



Research paper

Inter and intra-individual variation in skeletal DNA preservation in buried remains

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ABSTRACT

Our ability to identify skeletal remains often relies on the quality and quantity of DNA extracted from bone and teeth. Current research on buried remains has been retrospective, and no study to our knowledge has comprehensively assessed both intra-individual and inter-individual variation in human skeletal DNA from all representative skeletal element types recovered from a burial. Three individuals were interred together in a single grave for four years. Following disinterment, skeletal DNA was extracted, quantified, and GlobalFiler™ results were produced from 49 bones per skeleton, representing all bone types. Multiple sites per bone were also tested to determine intra-bone variability. Co-extracted bacterial and fungal DNA were quantified to determine microbial loads in the bones. Results show that the small, cancellous bones of the feet outperformed other bones in terms of DNA yield, measured as nanograms per gram of bone powder, and short tandem repeat (STR) profile completeness. The cuneiforms, in particular, had consistently high human DNA yields for all three individuals. DNA yield varied by individual and depth within the grave, with the shallowest individual demonstrating the highest DNA yields. While the feet exhibited the greatest variation in DNA yield across bone type and sampling site, they also demonstrated some of the highest DNA yields and the most complete STR profiles, evoking a re-evaluation of their use for skeletal DNA sampling and analysis.

1. Introduction

DNA extracted from bone or teeth is an important source for forensic identification [1–5], particularly when remains are decomposed or skeletonized. Though DNA testing has become routine in forensic practice, degraded DNA, which is common with extended postmortem intervals, requires specialized expertise found at few laboratories. Additionally, skeletal DNA does not degrade evenly across the human skeleton [6,7], and because of this, skeletal selection for DNA sampling can be challenging, informed by anecdotal evidence and limited research [e.g., 7–12].

Multiple recommendations and guidelines currently exist for DNA sampling, especially in the context of disaster victim identification (DVI) [13–16], however, to our knowledge, only one guideline [i.e., 16] provides detailed DNA sampling recommendations for skeletal remains. The International Commission of Missing Persons (ICMP) [16] provides information detailing which bones should be prioritized in sampling, namely teeth followed by femora, tibiae, and pelvic bones. The ICMP also provides regional sampling guidelines for partial remains,

describing which element to sample when only a particular region of the body (e.g., leg, arm, thorax, etc.) is present. Moreover, the ICMP works with remains from many different depositional contexts, and therefore, is one of few published guidelines that considers circumstances of buried and commingled remains.

Recommendations published by ICMP [16] are based on research conducted by Hines et al. [12], which ranked osseous remains as follows: 1) teeth, 2) talus, 3) other tarsals, 4) petrous portion of the temporal bone, 5) femur, 6) vertebrae, 7) tibia, and 8) metatarsals. This study benefited from a large sample size and less biased sampling practices – earlier DNA testing generally focused only on specific bone types, ignoring small or predominantly cancellous bones such as the tarsals. Research was based on 11,650 DNA tests conducted on remains recovered from the Western Balkans (18–21 years post-mortem) [12]. Despite observing high DNA typing success from small foot bones, the authors recommended the sampling of dense, weight bearing bones such as the femur due to other considerations including ease of sampling, diagnostic ability, and potential for re-association when working with large commingled collections, as foot bones are notoriously

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difficult to re-associate to the skeleton [12].

Others have also advocated for the prioritization of weight bearing bones (i.e., the femur and tibia) in skeletal DNA sampling and testing, including earlier research conducted on remains from the former Yugoslavia [11], as well as research generated from the Armed Forces DNA Investigative Laboratory (AFDIL), which regularly processes recovered remains of missing U.S. servicemen from WWII, Vietnam, and Korea for mitochondrial DNA testing and analysis [8–10]. However, these studies used a sampling approach that preferentially selected dense cortical bones, leading to biased sample sizes by bone type. For example, tarsals were not included in tests by Leney [10], Edson et al. [8], or Milos et al. [11]. Extraction protocols also varied between these studies, rendering results less comparable. Differences in extraction methodologies can impact all other downstream processes and can impact final human DNA yields [17].

Small bones of the hands and feet have shown utility in specific DVI scenarios. Sample types including knee cartilage, phalanges, and metatarsals have shown efficacy in DNA testing and identification from disasters in Rio de Janeiro, Brazil, including floods, mudslides, and plane crashes. These smaller elements were easy to remove from individuals using disposable equipment; further sampling did not require large bone saws and other equipment that necessitate decontamination protocols prior to each sampling event [18,19]. Mundorff et al. [20] also observed high DNA typing success in patellae and other small bones including the tarsals and metatarsals in identifying remains recovered from the World Trade Center disaster. In addition, in a study looking at both intraindividual and interindividual variation in human skeletal DNA preservation following surface decomposition, Mundorff and Davoren [7] found that small cancellous bones, on average, yielded greater amounts of better quality DNA than larger, denser cortical bones, a pattern that held true in remains with extended postmortem intervals, up to 21 years.

The extent to which this pattern persists in a burial environment remains unclear. Just as the relative ease of sampling and reduced risk of contamination lends support to the use of smaller bones in certain DVI circumstances, they may additionally show utility in the case of a single individual burial or in a mass grave lacking commingling. Therefore, the purpose of this research was to understand patterns of intra-individual and inter-individual skeletal DNA preservation in a multi-individual burial and how these patterns are shaped by burial factors, including moisture and microbial loading. Microbial DNA is often co-extracted with human DNA, and microbes are often implicated as important mediators of human DNA degradation and PCR inhibition [10,11,21,22]. Because of this, concentrations of human DNA were directly compared with gene abundances quantified from co-extracted bacterial and fungal DNA. Moreover, for comparability with previous research, the same full demineralization extraction procedure (i.e. [23],) used by Hines et al. [12] and Mundorff and Davoren [7] was used in the present study.

2. Materials and methods

2.1. Sample site and skeletal processing

In February 2013, a multi-individual grave was excavated at the Anthropology Research Facility (ARF) at the University of Tennessee, Knoxville (UTK) with the purpose of understanding patterns of skeletal DNA preservation in a buried environment. The grave was approximately two meters by two meters, with a depth of 0.7 m. One female and two males (Table S1) with similar ages at death, who had been donated to the Department of Anthropology's Body Donation Program, were placed inside the grave stacked in a cross formation. The bodies were placed as such because the grave was initially created for a research project designed to simulate a real-world mass grave. The individual at the base of the grave (A) was oriented West to East, with her head placed in the West. The middle individual (B) was placed North to

South, and the shallowest body, individual (C), was oriented West to East. Plastic mesh was placed between donors to prevent commingling. The grave was backfilled and left undisturbed until excavation and disinterment in March 2017.

During disinterment, soils were collected with depth throughout the grave and along lateral transects extending away from the grave. Two types of soil controls were also collected at depths of 0 and 0.3 m, as discussed in Keenan et al. [24]. Soil biogeochemical analyses and micro- and macro-faunal counts were conducted in the Department of Bioengineering and Soil Science and reported in Keenan et al. [24]. Mapping, including depth information, was collected via hand mapping, LIDAR scanning, and FARO 3-dimensional imaging technologies (Fig. S1). Human remains were collected in paper bags and stored at room temperature prior to processing. Decomposition material, debris, and soil were gently removed using a soft toothbrush and warm water at the Forensic Anthropology Center (FAC). Because of adipocere formation on individual A, bones of the upper and lower torso were removed by way of dissection and then cleaned with warm water. All skeletal elements were stored in cardboard boxes for approximately 4–6 months prior to skeletal sampling.

2.2. Skeletal selection and sampling

To represent all skeletal types, a total of 49 bones per skeleton were selected for DNA analysis based on previous research conducted by Mundorff and Davoren [7]. The carpals were represented by the capitate only. To understand how the selection of the specific site to be sampled on a single bone may result in variation in human DNA quality and quantity (inter-site variation), 19 bones per individual were sampled at 2 different places on the same bone (e.g., shaft and epiphysis), and 3 bones per individual were sampled from 3 different areas. To understand variation at a *single sampling site* (intra-site variation), replicate samples were also collected at a single site. Intra-site variation was examined at 3 sample sites on the humerus, tibia, and femur, for each individual (Table 1). Skeletal elements were inventoried, marked for sampling, and photographed prior to sampling.

A Dremel® rotary tool was used to remove approximately 1–2 mm from the outer surface of each bone at the area marked for sampling. The same area was then cleaned with 10 % bleach followed by 70 % ethanol; bones were set to dry for a minimum of 12 h prior to sampling. All bones were sampled in a designated clean lab facility, the Molecular Anthropology Labs at UTK. A standard drill with a 9.5 mm masonry bit was used to extract ~ 0.2 g of bone powder per sample. Samples were weighed and immediately frozen using liquid nitrogen and stored at –80 °C prior to DNA testing. Samples were sent to Bode Cellmark Forensics, Lorton, VA for human DNA testing including extraction, amplification, short tandem repeat (STR) fragment separation, and analysis.

2.3. DNA extraction

Skeletal DNA was extracted from ~0.2 g of bone powder using a full demineralization procedure, with the additional purification step as per Amory et al. (2012). This resulted in a total elution volume of 50 µL.

2.4. DNA quantification

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 quantification reactions were setup as half volume reactions (11.5 µL) with 2 µL of template. DNA degradation levels were assessed by dividing the quantity of DNA from the small 80 bp amplicon by that of the large 214 bp amplicon, resulting in a DNA degradation index (D.I.). The presence of PCR inhibitors was evaluated by the threshold cycle (C_T) value of the internal positive control. Quantities were normalized per gram of bone powder

Table 1
Skeletal Sampling Strategy.

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	Thoracic Vertebrae	1	3
	Lumbar Vertebrae	1	3
	11th or 12th Rib	1	3
	Sacrum	1	3
	Ilium	1	3
	Ischium	1	3
Leg	Pubis	1	3
	Femur	6	18
	Tibia	6	18
	Fibula	1	3
	Patella	2	6
	Humerus	6	18
Arm	Radius	2	6
	Ulna	2	6
	Metacarpal 1	1	3
Hand	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
	Metatarsal 1	1	3
	Metatarsal 2	2	6
Foot	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

(ng gbp⁻¹).

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. Samples were quantified in triplicate, while standards were measured in duplicate. At least 3 no template controls were included in each 96-well plate. Bacterial and fungal assays were performed in the Department of Biosystems Engineering and Soil Science at UTK on a BioRad CFX96™ Real-Time PCR Detection System. Quantities are reported as total gene abundances per gram of bone powder (gene gbp⁻¹).

Total DNA from bone was measured using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen). The assay was performed using reduced volumes of 200 µL and measured on a 96-well microplate reader. Samples were run in duplicate, including five standards, ranging from 0 µg mL⁻¹ to 1.0 µg mL⁻¹; DNA concentrations are reported as nanograms per gram of bone powder.

2.5. Short tandem repeat (STR) amplification, separation, and genotyping

All DNA extracts were amplified on a GeneAmp 9700 system using the GlobalFiler™ PCR Amplification Kit (Applied Biosystems). The amplification was performed at 29 cycles and targeted 1 ng of input DNA, where possible, or 15 µL for samples that had less than 0.06 ng/µL. Following amplification samples were prepared for capillary electrophoresis by taking 1 µL of amplified PCR product, mixing in 0.4 µL GeneScan™ 600 LIZ® dye Size Standard v2.0 (Applied Biosystems) and 9.6 µL HiDi™ formamide (Applied Biosystems) then heating for 5 min at 95 degrees and placing on ice for 5 min. Samples were injected on an ABI3500 XL fragment analyzer and genotyped using the GeneMapper® ID-X Software v.1.5 (Applied Biosystems®) using a peak detection threshold of 125 RFU.

2.6. Data analysis and statistics

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, described above. 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 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 (A, B, and C) 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) [25] and Levene's Test from the package car (v. 3.0.2) [26], 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) [27] 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 [28].

To develop a sampling rank order of bones similar to Mundorff and Davoren [7], 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 and the percentage of STRs recovered were also assessed as additional indicators of DNA quality. The RFU per allele was calculated as the sum of the heterozygous peak heights and homozygous peak heights divided by the total of successfully amplified alleles. The percentage of STRs recovered by sample was calculated by dividing the total number of alleles generated in an STR profile by the total number of alleles possible for an individual and then multiplying by 100 %.

3. Results

3.1. Decomposition state

The individuals were stacked in a single grave, which resulted in disparate states of decomposition relative to depth. Even though the grave was located in the unsaturated zone above the water table, the lower portion of the grave was saturated with water upon disinterment

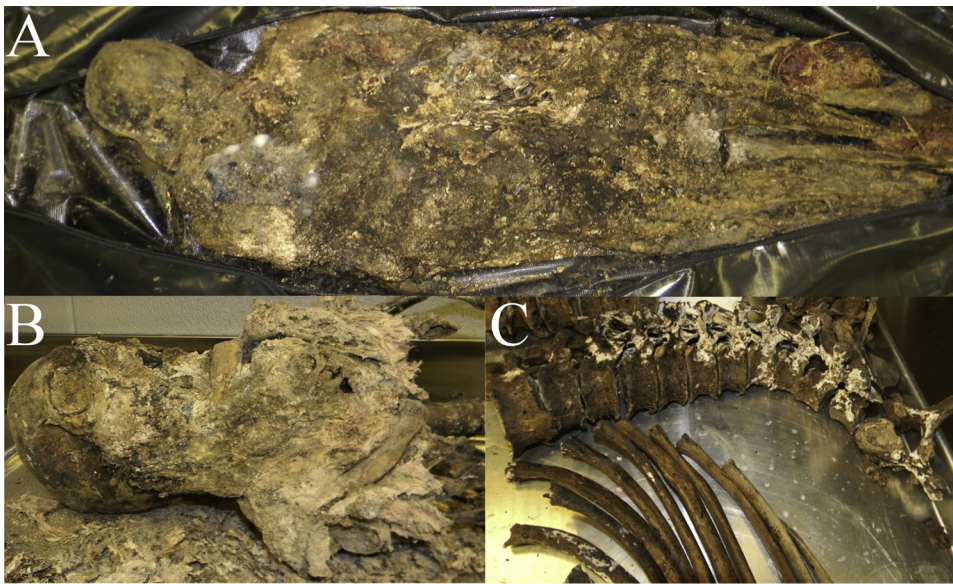


Fig. 1. Adipocere formation and soft tissue preservation on disinterred remains. (A) Individual A exhibited extensive adipocere formation; a hard shell formed with underlying preserved tissue. A bloom of white fungus can be seen in the image that occurred upon storage in a walk-in cooler ($\sim 2^{\circ}\text{C}$). (B) Individual B had moderate soft tissue preservation and adipocere development, especially on the torso. (C) Individual C was mostly skeletonized with minimal amounts of adipocere present on the hands, feet, and vertebral column.

due to a perched water table that had formed within the grave. Individual C, which was closest to the ground surface (shallowest) was skeletonized with no adipocere and little soft tissue present (Fig. 1A). The middle body, Individual B, was partially skeletonized, with some adipocere, skin, and soft tissue present, especially in the upper torso, hands, and feet (Fig. 1B). The deepest body, Individual A, was the least decomposed due to extensive adipocere formation. The upper and lower torso including the proximal one third of the femora and humeri, exhibited pinkish decomposing muscle tissue encased in skin and adipocere; the hands, feet, and lower segments of the arm (radius and ulna) and leg (tibia and fibula) were mostly skeletonized with minimal amounts of soft tissue or adipocere. The skull was primarily skeletonized, with preserved brain tissue (Fig. 1C).

3.2. DNA by individual and body region

Human DNA yield and total DNA concentrations were significantly different between individuals (Human: $\text{DF} = 2$, $F = 6.93$, $p = 0.001$; Total: $\text{DF} = 2$, $X^2 = 16.0$, $p < 0.001$) and body region (Human: $\text{DF} = 6$, $F = 15.437$, $p < 0.001$; Total: $\text{DF} = 6$, $X^2 = 41.4$, $p < 0.001$), with human DNA yield exhibiting a significant interaction effect (Human: $\text{DF} = 12$, $F = 2.21$, $p = 0.01$). Individual C had greater quantities of human DNA than either A ($p = 0.001$) or B ($p < 0.05$), while individual A had significantly lower total DNA concentrations than either C ($p < 0.001$) or B ($p = 0.001$) (Fig. 2A and D; Table S2). Human DNA concentrations from the foot were significantly greater than all body regions except for the lower torso ($p < 0.001$) (Fig. 3A). Bones of the lower torso from individual A had higher human DNA yields than those from B or C. Excluding individual A, bones of the hand ranked second highest, in terms of DNA yield (Fig. S2). Moreover, human DNA concentrations of the arm were significantly lower than human DNA concentrations from all body regions except the head ($p < 0.01$) (Fig. 3A–B). Bones from the arm and head consistently yielded low human DNA concentrations in each of the three individuals (Figs. S2A–S2B). Similarly, total DNA concentrations in the arm were significantly lower than total DNA concentrations from all other body regions, again, excluding the head ($p < 0.05$) (Fig. 3C–D).

Degradation indices significantly differed by individual ($\text{DF} = 2$, $X^2 = 93.9$, $p < 0.001$) (Fig. 2E); human DNA from individual B was significantly more degraded than human DNA from A ($p < 0.001$) or C ($p < 0.001$) (Table S2). The occipital and the first proximal phalanx of the hand from B had much higher degradation indices than all other bones (Fig. S3).

Bacterial gene abundances were significantly different by individual ($\text{DF} = 2$, $X^2 = 52.3$, $p < 0.001$) and body region ($\text{DF} = 6$, $X^2 = 18.2$, $p < 0.01$) (Figs. 2B, 3 E), though the only regions that were significantly different were the hand and the arm ($p < 0.01$). The hand, on average, had greater 16S rRNA gene copies (Fig. 3E–F). Fungal gene abundances were neither significant by individual ($p > 0.05$) nor body region ($p > 0.05$) (Fig. 2C). Neither bacterial DNA nor fungal DNA were significant predictors of human DNA concentrations when tested using linear regression ($p > 0.05$).

3.3. PCR inhibition

Samples were minimally affected by PCR inhibition; only a single sample, from the proximal tibia of individual B, had a threshold cycle (C_T) value greater than 30 for the internal positive control.

3.4. Multi-site differences in human DNA

Human DNA concentrations varied by site on a single bone (Fig. S4). The maximum difference in human DNA concentration for individual A between sampling sites on a single bone, when only two sampling sites were considered, was $83.23 \text{ ng gbp}^{-1}$ for the 2nd cuneiform. In contrast, the minimum difference in human DNA concentration was observed in the radius ($0.2803 \text{ ng gbp}^{-1}$), which was sampled at the distal end and at the midshaft. The ulna also showed only a small difference ($< 1 \text{ ng gbp}^{-1}$) between proximal and midshaft sampling sites for individual A. These patterns in the ulna and radius were consistent for individuals B and C. For individual B, the maximum difference in human DNA concentration by sampling site was observed in the calcaneus ($66.00 \text{ ng gbp}^{-1}$), while in C, the maximum difference was found for rib 1 ($326.8 \text{ ng gbp}^{-1}$). The middle rib of individual A also showed a large difference in human DNA concentration between sampling sites at the center and vertebral end ($65.09 \text{ ng gbp}^{-1}$), with the center having a larger concentration of human DNA; the opposite was observed in rib 1 of C; the vertebral end had a much larger concentration than the center.

When considering bones that were sampled from three sites (i.e., humerus, femur, and tibia), human DNA concentrations varied the greatest by sample site in the femur (Fig. 4). The head of the femur had higher concentrations of human DNA than the midshaft or distal end for all individuals. Total DNA concentrations were also variable by sampling site in the humerus and tibia. Like the femur, total DNA was greatest in the humeral head compared with the midshaft and distal

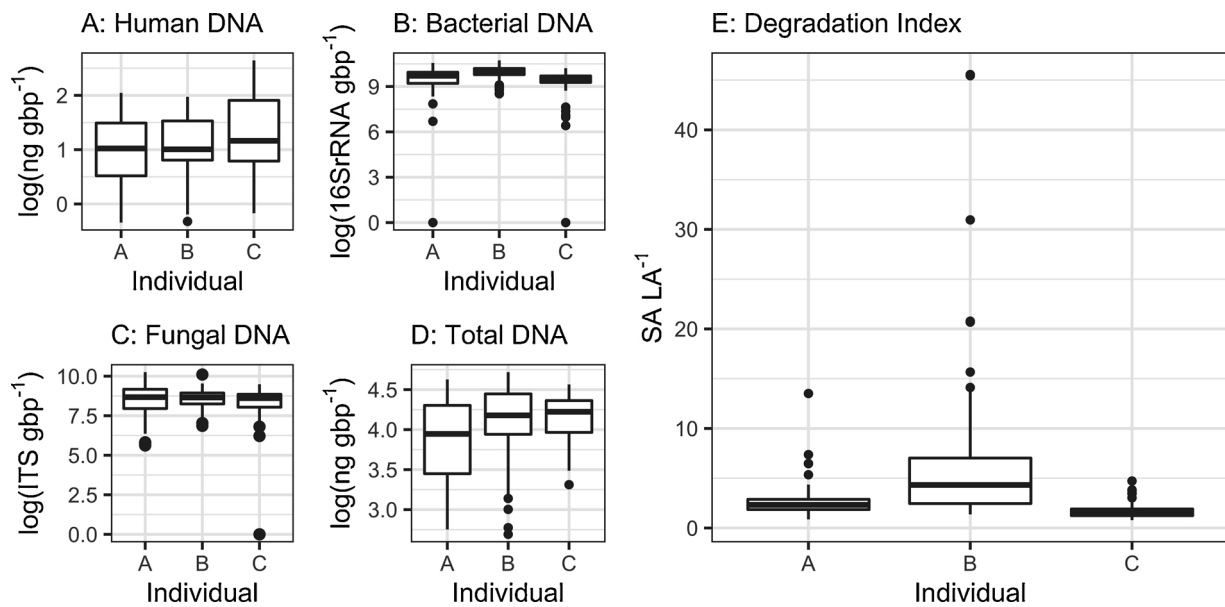


Fig. 2. DNA quantitation by individual. (A) Human DNA concentration by individual. (B) Bacterial and (C) fungal gene abundances by individual. (D) Total DNA concentration by individual. (E) Degradation Index, as the ratio between the small human autosomal target (SA) and the large human autosomal target (LA) by individual.

end.

Variation in human DNA concentration within a single sample site was generally low (standard deviation $< 5.0 \text{ ng gbp}^{-1}$), with the exception of the femoral heads of C and A, the superior articular facet (tibial plateau) of the tibia of B, the humeral head of C, and the mid-shaft of the tibia of B. Within-site differences were not substantial. Approximately 66.7 % of sites sampled twice at the same site, had a standard deviation less than 2.0 ng gbp^{-1} , and 44.4 % of sites with multiple sampling events had a standard deviation less than 1.0 ng gbp^{-1} .

The degradation index (D.I.) demonstrated the greatest variation in the humerus, femur, and tibia of individual B, with the greatest degradation index noted for the femoral head of B. The degradation index also varied within a single sampling site, with the greatest variation noted in the femoral head and superior articular facet of the tibia in B.

3.5. STR analysis

While the percentage of STRs recovered did not significantly differ by individual, they did significantly differ by body region, when data from all individuals were combined ($DF = 6$, $F = 4.997$, $p = 7.9\text{e-}05$), which following post-hoc analysis, was influenced by the arm. The arm had the poorest STR recovery rate (mean: 73.1 %, Std. Dev.: 22.58 %); this was significantly lower than all other body regions ($p < 0.01$), except for the head and the lower torso (Table 2; Fig. 5). The foot was the most successful body region, with the highest STR recovery rate (mean 94.15 %, Std. Dev.: 16.33 %) even though the 1st distal phalanx of the foot consistently performed poorly across all three individuals (Figs. 4; S5). RFUs/allele were also greatest in the foot (Mean 3357.07 RFU, Std. Dev. 1840.26 RFU) (Table 2).

Several samples (26.3 %; 65 out of 247) contained contaminant peaks or other artifacts within resulting STR profiles. The following autosomal STR loci showed artifact/contaminant intrusion: SE33, vWA, D13S317, TPOX, D2S1338, D3S1358, D18S51, D8S1179, TH01, D19S433, and DYS391. While the origin of these peaks remains unknown, at least two have been characterized as known GlobalFiler™ artifacts (GlobalFiler™, Applied Biosystems, Carlsbad, CA) (Fig. 6). Bones of the foot were impacted the most by contaminant intrusion.

3.6. Skeletal ranking by individual

A rank order of skeletal elements by percentage of STRs recovered and human DNA concentration was created by individual, with the top ten highest scoring bones (Table 3). Using STR completeness and human DNA yield as criteria, 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 at least two individuals (Table 3). Foot bones occupied nearly half of the top ranked bones, with 5/10, 3/10, and 7/10 for individuals A, B, and C, respectively. Other top-ranking bones were from the hand and upper and lower torso, with a rib and a hand bone ranked in the top 10 for each individual; of these, only the capitate was observed among the top-ten highest ranking bones across more than one individual. Not a single long bone ranked in the top 10 for any individual.

4. Discussion

Though individuals in this study showed significant differences in mean human DNA concentrations, consistent trends across individuals did present. When ranking bones by both percentage of STRs recovered and DNA concentration, similar to Mundorff and Davoren [7], not a single long bone ranked in the top 10 for any individual. Moreover, 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. In cases when this was not true (i.e., bones of the lower torso of A), extensive adipocere formation was present, potentially influencing results.

Small bones of the feet have also performed well in other research [12,20]. A microscopic analysis from surface remains indicated that this may be due to the survival of remnant tissues, including those of hematopoietic origin, which would increase the pool of available cells from which DNA would then be extracted [29]. Additionally, bones of the feet are weight bearing, and because of this, undergo extensive bone remodeling, not only because these bones experience external force more directly but also because they are impacted by high levels of pressure from interstitial fluids [30]. While this would certainly increase hematopoiesis in the feet, remodeling is also likely to result in the release of DNA into the newly forming bone matrix with the cyclical

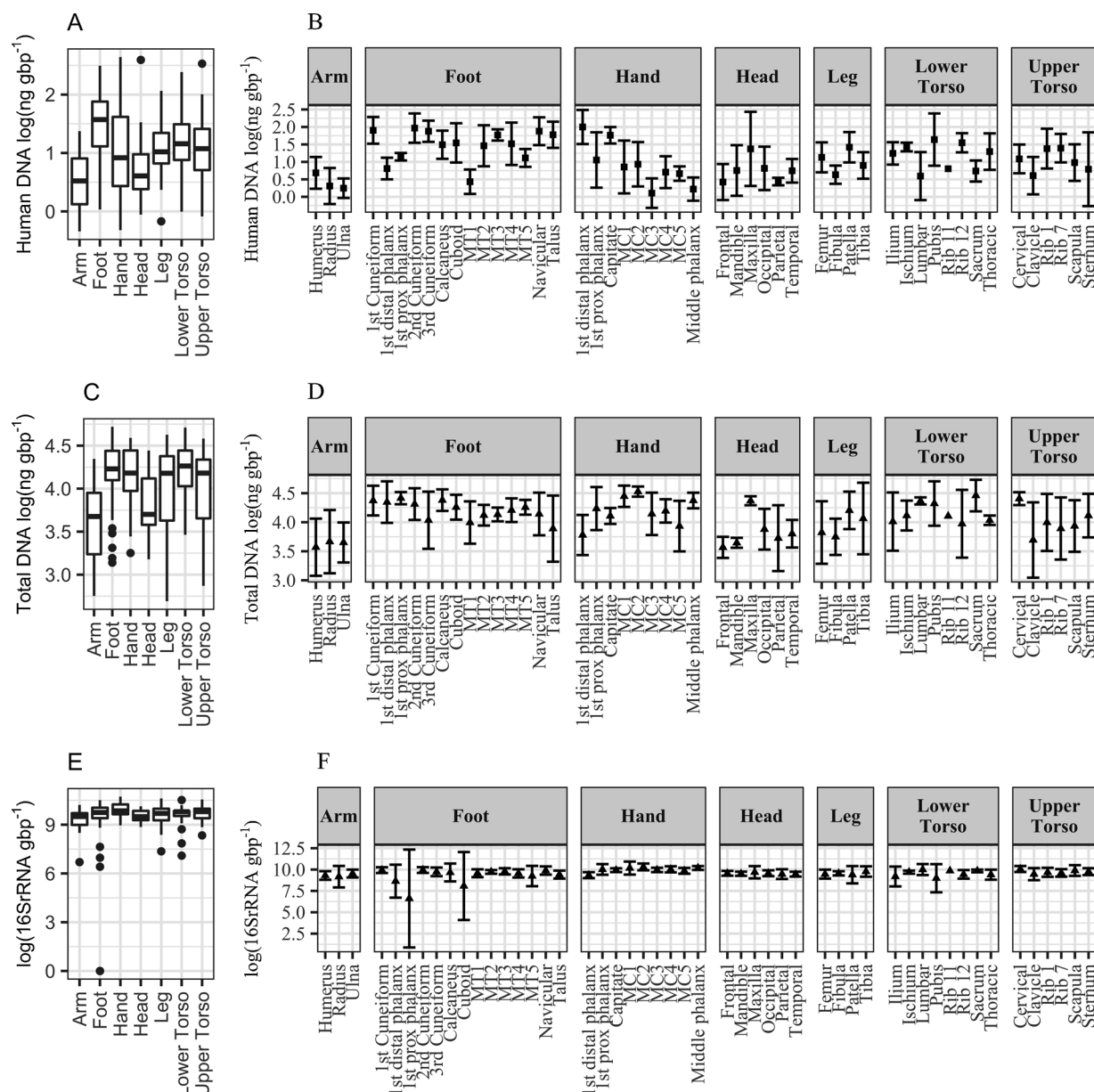


Fig. 3. DNA quantitation by body region. (A) Human DNA concentrations by body region (B) Human DNA concentrations averaged by bone across individuals and visualized by body region. (C) Total DNA concentrations by body region. (D) Total DNA concentrations averaged by bone across individuals and visualized by body region. (E) Bacterial gene abundances by body region. (F) Bacterial gene abundances averaged by bone and visualized by body region. Error bars indicate standard deviation.

cell death of osteoclasts and osteoblasts with continual bone destruction and formation, as first speculated by Campos et al. [17]. Unlike hematopoietic tissue, released DNA into the surrounding matrix during bone remodeling would be more likely to confer protection following death. Alternatively, there could be something unique about foot microbial communities that results in a greater degree of skeletal DNA preservation as previously suggested [31–33], the increased distance from the gut may relate to skeletal DNA survival. In addition, bones of the feet, as well as being exposed to mechanical stress, have less tissue biomass in life than other bones of the body. Because of this, they are prone to mummification rather than decomposition, conferring a level of protection exposed bones do not benefit from [20]. This would not only impact microbial motility but would also likely encourage the formation of a specific postmortem microbiome, potentially with reduced influence from both enteric and environmental microbes. In fact,

unclassified Dermacoccaceae, a bacterial family comprised of multiple taxa isolated from skin [34], has previously been linked to increased human DNA concentrations in postmortem human foot bones from the ground surface ([35], Under Review)).

The temporal bone was not among the top ten highest ranking bones for any single individual, which may be in part due to the location of sampling in the present study, above the external auditory meatus. Though human DNA yields from sampled temporal bones were low, unlike other cranial bones, the temporal bone resulted in complete and nearly complete STR profiles. Two individuals demonstrated drop-out of a single allele, while the third individual had a complete (100 %) STR profile. In addition, the degradation index remained below 2 for all three individuals, and in A and B, the temporal bone was among the top ten highest performing bones based solely on the degradation index. The petrous portion of the temporal bone has shown exceptional preservation in ancient DNA research, which has been ascribed to its

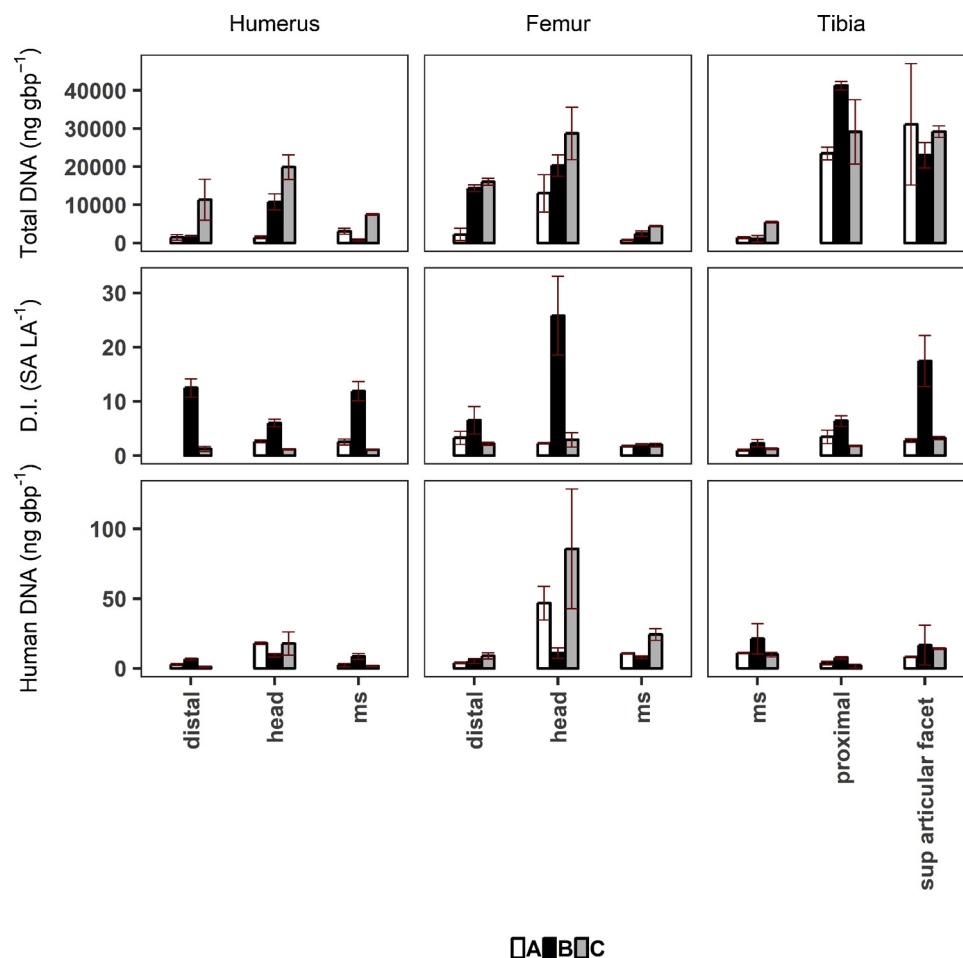


Fig. 4. Intra-bone variation in total DNA, degradation index (D.I.) and human DNA for the humerus, femur, and tibia. Data shown are the means and standard deviation for $n = 3$ technical replicates. Sampling sites are on the x-axis; “ms” refers to the mid-shaft, while “sup” refers to superior. The superior articular facet of the tibia is synonymous with the tibial plateau. “A”, “B”, and “C” are the individuals sampled.

Table 2
Mean STRs recovered (%) and mean RFUs/allele by body region.

Body Region	STR (%)	Std. Dev.	RFUs/allele	Std. Dev.
Arm	73.10	22.58	1124.43	1338.73
Foot	94.15	16.33	3357.07	1840.26
Hand	90.24	17.38	2148.45	1502.46
Head	87.79	19.57	1568.67	1477.16
Leg	89.44	17.54	1980.71	1294.20
Lower Torso	86.10	21.90	2095.41	1359.26
Upper Torso	91.73	17.65	2755.39	1809.47

increased density, the greatest of any human bone, which is required to protect the inner ear [36–39]. In relatively modern contexts, the petrous portion has shown greater DNA typing success than other cranial bones when testing for mtDNA [9] and was ranked highly, prioritized after tarsals, when considering nuclear DNA typing using STRs [12]. While the petrous portion of the temporal bone may yield high quality DNA from forensically relevant remains, more research is needed to determine its utility for decreased time intervals (< 20 years) and various contexts.

As supported across multiple studies [7,8,11,12,20], bones of the arm and skull, with the exclusion of the maxilla from individual C, yielded the poorest human DNA results, with low concentrations and reduced quality scores. Bones of the leg, including the femur and tibia, were not among the highest-ranking skeletal elements. Interestingly, the midshaft of the femur did not produce the highest human DNA

yields, rather the femoral head, which is more cancellous and likely contains more hematopoietic tissue, resulted in the greatest human DNA yields from the femur in all three individuals. The same holds true for the humeral head, which out-performed the midshaft and distal ends. Taken together, these results have implications for skeletal sampling from buried remains. First, for graves containing a single individual or multiple individuals who are not commingled, tarsals should be prioritized over other bones for DNA sampling and analysis. Moreover, if the femur is sampled, and the postmortem interval is under five years, and the femoral head is intact without exhibiting evidence of degradation, the femoral head should be prioritized for DNA sampling over the femoral midshaft.

Individuals were placed simultaneously, in the same grave, to replicate a realistic mass grave scenario and to minimize effects related to seasonality or differences by geographic region, which can affect decomposition rate. As a consequence, differences in decomposition states were likely influenced by depth within the burial, environmental and enteric microbes, and hydrolytic enzyme activity. The deposition environment is an important predictor of skeletal degradation and skeletal DNA degradation; low temperature, increased moisture, and microbial activity favor skeletal DNA preservation, while the opposite, including drastic environmental fluctuations, promote DNA degradation [40–42]. Because individuals were stacked, bones from each individual spanned multiple depths. Individual C was first observed at approximately 27 cm below surface (FARO) and extended to a depth of 55 cm (hand measurement); remains from individual B spanned 34 cm and 62 cm, while individual A spanned between depths of 42 cm and 62 cm (Fig. S1).

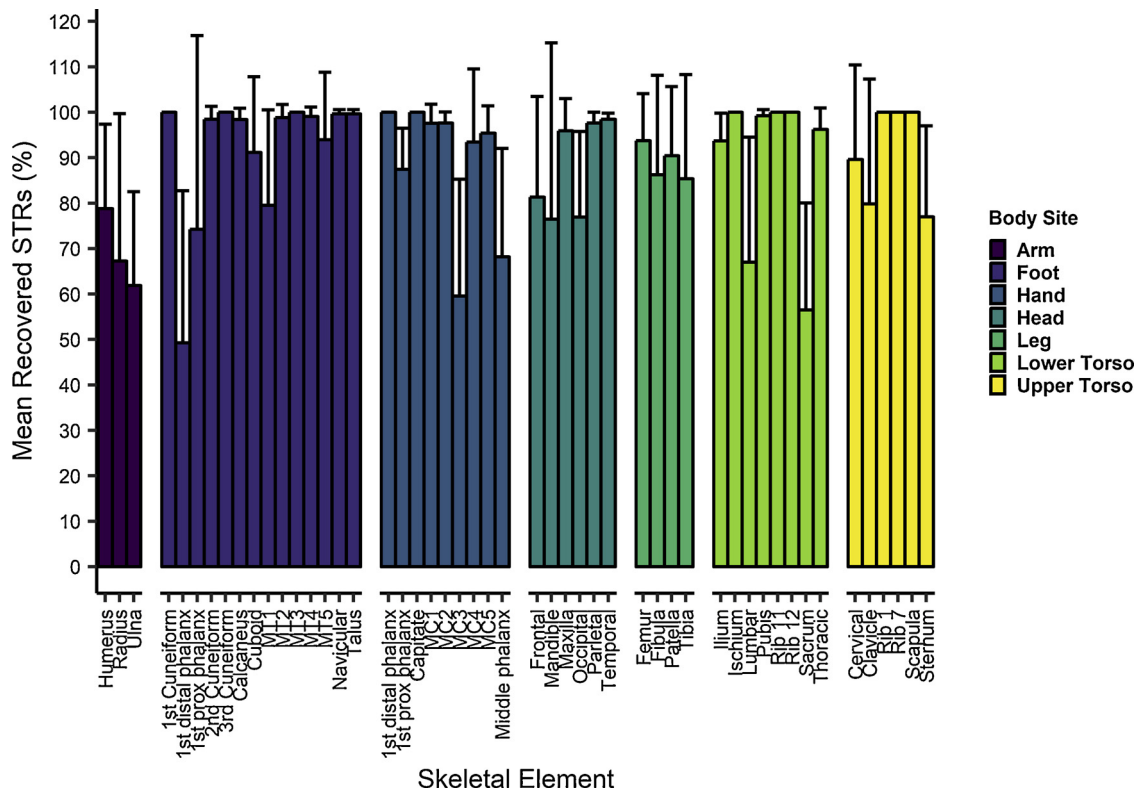


Fig. 5. Mean STR recovery (%) by bone type across three individuals (n = 3).

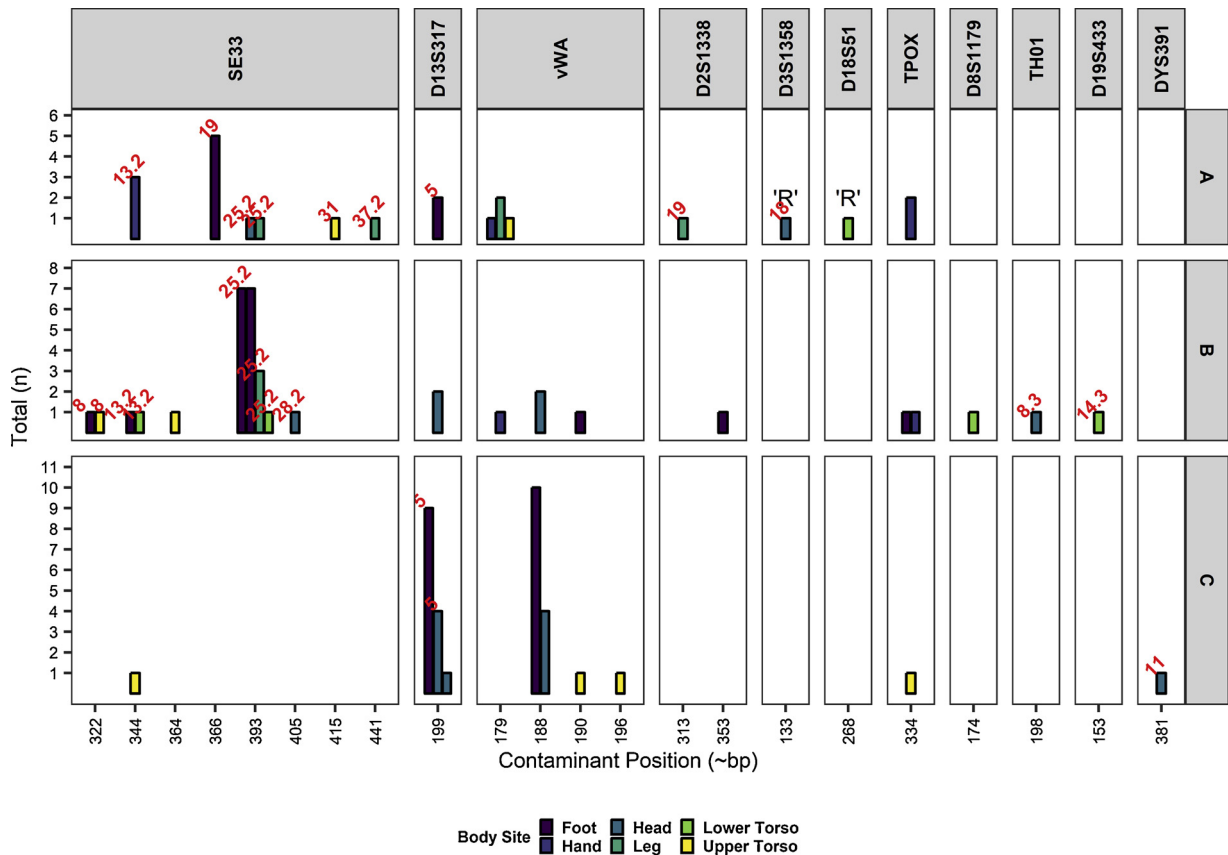


Fig. 6. Number of artifacts and contaminants present in STR profiles by body region and individual. “R” refers to reported artifacts known to the GlobalFiler™ kit. Resulting STR repeat lengths are reported in red; positions without red size indicators were “off-ladder”. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Rank order of skeletal elements by % STR recovered and human DNA (ng gbp⁻¹).

Individual	Body Region	Bone	Bacterial log(gene gbp ⁻¹)	Std. Dev.	Fungal log(gene gbp ⁻¹)	Std. Dev.	Human (ng gbp ⁻¹)	Std. Dev.	Total log(ng gbp ⁻¹)	Std. Dev.	D.I.	Std. Dev.	STR (%)	Std. Dev.
A	Upper Torso	Sternum	9.40	NA	5.82	NA	101.39	NA	3.68	NA	2.30	NA	100.00	NA
A	Lower Torso	Thoracic	9.80	NA	10.25	NA	78.57	NA	3.96	NA	4.17	NA	100.00	NA
A	Foot	2nd Cuneiform ^a	10.04	0.18	9.45	0.05	69.48	58.85	4.43	0.06	4.46	2.85	100.00	0.00
A	Foot	MT3 ^a	10.03	NA	8.34	NA	69.24	NA	4.17	NA	2.25	NA	100.00	NA
A	Lower Torso	Rib 12	9.09	NA	6.69	NA	55.03	NA	3.56	NA	3.75	NA	100.00	NA
A	Hand	Capitate ^a	9.77	NA	7.37	NA	49.18	NA	4.03	NA	3.02	NA	100.00	NA
A	Foot	1st Cuneiform ^b	9.77	0.07	9.17	0.03	47.44	3.29	4.29	0.22	1.78	0.38	100.00	0.00
A	Lower Torso	Pubis	9.74	NA	8.20	NA	43.61	NA	3.88	NA	2.15	NA	100.00	NA
A	Foot	3rd Cuneiform ^b	10.03	0.06	8.87	0.39	43.54	11.17	4.23	0.12	2.54	0.22	100.00	0.00
A	Foot	Navicular	9.58	0.51	8.62	0.16	40.09	16.29	3.78	0.43	2.03	0.53	100.00	0.00
B	Hand	1st distal phalanx ^a	9.73	0.14	8.45	0.08	75.88	25.63	4.03	0.09	1.73	0.10	100.00	0.00
B	Foot	3rd Cuneiform ^b	10.08	0.04	8.70	0.03	56.30	8.11	4.45	0.10	9.57	3.97	100.00	0.00
B	Foot	1st Cuneiform ^b	10.35	0.15	8.68	0.06	44.90	11.87	4.66	0.08	3.45	0.63	100.00	0.00
B	Hand	MC2	10.55	NA	9.05	NA	42.21	NA	4.59	NA	2.63	NA	100.00	NA
B	Foot	MT3 ^a	9.97	NA	8.67	NA	38.58	NA	4.23	NA	2.77	NA	100.00	NA
B	Hand	Capitate ^a	10.18	NA	7.27	NA	37.04	NA	4.02	NA	1.60	NA	100.00	NA
B	Lower Torso	Ischium	9.78	NA	8.96	NA	31.74	NA	4.27	NA	1.82	NA	100.00	NA
B	Lower Torso	Ilium	9.90	NA	7.60	NA	30.28	NA	4.12	NA	2.26	NA	100.00	NA
B	Upper Torso	Rib 7	10.05	0.13	7.93	0.56	24.73	20.96	4.18	0.05	1.65	0.12	100.00	0.00
B	Hand	MC1	10.66	NA	9.32	NA	23.33	NA	4.57	NA	3.33	NA	100.00	NA
C	Head	Maxilla	8.86	NA	7.48	NA	392.86	NA	4.29	NA	1.32	NA	100.00	NA
C	Hand	1st distal phalanx ^a	9.00	0.06	7.98	0.11	390.00	70.71	3.96	0.06	1.93	0.59	100.00	0.00
C	Foot	2nd Cuneiform ^a	9.55	0.06	8.67	0.06	272.40	56.21	3.98	0.05	1.44	0.18	100.00	0.00
C	Foot	1st Cuneiform ^b	9.68	0.14	9.17	0.15	246.56	40.21	4.16	0.08	1.55	0.22	100.00	0.00
C	Foot	Navicular	9.64	0.03	9.05	0.27	236.28	89.33	4.17	0.12	1.57	0.24	100.00	0.00
C	Foot	3rd Cuneiform ^b	9.08	0.05	8.19	0.44	180.82	44.02	3.43	0.16	1.41	0.12	100.00	0.00
C	Foot	Talus	9.40	0.00	8.88	0.30	179.55	80.63	4.17	0.09	1.80	0.41	100.00	0.00
C	Upper Torso	Rib 1	9.87	0.03	9.08	0.35	174.92	231.08	4.35	0.16	1.99	1.12	100.00	0.00
C	Foot	MT2	9.51	0.04	8.59	0.15	153.18	152.30	4.07	0.24	1.46	0.14	100.00	0.00
C	Foot	Cuboid	9.22	0.28	8.66	0.33	149.20	103.82	4.15	0.03	1.52	0.52	100.00	0.00

^a Shared across two individuals.

^b Shared across all three individuals.

Depth is an important indicator of microbial composition, structure, and function, related to changes in edaphic features including soil composition, oxygen availability, carbon source, temperature, moisture, etc. [43,44]. Human DNA concentrations did not correlate with microbial gene abundances; though, this may have been influenced by a delay between the time at which the remains were disinterred and the time at which the bones were sampled for DNA. The quantity of microbes able to colonize bone may be of little importance; rather, changes in community composition and microbial interactions may be more indicative of human DNA preservation in buried remains.

In a separate study, we examined changes in soil geochemistry within the grave, including pH, moisture, microbial respiration, dissolved organic carbon, dissolved organic nitrogen, and extracellular enzyme potentials, among others [24]. Despite observing high concentrations of labile organic sources including dissolved organic carbon and nitrogen at the grave base (70–75 cm), likely as pooled inputs from all three sets of remains, shifts in enzyme potentials – including increases in N-acetyl- β -glucosaminidase and decreases in phosphodiesterase and leucine aminopeptidase – indicate a more recalcitrant functionality for microbial communities closer to the grave base. This activity was likely mediated by oxygen availability. With the base of the grave saturated, anoxia was prevalent here. In contrast, at 40 cm and above the saturated zone, soils were oxic. In addition, potential collagenase activity, as an indicator of collagen degradation, which makes up a large portion of the organic content of bone, demonstrated increased concentrations at depths of 30 and 40 cm, indicating an increase in potential collagen degradation [24]. Collagen degradation facilitates the release of bound DNA from either collagen fibrils or hydroxyapatite [17,45], which is why collagen degradation has been used as an indicator of skeletal DNA degradation [6,46,47].

Together, these observations would lead us to expect lower human DNA concentrations in the bones of C and B than in the bones of A, yet paradoxically, we saw significantly greater human DNA concentrations in C, the only fully skeletonized individual. The probable cause of this pattern is the increase in moisture with depth in the grave. The base of the grave was completely saturated at the time of excavation, and several elements from A and B were submerged. Water can be devastating to skeletal preservation [47,48]. Increased moisture encourages microbial growth, though complete saturation deters growth [49]. Moreover, water augments dissolution of the inorganic component, hydroxyapatite, of bone and the subsequent degradation of the organic component, collagen, releasing bound DNA from both hydroxyapatite crystallites [17,48,50] and mineralized collagen fibrils [17]. With completely submerged bones, the possibility of leaching of nucleic acids into the external environment cannot be ignored, as nucleic acids are soluble in water [48]. The fluctuation of the water table and hydraulic potential of the burial in this study over time remain unknown. DNA from non-mineralized osteoid containing osteoblasts, osteoclasts, and bone lining cells [17], is likely consumed early in the decomposition process by colonizing microbes, due to ease of access, and likely is not the primary source of skeletal DNA in this study.

5. Conclusion

Results indicate that bones of the feet should be considered priority sources for skeletal DNA sampling and analysis from buried non-commingled remains. While the sample size in the current study was small, the patterns observed align well with those in previous research [7,12,20]. Though bones of the feet may be more challenging to re-associate in cases of commingled individuals, there are other circumstances in which this would not be problematic, such as the case of a single-person burial or multiple person burial without commingling. Moreover, we acknowledge that the mechanism(s) of skeletal DNA preservation likely shift(s) with time, as does the diagenetic profile of bone [46], and so the observed patterns may not be reflective of post-mortem intervals greater than four years. However, patterns from

surface and buried remains suggest otherwise [7,12].

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.fsigen.2019.102193>.

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