Document Title:	Intra-Bone Variation of Recoverable Nuclear and Mitochondrial DNA from Human Skeletal Material
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Intra-bone Variation of Recoverable Nuclear and Mitochondrial DNA

from Human Skeletal Material[1]

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Draft Summary Overview

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Skeletal material often forms the basis of forensic investigation, given that it is among the most durable tissue in human remains. Forensic anthropologists' analysis of osseous tissues can help determine the identification of the deceased, however anthropological identifications are often limited by the bone available for analysis, its condition, and a lack of individualizing characteristics or features that can be compared to ante-mortem records such as dental X-rays. In such instances genetic analyses of the skeletal remains may be indicated, as DNA identifications are both highly precise and can confer statistical certainty on the identification of an individual, which other forensic techniques cannot. Owing to this, DNA-based analyses of skeletal material has become the *de rigueur* method for human identification from skeletal remains that do not have a direct ante-mortem method for comparison.

The default skeletal elements used for human identification are weight bearing long bones such as the femur or tibia[2]. It is presumed that the dense cortical material in these elements 'protect' the DNA from environmental insult, and aid in its preservation. However, this hypothesis relies on anecdotal evidence, and had not been tested using objective experimental data. In 2013 Mundorf and colleagues noted that other, less dense skeletal elements, such as the phalanx, patella, and tarsals produced as good or better genetic identification data than did long bones[3]. Following this, work in our laboratory indicated that substantial differences in both nuclear and mitochondrial DNA quantity exist *within* animal femora[4]. Next, Antinick showed that not only were there significant differences in mitochondrial and nuclear DNA quantity and quality within both cow and pig femora, but that the greatest amounts of DNA were not in the

[2] e.g., Edson SM, Ross JP, M, Coble D, Parsons TJ, Barritt SM. 2004. Naming the dead—Confronting the realities of rapid identification of degraded skeletal remains. *Forensic Sci Rev*. 16:63–90

[3] Mundorff AZ, Davoren J, Weitz S. 2013. Developing an empirically based ranking order for bone sampling: Examining the differential DNA yield rates between human skeletal elements over increasing post mortem intervals. Final Technical Report submitted to the National Institute of Justice.

[4] Hebda LM, Foran DR. 2015. Assessing the utility of soil DNA extraction kits for increasing DNA yields and eliminating PCR inhibitors from buried skeletal remains. *J Forensic Sci* 60, 1322–1330

midshaft diaphysis, but instead existed at the proximal and distal ends of the bones, in stark contrast to accepted doctrine (since published in [5]). Based on this, we proposed to NIJ that we perform a similar study examining unpreserved human bone, both fresh and following surface and sub-surface interment. Nuclear and mitochondrial DNA quantity and quality would be assessed, with a goal of determining if the mid-shaft diaphysis of femora is the optimal location for DNA recovery from human skeletal remains.

Methods

Five sets of fresh human remains were obtained from the Anatomical Donations Program at the University of Michigan (Ann Arbor, MI). When appropriate remains[6] became available, both legs were detached and the bulk of the soft tissue was removed. The limbs were returned to the Forensic Biology Laboratory at Michigan State University, and femora, patellae, tali, and calcanei [7] were macerated as described in [8]. The 14 bone regions analyzed are shown in Figure 1. Each region was drilled as described in [4], producing 20 – 30 mg of bone powder. The drilled hole(s) was filled in with dental stone, and the bones were alternately [9] placed on the ground surface or buried one foot directly beneath them, at the edge of a woodlot on the MSU campus (*ca.* coordinates 42°41'30.3"N 84°29'36.2"W). The bones were covered with staked fencing to prevent scavenging. The bones were exhumed after: 4 days, 1 week, 11 days, 2 weeks, 3 weeks, 1 month, 4 months, 6 months, 8 months, rinsed and re-drilled, and reinterred. All skeletal material was subsequently returned to the Anatomical Donations Program.

[5] Antinick TC, Foran DR. 2019. Intra- and inter-element variability in mitochondrial and nuclear DNA from fresh and environmentally exposed skeletal remains. *J Forensic Sci* 64, 88–96 doi:10.1111/1556-4029.13843
[6] Defined as having no alterations such as skeletal prostheses, and no disease directly affecting bone
[7] In a separate and much less extensive undergraduate research project, tibiae were analyzed similarly
[8] Rennick SL, Fenton TW, Foran DR. 2005. The effects of skeletal preparation techniques on DNA from human and nonhuman bone. *J Forensic Sci* 50, 1016–1019
[9] Individual 1: left leg surface, right leg buried; Individual 2: right leg surface, left leg buried, etc.

1	Midshaft diaphysis (MD)	8	Greater trochanter (GT)
2	Proximal diaphysis (PD)	9	Patellar grove (PG)
3	Distal diaphysis (DD)	10	Neck (N)
4	Proximal metaphysis (PM)	11	Lateral condyle (LC)
5	Distal metaphysis (DM)	12	Patella (P)
6	Lesser trochanter (LT)	13	Talus (T)
7	Medial condyle (MC)	14	Calcaneus (C)
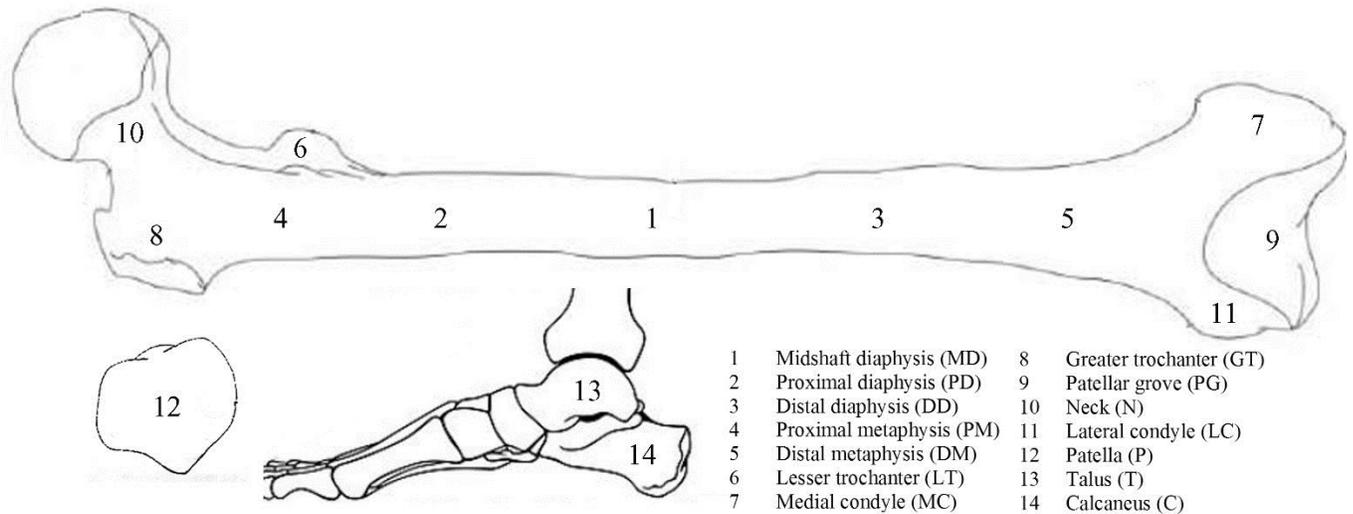
Figure 1 The fourteen bone regions sampled in this study (not to scale).

DNA from the bone powder was isolated using a high EDTA method as described in [4]. Nuclear DNA was quantified using an ABI Quantifiler kit. Mitochondrial DNA was quantified using an in-house TaqMan assay, based on a 5' HEX dye and a 3' Black Hole quencher. The determined mitochondrial DNA yields are relative not absolute, as they were based on the mitochondrial DNA levels of the human DNA standard in the Quantifiler kit, whose absolute mitochondrial DNA levels are not know. Nuclear DNA quality was assessed through amplification of non-overlapping segments of the human cystic fibrosis gene, ranging from 170 ~ 600 bp. Mitochondrial DNA quality was assessed through amplification of non-overlapping segments of human mitochondrial DNA, ranging from 100 ~ 600 bp.

Results

Nuclear and mitochondrial DNA quantity data from 0 – 8 months for surface and sub-surface interred bones are displayed in Figures 2 – 5 [10]. In general, the trochanters had the

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[10] Months 4 – 8 data are also presented so as to display finer details that are not resolvable in the full temporal charts, given the range of the Y axis
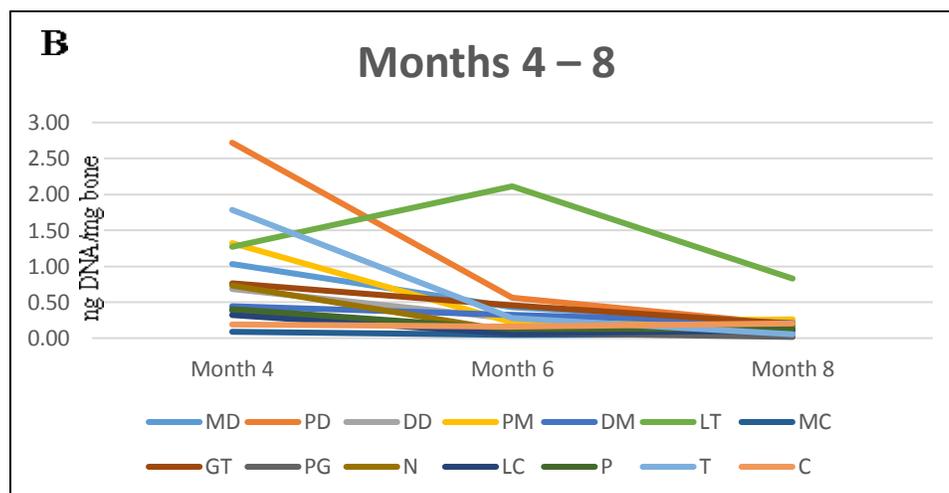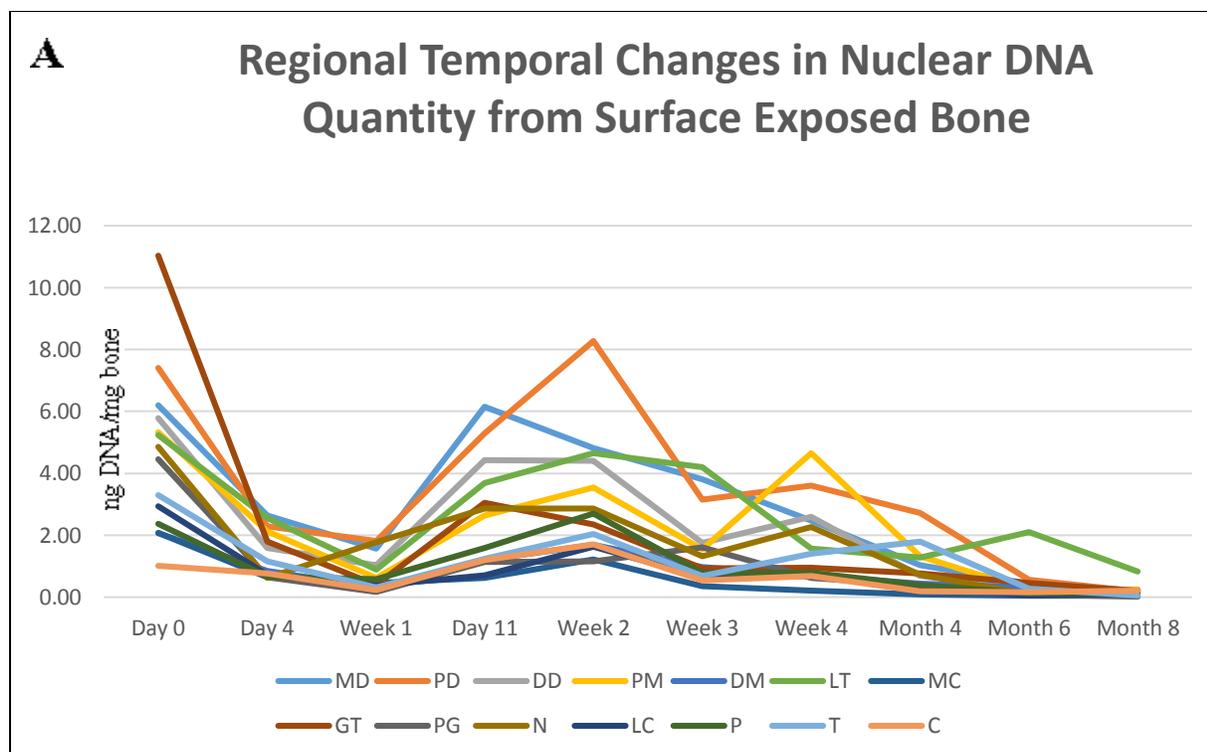
Figure 2 Changes in nuclear DNA quantity among bone regions in surface exposed bones over time. A) Day 0 through 8 months. B) Details of months 4 – 8. Bone locations tested (colored lines) are detailed in Figure 1.

greatest starting amount of both nuclear and mitochondrial DNA. Nuclear DNA levels for both surface and buried bone dropped off dramatically after just four days (the first time point tested,
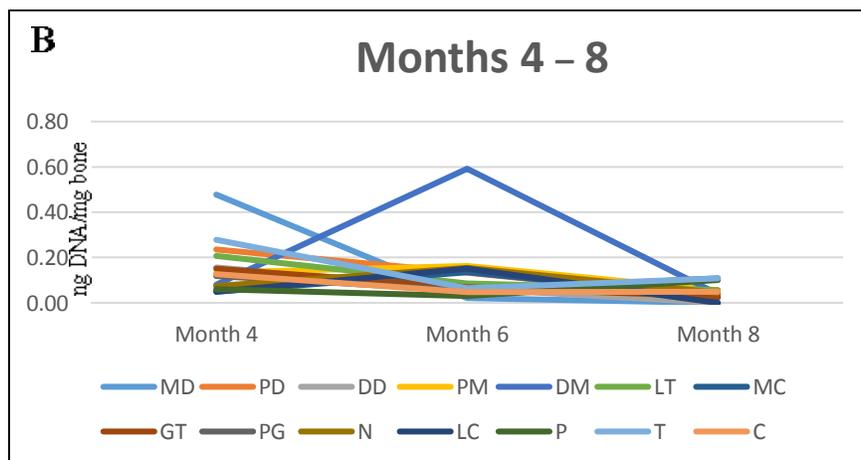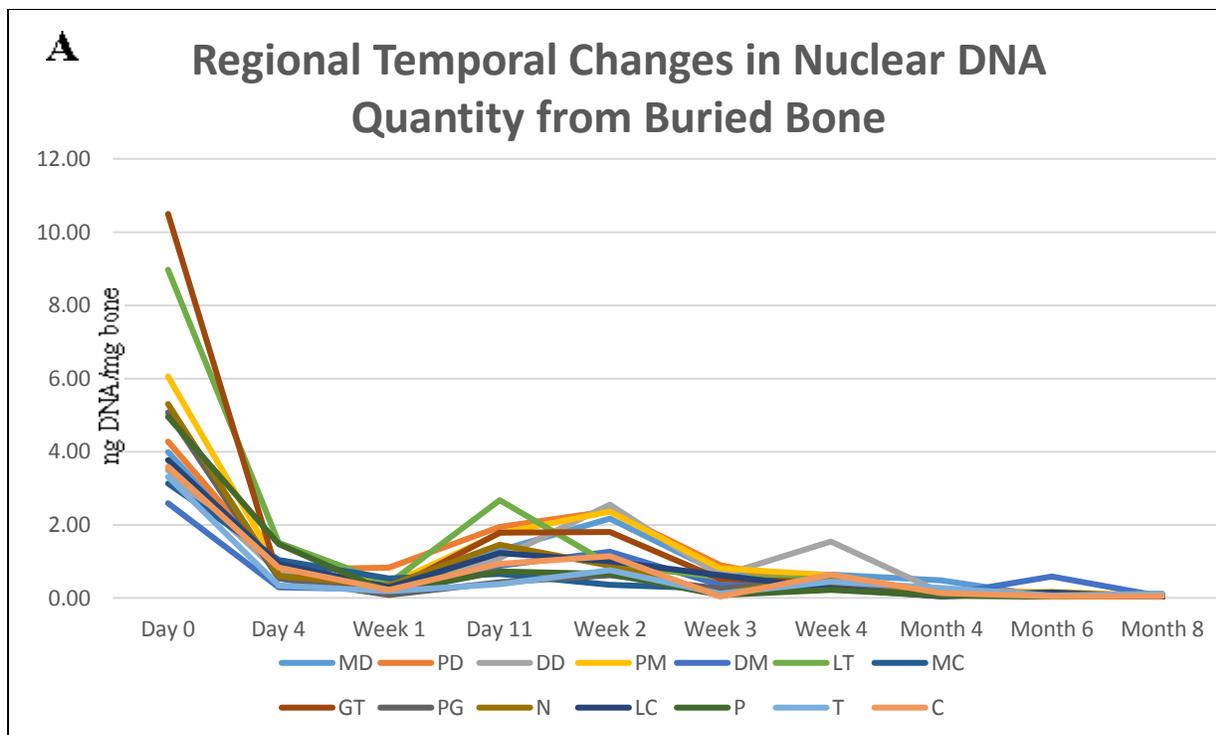
Figure 3 Changes in nuclear DNA quantity among bone regions in buried bones over time. A) Day 0 through 8 months. B) Details of months 4 – 8. Bone locations tested (colored lines) are detailed in Figure 1.

Figures 2A and 3A), as did mitochondrial DNA from buried bones (Figure 5A), and overall,

buried bones had 70 – 90% lower DNA yields after just 4 days of interment. In contrast, many

regions of surface bones showed a slight increase in mitochondrial DNA from day 0 to day 4

(Figure 4A), and virtually all bone locations showed increased DNA yields between day 4 and
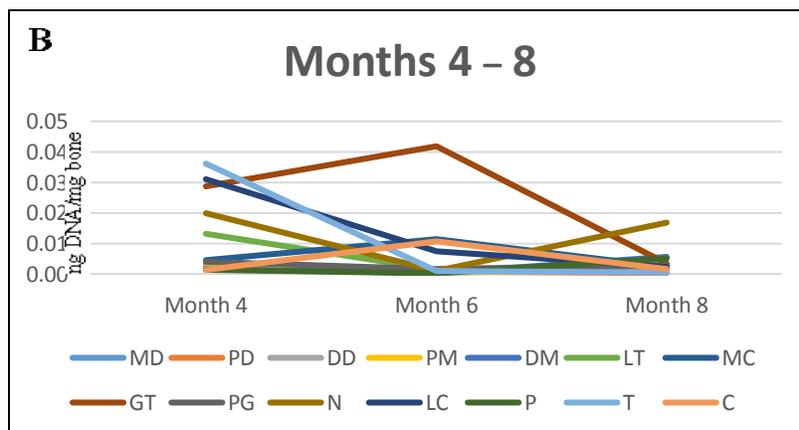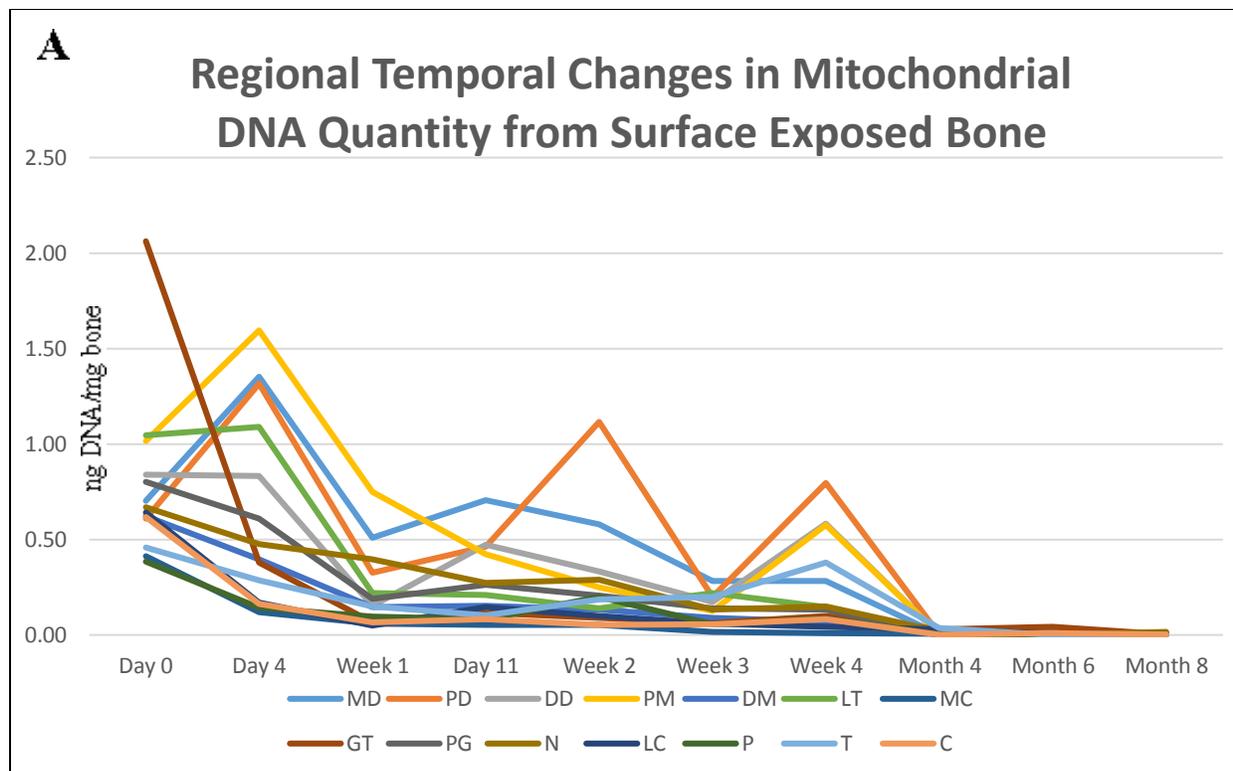
Figure 4 Changes in mitochondrial DNA quantity among bone regions in surface exposed bones over time. A) Day 0 through 8 months. B) Details of months 4 – 8. Bone locations tested (colored lines) are detailed in Figure 1.

week 3 (readily apparent in Figures 2A – 4A). This latter result is consistent with those in [3] and [4], wherein nuclear DNA yields, in particular, tended to increase after short intervals of interment, most prominently for surface exposed material. The reason for this is not known, however it seems likely that interment helped soften the bone, making DNA retrieval more effective, which
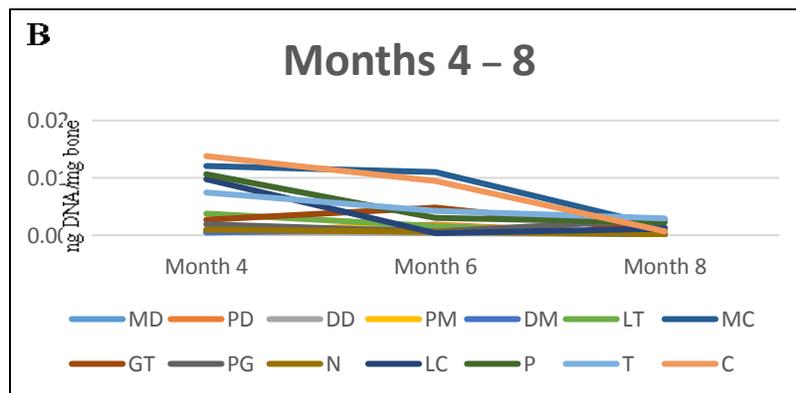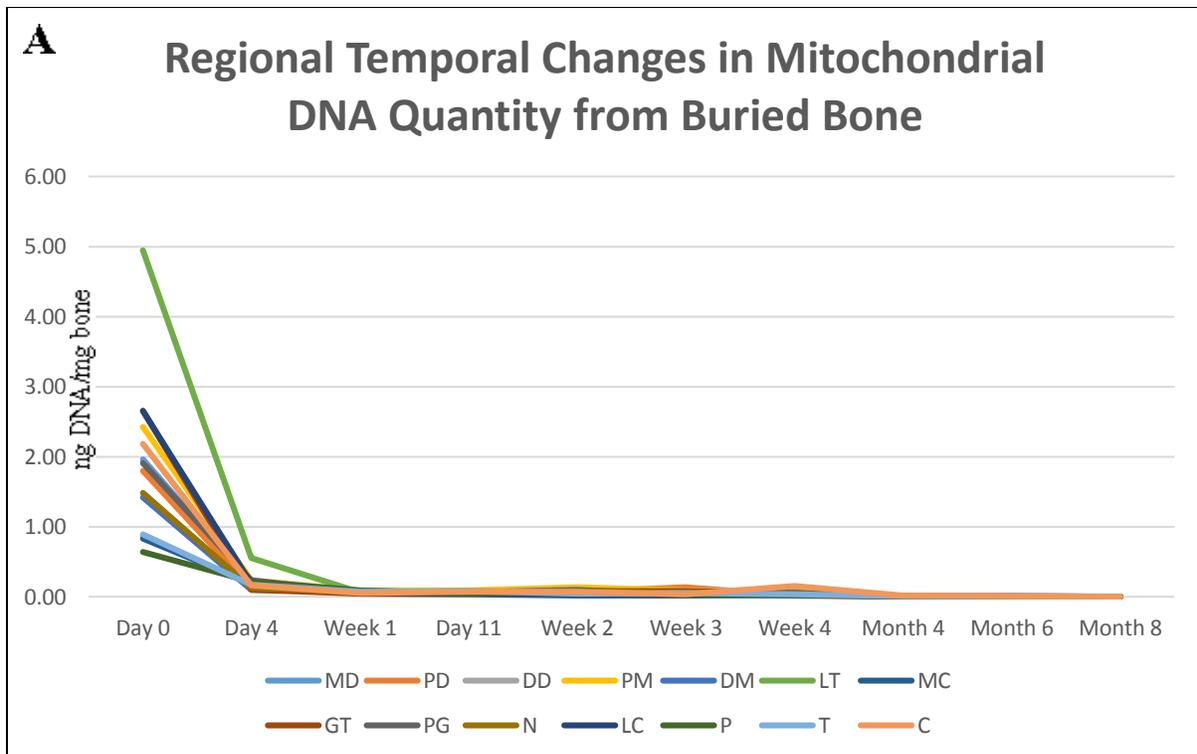
Figure 5 Changes in mitochondrial DNA quantity among bone regions in buried bones over time. A) Day 0 through 8 months. B) Details of months 4 – 8. Bone locations tested (colored lines) are detailed in Figure 1.

was then overshadowed as DNA degradation became more substantial after a few weeks.

Because the DNA in buried bones degraded even more quickly, this effect, while present, was

less pronounced. In general, regions of the diaphyses (regions 1 – 3) had higher median levels of

nuclear and mitochondrial DNA over short periods, which differs from the findings in [4], wherein the more proximal and distal regions had highest DNA yields (discussed below).

Over longer periods (4 – 8 months; Figures 2B – 5B), the highest nuclear and mitochondrial DNA yields varied widely depending on DNA type and burial condition. Overall, regions of the femur produced the highest DNA levels, while bones 12 – 14 did not. Of these latter three, the talus was the most likely to have relatively high median DNA levels.

Finally, DNA quality followed an expected pattern of decreasing with increased interment time, with mitochondrial DNA maintaining quality far better than nuclear DNA (data not shown). By 4 – 8 months, even the smallest region of nuclear DNA tested often failed to amplify, while segments of mitochondrial DNA from 200 – 400 bp did amplify. There were no bone regions/types that produced higher quality DNA temporally than did others.

Discussion

Our previous work showed that, in animal models, nuclear and mitochondrial DNA quantity and quality from the proximal and distal ends of femora, as well as from bones of the ankle, are higher than those from the midshaft diaphysis. The goal of the current research was to examine if this held true in human skeletal material. The standard belief in human forensics is that the densest portion of weight bearing bones is the optimal region for obtaining useful genetic data, however this has recently been called into question (see extensive references in [4]). The results presented here and elsewhere ([11,12]) do not indicate that the femoral diaphysis produces

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[11] Bermudez BB and Foran DR. The Short-Term Effects of Surface and Subsurface Burial on DNA from Human Skeletal Remains**.** The American Academy of Forensic Sciences Annual Meeting, Feb. 2017, New Orleans, LA
[12] Landhuis Z, Siewart M and Foran DR. Changes in DNA Quantity and Quality in the Human Tibia After Short-Term Surface and Subsurface Burial. The American Academy of Forensic Sciences Annual Meeting, Feb. 2018, Seattle, WA

either higher quantity or quality of nuclear or mitochondrial DNA than do other regions of the femur, or than the patella, talus, or calcaneus.

However, the results also did not show that femoral regions proximal or distal to the femur midshaft diaphysis had higher quantity or quality DNA, as has been reported previously ([4]). One cannot be sure why this was the case, but an important possibility is the differences in the origins of the skeletal material tested. Of course humans differ from the earlier model species tested, but the form and development of the femur is quite similar. What was not similar was the relative age of the individuals tested. The cattle and swine utilized in [4] were healthy individuals at an age optimal for slaughter, which is the earliest age where full body size, and thus market value, is reached. In stark contrast, the human remains tested in the current study were limited to those available from donors, which only included elderly females who had recently died of illness [13]. Given this, it seems likely that the model system individuals that were young adults had areas of growth (the metaphyses) and stress (such as tendon/ligament attachments) that had high cellular activity/presence, and therefore had high levels of intact DNA, while the skeletal material from the elderly and unhealthy human subjects did not possess those same traits, and that very different results would have been obtained had access to a more diverse set of human subjects been available [14].

From a forensic or human identification standpoint, the results presented here further confirm that the human midshaft femoral diaphysis does not represent a superior skeletal region for obtaining DNA identification data when compared to other femoral regions. Likewise, the

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[13] The five human donors in this study were all females in their 6th to 8th decade of life, and were not in good health, having died from disease. All showed substantial osteoporosis.

[14] To begin to test this hypothesis, we are conducting a similar study on deer (*Odocoileus virginianus*) femora, whose skeletal development ceases at approximately 3 years of age. Fresh, healthy animals ranging from fawns through very old (>10 years) are available through the Michigan State University Veterinary Diagnostics Laboratory, which test deer from throughout the state for illnesses such as chronic wasting disease.

patella, calcaneus, and talus showed very similar levels of nuclear and mitochondrial DNA quantity and quality as the midshaft femora, again indicating no advantage to the latter. Overall, in no instances did region 1 (Figure 1) produce superior results to other regions, either immediately or following surface or subsurface interment ranging from 4 days to 8 months. In contrast, regions of long bones, particularly those that are undergoing growth or remodeling and are thus likely to harbor cells that contain DNA, may be far more useful for forensic DNA typing. In instances where an unidentified individual, based on skeletal appearance or other factors, is likely to have been young, active, or generally healthy, the distal and proximal regions of long bones, as well as other skeletal elements, may produce better DNA typing results than do midshaft diaphyses, and should be considered for their forensic utility. [15]

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[15] It is anticipated that findings from this research will be submitted in early 2020 to the Journal of Forensic Sciences for publication. A likely title is: Intra-bone Variation of Recoverable Nuclear and Mitochondrial DNA from Human Skeletal Material. A related publication, tentatively titled The Influence of Age and Sex on DNA Heterogeneity within Bone, should be submitted to JFS later in 2020.