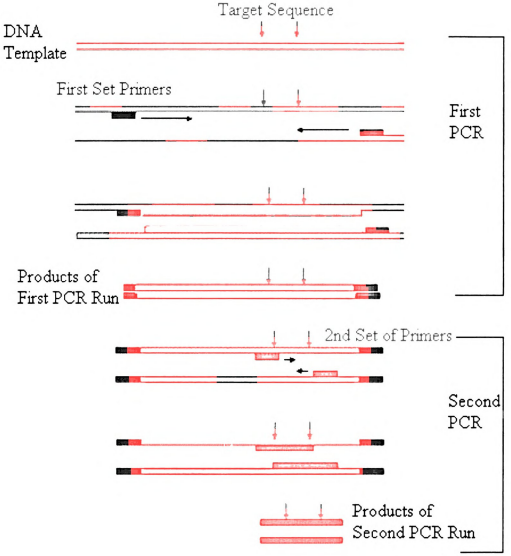
The powder was incubated overnight at 56°C in 400µl digestion buffer (20mM Tris, 100mM EDTA, 0.1% SDS) and 0.4mg/ml proteinase K. After digestion, one

volume of phenol was added, the tube vortexed, and centrifuged at 18,000 rcf for five minutes. The aqueous layer was transferred to a sterile, labeled microcentrifuge tube, and an equal volume of chloroform added. The sample was vortexed and centrifuged, and the aqueous layer transferred to a new, sterile, labeled microcentrifuge tube. The DNA was precipitated by the addition of 1/10 volume (approximately 40µl) of 3M sodium acetate (NaAc) and two volumes (approximately 800µl) of cold 95% ethanol. The tube was vortexed and stored at -20°C overnight. The following morning, the tube was centrifuged at 18,000 rcf for 20 minutes. The liquid was aspirated off and discarded. The remaining pellet was vacuum-dried for 30 – 40 minutes. The DNA was resuspended in 15µl TE buffer (10mM Tris, 1mM EDTA) and stored at -20°C.

DNA Amplification

DNA amplification was attempted for each sample using semi-nested polymerase chain reaction (semi-nested PCR) which involved two PCR preparations as depicted in Figure 2. The first round of PCR included one set of primers, while the second round contained the same reverse primer, but a different forward primer. The first reaction of semi-nested PCR was a 20µl solution containing 1unit Hot Master Taq (Eppendorf), 0.2mM of each dNTP (Promega), one-tenth volume of 10X Hot Master Buffer (containing Mg^{2+}) (Eppendorf), and 2µmol of the primers (F16144 and R16410) (Table 2). In addition, 4µl of 10µg/µl bovine serum albumin (BSA) was added to each sample. Two reactions were prepared for each DNA extraction, one using 1µl of undiluted DNA and the other a 10X dilution of the first reaction.

Figure 2: Nested PCR



In nested PCR, the first PCR uses two primers. The second PCR uses two internal primers to obtain the final desired product. Semi-nested PCR uses two primers for the second reaction, but one primer is the same as the first PCR. For the Voegtly samples, the first PCR used forward primer F16144 and reverse primer R16410. The second PCR used R16410 and F16190. (Image design from pcrstation.com)

Table 2: Primer Sequences

F16144	TGACCACCTGTAGTACATAA
F16190	CCCCATGCTTACAAGCAAGT
R16410	GAGGATGGTGGTCAAGGGAC

The nucleotide sequences for the primers used for semi-nested PCR (Edson *et al.* 2004).

Following an initial denature period at 94°C for 2 minutes, PCR parameters were as follows: DNA was denatured at 94°C for 45 seconds, primers annealed at 56°C for 1 minute, and sequences extended at 72°C for 1 minute. The cycle was repeated 38 – 40 times with an additional 5 minute extension at 72°C following the last cycle. A 4µl aliquot of each sample was electrophoresed on a 3% agarose gel and visualized using ethidium bromide staining.

For the second reaction of semi-nested PCR, 1µl from the first amplification reaction was added to the second. The thermocycler parameters were the same as those above except that the number of cycles was reduced to 10 – 15. The number of cycles was based on how much additional amplification was desired. Another 3% agarose gel, with ethidium bromide staining, was used to visualize 5µl of each reaction.

DNA Sequencing

The amplified product was transferred to a Montage PCR Purification Column (Millipore) and the volume increased to 400µl using TE. Columns were centrifuged per the manufacturer's instructions. TE was then added to each purified product to bring the final volume of amplified DNA up to 15µl.

DNA was sequenced according to the manufacturer's recommendations using a CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter) except the final volume was reduced to 10µl for each reaction. The primers used for the second

round of PCR (F16190 and R16410) were used for sequencing. Twenty-five to 100fmol of amplified DNA were added to the sequencing reaction. Thermocycler parameters were based on the kit's guidelines: denaturation at 96°C for twenty seconds, primer annealing at 50°C for twenty seconds and extension at 60°C for four minutes, with the entire process repeated for thirty cycles.

Following sequencing, 2.5µl of "stop mix" (40µl of 3M NaAc, 8µl of 500mM EDTA, 20µl of 20mg/ml glycogen and 32µl of water to make 100µl of stop mix solution) and 30µl cold 95% ethanol were added to the sequenced products and centrifuged for 15 minutes at 20,000 rcf. The supernatant was removed and the pellet washed with 200µl cold 70% ethanol followed by a 20,000 rcf centrifugation for five minutes. The supernatant was removed and the wash step repeated. The pellet was vacuum-dried for approximately 30 minutes. The pellet was then resuspended in 40µl Sample Loading Solution (Beckman Coulter).

Sequences were generated using a Beckman Coulter CEQ 8000 Genetic Sequence Analyzer using the LFR-1-45 program (capillary temperature: 50°C, denature: 90°C for 120 seconds, inject: 15 seconds at 2.0kV, separate: 45 minutes at 4.2kV). Sequences were aligned to the Anderson reference sequence (Anderson et al. 1981) and compared using the BioEdit Sequence Alignment Editor (Hall, 2004). Sequence information was also compared to the researcher's mtDNA sequence.

RESULTS

Sample Description

Thirty-one adult teeth were analyzed in this study. No preference was given to the type of tooth (incisor, molar, premolar, or canine) and at least one of each was examined. The samples were part of a collection excavated from a single cemetery, and consequently, some skeletal weathering stages were under-represented, meaning it was not possible to select the same number of samples from each stage. As such, there were fewer teeth in Weathering Stages 1 and 5 and more in Stages 2, 3, and 4.

Observations During Tooth Preparation

In general, the teeth were soiled (discolored from burial) upon receipt from The Smithsonian. The cleaning procedures described above (see Methods) removed much of this discoloration. After the hour incubation in the wash buffer, the buffer was brownish in color and contained specks of what appeared to be dirt and debris. The subsequent water rinses removed these, and by the last two washes, samples left the wash water clear.

A few of the samples had dental carries in which part of the tooth was missing. These areas were avoided during the drilling processes. The half containing the imperfection was returned to storage while the other half of the tooth was selected for further processing. There was no difference in the ability to extract, amplify, or sequence DNA between the teeth that originally contained a defect and those in which no defect was seen.

When sectioning the tooth with the Dremel Tool, the root was relatively easy to cut through, while the crown of the tooth was more difficult to section. As the Dremel

Tool touched the enamel surface small pieces flaked off from the area closest to the root. It appeared that this was more prevalent in the higher weathering stages. The enamel flakes were not collected for further processing—the larger pieces of enamel were returned to the storage container and the smaller pieces discarded. The presence or absence of enamel flaking did not have an effect on the ability to extract, amplify, or sequence DNA from the crown or root of a tooth.

During the drilling processes, it was important to keep drafts away from the freshly drilled bone dust. The drill press was too large to be contained inside a PCR hood; consequently, walking by the machine at a brisk pace while the powder was still on the platform would cause it to be blown away. In order to decrease the possibility of contamination and loss, teeth were drilled when others were not in the laboratory.

DNA Extraction

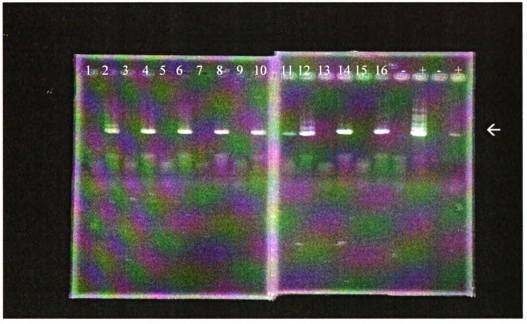
DNA was extracted from each tooth using one phenol extraction. In previous research by Misner (2004), Voegtly bone samples required two or three phenol extractions before the solution was no longer brown. The teeth samples did not require these additional phenol extractions. After phenol/chloroform extraction, the resulting DNA was clear or a very pale yellow. The coloration of the DNA after extraction did not to have an effect on further processing.

DNA Amplification Success

DNA from 10 teeth (22 crown, 22 root, 23 root, 280 crown, 539 crown, 539 root, 546 crown, 583 root, 593 root, and 622 crown) amplified using standard PCR, however, with the exception of DNA from the root of tooth 583, the DNA was only faintly visible after gel electrophoresis. DNA from 60 of the 62 teeth amplified using semi-nested PCR.

Typical results following semi-nested PCR are shown in Figure 3. The reactions that contained DNA diluted prior to the first PCR amplified more often than the non-diluted DNA and are shown in the even numbered lanes. The odd numbered lanes contain DNA that was not diluted and, with the exception of lane 11, did not adequately amplify.

Figure 3: Gel Electrophoresis Following Semi-Nested PCR Amplification



Results of semi-nested PCR. DNA amplification was attempted during the first round of PCR using 1µl DNA and a 10X dilution. Products of the first PCR were used as template DNA during the second round of PCR. Products of the second round PCR were electrophoresed in adjacent lanes (diluted DNA in even lanes). Positive results are seen in the lanes containing a band at the target fragment length depicted by the arrow. Lanes in which bands cannot be seen were considered negative and were not used for subsequent DNA sequencing. Negative and positive controls (depicted by "-" and "+") were initiated during the first PCR and continued during the second PCR. Separate controls were included for the second amplification of semi-nested PCR.

Table 3 displays amplification and sequencing results for each tooth. There was no difference in amplification success between DNA extracted from the crown and root of a tooth. Amplification was successful from 30 of 31 crown DNAs and 30 of 31 root

DNAs. There was no relationship between DNA amplification success and the weathering stage of the skeleton, the type of tooth assayed, nor the anthropologically estimated skeleton age and sex. The DNAs that failed to amplify originated from a premolar of a 35 – 45 year old male and a molar from an 11 year old female.

Table 3: Amplification and Sequencing Success of Voegtly Teeth Samples

Sample Number	Weathering Stage	Crown Amplification Success	Crown Sequence Success	Root Amplification Success	Root Sequence Success
545	1	+	-	+	+
280	1	+	+	+	-
281	1	+	+	+	+
167	2	+	+	+	+
322	2	+	+	+	+
111	2	+	+	+	+
132	2	+	+	+	+
381	2	+	+	+	+
328	2	+	+	+	+
259	2	+	+	+	+
348	3	+	+	+	+
126	3	+	+	+	+
345	3	+	+	+	+
449	3	+	-	+	-
409B	3	+	+	+	-
586	3	+	+	+	+
583	3	+	+	+	+
593	3	+	+	+	+
622	3	+	+	+	+
34	4	+	+	-	-
47	4	-	-	+	+
539	4	+	+	+	+
546	4	+	+	+	+
33	4	+	+	+	-
23	4	+	+	+	+
3	4	+	-	+	+
22	4	+	+	+	-
690	4	+	+	+	-
590	4	+	+	+	+
26	5	+	+	+	+
30	5	+	-	+	+

DNA amplification and sequencing success for each tooth. Positive results are represented by "+" and negative results are represented by "-".

DNA Sequencing Success

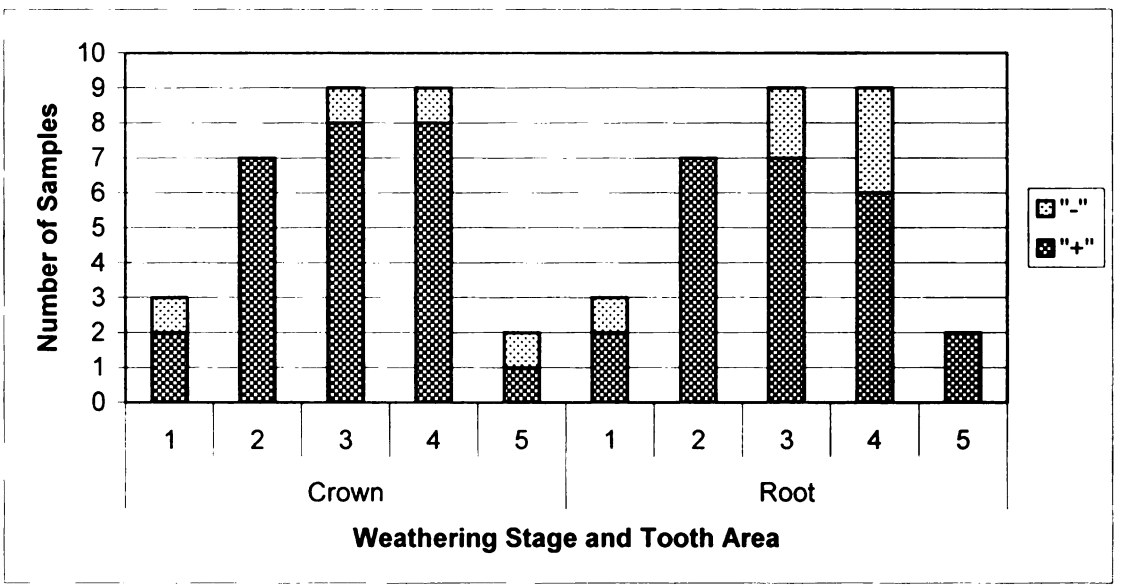
Sequence data were obtained from 83% (50 of 60) of the amplified DNAs, including 87% (26 of 30) of crown DNAs and 80% (24 of 30) of root DNAs. In only one case (Burial 449), neither crown nor root returned usable sequence data. The difference between the number of crown and root DNAs that were successfully sequenced was not significant based on a two-way chi square test for independence (χ^2=0.4894).

The weathering stage of each burial was also compared to DNA sequencing success. All DNA extracted from teeth assigned to Weathering Stage 2 generated sequences (14 of 14). Sequence data were obtained from 67% of DNAs from Stage 1 (4 of 6), 83% from Stage 3 (15 of 18), 78% from Stage 4 (14 of 18), and 75% from Stage 5 (3 of 4). The success of DNA sequencing was not related to the skeletal weathering stage based on a two-way chi square test for independence (χ^2=5.15).

Sequencing success for each weathering stage separated by crown and root are displayed in Figure 4. Sequence data were obtained from all DNAs extracted from the tooth crown from Weathering Stage 2 skeletons (7 of 7). Sixty-seven percent of Stage 1 (2 of 3), 89% of Stage 3 (8 of 9), 89% of Stage 4 (8 of 9), and 50% of Stage 5 (1 of 2) crown DNAs were successfully sequenced. When extracted from the root, all DNAs from Stage 2 (7 of 7) and Stage 5 (2 of 2), 67% from Stage 1 (2 of 3), 78% from Stage 3 (7 of 9), and 67% from Stage 4 (6 of 9) produced sequence data. There was no significant difference in sequencing success between the crown and root of the tooth based on weathering stage using a two-way chi square test for independence (χ^2=0.607).

In addition, there was no significant difference between weathering stage and sequencing success within the crown (χ^2=4.51) or the root (χ^2=3.66).

Figure 4: Sequencing Success Based on Weathering Stage and Area of Tooth



The success (+) and failure (-) of sequencing for each section of tooth, as well as the weathering stage of each burial

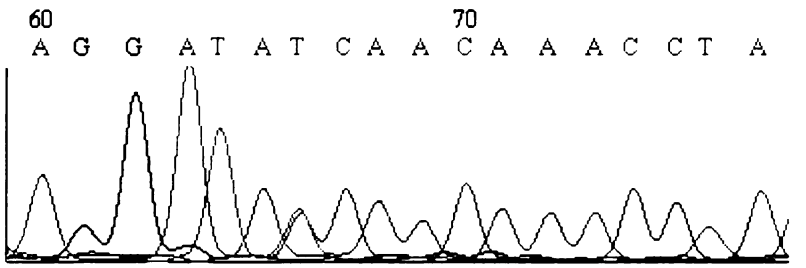
Alignment of Teeth DNA Sequences to the Reference Sequence

Forward and reverse sequence data were aligned to the Anderson *et al.* (1981) reference sequence and compared to the other samples (see Appendix I). Sequence information for the entire 220bp region assayed was only obtained from 12 teeth, while fewer basepairs were obtained from the remainder. The most common polymorphism was a C to T transition at position 16294, which was found in 19 of the 60 samples (32%). Eleven sequences (18%) required the use of the optional sequence code "Y" (C/T) at 16294.

Optional sequence codes were not required for 6 sequences (132 root, 281 crown, 409B crown, 583 root, 586 crown, and 690 crown). Two of these teeth were from

13 to 30 – 45. The rest of the sequences had ambiguous base calls. Figure 5 depicts a portion of an electropherogram in which 2 nucleotides were present at one location, wherein the allele at location 66 was called a thymine by the software, but peaks for both thymine and cytosine are seen. Forward and reverse sequence data were compared to eliminate confounding results, but ambiguity was still present.

Figure 5: Ambiguity within a Portion of a mtDNA Sequence



Using the ruler at the top of the figure, two peaks are present at position 66. The software called the position thymine, but both thymine and cytosine peaks were present at the same intensity. The reverse strand was analyzed concurrently to confirm the presence of both nucleotides at this location.

Sequence ambiguity made analysis difficult. For 11 teeth, the optional sequence code used to depict the presence of two nucleotides at one location in the sequence obtained from the crown matched a single nucleotide at the same position in the sequence from the root (or from the root to the crown respectively). Sequence comparison was not possible for 10 teeth because data were only obtained from one section of the tooth, 6 crown and 4 roots. Nine teeth had sequence differences between the crown and the root. Table 4 shows the locations of disparity for these teeth.

Table 4: Teeth Containing DNA Sequence Differences Between Crown and Root

Tooth	16249	16278	16288	16290	16291	16292	16293	16294	16301	16311
Reference Sequence	T	C	T	C	C	C	A	C	C	T
23 Crown								T		
23 Root		Y					R			Y
26 Crown							W	Y		
26 Root		T					R	Y		Y
126 Crown		Y					M			Y
126 Root								T		
132 Crown										
132 Root								T		
167 Crown								Y		Y
167 Root		T								
259 Crown								T	T	
259 Root										
345 Crown		T								
345 Root		T					G			C
348 Crown	C					T				
348 Root								Y		
586 Crown					T					C
586 Root			C	T			C		Y	C

Sequence data for teeth that had differences between the crown and root. Sequence data for the locations not displayed were the same for both the crown and root and matched the reference sequence. The areas that are grey are sites that were not sequenced for that tooth.

Teeth Sequence Differences from Reference Sequence

The obtained sequences were all compared to the standard reference sequence (Anderson *et al.* 1981). The 9 teeth that had disparities between crown and root sequence data were not included in this analysis. For the rest of the teeth, 6 sequences did not have any differences from the reference sequence. Eleven contained 1 alteration from the reference sequence, 2 teeth had 2 discrepancies, and 2 teeth had 3 inconsistencies. However, none of the teeth sequences had 4 or more differences from the reference sequence. Table 5 displays these data. Ambiguous basepairs within the sequences were not included because it could not be determined from the data which nucleotide was present. Only the nucleotides that were clearly depicted in the electropherogram are represented.

Table 5: Sequence Differences from the Reference Sequence

Number of Differences	Number of Sequences
0	6
1	11
2	2
3	2
4 or more	0

The number of differences from the Anderson *et al.* (1981) reference sequence and the number of teeth sequences that contained that number of variations.

DISCUSSION

One goal of this study was to determine if there is a relationship between the appearance of a tooth (degree of weathering) and the ability to obtain a mitochondrial profile. A second goal was to establish which area of the tooth, crown or root, was more likely to contain mtDNA. This knowledge would allow the forensic scientist to predict which section to select for DNA extraction.

DNA Amplification and Sequencing

Preliminary work with the Voegtly samples demonstrated that the combination of diluted DNA and the addition of BSA increased the success of amplification (data not shown). DNA amplification was attempted using the DNA from each tooth, as well as a dilution of the extracted DNA, and for 83% of the teeth, the diluted DNA amplified successfully while the undiluted DNA did not. The increase in amplification success of the diluted DNA may have been partly due to a decrease in PCR inhibitors. The dilution of the DNA added to the PCR also diluted the inhibitors, decreasing the concentration of the components that could compete with the DNA for the polymerase. In addition, Kreader (1996) suggested that BSA acts as a competitor for protein-binding inhibitors. Amplification success of the DNAs may have increased due to the addition of BSA because it binds inhibitors during PCR that would typically bind to the *Taq* polymerase. Alternatively, BSA may minimize free space in the reaction, which, in turn, increases the concentration of required PCR components (Eilert, 2007), improving DNA amplification. Regardless of the mechanism, diluted DNA from the teeth amplified successfully when BSA was included in the PCR.

The DNA sequence length obtained from the teeth varied due to poor quality data at the 5' end of the sequences. During electrophoresis unincorporated ddNTPs elute first, masking the actual sequence, and the amount of usable data is reduced. It is likely that better cleanup methods would remove residual ddNTPs and make sequence analysis more straightforward. Several companies offer products designed to remove superfluous dNTPs and ddNTPs prior to sequencing. The ZR DNA Sequencing Cleanup Kit (Zymo Research) is designed to bind and remove, during subsequent washes, remaining ddNTPs. Montage Cleanup Kits (Millipore) use size exclusion membranes to retain sequencing products but remove excess dye terminator molecules, whereas, a similar product from Sigma, the UltraClear Clean-Up Kit, utilizes an ultrafiltration membrane to separate low molecular weight containments from the desired DNA sequences. Additionally, Wizard MagneSil Clean-Up System (Beckman Coulter) and Dynabeads (Dynal Biotech) rely on proprietary particles to bind nucleic acids while unbound material is washed away. The use of one of these products for the Voegtly teeth DNAs may have reduced the number of unincorporated ddNTPs that eluted during electrophoresis and increased the length of usable sequence data.

Sequence Ambiguity

The sequences obtained from the teeth were not pristine and optional sequence codes were included when the presence of a single nucleotide could not be ascertained. The ambiguity of the sequences could be due to the misincorporation of nucleotides by the polymerase. For the Voegtly teeth, the location with the highest percentage of variance was 16294. Adjacent to this, the mitochondrial sequence (Anderson *et al.* 1981) contains several cytosine nucleotides in a row with an adenine in the middle. It is

possible that along this stretch of cytosine bases, the DNA template was released from the polymerase temporarily and the wrong nucleotide was incorporated. Alternatively, sequence ambiguity could result from heteroplasmy, in which an individual contains more than one mtDNA type. Heteroplasmy is thought to occur rarely in the mtDNA genome, but Grzybowski (2000) concluded that it is present more often than originally believed based on his analysis of hair roots. Budowle *et al.* (2003), however, disagreed stating that the increased presence of heteroplasmy observed by Grzybowski (2000) was probably due to Grzybowski (2000) using more template DNA in his PCR than is typically added and utilizing a greater number of cycles during PCR. However, Budowle *et al.* (2003) conceded that more research needs to be completed before scientists can agree on the prevalence of heteroplasmy. The ambiguity observed in the teeth DNA sequences could be the result of heteroplasmy especially since one of the areas Grzybowski (2000) termed a heteroplasmy "hot-spot" is location 16294—the position of much of the Voegtly teeth sequence ambiguity.

Treatments to Eliminate or Reduce Postmortem Damage in Ancient Material

The most common polymorphism seen in the teeth DNA sequences were C to T transitions. These disparities, which made sequence comparisons difficult and created uncertainty within the sequences, may have been due to the breakdown of the DNA within the teeth. As an organism decays, its DNA accumulates chemical damage (Lindahl, 1993). DNA deamination is the most common form of damage (Hofreiter *et al.* 2001) and when present during amplification, the polymerase incorrectly incorporates thymine and adenine where cytosine and guanine should have been inserted.

Chemical damage or modification to DNA that occurs during decomposition may not be preventable, but it can be treated prior to sequencing. Using ancient cave bear DNA from bone and teeth, Hofreiter *et al.* (2001) proposed that the amplification of ancient DNA contained cytosine to thymine and guanine to adenine substitutions as a result of the deamination of deoxycytidine during the decomposition process. The authors suggested adding uracil N-glycosylase (UNG) to template DNA to remove the deaminated cytosine residues. Treatment of the cave bear DNA with UNG eliminated G/C → A/T substitutions. The addition of UNG to the teeth DNA may have reduced sequence ambiguity and increased sequence clarity.

Alternatively, the PreCR Repair Mix (New England BioLabs) repairs DNA damage prior to amplification, including gaps, nicks, and thymine dimers, as well as alterations caused by cytosine deamination (PreCR Repair Mix Technical Bulletin). The addition of the repair mix to DNA extracted from the teeth may have repaired many types of DNA damage and made further sequence analysis and comparison possible.

Teeth With Sequences Differences

As displayed above, nine teeth contained sequence differences between the DNA extracted from the crown and root. In all cases except one, the reference sequence contains cytosine or thymine, where as for the teeth DNA, the crown contained either C or T, but the root contained the other nucleotide at the same location. It is possible that a small fragment of exogenous DNA was amplified and once amplified, masked the tooth DNA sequence. Re-extracting, amplifying, and sequencing DNA from the crown and root of these teeth may eliminate the observed differences. Utilization of one of the

previously mentioned repair mixes may have also eliminated the differences observed between the two sequences from the same tooth.

Methods to Obtain Powdered Skeletal Material

Mitochondrial DNA is generally extracted from bone and teeth by reducing the sample to powder. The standard procedure at AFDIL is to use a sterilized Waring MC2 blender cup and laboratory blender to pulverize bone samples prior to DNA extraction (Edson *et al.* 2004), while Misner (2004) used an IKA A11 Basic Grinder with a tungsten blade to crush the Voegtly Cemetery bones. In both situations, the blender must be decontaminated between samples. Misner (2004) disassembled the entire mill in order to ensure it was clean and free of exogenous DNA between each sample processed. This translated into extra time spent cleaning the equipment and less time processing samples. This project used a drill press to obtain powdered material from each sample (as in Holland *et al.* 2003). The only point of contact with a tooth was the drill bit, which was easily exchanged. The simple substitution of drill bits between samples greatly increased the speed at which the Voegtly teeth could be analyzed. The powder produced using the drill press was contained to a piece of weigh paper and did not have to be collected from all around the mill cup. This helped reduce the risk of contamination as there was less need to manipulate the powdered tooth in order to collect it.

Both the milling of bone and the use of a drill press resulted in sufficient powdered bone for mtDNA analysis. The powder generated by the drill press was of uniform consistency, whereas processed bone from the mill used by Misner (2004) was irregular in size—a continuum from powdered bone to small fragments. Future research may establish if the use of a mill or drill would be the most beneficial for subsequent

mtDNA extraction depending on the size of the bone fragment. Some bone fragments, for example, may prove to be too small to be drilled and require alternative processing. However, an advantage to the drill is that it does not consume the entire bone fragment during processing. Instead, small holes are produced while the majority of the sample is left undisturbed. In addition, it would be possible to drill teeth samples without sectioning the sample first. This study demonstrated that DNA could be obtained after drilling into the interior of the tooth. It is possible that sufficient DNA could be recovered simply by drilling into the exterior of the root, eliminating the time spent sectioning the sample, and more importantly, preserving the structure of the tooth. Any method used to process skeletal material that retains more of the original bone is advantageous to those techniques that consume the entire fragment. Additionally, in situations where only single bone fragments are recovered, drilling would allow at least some of the bone fragment to be preserved for further processing or future burial.

Weathering Stage Problems

When the Voegtly Cemetery was discovered, each grave was excavated and the remains were assigned a weathering stage by the researchers at The Smithsonian (see Introduction). When Misner (2004) analyzed the remains at Michigan State University, she found that the weathering stage of some of the individual bones was not in line with that of the overall skeleton. Misner (2004) developed a separate, but similar, classification system and re-assigned individual bones to weathering stages.

Compared to most of the bones from the Voegtly Cemetery, the teeth were less weathered. One potential reason for such a disparity is that teeth, in general, withstood decomposition better than bone. As previously discussed, the enamel covering the crown

of the tooth is one of the hardest substances in the body and may protect the tooth from environmental insult. A second possibility is that the teeth sent from The Smithsonian were not selected randomly. The most well preserved tooth could have been selected for additional analysis, instead of a tooth that was more representative of the weathering stage of the overall skeleton. None of the teeth analyzed were appreciably weathered, and this may have contributed to the success of DNA amplification. Conclusions about amplification and sequencing success based on the outward appearance of a tooth are limited for this work due to the lack of weathering among the teeth analyzed.

Discussion of Other Voegtly Cemetery Research

Ancient bone research undertaken by Schwartz *et al.* (1991), Hagelberg and Clegg (1991), Hagelberg *et al.* (1991), Garcia *et al.* (1996), and Gaytmenn and Sweet (2003) contained many confounding variables: differences in sample age, soil, burial condition, temperature, humidity, as well as other environmental factors. Analysis of the bones obtained from the Voegtly Cemetery reduced many of these. Variations in burial conditions, internment length, and ethnicity were minimized and differences among the DNAs were thought to be from the type of bone from which the it was extracted. Misner (2004) found that the degree of weathering present in a particular skeleton or individual bone was not useful in predicting if mtDNA amplification would be successful. Additionally, Halvorson (2005) did not find a significant difference in the amount of DNA extracted from the Voegtly Cemetery samples based on the skeletal weathering stage, however, the p-value obtained (0.06) was very close to the significance level (0.05). Halvorson (2005) stated that due to the obtained p-value, it was possible that there was a real difference among the skeletal stages.

The success rate for DNA amplification and sequencing was very high for the teeth analyzed, but it is difficult to conclude that a tooth is the best skeletal material from which to attempt DNA extraction and analysis, especially since the teeth used, as a whole, were less weathered than the bones examined by Misner (2004). In addition, Misner (2004) used only standard PCR to amplify DNA from the bones, whereas the DNA from the teeth could not be amplified using this method. Semi-nested PCR may have been necessary to amplify the teeth DNAs for several reasons. First, they might not have contained as much DNA as the bones used by Misner (2004) and Halverson (2005). A second explanation was that the chemical composition of the teeth made DNA extraction and amplification more difficult. The enamel covering of teeth may protect the DNA from environmental damages, but it may also impede the amplification process.

Future Research

The project described above established that powder from a hole drilled into a tooth can provide sufficient mtDNA for sequence analysis. Future research might demonstrate if the drilling location has any effect on DNA amplification and sequencing. The teeth used here were sectioned prior to drilling so that the hole was drilled from the interior of the tooth. It is possible that DNA may also be adequately obtained if the sample is not sectioned prior to drilling. The elimination of the sectioning step would not only increase the speed of sample processing, it would further reduce alteration to the skeletal material. However, an advantage to sectioning the bone or tooth prior to drilling is that there are fewer sources of exogenous DNA on the interior of a bone fragment than on the exterior.

Conclusions

DNA has been obtained from teeth by many researchers and the work detailed here confirms that teeth are a reliable source of mtDNA. Neither area of the tooth, crown or root, emerged as a better source of mtDNA as it was obtained from both areas with a nearly equal success rate. Additionally, this study has shown that if the only skeletal material recovered is a single tooth, powder from a small, drilled hole may be enough to extract, amplify, and sequence DNA, leaving much of the original sample intact. Future work might demonstrate if the quantity of DNA extracted from a tooth is similar to that of bone and if sufficient DNA can be obtained from more weathered teeth than the ones analyzed here. This knowledge allows the forensic biologist to attempt DNA extraction from the skeletal material most likely to yield a DNA profile, which could eliminate time and resources spent testing samples where the probability of obtaining useful sequence information is less predictable.

APPENDIX

Sequence data for each tooth are shown in the following table. The table displays polymorphisms at specified cites. Locations that are not depicted were found to be the same as the Anderson *et al.* (1981) reference sequence. The areas that are grey are sites that were not sequenced for that tooth. Optional sequence codes are included where two or more nucleotides were depicted in the sequence (Y=C/T, R=A/G, M=A/C, W=A/T, S=C/G, K=G/T, V=A/C/G, D=A/G/T, H=A/C/T, B=C/G/T, N=A/C/G/T).

Table 6: Sequence Data

Tooth	16190	16192	16193	16199	16217	16224	16225	16232	16235	16239	16246
Reference Sequence	C	C	C	T	T	T	C	C	A	C	A
3 Root Forward											
22 Crown Forward											
22 Crown Forward										S	
23 Crown Forward											
23 Crown Reverse											
23 Root Forward											
23 Root Reverse											
26 Crown Forward										S	
26 Crown Reverse											
26 Root Forward											
26 Root Reverse											
30 Root Forward											
30 Root Reverse											
33 Crown Forward											
34 Crown Forward										S	
34 Crown Reverse											
47 Root Forward											
47 Root Reverse						C					
111 Crown Forward											
111 Root Forward											
126 Crown Forward											
126 Crown Reverse											
126 Root Forward											
126 Root Reverse											
132 Crown Forward											
132 Crown Reverse				R			S				
132 Root Forward											
167 Crown Forward											
167 Crown Reverse											
167 Root Forward											
167 Root Reverse											
259 Crown Reverse											
259 Root Forward											
259 Root Reverse					Y						
280 Crown Forward											

Table 6: Continued

Tooth	16190	16192	16193	16199	16217	16224	16225	16232	16235	16239	16246
Reference Sequence	C	C	C	T	T	T	C	C	A	C	A
281 Crown Forward											
281 Crown Reverse											
281 Root Forward											
281 Root Reverse											
322 Crown Forward											
322 Crown Reverse											
322 Root Forward											
322 Root Reverse											
328 Crown Forward											
328 Crown Reverse											
328 Root Forward											
328 Root Reverse											
345 Crown Forward											
345 Crown Reverse											
345 Root Forward											
345 Root Reverse						Y					
348 Crown Forward											
348 Root Reverse		T									
381 Crown Forward								Y			
381 Crown Reverse											
381 Root Forward											
381 Root Reverse											
409B Crown Reverse											
539 Crown Forward											
539 Root Forward											
539 Root Reverse			A								
545 Root Reverse											
546 Crown Reverse											
546 Root Forward											
546 Root Reverse											

Table 6: Continued

Tooth	16190	16192	16193	16199	16217	16224	16225	16232	16235	16239	16246
Reference Sequence	C	C	C	T	T	T	C	C	A	C	A
583 Crown Reverse											
583 Root Forward											
586 Crown Forward											
586 Crown Reverse						C					
586 Root Forward											
590 Crown Forward											
590 Crown Reverse											
590 Root Forward											
590 Root Reverse											
593 Crown Forward											
593 Crown Reverse									R		
593 Root Reverse											
622 Crown Forward											
622 root Forward											
622 Root Reverse											N
690 Crown Forward											
Researcher		T									

Table 6: Continued

Tooth	16249	16250	16251	16252	16260	16261	16265	16267	16269	16275	16277
Reference Sequence	T	C	C	A	C	C	A	C	A	A	A
3 Root Forward											
22 Crown Forward											
22 Crown Forward											
23 Crown Forward											
23 Crown Reverse											
23 Root Forward											
23 Root Reverse											
26 Crown Forward											
26 Crown Reverse											
26 Root Forward											
26 Root Reverse											
30 Root Forward											
30 Root Reverse											
33 Crown Forward											
34 Crown Forward											
34 Crown Reverse											
47 Root Forward											
47 Root Reverse											
111 Crown Forward			Y								
111 Root Forward			N								
126 Crown Forward											
126 Crown Reverse											
126 Root Forward		Y									
126 Root Reverse											
132 Crown Forward											
132 Crown Reverse				M							
132 Root Forward											
167 Crown Forward											
167 Crown Reverse											
167 Root Forward											
167 Root Reverse											
259 Crown Reverse											
259 Root Forward											R
259 Root Reverse											
280 Crown Forward											

Table 6: Continued

Tooth	16249	16250	16251	16252	16260	16261	16265	16267	16269	16275	16277
Reference Sequence	T	C	C	A	C	C	A	C	A	A	A
281 Crown Forward											
281 Crown Reverse											
281 Root Forward											
281 Root Reverse											
322 Crown Forward			M								
322 Crown Reverse											
322 Root Forward			M								
322 Root Reverse											
328 Crown Forward			M								
328 Crown Reverse											
328 Root Forward			M								
328 Root Reverse											
345 Crown Forward			M								
345 Crown Reverse								S			
345 Root Forward											
345 Root Reverse							M		M	M	M
348 Crown Forward	C										
348 Root Reverse											
381 Crown Forward											
381 Crown Reverse						Y					
381 Root Forward			M		M						
381 Root Reverse											
409B Crown Reverse											
539 Crown Forward											
539 Root Forward											
539 Root Reverse											
545 Root Reverse											
546 Crown Reverse											
546 Root Forward											
546 Root Reverse											

Table 6: Continued

Tooth	16249	16250	16251	16252	16260	16261	16265	16267	16269	16275	16277
Reference Sequence	T	C	C	A	C	C	A	C	A	A	A
583 Crown Reverse											
583 Root Forward											
586 Crown Forward											
586 Crown Reverse											
586 Root Forward											
590 Crown Forward											
590 Crown Reverse										M	
590 Root Forward											
590 Root Reverse											
593 Crown Forward											
593 Crown Reverse						Y					
593 Root Reverse											
622 Crown Forward											
622 root Forward											
622 Root Reverse											
690 Crown Forward											
Researcher											

Table 6: Continued

Tooth	16278	16280	16281	26284	16288	16290	16291	16292	16293	16294	16296
Reference Sequence	C	A	A	A	T	C	C	C	A	C	C
3 Root Forward											
22 Crown Forward											
22 Crown Forward											
23 Crown Forward										T	
23 Crown Reverse										T	
23 Root Forward	Y								R		
23 Root Reverse	Y								R		
26 Crown Forward									W	Y	
26 Crown Reverse										T	
26 Root Forward											
26 Root Reverse	T								R	Y	
30 Root Forward										T	
30 Root Reverse										T	
33 Crown Forward											
34 Crown Forward											
34 Crown Reverse										T	
47 Root Forward											
47 Root Reverse											
111 Crown Forward	Y									T	Y
111 Root Forward										Y	
126 Crown Forward	Y								M		
126 Crown Reverse	Y								M		
126 Root Forward										T	
126 Root Reverse										T	
132 Crown Forward											
132 Crown Reverse											
132 Root Forward										T	
167 Crown Forward										Y	
167 Crown Reverse											
167 Root Forward	Y										
167 Root Reverse	T										
259 Crown Reverse										T	
259 Root Forward											
259 Root Reverse											
280 Crown Forward										Y	

Table 6: Continued

Tooth	16278	16280	16281	26284	16288	16290	16291	16292	16293	16294	16296
Reference Sequence	C	A	A	A	T	C	C	C	A	C	C
281 Crown Forward										T	
281 Crown Reverse										T	
281 Root Forward											
281 Root Reverse										T	
322 Crown Forward										Y	
322 Crown Reverse										Y	
322 Root Forward										T	
322 Root Reverse										T	Y
328 Crown Forward										T	
328 Crown Reverse										T	
328 Root Forward										T	
328 Root Reverse										T	
345 Crown Forward	T										
345 Crown Reverse	T	R									
345 Root Forward	T								G		
345 Root Reverse	T								G		
348 Crown Forward								T			
348 Root Reverse										Y	
381 Crown Forward										T	T
381 Crown Reverse										T	T
381 Root Forward										T	T
381 Root Reverse										T	T
409B Crown Reverse										T	
539 Crown Forward										T	
539 Root Forward										Y	
539 Root Reverse										Y	
545 Root Reverse											
546 Crown Reverse			W	W					R	Y	
546 Root Forward										T	
546 Root Reverse										T	

Table 6: Continued

Tooth	16278	16280	16281	26284	16288	16290	16291	16292	16293	16294	16296
Reference Sequence	C	A	A	A	T	C	C	C	A	C	C
583 Crown Reverse										Y	
583 Root Forward										T	
586 Crown Forward							T				
586 Crown Reverse							T				
586 Root Forward					C	T			C		
590 Crown Forward											
590 Crown Reverse											
590 Root Forward	Y										
590 Root Reverse											
593 Crown Forward											
593 Crown Reverse											
593 Root Reverse										Y	Y
622 Crown Forward	Y								R	Y	
622 root Forward						Y				Y	
622 Root Reverse										Y	
690 Crown Forward										T	T
Researcher										T	

Table 6: Continued

Tooth	16299	16301	16303	16304	16305	16306	16307	16308	16309	16310	16311
Reference Sequence	A	C	G	T	A	C	A	T	A	G	T
3 Root Forward											
22 Crown Forward									G		
22 Crown Forward									G		
23 Crown Forward											
23 Crown Reverse			K								
23 Root Forward											Y
23 Root Reverse											Y
26 Crown Forward											
26 Crown Reverse											
26 Root Forward											Y
26 Root Reverse						N	N	N	N	N	N
30 Root Forward											Y
30 Root Reverse											Y
33 Crown Forward	G										
34 Crown Forward											
34 Crown Reverse											
47 Root Forward											C
47 Root Reverse											C
111 Crown Forward											Y
111 Root Forward											
126 Crown Forward											Y
126 Crown Reverse											Y
126 Root Forward											
126 Root Reverse											
132 Crown Forward											
132 Crown Reverse											
132 Root Forward											
167 Crown Forward											Y
167 Crown Reverse											
167 Root Forward											
167 Root Reverse											
259 Crown Reverse		T									
259 Root Forward											
259 Root Reverse											
280 Crown Forward											

Table 6: Continued

Tooth	16299	16301	16303	16304	16305	16306	16307	16308	16309	16310	16311
Reference Sequence	A	C	G	T	A	C	A	T	A	G	T
281 Crown Forward											
281 Crown Reverse											
281 Root Forward											
281 Root Reverse											
322 Crown Forward											
322 Crown Reverse											
322 Root Forward											
322 Root Reverse											
328 Crown Forward											
328 Crown Reverse											
328 Root Forward											
328 Root Reverse											
345 Crown Forward											
345 Crown Reverse											
345 Root Forward											C
345 Root Reverse					M						C
348 Crown Forward											
348 Root Reverse											
381 Crown Forward				Y							
381 Crown Reverse				Y							
381 Root Forward											
381 Root Reverse											
409B Crown Reverse											
539 Crown Forward											
539 Root Forward											
539 Root Reverse											
545 Root Reverse											
546 Crown Reverse											
546 Root Forward											
546 Root Reverse											

Table 6: Continued

Tooth	16299	16301	16303	16304	16305	16306	16307	16308	16309	16310	16311
Reference Sequence	A	C	G	T	A	C	A	T	A	G	T
583 Crown Reverse											
583 Root Forward											
586 Crown Forward											C
586 Crown Reverse											C
586 Root Forward		Y									C
590 Crown Forward											
590 Crown Reverse											
590 Root Forward											
590 Root Reverse											
593 Crown Forward											Y
593 Crown Reverse											Y
593 Root Reverse											Y
622 Crown Forward											Y
622 root Forward											
622 Root Reverse											
690 Crown Forward				C							
Researcher											

Table 6: Continued

Tooth	16317	16318	16319	16330	16335	16339	16350	16351	16352	16355	16357
Reference Sequence	A	A	G	T	A	C	A	A	A	C	T
3 Root Forward											
22 Crown Forward											
22 Crown Forward											
23 Crown Forward											
23 Crown Reverse											
23 Root Forward											
23 Root Reverse											
26 Crown Forward											
26 Crown Reverse										N	
26 Root Forward											
26 Root Reverse				Y							
30 Root Forward											
30 Root Reverse								M			
33 Crown Forward											
34 Crown Forward											
34 Crown Reverse											
47 Root Forward											
47 Root Reverse											
111 Crown Forward						M					
111 Root Forward											
126 Crown Forward											
126 Crown Reverse							N	N	N		
126 Root Forward											
126 Root Reverse							N	N	N		
132 Crown Forward											
132 Crown Reverse											
132 Root Forward											
167 Crown Forward											
167 Crown Reverse											
167 Root Forward											
167 Root Reverse											
259 Crown Reverse											
259 Root Forward											
259 Root Reverse											
280 Crown Forward											

Table 6: Continued

Tooth	16317	16318	16319	16330	16335	16339	16350	16351	16352	16355	16357
Reference Sequence	A	A	G	T	A	C	A	A	A	C	T
281 Crown Forward											
281 Crown Reverse											
281 Root Forward										S	
281 Root Reverse											
322 Crown Forward											
322 Crown Reverse											
322 Root Forward											
322 Root Reverse											
328 Crown Forward											
328 Crown Reverse							N	N	N		
328 Root Forward											
328 Root Reverse											
345 Crown Forward											
345 Crown Reverse											
345 Root Forward											
345 Root Reverse				Y					N		
348 Crown Forward			R								
348 Root Reverse					N		N	N			
381 Crown Forward											
381 Crown Reverse											
381 Root Forward											
381 Root Reverse							N	N			
409B Crown Reverse											
539 Crown Forward											Y
539 Root Forward											Y
539 Root Reverse											
545 Root Reverse	N	N									
546 Crown Reverse											
546 Root Forward											
546 Root Reverse											

Table 6: Continued

Tooth	16317	16318	16319	16330	16335	16339	16350	16351	16352	16355	16357
Reference Sequence	A	A	G	T	A	C	A	A	A	C	T
583 Crown Reverse											
583 Root Forward											
586 Crown Forward											
586 Crown Reverse											
586 Root Forward										Y	
590 Crown Forward											
590 Crown Reverse											
590 Root Forward											
590 Root Reverse											
593 Crown Forward											
593 Crown Reverse								M			
593 Root Reverse											
622 Crown Forward											
622 root Forward											
622 Root Reverse											
690 Crown Forward											
Researcher											

Table 6: Continued

Tooth	16358	16362	16367	16368	16371	16372	16373	16374	16381	16383	16389
Reference Sequence	C	T	A	T	A	T	G	A	T	A	G
3 Root Forward											K
22 Crown Forward											
22 Crown Forward											
23 Crown Forward											
23 Crown Reverse											
23 Root Forward									K		
23 Root Reverse											
26 Crown Forward											
26 Crown Reverse											
26 Root Forward											
26 Root Reverse											
30 Root Forward									K		
30 Root Reverse											
33 Crown Forward											
34 Crown Forward											
34 Crown Reverse											
47 Root Forward		C							K		K
47 Root Reverse		C									
111 Crown Forward											
111 Root Forward											
126 Crown Forward											
126 Crown Reverse											
126 Root Forward											
126 Root Reverse											
132 Crown Forward											
132 Crown Reverse				M							
132 Root Forward											
167 Crown Forward											
167 Crown Reverse											
167 Root Forward											
167 Root Reverse											
259 Crown Reverse	N										
259 Root Forward											
259 Root Reverse											
280 Crown Forward											

Table 6: Continued

Tooth	16358	16362	16367	16368	16371	16372	16373	16374	16381	16383	16389
Reference Sequence	C	T	A	T	A	T	G	A	T	A	G
281 Crown Forward											
281 Crown Reverse											
281 Root Forward			N		N			N		N	
281 Root Reverse											
322 Crown Forward											
322 Crown Reverse											
322 Root Forward											
322 Root Reverse						V					
328 Crown Forward											
328 Crown Reverse											
328 Root Forward											
328 Root Reverse											
345 Crown Forward											
345 Crown Reverse											
345 Root Forward		Y									
345 Root Reverse											
348 Crown Forward											
348 Root Reverse											
381 Crown Forward											
381 Crown Reverse											
381 Root Forward											
381 Root Reverse											
409B Crown Reverse											
539 Crown Forward								K			
539 Root Forward								K			
539 Root Reverse							N				
545 Root Reverse											
546 Crown Reverse											
546 Root Forward											
546 Root Reverse							N				

Table 6: Continued

Tooth	16358	16362	16367	16368	16371	16372	16373	16374	16381	16383	16389
Reference Sequence	C	T	A	T	A	T	G	A	T	A	G
583 Crown Reverse											
583 Root Forward											
586 Crown Forward											
586 Crown Reverse											
586 Root Forward				Y							
590 Crown Forward											
590 Crown Reverse											
590 Root Forward											
590 Root Reverse											
593 Crown Forward											
593 Crown Reverse											
593 Root Reverse											
622 Crown Forward											
622 root Forward											
622 Root Reverse											
690 Crown Forward											
Researcher											

Table 6: Continued

Tooth	16393	16409	16410
Reference Sequence	C	T	C
3 Root Forward	M		
22 Crown Forward			
22 Crown Forward			
23 Crown Forward			
23 Crown Reverse			
23 Root Forward			
23 Root Reverse			
26 Crown Forward			
26 Crown Reverse			
26 Root Forward			
26 Root Reverse			
30 Root Forward			
30 Root Reverse			
33 Crown Forward			
34 Crown Forward			
34 Crown Reverse			
47 Root Forward			
47 Root Reverse			
111 Crown Forward			
111 Root Forward			
126 Crown Forward			
126 Crown Reverse			
126 Root Forward			
126 Root Reverse			
132 Crown Forward			
132 Crown Reverse			
132 Root Forward			
167 Crown Forward			
167 Crown Reverse			
167 Root Forward		C	T
167 Root Reverse			
259 Crown Reverse			
259 Root Forward			
259 Root Reverse			
280 Crown Forward	A		

Table 6: Continued

Tooth	16393	16409	16410
Reference Sequence	C	T	C
281 Crown Forward			
281 Crown Reverse			
281 Root Forward			
281 Root Reverse			
322 Crown Forward			
322 Crown Reverse			
322 Root Forward			
322 Root Reverse			
328 Crown Forward			
328 Crown Reverse			
328 Root Forward			
328 Root Reverse			
345 Crown Forward			
345 Crown Reverse			
345 Root Forward			
345 Root Reverse			
348 Crown Forward			
348 Root Reverse			
381 Crown Forward			
381 Crown Reverse			
381 Root Forward			
381 Root Reverse			
409B Crown Reverse			
539 Crown Forward			
539 Root Forward			
539 Root Reverse			
545 Root Reverse			
546 Crown Reverse			
546 Root Forward			
546 Root Reverse			

Table 6: Continued

Tooth	16393	16409	16410
Reference Sequence	C	T	C
583 Crown Reverse			
583 Root Forward			
586 Crown Forward			
586 Crown Reverse			
586 Root Forward			
590 Crown Forward			
590 Crown Reverse			
590 Root Forward			
590 Root Reverse			
593 Crown Forward	M		
593 Crown Reverse			
593 Root Reverse			
622 Crown Forward			
622 root Forward			
622 Root Reverse			
690 Crown Forward			
Researcher			

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