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The Correlation Between Skeletal Weathering and DNA Quality and Quantity*

ABSTRACT: Mitochondrial DNA analysis of skeletal material is invaluable in forensic identification, although results can vary widely among remains. Previous studies have included bones of different ages, burial conditions, and even species. In the research presented, a collection of human remains that lacked major confounders such as burial age, interment style, and gross environmental conditions, while displaying a very broad range of skeletal degradation, were examined for both mitochondrial DNA (mtDNA) quality and quantity. Overall skeletal weathering, individual bone weathering, and bone variety were considered. Neither skeletal nor bone weathering influenced DNA quality or quantity, indicating that factors that degrade bone do not have the same effect on DNA. In contrast, bone variety, regardless of weathering level, was a significant element in DNA amplification success. Taken together, the results indicate that neither skeletal nor individual bone appearance are reliable indicators of subsequent mtDNA typing outcomes, while the type of bone assayed is.

KEYWORDS: forensic science, mitochondrial DNA, bone, DNA degradation, human skeletal remains, Voegtly Cemetery, skeletal weathering

DNA analysis has become an invaluable tool for the identification of human skeletal remains (1–3), aiding in the identification of missing individuals, U.S. military personnel (4), and the victims of the September 11th attacks (5). Unfortunately, DNA identification from bone, particularly if it has undergone extensive environmental insult, is not always successful; in this regard thousands of skeletal samples from 9/11 have not been useful for DNA-based identification, leaving a substantial percentage of the World Trade Center victims unidentified (6). In part, inconsistencies in skeletal DNA typing success stem from an inability to predict the quality or quantity of DNA contained within a bone sample. Without knowing if a sample contains DNA, or how degraded that DNA is, the analyst may have to repeat a test multiple times or resort to alternate PCR primers before results are obtained or the samples are abandoned. In contrast, if the quality and/or quantity of DNA is correlated with the visual condition of a bone or skeleton, the best avenue to take for successful DNA typing may be predicted in advance.

As skeletal material weathers, the organic molecules within the bone, including DNA, also degrade (7); however, few studies have detailed how bone and DNA degradation overlap. Factors such as bone gross morphology, surrounding environment, and microscopic preservation may influence DNA degradation. At the macroscopic level, researchers have generally found that harder, intact bones provide typable DNA more often than softer or brittle bones (8–10). Others have noted a correlation between the microscopic preservation of bone and DNA recovery, with DNA being present in bones with better preserved microstructure (reviewed in Ref. [11]). This may be related to the preservation of collagen or the crystallinity of hydroxyapatite within aged bone, as the ability to amplify extracted DNA decreases as the crystallinity of hydroxyapatite increases (7,12). Likewise, environmental factors such as soil pH, moisture levels, attack by microorganisms, and the time the remains had been in/on the ground can have a direct influence on not only the bone itself, but the DNA within (reviewed in Ref. [8]).

While these and other studies have begun to address correlations between bone weathering and DNA degradation, they tend to have numerous confounders that make drawing conclusions, particularly statistically significant ones, difficult or impossible. In general, they suffer from small sample sizes, compare remains from diverse geographic locations or variable habitats, have highly variable times since death, and even use multiple species. Indeed, much of the skeletal material examined in the more objective studies has been of animal origin (e.g., Ref. [12]) and in some cases long-term weathering was mimicked in a laboratory setting (e.g., Ref. [7]), which may or may not accurately reflect how bone and DNA degrade. Owing to this variability, general conclusions drawn from the studies can be called into question, whereas removing as many variables as possible would be advantageous in uncovering correlations between bone weathering and DNA quality and quantity.

The goal of the research presented here was to determine if the degradative state of skeletal remains is a useful predictor of

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subsequent DNA analysis success. Many of the confounders detailed above were eliminated by testing a large set of human skeletal material from the Voegtly Cemetery, located in what is now northern Pittsburgh, Pennsylvania. The cemetery, associated with the First Evangelical Church of Allegheny, was utilized between 1833 and 1861, after which all remains were reported to have been transferred to a new cemetery atop nearby Troy Hill. The church grounds were in a highway right-of-way, and when highway construction began in 1987, human skeletal remains were unearthed. A full-scale archeological excavation was carried out from June to September of that year, revealing over 700 burials. All were unembalmed, had been interred in similar six-sided wooden coffins, were tightly packed into the small graveyard, and otherwise were treated similarly. The remains were recorded, removed, packaged, and transferred to the Smithsonian Institution for anthropological analysis. Skeletal data collection at the Smithsonian began in 1993 following the *Standards for Data Collection from Human Skeleton Remains* (13). Soil (a clay loam) was removed by brush or water rinsing (if a bone was intact enough), and estimations of sex, age, and stature were recorded to the extent possible (14). In addition, the degree of skeletal weathering was assessed (see Materials and Methods). Extensive detail on each burial, including its associated artifacts, biological profile, bone anomalies, and skeletal weathering, as well as the history of the Voegtly Cemetery, the community, and methods used for anthropological analyses, can be found in (14).

The Voegtly remains, which do not harbor many of the confounding variables outlined above, were an ideal sample set for examining the relationship between human skeletal weathering and the DNA found in those bones. Mitochondrial DNA (mtDNA) analysis was featured because it is the primary mode of DNA typing of weathered skeletal material (4,7,10,15–18). Both DNA quality (mtDNA amplicon length), and DNA quantity were considered. Skeletons at all levels of degradation were tested, as were different types of bone, allowing for an objective and statistically relevant analysis of the relationship between skeletal weathering and DNA quality and quantity.

Materials and Methods

The Voegtly Church Skeletal Remains

Skeletons had been assigned consecutive identification numbers upon discovery and were given an overall weathering score at the Smithsonian (14) based on a classification system taken from Behrensmeyer (19). The system uses a 0 to 5 scale detailed as:

Stage 0: Bone surface shows no signs of cracking or flaking resulting from aging (weathering).
Stage 1: Bone shows some cracking, usually longitudinal in long bones.
Stage 2: Some cracking and flaking is apparent, especially on the outermost concentric thin layers of the bone.
Stage 3: Bone surface has rough patches of weathered compact bone; external concentric layers have been removed, but weathering does not penetrate deeper than 1.0–1.5 mm.
Stage 4: Bone surface is coarse and splinters may exist; weathering reaches into inner cavities.
Stage 5: Bone has large splinters and is easily broken; original bone shape may be undeterminable.

A complete record of how weathering stages were applied at the Smithsonian is available in (14).

During DNA isolation it became apparent that the weathering level of individual bones did not always correspond to the weathering of the overall skeleton (e.g., obtaining a solid piece of femur from a stage 5 skeleton). To examine if the weathering level of individual bones influenced DNA typing results, a second weathering staging system was developed, which was divided into four categories:

Stage 1: Bone surface shows minimal flaking. The bone section is generally intact.
Stage 2: Bone surface shows some flaking, pieces of bone come off in sheets.
Stage 3: The bone is fragmented into several pieces. At least one large (approaching 1 cm) section is still present.
Stage 4: The bone is extensively fragmented. No large pieces are present.

Bone Selection and Processing

The 36 skeletons utilized spanned five of the six weathering stages assigned in (19): six bones were tested from stage 1 skeletons, 23 from stage 2, 22 from stage 3, 18 from stage 4, and 20 from stage 5 (Table 1). Five skeletons from weathering stage 5 (burials 30, 114, 349, 402, 686) were analyzed although only one bone variety was available from each; these were included to increase the sample size of that stage. Just one stage 0 skeleton existed, thus it was not tested as no statistical analyses could be conducted. Major bone varieties examined included femur (28 samples), pelvis (25), and rib (33). Also included was a fibula from a skeleton with no femur available; it was categorized with the femora.

Adjacent 1- to 2-cm fragments (for long bones these were wedges) were removed from each bone; one was used for histological examination (20), while the other was reserved for DNA analysis. When processing the bones for DNA, a mask, lab coat, and gloves were worn. One-quarter to 1 cm of bone was cut from the source material using a Dremel MultiPro Tool (Dremel, Mount Prospect, IL) with cut-off wheel attachment number 409. The segment was placed in a sterile 15 mL tube, immersed in 1–2 mL of filter-sterilized wash buffer (1% SDS, 25 mM EDTA, 0.1 mg/mL proteinase K) and incubated for 1 h at room temperature, using a method shown to remove exogenous cells/DNA (21). Wash buffer was removed and bones were rinsed with 1 mL of sterile dH_2O six times. Water was removed and bone fragments were dried using compressed air passed through a 0.45 μm vent filter (Millipore, Billerica, MA) for 15–30 min.

Bone fragments were ground to a powder in an IKA A11 Basic Grinder mill with a tungsten blade (IKA Works, Inc., Wilmington, NC). The ground bone was collected in a 2 mL microcentrifuge tube and bone mass was recorded. The mill was cleaned with 10% bleach followed by 70% EtOH between bones, and exposed to UV light for 300 sec (*c.* 2.3 J/cm^2). Between burials the mill was disassembled and cleaned with 10% bleach followed by 70% EtOH, then reassembled and exposed to UV light for 500 sec (*c.* 3.8 J/cm^2). A reagent blank was initiated by placing extraction buffer into the grinder cup, and processing it as detailed below.

DNA Extraction

Four hundred microliters of digestion buffer (20 mM Tris pH 7.5, 100 mM EDTA, 0.1% SDS) and 0.4 mg/mL proteinase K were added to each ground bone sample, which was incubated at 56°C overnight. One volume of saturated phenol (pH 6.5) was

TABLE 1—*Samples analyzed, weathering level and amplicon generated.*

Burial No.	Bone	Age	Sex	SW	BW	107 bp	220 bp	329 bp
27	Rib	22	M	5	3		X	
27	Femur	22	M	5	1		X	
30	Femur	40–55	M	5	3		X	
34	Pelvis	35–45	M	4	2			
34	Rib	35–45	M	4	4		X	
34	Femur	35–45	M	4	1	X*		
47	Pelvis	10.5–12.5	F	4	2		X	
47	Rib2	10.5–12.5	F	4	4			
47	Rib1	10.5–12.5	F	4	1		X*	
47	Femur	10.5–12.5	F	4	2			
111	Pelvis	30–35	M	2	1	X		
111	Rib	30–35	M	2	3	X		
111	Femur	30–35	M	2	1	X		
114	Femur	10–10.5	F	5	2		X	
124	Pelvis	28–35	M	3	3	X		
124	Rib	28–35	M	3	4		X*	
124	Femur	28–35	M	3	2	X		
126	Femur	25–30	F	3	1			
126	Pelvis	25–30	F	3	3			
126	Rib	25–30	F	3	3			
132	Rib	25–30	M	2	3	X		
132	Femur	25–30	M	2	1	X		
132	Pelvis	25–30	M	2	2		X*	
164	Femur	22–26	M	3	1			X
164	Rib	22–26	M	3	3			X
164	Pelvis	22–26	M	3	2			
167	Rib	15–16	M?	2	2			
167	Pelvis	15–16	M?	2	2			
167	Femur	15–16	M?	2	1		X	
192	Rib	60–80	M	2	1			
192	Pelvis	60–80	M	2	2		X	
192	Femur	60–80	M	2	2		X	
203	Fibula	infant		1	2		X	
203	Cranium	infant		1	2			X
203	Rib	infant		1	2			
256	Pelvis	35–45	M	3	2			
256	Rib	35–45	M	3	4			
256	Femur	35–45	M	3	3			
260	Rib	2.5–3.5		4	2	X		
260	Femur	2.5–3.5		4	2	X		
322	Femur	20–24	M	2	2	X		
322	Rib	20–24	M	2	2	X		
322	Pelvis	20–24	M	2	2		X	
328	Pelvis	40–45	M	2	2			
328	Rib	40–45	M	2	2		X*	
328	Femur	40–45	M	2	1	X		
331	Rib	15	M	5	3	X		
331	Femur	15	M	5	1	X		
345	Rib1	Adult	M	3	4			X
345	Femur	Adult	M	3	1			
345	Rib2	Adult	M	3	2			X
345	Pelvis	Adult	M	3	3			
348	Rib	27–35	M	3	4	X		
348	Femur	27–35	M	3	1			
348	Pelvis	27–35	M	3	3			
349	Femur	4.7–6.5		5	3		X	
355	Rib	4		5	4			
355	Femur	4		5	3			
381	Femur	25–30	M	2	1	X		
381	Rib	25–30	M	2	2	X		
381	Pelvis	25–30	M	2	3			
389	Rib			5	3			
389	Pelvis			5	3			
402	Pelvis	25–40	F	5	3			
409B	Femur	30–40	M	3	2	X		
409B	Rib	30–40	M	3	3		X*	
409B	Pelvis	30–40	M	3	3			
447	Rib	30–40	F	4	3		X	
447	Femur	30–40	F	4	2		X	
448	Femur	25–35	F	5	2		X	
448	Rib	25–35	F	5	4		X	
489	Pelvis	1.5		2	1			
489	Rib	1.5		2	1			
529A	Rib	18–25	F	4	2		X	
529A	Femur	18–25	F	4	1		X	
529A	Pelvis	18–25	F	4	2			
539	Rib	30–40	F	4	4			
539	Pelvis	30–40	F	4	2			
540	Rib	32–45	F	5	4			
540	Pelvis	32–45	F	5	2		X	
545	Femur	25–32	F	1	2	X		
545	Pelvis	25–32	F	1	3			
545	Rib	25–32	F	1	4		X*	
546	Pelvis	18–21	F	4	2		X	
546	Rib	18–21	F	4	3			
686	Cranium	4–5.5		5	2		X	
704	Femur	45–60	M	5	2		X	
704	Pelvis	45–60	M	5	1	X*		
704	Rib	45–60	M	5	3		X*	
Total						21	29	5

Burial numbers, age, sex, and skeletal weathering stage (SW) are from (14). Estimated age of individuals at time of death is shown in years, unless there was inadequate skeletal material present. Individual bone weathering (BW) stages were determined as described in Materials and Methods. The largest mtDNA amplicon obtained is denoted by an X, or left blank if no product was produced. *Sample amplified only after purification using a Microcon YM-100 column.

added, the sample was vortexed, and centrifuged at 20,000 × *g* at 4°C for 5 min in a microcentrifuge. The aqueous layer was transferred to a sterile microcentrifuge tube; if the organic layer was dark brown, a second or third phenol extraction was conducted as needed. The sample was then extracted with chloroform, and DNA precipitated by addition of 40 μL of 3 M sodium acetate and 800 μL of 95% EtOH, which was stored at −20°C for at least 1 h. Samples were centrifuged at 20,000 × *g* at 4°C for 20 min, after which the supernatant was removed and DNA vacuum-dried for *c.* 20 min. DNAs were resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.5), based on starting bone mass, using 1 μL/mg of ground bone, and stored at −20°C.

Mitochondrial DNA Amplification from Bone Samples

Preliminary amplification reactions included 1 U *Taq* DNA polymerase (Promega, Madison, WI) or HotMaster Taq (Eppendorf, Hamburg, Germany), 0.2 mM of each dNTP (Promega), 2 μM of primers, 1× PCR buffer (Promega/Eppendorf), 2.5 mM $MgCl_2$ (Promega Taq only), in a 20 μL volume. One × HotM Enhancer (Eppendorf) was later incorporated into the standard HotMaster Taq reactions to help alleviate PCR inhibition. Two quantities of DNA were used in the preliminary experiments: 1 μL neat and 1 μL of a 1:20 dilution. Based on these results the 1:20 dilution was used for subsequent reactions. Positive and negative controls were included in all amplification experiments. PCR parameters were denatured at 94°C for 2 min, followed by 38 cycles of denaturation at 94°C for 30 sec, primer annealing at 56°C for 45 sec, and extension at 72°C for 45 sec, then an extension step at 72°C for 5 min. Amplification success was examined by electrophoresing 5 μL of PCR product on a 2% agarose gel followed by ethidium bromide staining and UV visualization.

Mitochondrial DNA amplification was performed using primers F16190 and R16410 (see [18] for primer sequences; primers were produced at the Michigan State University genomics facility). If a

sample did not generate the 220 bp amplicon, F16144 and R16251 were used, resulting in a 107 bp amplicon. When amplification of the 220 bp amplicon was successful, progressively larger fragments (329 bp followed by 402 bp) were assayed using primers F155/R484 and F82 (5′-ATAGCATTGCGAGACGCTGG-3′)/R484 respectively. Amplification success was judged solely by the presence or absence of a band.

DNA quality/quantity could not be ascertained if PCR reactions were inhibited. Thus, DNAs that showed inhibition (neither an amplicon nor primer activity [primer–dimer] was observed), were purified using a Microcon YM-100 column (Millipore) with 300 μL of TE, at the manufacturer's recommended speed and time. The sample was washed twice more with TE and the retenate brought to the starting volume using TE. The amplification process was then repeated.

Mitochondrial DNA Sequencing of Skeletal DNA

When amplification was successful from more than one bone from a skeleton, the product was sequenced to confirm all bones generated a single haplotype, helping to authenticate the origin of the DNA. Following gel electrophoresis, the remaining 15 μL of PCR product was purified using a Microcon YM-30 column (Millipore) with 300 μL of TE and centrifuged per the manufacturer's instructions. The DNA was washed two additional times with TE and the retentate was brought back to 15 μL with TE. 50–100 fg of DNA, based on the gel electrophoresis detailed above, was sequenced using a CEQ DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA), in a volume of 10 μL. Primers for sequencing were the same as those for amplification, and the Quick Start Kit protocol was followed. Sequences were generated on a CEQ 8000 Genetic Sequence Analyzer (Beckman Coulter), following the LFR-1-60 program (capillary temperature 50°C, denature 120 sec at 90°C, inject 15 sec at 2.0 kV, and separate 60 min at 4.2 kV). Resulting sequences were aligned with BioEdit Sequence Alignment Editor (22) and compared to the Anderson reference sequence (23).

If a PCR amplicon was observed that did not contain enough DNA for sequencing, 1 μL of the PCR product acted as template for an additional 20 cycles of PCR using the same master mix, followed by another round of gel electrophoresis. This reamplification was not used to assess DNA quality, but only to produce adequate product for sequencing.

Quantitative PCR

Real time PCR primers were designed in house using Primer Express software (Applied Biosystems, Foster City, CA), targeting a 118 bp section of hypervariable region 1 (HV1) of human mtDNA. F16400 (5′-ACCATCCTCCGTGAAATCAA-3′) acted as the forward primer and the universal primer "D-loop" (5′-ACCCTGAAGTAGGAACCAGA-3′; 24) as the reverse primer. A TaqMan probe (5′-FAM-CCTCGCTCCGGGCCCATAAC-TAMRA-3′) internal to the primers was utilized (25). Reactions were run in MicroAmp 96-well plates (Applied Biosystems) with an optical adhesive cover and compression pad, on an ABI Prism 7700 (Applied Biosystems).

The manufacturer's cycling parameters were utilized with slight modifications, including primer concentrations optimized at 200 nM forward, 500 nM reverse, 250 nM probe, and addition of 2 ng/μL bovine serum albumin. Reaction volumes were reduced to 10 μL. Standards were 10-fold mtDNA dilutions from 6×10^7 to 6 copies/μL. 1:10, 1:20, and 1:100 dilutions of 1 μL of DNA were first tested from eight bones. Based upon successful amplification of the 1:20 and 1:100 dilutions, a 1:50 DNA dilution was used for quantification experiments. The reactions were run for 50 cycles, in replicates of five.

Statistical Analysis

Single factor analysis of variance (ANOVA) was used to examine the relationship between skeletal weathering, individual bone weathering, and bone variety on DNA quality and quantity. DNA quality statistics were based on production of a PCR product of any size class, as dividing each skeletal or bone weathering stage into multiple amplicon lengths made sample sizes too small for statistical analysis. Quantitative PCR data were based on the average of five replicates for each DNA sample. These data were then averaged based on skeletal weathering, individual bone weathering, or bone type. Results were considered significant at $p < 0.05$. Statistical analyses were performed using Microsoft Excel.

Results

Skeletal DNA Amplification and Controls

Multiple DNA samples showed evidence of PCR inhibition based on the lack of both an amplicon and primer activity (exemplified in Fig. 1). These were purified further (see Materials and Methods), after which four samples retained inhibition; all others produced a PCR product or showed only primer activity and were considered negative. Full sequences were obtained from a large

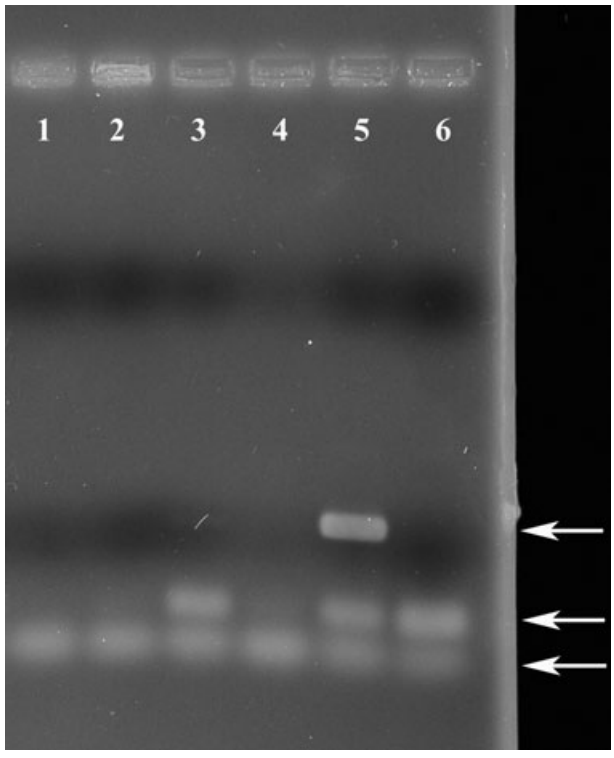

FIG. 1—*Sample agarose gel of postamplification mtDNA products. The upper arrow denotes the target amplicon, the middle arrow denotes primer activity (''primer–dimer''), while the lower arrow denotes primers. Lane 5 contains a positive result, while lanes 3 and 6 are negative. Because lanes 1, 2, and 4 do not show primer activity, PCR was inhibited and the DNA samples were subjected to additional purification to ascertain if DNA was present.*

majority (*c.* 90%) of the bigger amplicons, while the 107 bp product produced little useful sequencing information, as only 20% yielded even partial sequence data. Negative controls and reagent blanks did not produce PCR products, with the exception of a small number of reagent blanks that produced a very weak band. Each was sequenced and compared to bone DNAs processed with it. In no instances were reagent blank sequences the same as skeletal sequences, laboratory personnel sequences, or repeatable. Likewise, DNA sequences from different bones of a skeleton produced identical sequences in all instances, which included 11 different haplotypes that again did not match laboratory personnel, the exception being the most common Caucasian haplotype, which was considered to match by chance. Thus, amplicons produced were assumed to have originated from the skeletal material.

Skeletal Weathering Versus DNA Quality and Quantity

The success of DNA amplification for all samples is displayed in Table 1. None produced the largest (402 bp) amplicon, while five generated the 329 bp product, 29 produced the 220 bp amplicon, 21 were limited to the 107 bp product, and 29 did not produce an amplicon. The level of successful amplification based on skeletal weathering (regardless of amplicon size, which maximized sample size) is shown in Table 2. Skeletal weathering had no influence on the ability to amplify mtDNA ($p = 0.46$). The relationship between skeletal weathering and mtDNA quantity (Table 2) was also not significant ($p = 0.06$), although a potential trend existed in that more weathered skeletons produced larger quantities of DNA. For both DNA quality and quantity there was no influence of anthropologically estimated age or sex (data not shown).

Individual Bone Weathering Versus DNA Quality and Quantity

DNA amplification was next measured based on individual bone weathering criteria (Tables 1 and 2). Amplification results across the stages were similar overall, with the highest success at stage 2 (71.4%) and the lowest at stage 3 (45.8%). There was no statistical difference in amplification among bone weathering stages ($p = 0.269$). Likewise, the quantity of mtDNA recovered showed no correlation with individual bone weathering ($p = 0.71$).

TABLE 2—*Mitochondrial DNA (mtDNA) amplification and yield.*

Weathering Stage/Variety	Amp Success	Amp Failure	Percent	Yield
Skeletal 1	4	2	66.7	54.7
Skeletal 2	16	7	69.6	61.6
Skeletal 3	10	12	45.5	101.6
Skeletal 4	11	7	61.1	128.3
Skeletal 5	14	6	70	122.5
Bone 1	12	7	63.2	105.2
Bone 2	25	10	71.4	85.4
Bone 3	11	13	45.8	76.8
Bone 4	7	4	63.6	107.4
Femur	23	6	79.3	103.2
Rib	21	12	63.6	87.4
Pelvis	9	16	36	77.6

Samples are categorized by skeletal weathering, bone weathering, or the variety of bone. Amp, PCR amplification. Percent is the percent of samples that produced a mtDNA amplicon. Yield is average mtDNA copies/μL, with DNA having been originally resuspended at 1 μL/mg of ground bone. Skeletal weathering showed a (not statistically significant) trend in mtDNA yield, although this was not borne out by individual bone weathering. Only bone variety had a statistically significant influence on amplification success.

Bone Variety Versus DNA Quality and Quantity

The greatest influence on mtDNA amplification success correlated with the type of bone tested (Tables 1 and 2). DNA amplification was observed in 23 of 29 femora/fibula (79.3%), 21 of 33 ribs (63.6%), and nine of 25 pelves (36%). Across bone varieties there was a large statistical difference in amplification success ($p = 0.006$), with the greatest difference occurring between femora and pelves ($p = 0.0009$), followed by ribs and pelves ($p = 0.037$). There was no significant difference between femora and ribs ($p = 0.181$), although the former performed better. The same trend was observed for mtDNA quantity, wherein femora (10–305 copies/μL, average 103.2) had greater quantities of mtDNA than ribs (1–175 copies/μL, average 87.4) which in turn had greater quantities than pelves (11–283 copies/μL, average 77.6), although these did not differ significantly ($p = 0.55$).

Discussion

The goal of this study was to determine if the visual degradative state of skeletal remains is indicative of their DNA quality and quantity, information that might aid in DNA analysis success. Further, different types of bones were analyzed to establish what role bone variety plays in mtDNA recovery. The skeletal material obtained from the Voegtly Cemetery was ideal in this regard, as many variables that could be confounding in this type of research, including the age of the remains, the method of burial, and the general environment in which they were interred, were controlled. DNA was obtained using a standard organic extraction, which may not have been optimal given the amount of PCR inhibition that was originally seen; however, once a Microcon column was employed for inhibited samples, all but four could be amplified (based on a PCR product or primer activity). Still, other DNA isolation methods may result in even better results.

The quality of mtDNA in each sample was assessed by determining the largest amplifiable mtDNA fragment within a sample. The highly degraded state of the DNA (no 402 bp amplicons were produced) reflects the age and condition of the samples and is in line with fragment lengths typically isolated from aged skeletal material (10,11,18). Somewhat surprisingly, there was no correlation between the weathering level of a skeleton and the quality of the DNA within. Likewise, the quantitative PCR experiments showed no significant correlation between skeletal weathering and DNA quantity. A rather counterintuitive trend was seen in which highly weathered skeletons produced greater amounts of DNA, although this was not statistically significant, and was not maintained when the weathering of individual bones was considered, thus it seems more likely that it was circumstantial than widely applicable.

Because the visual condition of individual bones often did not match the weathering state of the entire skeleton from which they originated, and because the structure among bone varieties differs, weathering of each bone was considered. Femora and other long bones are composed of compact bone from the cortex to the medullary cavity along the entire diaphysis, with spongy bone beneath the cortex at the articular ends. In contrast, ribs and pelves have compact bone only in the cortex, with spongy bone beneath the cortex throughout. Among bone varieties in the Voegtly material, femora, regardless of skeleton condition, tended to be in reasonably good shape (none were placed in the poorest individual bone weathering class), while pelves were usually much more degraded, with the outer cortical bone missing in virtually all instances, and only spongy bone, frequently disintegrating, remaining. Rib sections were generally classified between the two (retaining some cortical

material), although these too were often highly degraded. However, even when individual bone weathering was taken into account, no correlation between it and mtDNA quality/quantity was observed, meaning that much like skeletal weathering, the visual appearance of the bone itself is not a useful predictor of DNA typing results.

The differences in bone makeup led to the most consistent findings from this research, wherein the kind of bone assayed had a notable and statistically significant influence on typing success, a finding observed by others (e.g., Ref. 18), and one seemingly consistent with differences in the amount of cortical bone noted above. The largest difference existed between femora and pelves (regardless of weathering levels), followed by ribs and pelves, with the pelvic material producing the poorest results in all cases. This likely stems from microstructural differences between compact and spongy bone. The osteocytes of compact bone are embedded in spaces between concentric layers of hydroxyapatite and collagen, while in spongy bone they are found in spaces within the trabeculae, which occur without the layers of protective bone matrix. Such an open structure most likely allows degradative entities, be they chemical (e.g., acidic moisture) or biological (microorganisms), access to the DNA. Once the thin cortical layer on spongy bone is breached, DNA within is highly vulnerable to degradation. In contrast, the compact microstructure of long bones such as femora extends beyond the cortical layer, providing a larger region of protected DNA. This also indicates that other types of bone with a high level of cortical material will act as a better source of DNA (18). In this regard, since the research presented here was conducted, when testing aged skeletal remains we have begun to regularly sample the hardest/densest bone in the body, the petrous portion of the skull's temporal bone, which houses the internal segments of the ear. The irregular shape of the bone makes its cleaning somewhat challenging, but DNA typing success has been high, including among infant remains, where other bones may be difficult to process (data not shown).

It seems clear from this study that skeletal material and DNA do not degrade via the same mechanisms or in a parallel manner. In some cases this might be obvious: for instance, buried bone can be damaged by mechanical forces (e.g., crushing) that have no direct effect on DNA, while nuclease activity would affect DNA but not bone. Environmental conditions can have an impact on each, but again may do so differentially. As an example, pH is known to directly influence both bone and DNA degradation, with acidic conditions being detrimental to each. However, the dissolution of bone begins at just slightly acidic conditions, while nicking DNA requires a substantially lower pH. In this regard it is interesting to note the very broad range of weathering observed in the Voegtly skeletal remains, even though they were all exhumed from one small cemetery. Apparently, while the remains experienced the same *general* environmental conditions over time, including temperature (e.g., freeze thaw cycles), moisture/precipitation, general soil type, etc., as well as the burial style used at the church, environmental microhabitats existed that affected them to a large degree. We are currently undertaking detailed analyses of soils specifically associated with the remains, in an attempt to understand those factors that differentially influence skeletal and DNA degradation. Given the results detailed here, microhabitats appear to have a strong influence on bone weathering—one that does not necessarily correlate with DNA degradation.

Disclaimer

Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice.

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References

1. Boles T, Snow C, Stover E. Forensic DNA testing on skeletal remains from mass graves: a pilot project in Guatemala. J Forensic Sci 1995;40:349–55.
2. Hagelberg E, Gray IC, Jeffreys AJ. Identification of the skeletal remains of a murder victim by DNA analysis. Nature 1991;352:427–9.
3. Primorac D, Andelinovic S, Definis-Gojanovic M, Drmic I, Rezic B, Baden MM, et al. Identification of war victims from mass graves in Croatia, Bosnia, and Herzegovina by use of standard forensic methods and DNA testing. J Forensic Sci 1996;41:891–4.
4. Holland MM, Parsons TJ. Mitochondrial DNA sequence analysis—validation and use for forensic casework. Forensic Sci Rev 1999;11: 21–50.
5. Holland MM, Cave CA, Holland CA, Bille TW. Development of a quality, high throughput DNA analysis procedure for skeletal samples to assist with the identification of victims from the World Trade Center attacks. Croatian Med J 2003;44:264–72.
6. Gill JR. 9/11 and the New York City Office of Chief Medical Examiner. Forensic Sci, Med, Path 2006;2:29–32.
7. Gotherstrom A, Collins MJ, Angerbjorn A, Liden K. Bone preservation and DNA amplification. Archaeometry 2002;44:395–404.
8. Kaestle FA, Horsburgh KA. Ancient DNA in anthropology: methods, applications and ethics. Am J Phys Anth 2002;119(Suppl. 35):92–130.
9. Parsons TJ, Weedn VW. Preservation and recovery of DNA in postmortem specimens and trace samples. In: Haglund WD, Sorg MH, editors. Forensic taphonomy: the postmortem fate of human remains. Boca Raton: CRC, 1997;109–38.
10. Just RS, Irwin JA, O'Callaghan JE, Saunier JL, Coble MD, Vallone PM, et al. Toward increased utility of mtDNA in forensic identifications. Forensic Sci Int 2004;146(Suppl):S147–9.
11. O'Rourke DH, Hayes GM, Carlyle SW. Ancient DNA studies in physical anthropology. Ann Rev Anth 2000;29:217–42.
12. Tuross N. The biochemistry of ancient DNA in bone. Experentia 1994;50:530–5.
13. Buikstra JE, Ubelaker DH, editors. Standards for data collection from human skeletal remains, Proceedings of a Seminar at The Field Museum of Natural History. Fayetteville, AR: Arkansas Archeological Survey Research Series, 1994;44.
14. Ubelaker DH, Jones EB, Landers DB, editors. Human remains from Voegtly Cemetery, Pittsburgh, Pennsylvania. Smithsonian Contributions to Anthropology Series, Washington, DC: Smithsonian Inst Pr, 2003;46.
15. Gilbert MT, Willerslev E, Hansen AJ, Barnes I, Rudbeck L, Lynnerup N, et al. Distribution patterns of postmortem damage in human mitochondrial DNA. Amer J Hu Genet 2003;72:32–47.
16. Kalmar T, Bachrat CZ, Marcsik A, Rasko I. A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. Nucleic Acids Res 2000;28:e67.
17. Pääbo S, Higuchi RG, Wilson AC. Ancient DNA and the polymerase chain reaction: the emerging field of molecular archaeology. J Bio Chem 1989;264:9709–12.
18. Edson SM, Ross JP, Coble MD, Parsons TJ, Barritt SM. Naming the dead—confronting the realities of rapid identification of degraded skeletal remains. Forensic Sci Rev 2004;16:63–90.
19. Behrensmeyer AK. Taphonomic and ecologic information from bone weathering. Paleobiology 1978;4(2):150–62.
20. Braz VS, Ubelaker DH, Foran DR. Morphological analysis of differential bone survival. Anthropologie 2006;44:123–7.
21. Cline RE, Laurent NM, Foran DR. The fingernails of Mary Sullivan: developing reliable methods for selectively isolating endogenous and exogenous DNAs from evidence. J Forensic Sci 2003;48:328–33.
22. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999;41:95–8.

23. Anderson S, Bankier AT, Barrell BG, deBruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature 1981;290:457–65.
24. Shields F, Kocher T. Phylogenetic relationships of North American Ursids based on analysis of mitochondrial DNA. Evolution 1991;45:218–21.
25. Gehring ME. The recovery and analysis of mitochondrial DNA from exploded pipe bombs (thesis). East Lansing (MI): Michigan State Univ, 2004.

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