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Final Summary Overview

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Establishing a Rank Order for Skeletal Element Sampling: Examining Differential DNA Yield Rates Among and Between Buried Human Skeletal Elements as Compared to Surface Recovered Skeletal Elements

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Summary of Research Project

DNA analysis plays a critical role in forensic identification, particularly when lengthy post-mortem intervals and advanced decay are considered (e.g., Hagleberg, 1991; Holland, 1993; Boles et al., 1995; Anđelinović et al., 2005, etc.). In such instances, extracting DNA from skeletal or dental remains is often necessary to obtain an identification. However, this process is not without its shortcomings. Extracting DNA of sufficient quality for analysis from degraded skeletal remains is challenging and few laboratories possess the necessary expertise to deal with remains of this type in significant numbers. Recent research demonstrates that all skeletal elements do not start out with the same reservoir of DNA, and because DNA does not degrade evenly throughout the skeleton, some bones may prove more reliable for yielding DNA over increased post mortem intervals than others (Frank et al. 2015; Coulson-Thomas et al. 2015; Mundorff & Davoren 2014).

Human decomposition is highly variable and influenced by a number of environmental factors, including burial environment. Patterns established from above-the-ground decomposition may not be representative of a burial context (Mundorff & Davoren 2014). In addition, we know that the burial environment strongly influences decomposition, so it is likely that differences in DNA yield between aboveground remains and buried remains are due to differences in degradation pressures from soil microbes and the intrinsic qualities of bone that contribute to DNA protection from degradation. Our objectives for this research were threefold: (1) Determine whether the patterns of DNA preservation previously observed in skeletal remains associated with above-the-ground processes of decomposition hold true in a burial context; (2) Develop a rank order of skeletal elements based on DNA quantity and quality; and (3) Determine if skeletal DNA degradation is related to bacterial colonization.

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Project Subjects

Project subjects consisted of three donated human cadavers from the University of Tennessee, Forensic Anthropology Center (FAC) Body Donation Program. Three individuals were interred together in a single grave in February 2013, and left undisturbed until March 2017. The grave was located on the newest parcel of land at University of Tennessee's Anthropology Research Facility (ARF). This location was deliberately chosen because the land had previously never been used by the ARF for decomposition research. Having been left undisturbed for four years, the grave was excavated and the individuals were disinterred March 9-10 2017, for the purpose of this project. The bone samples comprised 49 skeletal elements from each of the three individuals.

Project Design and Methods

To empirically assess patterns of DNA degradation from buried skeletal remains and to determine how such patterns relate to site-specific taphonomic variables, including both physical (bone tissue type and size) and biological (microbial degraders), we disinterred a four-year-old grave containing three individuals. Human remains were mapped (hand, FARO, LIDAR) and photographed *in situ* prior to individual disinterment, then placed in paper bags and taken to the FAC laboratory where the bones were brushed with a new sterile toothbrush and room temperature water to remove adherent soil and debris prior to inventory. The individuals were stacked in the grave, which resulted in disparate states of decomposition relative to depth. Upon disinterment, the lower portion of the grave was saturated with water, however, at the time of internment the water table had not been reached. The individual closest to the ground surface (shallowest) was completely skeletonized. The middle body was mostly skeletonized, with small amounts of adipocere, skin and soft tissue present, especially in the upper torso, hands, and feet. The deepest

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body exhibited the most variation in decomposition. The torso, including the proximal ends of femora and humeri, were encased in adipocere and covered with skin, while the hands, feet, and lower segments of the arm (radius and ulna) and leg (tibia and fibula) were mostly skeletonized. The skull was primarily skeletonized, with preserved brain tissue.

Bone Sampling

To directly compare DNY yield from the disinterred skeletal remains, to results from previously tested remains that decomposed on the ground surface, the same methodologies were employed (Mundorff & Davoren 2014), including sampling site location, sampling procedures and amounts, and DNA protocols. To assess *inter*-site variation in DNA quality and quantity, 19 bones per individual were sampled at 2 different sites on the bone, and 3 bones per individual were sampled from 3 sites on the bone. To assess *intra*-site variation, replicate samples were collected from three sites on the same three bones for each individual (Table 1). Each sampling site was marked, photographed, and later photographed again after sampling. Approximately 1-2 mm of the bone's outer surface was removed with a Dremel® rotary tool, then the bones were cleaned with a 10% bleach solution followed by sterile water and 70% ethanol and allowed to air dry. Using a 3/8" drill bit, ~0.2 g of bone powder was collected from each sampling site.

Soil Sampling

In addition to bone, it was our aim to also document the burial environment and to evaluate the impact of human decomposition in a multi-individual grave on soil biogeochemistry and soil microbial communities. Soil samples were collected prior to exhumation using a 10 cm-diameter auger from three linear transects radiating 2 m from the grave at 0.5 m intervals from depths of 0-5 cm and 30-35 cm (Kennan et al. 2018). Control samples were also collected from two undisturbed regions up to 5 m from the grave. Surface soil collections were taken over the grave prior to excavation and during exhumation three samples were collected immediately adjacent to

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the bodies as 30-35 cm. Following disinterment, four samples were collected directly below the bottom individual at the base of the grave ~70-75 cm, and three from 85 cm depth, which was below the grave floor in sterile soil.

At each location, soils were collected using a sterilized probe and bucket: replicate cores were combined and homogenized in the bucket; plant roots and insects were removed manually prior to transferring approximately 500 g samples to plastic bags for storage. All equipment was re-sterilized between samples to prevent cross contamination. Samples were transferred to the Soil Science lab for basic soil chemistry analyses: gravimetric soil moisture, pH, and nutrients were determined using standard measures. Approximately 10 g of the homogenized sample was flash-frozen in liquid nitrogen and stored at -80 for DNA extraction and enzyme assays.

Data Analysis

Bone

The human DNA quantification was performed on a 7500 Real-Time PCR System with the Quantifiler™ Trio system and the HID 1.2 analysis software (Applied Biosystems™). The Femto™ Bacterial DNA Quantification Kit and Femto™ Total Fungal Quantification Kit (Zymo Research) were used to quantify total bacterial and total fungal DNA, respectively, per manufacturer's instructions. Total DNA from bone was measured using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen). All DNA extracts were amplified on a GeneAmp 9700 system using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems).

Human DNA yields were reported based on the measured quantity of the small autosomal target from the Quantifiler™ Trio DNA Quantification Kit; these yields per gram of bone powder were then used to establish a relative ranking of bone samples based on human DNA quantity. DNA quality was determined using the degradation index (DNA degradation levels were assessed

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by dividing the quantity of DNA from the small 80 bp amplicon by that from the large 214 bp amplicon). To further examine DNA quality, the percentage of recovered alleles, defined as the number of successfully amplified alleles in a sample divided by the total number of alleles possible for a given individual, was calculated by bone type for each individual and averaged across individuals. A two-factor analysis of variance test (ANOVA) was used to assess statistical differences in log transformed human DNA concentrations between individuals and body region (skull, arm, hand, upper torso, lower torso, leg, foot). Statistical assumptions including normality and homogeneity of variance were calculated using the D'Agostino Normality Test from the package fBasics (v. 3042.89) and Levene's Test from the package car (v. 3.0.2), respectively. Tukey's HSD test for multiple comparisons was used post-hoc to the two-factor ANOVA to determine group differences. Non-parametric Kruskal-Wallis tests were used to assess statistical differences in log transformed bacterial gene abundances, log transformed fungal gene abundances, log transformed total DNA concentrations, degradation indices, and the percentage of recovered alleles between individuals and body regions. Conover's test for multiple comparisons was used as a post-hoc to Kruskal-Wallis using the package PMCMR (v. 4.3) to determine group differences; a Bonferroni correction was applied to reduce the effects of type I error. All analyses were performed in R v. 3.5.0.

To develop a sampling rank order of bones similar to Mundorff and Davoren (2014), the mean DNA yield by bone type was calculated, and means were sorted by the percentage of STRs recovered followed by human DNA concentration, thereby considering both the quantity and quality of recovered human DNA rather than quantity alone. The average relative florescent units (RFUs) per allele were also assessed as an additional indicator of STR quality. The RFU per allele

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was calculated as the sum of the heterozygous peak heights and homozygous peak heights divided by the total of successfully amplified alleles.

Soil and Bacteria

Soil chemical analyses included nutrient (nitrate and ammonium) concentrations using established microplate methods (Keenan et al. 2018). DNA was extracted from frozen soil samples using a PowerLyzer PowerSoil DNA Isolation Kit and PowerLyzer homogenizer instrument (MoBio Laboratories, Inc.). Total DNA was quantified with a Quant-iT™ PicoGreen® dsDNA quantification kit (Life Technologies). Enzyme activities were also determined using standard methods (Keenan et al. 2018).

Bacterial communities in the soils and the bones were characterized using 16S rRNA gene libraries. Purified DNA samples were sent to the Center for Environmental Biotechnology (UTK) for 16S rRNA library preparation and sequencing. All skeletal samples were submitted for 16S rRNA sequencing (n = 256); in addition, soil samples from within the grave and at 0.5 m outside the grave were submitted for sequencing, including two soil controls (disturbed and undisturbed) (n = 27). Universal primers were used to amplify the V4 region of 16S rRNA gene; libraries were quality checked and pooled, then sequenced on a MiSeq Instrument (Illumina) at a depth of approximately 150,000 reads per sample. Sequence reads were processed using the QIIME 2™ next-generation microbiome bioinformatics platform (v. qiime2-2018.6; v. qiime2-2019.1) (QIIME2 Development Team, 2018) (Bolyen et al., 2018). Read quality was assessed using QIIME2 demux; reads were quality filtered, denoised, chimeras removed, and demultiplexed using DADA2 (Callahan et al., 2016). Features, or amplified sequence variants (ASVs), were classified using a fitted classifier (classify-sklearn) using SILVA v132 (silva-132-99-515-806-nb-classifier.qza) (Pedregosa et al., 2011). Feature and taxonomic data were exported to R (v. 3.4.1) (R Core Team, 2018) for statistical analyses and visualization using phyloseq (v.1.20.0)

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(McMurdie and Holmes, 2013).

Findings

- While the percentage of STRs recovered did not significantly differ by individual, they did significantly differ by body region. Similar to Mundorff and Davoren's (2014) results examining surface remains, the quantity and quality of human DNA recovered from bones of the feet, on average, were much higher than bones from other regions of the body (Emmons et al. 2019, *In prep)*. The foot was the most successful body region and the cuneiforms exhibited the best performance; the 1st cuneiform and 3rd cuneiform ranked among the top ten highest performing bones for all individuals, while the 2nd cuneiform ranked high for two individuals. In fact, foot bones occupy nearly half of the top ranked bones for each individual (5/10, 4/10, and 7/10) (Table 2).
- Bones of the arm and skull, with the exclusion of one maxilla, yielded the poorest human DNA results, with low concentrations and reduced quality scores. Not a single long bone ranked in the top 10 for any of the three individuals.
- When considering bones that were sampled from three sites, total DNA was greater in both the humeral and femoral heads compared with their respective midshaft and distal end.
- Analysis of the soils in the grave area showed minimal lateral transfer of decomposition products. A clear change of soil physicochemical parameters was noted at the base of the grave, where the perched water lens formed: here, anoxic conditions and high ammonia concentrations may have affected the state of decomposition of the bottom-most individual, which had significantly more adipocere compared to the middle individual, while the individual closest to the surface was completely skeletonized. In addition, soil microbial communities at the base of the grave were more similar to bone microbial communities than those found in other soil samples, likely

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related to the pooling of decomposition materials at the grave base.

- The total abundance of bacteria in bone was not a reliable predictor of human DNA concentration, as hypothesized. However, human DNA concentrations are inversely related to the relative abundance of several species belonging to the genus *Clostridium*, which include known collagen degraders, as well as a suite of other taxa from the phyla Bacteroidetes, Proteobacteria, Planctomycetes, Firmicutes, and Actinobacteria (Emmons et al. 2019, *In prep)*.
- Human DNA testing from the foot bones showed similar success across surface (Mundorff and Davoren 2014) and subsurface bone samples. Though the mechanism driving the pattern of successful foot bones remains unknown, there are two plausible causes: (1) bones of the feet undergo extensive remodeling, which increases hematopoiesis and osteoclastic activity that increases the release of cellular DNA into the surrounding bone matrix for eventual stabilization by hydroxyapatite; (2) the bones of the feet harbor a unique foot microbiome following death, influenced by their distance from the gut and their tendency to mummify rather than liquify during decomposition (Emmons et al. 2019, *In prep)*. The foot was the only anatomical region that showed distinct microbial communities in subsurface bone samples.

Implications for Criminal Justice Policy and Practice in the United States

Implications from this research to criminal justice policy and practice in the United States include advancing a DNA sampling rank-order of success for all skeletal element types particular to human remains recovered from a buried context. Selecting the best sample for DNA testing is critical to our ability to identify skeletal remains. Missing person investigations place hefty demands on the U.S. criminal justice system financially and in human effort; not to mention the toll it puts on the family, friends, and communities of missing persons. This research advances

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policy and practice by refining skeletal sampling protocols for DNA analysis, thus reducing time, labor, and financial costs while maximizing identification rates of buried skeletonized remains.

Until this research, a skeletal sampling protocol specific to individual buried remains did not exist and there were significant gaps in the existing body of knowledge regarding skeletal DNA degradation and the effects of the burial environment. This research illuminated DNA degradation patterns to specific taphonomic and microbial influences, which are crucial to the wider applicability of bone sampling. DNA sample failure, due to insufficient preservation of DNA influenced by burial conditions, results in labor-intensive and costly resampling or worse, our inability to identify the individual. The results from this project – a rank order of skeletal elements by success based on the quality and quantity of DNA yielded – can be used to guide human identification efforts of remains recovered from a burial context. An empirically based ranking will focus investigators on those elements most likely to result in complete profiles, streamlining DNA sampling from unidentified remains, and reducing the time and resources dedicated to resampling.

Medical Examiner and Coroner's offices operate on limited and restricted budgets. Smaller jurisdictions in particular, have less leeway with strictly allocated money. When these jurisdictions encounter unidentified buried remains, they may only be able to afford testing a single bone. The results of this research will help maximize their chances of successfully accessing DNA from degraded and potentially contaminated remains.

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Appendices

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Tables

Table 1: Skeletal Sampling Strategy (from Emmons et al. 2019, *In prep*).

Body Region	*Skeletal Element*	*Sample / Individual*	*Total DNA Samples*
Skull	Frontal	1	3
	Temporal	1	3
	Maxilla	1	3
	Parietal	1	3
	Occipital	1	3
	Mandible	1	3
Upper Torso	Cervical Vertebrae	2	6
	1st Rib	2	6
	Middle Rib	2	6
	Sternum	1	3
	Clavicle	2	6
	Scapula	1	3
Lower Torso		1	3
		1	3
		1	3
		1	3
		1	3
		1	3
		1	3
Leg	Femur	6	18
	Tibia	6	18
	Fibula	1	3
	Patella	2	6
Arm	Humerus	6	18
	Radius	2	6
	Ulna	2	6
Hand	Metacarpal 1	1	3
	Metacarpal 2	1	3
	Metacarpal 3	1	3
	Metacarpal 4	2	6
	Metacarpal 5	1	3
	1st Proximal Phalanx	1	3
	2nd Middle Phalanx	1	3
	1st Distal Phalanx	2	6
	Capitate	1	3
Foot	Metatarsal 1	1	3

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	Metatarsal 2	2	6
	Metatarsal 3	1	3
	Metatarsal 4	2	6
	Metatarsal 5	2	6
	1st Proximal Phalanx	1	3
	1st Distal Phalanx	1	3
	Calcaneus	2	6
	Talus	2	6
	Navicular	2	6
	Cuboid	2	6
	1st Cuneiform	2	6
	2nd Cuneiform	2	6
	3rd Cuneiform	2	6

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Table 2: Rank order of skeletal elements by % STR recovered and human DNA (ng gbp^{-1}) (from Emmons et al. 2019, *In prep*).

Individual	Body Region	Bone	Human (ng gbp^{-1})	Std. Dev.	Total log(ng gbp^{-1})	Std. Dev.	D.I.	Std. Dev.	STR (%)	Std. Dev.
A	Upper Torso	Sternum	101.39	NA	3.68	NA	2.30	NA	100.00	NA
A	Lower Torso	Thoracic	78.57	NA	3.96	NA	4.17	NA	100.00	NA
A	Foot	2nd Cuneiform*	69.48	58.85	4.43	0.06	4.46	2.85	100.00	0.00
A	Foot	MT3*	69.24	NA	4.17	NA	2.25	NA	100.00	NA
A	Lower Torso	Rib 12	55.03	NA	3.56	NA	3.75	NA	100.00	NA
A	Hand	Capitate*	49.18	NA	4.03	NA	3.02	NA	100.00	NA
A	Foot	1st Cuneiform**	47.44	3.29	4.29	0.22	1.78	0.38	100.00	0.00
A	Lower Torso	Pubis	43.61	NA	3.88	NA	2.15	NA	100.00	NA
A	Foot	3rd Cuneiform**	43.54	11.17	4.23	0.12	2.54	0.22	100.00	0.00
A	Foot	Navicular	40.09	16.29	3.78	0.43	2.03	0.53	100.00	0.00
B	Hand	1st distal phalanx*	75.88	25.63	4.03	0.09	1.73	0.10	100.00	0.00
B	Foot	3rd Cuneiform**	56.30	8.11	4.45	0.10	9.57	3.97	100.00	0.00
B	Foot	1st Cuneiform**	44.90	11.87	4.66	0.08	3.45	0.63	100.00	0.00
B	Hand	MC2	42.21	NA	4.59	NA	2.63	NA	100.00	NA
B	Foot	MT3*	38.58	NA	4.23	NA	2.77	NA	100.00	NA
B	Hand	Capitate*	37.04	NA	4.02	NA	1.60	NA	100.00	NA
B	Lower Torso	Ischium	31.74	NA	4.27	NA	1.82	NA	100.00	NA
B	Lower Torso	Ilium	30.28	NA	4.12	NA	2.26	NA	100.00	NA
B	Upper Torso	Rib 7	24.73	20.96	4.18	0.05	1.65	0.12	100.00	0.00
B	Hand	MC1	23.33	NA	4.57	NA	3.33	NA	100.00	NA
C	Head	Maxilla	392.86	NA	4.29	NA	1.32	NA	100.00	NA
C	Hand	1st distal phalanx*	390.00	70.71	3.96	0.06	1.93	0.59	100.00	0.00
C	Foot	2nd Cuneiform*	272.40	56.21	3.98	0.05	1.44	0.18	100.00	0.00
C	Foot	1st Cuneiform**	246.56	40.21	4.16	0.08	1.55	0.22	100.00	0.00
C	Foot	Navicular	236.28	89.33	4.17	0.12	1.57	0.24	100.00	0.00
C	Foot	3rd Cuneiform**	180.82	44.02	3.43	0.16	1.41	0.12	100.00	0.00
C	Foot	Talus	179.55	80.63	4.17	0.09	1.80	0.41	100.00	0.00
C	Upper Torso	Rib 1	174.92	231.08	4.35	0.16	1.99	1.12	100.00	0.00
C	Foot	MT2	153.18	152.30	4.07	0.24	1.46	0.14	100.00	0.00
C	Foot	Cuboid	149.20	103.82	4.15	0.03	1.52	0.52	100.00	0.00

*Shared across two individuals. **Shared across all three individuals.

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