

The Preservation and Persistence of Human DNA in Soil during Cadaver
Decomposition

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Abstract

Recent decades have seen a marked increase in the amount of research concerning the impact of human cadaveric decomposition on the grave soil environment; however, despite such advances, the fate of important biological correlates in grave soil, including human DNA, have remained relatively understudied. This study redresses the current lack of knowledge regarding the preservation and persistence of human DNA in the soil during cadaveric decomposition, with the purpose of enhancing forensic identification efforts including the detection of primary burial sites. This study assessed the preservation (i.e., presence or absence) of human nuclear and mitochondrial DNA and evaluated the quantity and quality of recovered DNA from grave soil over the course of decomposition of four human cadavers placed at the University of Tennessee's Anthropological Research Facility (ARF). End-point and real-time quantitative PCR were used to assess quality and quantity of human DNA extracted from the grave soil environment. The presence of human cadaveric DNA from soil samples was verified by aligning and comparing sequences from the human mitochondrial DNA control region (HVI and HVII) between cadaver blood samples taken prior to placement at the ARF and soil samples taken from below each cadaver following placement and the initiation of decomposition processes.

Results indicate that human nuclear DNA from the cadavers was largely unrecoverable throughout decomposition. Conversely, cadaver mitochondrial DNA was detectable in the grave soil throughout all decomposition stages. Mitochondrial DNA copy number increased as decomposition progressed, peaked during the "Active Decay" stage of decomposition, and declined throughout the remainder of the decomposition process. The results of this study suggest that human mitochondrial DNA can be recovered from soil and is of a high enough quality to be used for exclusionary purposes during identification efforts. Therefore, the presence and persistence of this biomarker will prove useful in those forensic contexts in which DNA is not recoverable from a discovered set of remains or in the event that a cadaver is removed from its burial location.

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Chapter I

Introduction

The term ‘taphonomy’ was first coined in the early 20th century by the paleontologist Ivan Efremov. Efremov described taphonomy as an interdisciplinary study focusing on “the science of the laws of embedding,” citing taphonomy’s importance in various disciplines such as biology, geology, paleogeography, sedimentology, and archaeology (Efremov 1940: 93). More specifically, taphonomy deals with the laws of burial, relating to the transformation of organic material in a given environment following the cessation of life (Lyman, 1994). Since Efremov’s original formulation, the field’s scope has extended into the criminal legal context as forensic taphonomy, with the purpose of aiding discovery of clandestine burials and providing assistance in human identification efforts. Forensic taphonomy can be differentiated from Efremov’s formulation by the specificity of its goals, which include determination of postmortem interval (PMI; time between death and discovery) and assignment of cause/manner of death (Carter and Tibbet, 2008). To properly achieve these goals, Carter and Tibbett (2008) stress the need to understand basic issues surrounding taphonomy, with particular emphasis on changes in site ecology with the release of organic material into the soil during cadaver decomposition. It is with this focus in mind that the current study was undertaken.

Over the past two decades, human decomposition processes have been studied extensively via experimental work in forensic taphonomy. The majority of this work has focused on aboveground processes, leaving much to be understood regarding the impact these processes impose on the underlying soil (i.e., grave soil) (Tibbet and Carter, 2008). Nevertheless, recent decades have seen a marked increase in the amount of research concerning the impact of human cadaveric decomposition on the grave soil environment (e.g., Rodrigues and Bass, 1985; Vass et al., 1992; Hopkins et al., 2000; Carter et al., 2008a, b; Parkinson et al., 2009), including the fate of certain cadaveric biological correlates once they enter the soil (Tuller, 1991; Vass et al., 2002; Damann et al., 2012). For example, Tuller (1991) examined the presence of volatile fatty acids and blood proteins in soil associated with mass graves in Kosovo and Croatia. Vass et al. (2002) examined a number of biomarkers, including but not limited to amino acids, amines, and carboxylic acids, present in the soil during decomposition in an attempt to identify useful indicators for PMI estimation. Most recently, Damann et al. (2012) described the presence of various chemical/biological correlates in the context of possible carcass enrichment at a decomposition facility.

Despite these advances, the fate of another important biological correlate in grave soil –human DNA – has been relatively understudied. Though the persistence of microbial and plant DNA has been demonstrated by a number of studies (c.f., Nielsen et al., 2007), the number of microbial species in the soil and the sheer amount of plant biomass continually released into the soil likely outweigh the amount of material released from a single mammalian cadaver. Moreover, the way DNA is stored within an organism, the environment surrounding an organism, and the mechanisms of cell death

and DNA degradation can all lead to differences in the preservation of DNA in the soil. Therefore, environmental DNA of microbial, plant, or mammalian origin may not show equivalent degrees of preservation and persistence. In the majority of prokaryotes, DNA is circular and is not stored within a nucleus, as there are no membrane-bound organelles in prokaryotes (Griswold, 2008). Prokaryotes can also incorporate plasmids into their genome, which may either be linear or circular (Griswold, 2008); circular DNA is likely more protected from enzymatic breakdown than linear DNA (Nielsen et al., 2007). One form of eukaryotic DNA of both animals and plants is linear and stored within the nucleus of the cell (nuclear DNA). Eukaryotes have additional forms of DNA stored within various organelles such as chloroplasts (in plants) and mitochondria (in plants and animals). However, cell death and processes affecting DNA degradation are not uniform among tissue types within a single organism and can also differ between taxonomic kingdoms of organisms (e.g., animals and plants). Therefore, evidence of the persistence of DNA of a particular organism does not necessarily apply to all other organisms, as many of the processes leading to the release of extracellular DNA are not fully understood.

Thus, a clear need exists for a dedicated study of the presence and persistence of human DNA in soil, as the mere presence of human DNA in the soil can provide useful information in a number of circumstances. For example, in the event that a clandestine grave is located but is devoid of a body, the recovery of trace amounts of human DNA of a high enough quality could provide information regarding the identity of the individual/victim associated with the burial (Moreno et al., 2011). This would be particularly useful in situations where a body is purposefully relocated in an effort to evade criminal prosecution. In the context of mass graves, trace amounts of human DNA at a primary burial site could indicate post-burial disturbance and help establish intent to conceal evidence. The study of the persistence of human DNA in grave soil may also be useful in archaeological contexts. For example, in circumstances where a burial has been ransacked or a discovered set of remains lacks recoverable DNA, human DNA may remain in the soil and provide researchers with important information regarding the individual that was once present. Similarly, in light of Native American Graves Protection and Repatriation Act (NAGPRA) guidelines, the ability to extract human DNA from soil sediments may provide an important nondestructive alternative for ancient DNA analyses of archaeological mortuary samples.

This study seeks to redress the current lack of knowledge regarding the preservation and persistence of human DNA in the soil during cadaveric decomposition, with the purpose of enhancing forensic identification efforts to detect primary burial sites. This study assesses the presence or absence of two human DNA sources: nuclear (nDNA) and mitochondrial (mtDNA) and evaluates the quantity and quality of recovered DNA. The following questions are addressed: Does human DNA persist in the soil environment during and after human cadaveric decomposition, and if so, for how long? Does the quantity of any remaining human DNA in the soil relate to stage of cadaveric decomposition? Moreover, if human DNA can be recovered from the burial environment, what is the quality, or state of degradation, of this DNA and does it allow for forensic human identification?

To address these questions, this project uses real-time quantitative PCR (qPCR) to assess the quantity of human DNA (as copy number) extracted from the soil environment during human decomposition. Additionally, end-point PCR is used to amplify portions of the human mtDNA control region, specifically, Hypervariable Regions I and II (HVI and HVII). These target regions will be compared between blood samples taken from four human cadavers prior to placement within a decomposition facility and soil samples taken from beneath the cadavers throughout the decomposition process. The results of this project are expected to increase our overall understanding of the persistence and degradation of human DNA in soil, thereby expanding upon our current understanding of the interplay between chemical, physical, and biological processes occurring in the soil in concert with human decomposition.

Organization

This thesis is organized as follows: Chapter II reviews relevant empirical research, including information on mammalian decomposition, cell death and intracellular DNA degradation, extracellular DNA degradation and the role of microorganisms, the preservation and persistence of DNA in soil, and methods of forensic DNA identification. Chapter III discusses the goals, hypotheses, materials, and methods of the project. The location of the project, sample type and storage, extraction, amplification, and sequencing protocols will be covered in depth. Chapter IV outlines the study's results and Chapter V discusses results in light of the questions presented in this introduction and Chapter III. A summary of the main conclusions will be presented in Chapter V.

Chapter II

Background

This section provides a necessary review of not only what is known and unknown regarding the persistence of human DNA in the soil, but also provides information necessary to understand the research design, methodology, and hypotheses outlined in the subsequent chapter. A review of the stages of human decomposition is presented to provide information crucial to understanding expectations regarding the persistence of human DNA in the soil; it is during these stages that DNA is released from the cadaver into the soil environment. Additionally, to gain an initial understanding of what to expect in terms of DNA presence and recovery, both intracellular and extracellular processes of DNA degradation are described and research on the preservation of non-human DNA is summarized. Lastly, because this project attempts to discover the utility of human DNA recoverable from soil for human identification purposes, the final section on human identification was included to review current DNA methodologies used in human identification.

Stages of Human Decomposition and the Cadaver Decomposition Island (CDI)

Classically, decomposition is marked by six stages, originally observed by Payne (1965) while assessing the decomposition of *Sus scrofa*, the domestic pig: Fresh, Bloated, Active Decay, Advanced Decay, Dry, and Remains. It is important to recognize that the decomposition stages are largely assigned through subjective analysis, but when assigned appropriately, the six stages can provide a reasonable indication of physiochemical changes at different time points throughout the decomposition process (Carter et al., 2007; Carter and Tibbet, 2008). For a summary of the key characteristics of each stage, consult Table 1.

During decomposition, DNA and other cadaveric materials are released into the surrounding environment as a result of normal decay processes. Mammalian decomposition commences shortly after death. For example, Payne (1965) observed signs of decomposition within five minutes following the removal of pig carcasses from a freezer; others have reported the onset of cadaver decomposition as early as four minutes (Vass, 2001; Vass et al., 2002). DNA degradation initiates with the beginning of decomposition directly following death. As blood –and consequently oxygen – cease to flow to the body's various organ systems and ATP (adenosine triphosphate) production comes to a halt, the shift to anaerobic processes catalyzes cellular death including apoptosis and necrosis (Alaeddini et al., 2010). These mechanisms occur at diverse rates for different organ systems due to dissimilarities in energy requirements in various tissue types, leading to differential rates of cell death. For example, using rats, Tomita et al. (2004) noted postmortem ultrastructural changes in the liver and pancreas in as early as one hour, in three hours in the kidneys and heart, and in ten to twenty-four hours in skeletal muscle.

Table 1: The stages of human decomposition

Stage	Description	References
Fresh	Shortly following death Autolysis Initial fly colonization	Payne, 1965; Vass, 2001; Vass et al., 2002; Carter et al., 2007
Bloated	Bloating Color change Foul odor Initial purging of cadaveric fluids	Vass et al., 2002; Carter et al., 2007; Carter and Tibbet, 2008
Active Decay	Breaks in the Skin Extensive fluid release Increased maggot activity Mass loss Presence of anaerobic and aerobic bacteria Presence of a single CDI due to union of purging fluids	Carter et al, 2007; Carter and Tibbet, 2008
Advanced Decay	Coincides with maggot migration Continuation of the purging of liquids Increased microbial activity Nutrient-rich CDI	Carter et al., 2007
Dry	Tissues become dry and leathery	Swann et al., 2010
Remains	Loss of all remaining flesh and soft tissue	Payne, 1965

Enzymatic activity plays a major role in these processes, as endogenous and exogenous lipases, nucleases, and proteases aid in cellular degradation and the subsequent degradation of DNA; this enzymatic digestion of cellular materials is known as autolysis and is associated with the 'Fresh' stage of decomposition (Vass et al., 2002; Alaeddini et al., 2010). The Fresh stage is accompanied by the appearance of different fly species such as blowflies and flesh flies; colonization by such insects and subsequent maggot activity are important for the breakdown of soft tissue, especially when scavenging is minimal (Carter et al., 2007).

As oxygen depletion persists within the body, anaerobic microbes begin to flourish. These microorganisms use the body's existing organic materials to create various organic acids (butyric, propionic, and lactic acids) and gases (hydrogen sulfide, carbon dioxide, methane, ammonia, sulfur dioxide, and hydrogen) that cause visible bloating, color change (e.g., a greenish hue to the skin), and foul odors through a process known as putrefaction (Carter et al., 2007; Carter and Tibbet, 2008; Vass et al., 2002). Such changes define the 'Bloated' stage of human decomposition. During this stage, there is an initial flow of cadaveric substances into the soil as the pressure from the gases pushes the materials from the body's orifices. This flow of materials intensifies in the 'Active Decay' stage, as splits in the skin develop as a result of the above processes and overall increases in microbial (both aerobic and anaerobic) and insect activity. The substantial amount of cadaveric material percolating into the soil during this stage ultimately creates a 'cadaver decomposition island,' or CDI, which is characterized by a considerable change in the soil ecology surrounding the cadaver (Carter et al., 2007; Carter and Tibbet, 2008). Intense mass loss, increased maggot activity, and extensive fluid release are among the most noticeable changes seen during the Active Decay stage. This stage persists until the maggots migrate from the cadaver, commencing the 'Advanced Decay' stage.

According to Carter et al. (2007:16), the Advanced Decay stage is associated with a CDI with the highest levels of nutrient-rich materials from the cadaver, representing "an area of increased soil carbon, nutrients, and pH." Therefore, any trace amounts of human DNA extracted from the soil should be highest during 'Active Decay' and 'Advanced Decay' stages. The final two stages, 'Dry' and 'Remains,' are both associated with a slow loss of bodily materials (i.e., various organic substances) into the surrounding soil (Carter et al., 2007). In the Dry stage remaining tissues lose lingering moisture, causing them to take on a leathery appearance (Swann et al., 2010). The Dry and Remains stages are not easily divisible; however, in the Remains stage the cadaver has lost all remaining flesh and soft tissue, and the surrounding environment, excluding the underlying soil, has mostly reverted back to its original state (Payne, 1965).

It is important to clarify that the rate and pathway (general progression) of decomposition is highly variable. Because of differences in taphonomy, not all corpses follow the same pattern (Vass, 2001). Temperature, moisture, position of the body (e.g., above or below the ground), differential exposure to carnivores, etc. are all important variables.

Cellular Breakdown and DNA Degradation

By the end of decomposition, the complex macromolecules that make up the human body are reverted back to their simplest, monomeric form (Vass, 2001). Therefore, it is important to describe what is occurring at the cellular level and how this relates to DNA degradation. As discussed above, after organismal death and with the onset of decomposition, cell death follows one of two different paths: apoptosis or necrosis. The route taken depends on a number of factors, including differences in signaling mechanisms, the developmental stage/characteristics of a particular tissue type (Alaeddini et al., 2010), and the amount of ATP available (Leist et al., 1997). Both pathways occur simultaneously throughout the body during human decomposition.

Apoptosis is an ATP-dependent method of cell death, while necrosis follows a less energy-consuming pathway. Apoptosis is characterized by cytoplasmic condensation, removal of plasma membrane microvilli, and DNA fragmentation into segments of fewer than 200 nucleotides; necrotic changes include increased cell volume via swelling of cytoplasmic organelles, chromatin condensation, and eventual membrane rupture, organelle breakdown, and enzyme release (Alaeddini et al., 2010). Though these processes are distinct and can lead to dissimilar patterns of DNA degradation, they are difficult to differentiate by means of any single experimental method.

Regardless of the pathway taken – apoptosis or necrosis – cell death leads to increased activity among specific classes of intracellular enzymes important for cellular breakdown and DNA degradation, among which include lipases, nucleases, and proteases (Alaeddini et al., 2010). Lipases are responsible for the breakdown of lipids, nucleases for nucleic acids (involving RNA and DNA degradation), and proteases for proteins. In the context of DNA degradation, there has been a particular emphasis in the literature regarding nuclease activity during apoptotic cell death, most likely due to the fact that DNA degradation is a hallmark indicator of this pathway (Samejima and Earnshaw, 2005).

Unlike what is observed in necrotic cell death, during apoptotic cell death, cells manage to retain membrane integrity. Rather than spilling cellular debris into the extracellular space, apoptotic cells are rapidly phagocytized by neighboring cells (He et al., 2009). Throughout this process, DNA becomes cleaved both within the apoptotic cell and within the phagocytic cell. There are two main categories of nucleases involved in DNA cleavage: cell-autonomous nucleases and waste-management nucleases (Figure 1; Samejima and Earnshaw, 2005). Cell-autonomous nucleases, primarily caspases, are responsible for DNA cleavage directly inside the cell (and inside the cell's nucleus), and thus specifically target nuclear DNA leaving mitochondrial DNA intact (Murgia et al., 1992; Samejima and Earnshaw, 2005); they are a crucial component of apoptotic cell death.

Waste-management nucleases are responsible for the degradation of extracellular DNA and intracellular DNA outside of the cell's nucleus. Rather than being synthesized inside the nucleus, waste-management nucleases are made within the endoplasmic reticulum (ER) and are housed in lysosomes or released into the extracellular space. These nucleases are responsible for cleaning up DNA released during both apoptotic and

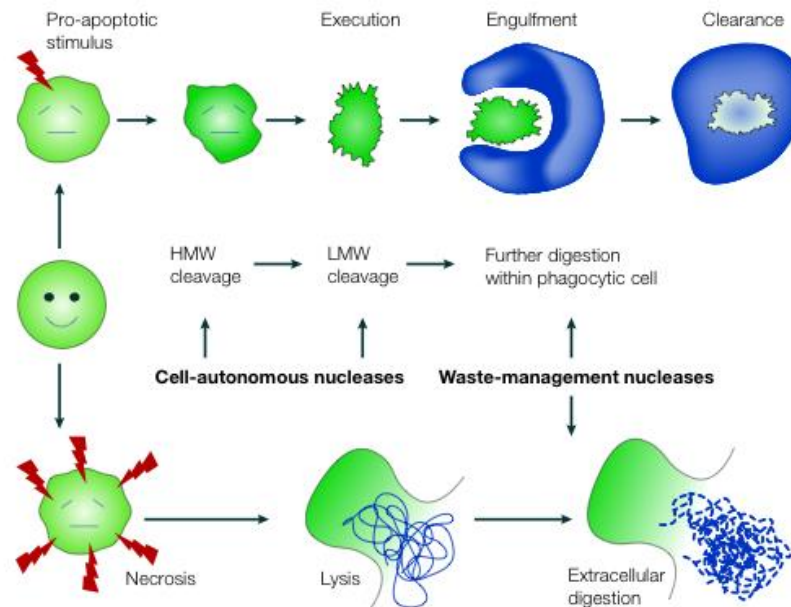


Figure 1: Apoptotic vs necrotic cell death and the two classes of nucleases involved (from Samejima and Earnshaw, 2005). Following initiation of apoptosis, the cell, which has retained membrane integrity, eventually becomes engulfed or phagocytized by a neighboring cell. The DNA becomes cleaved both within the apoptotic cell (high/low molecular weight (HMW/LMW) cleavage via cell-autonomous nucleases) and within the phagocytic cell (via waste-management nucleases). During necrotic cell death intracellular components including DNA are released into the extracellular space as the membranes of the cell and organelles rupture; waste-management nucleases, synthesized within the ER, work at cleaving and degrading this extracellular DNA.

necrotic forms of cell-death. They are released by the lysosomes in cells that have phagocytized apoptotic bodies, or they are released for the purpose of degrading DNA released into the extracellular environment following cell-death. Therefore, there are three consecutive fragmentation events associated with apoptosis: high molecular weight (HMW) cleavage, low molecular weight (LMW) cleavage, and single stranded DNA breaks (Samejima and Earnshaw, 2005). HMW cleavage occurs early on during apoptosis and results in fragment sizes between 50 and 300 kb. LMW cleavage occurs late in the stages of cellular degradation, yielding DNA fragments of less than 200 nucleotides (Samejima and Earnshaw, 2005). These events together result in a ladder-like pattern of DNA degradation observable during gel electrophoresis (Alaeddini et al., 2010).

In contrast to apoptotic patterns of DNA degradation, DNA cleavage in necrosis is much less predictable. Generally, necrotic cell death has been associated with a smear pattern during gel electrophoresis, indicative of extensive, non-patterned fragmentation, which has been described as the result of “nonspecific lysosomal nucleases” in the late stages of necrotic cell death (Dong et al., 1997; Didenko et al., 2003; Alaeddini et al., 2010). However, there has been evidence that necrotic DNA degradation can be both random and systematic (Dong et al., 1997). Didenko et al. (2003) demonstrated that early necrotic cell death involves 3’ to 5’ exonuclease activity, which causes 5’ overhangs. Though this pattern is also seen in apoptosis, the mechanism through which these breaks occur is not fully understood. Nevertheless, it is evident that following cell death, no matter the pathway at work, DNA is undergoing rapid degradation. As put best by Nielsen, “degradation of DNA in dead cells is a complex process that is dependent on a specific organism’s physiology and the conditions leading to cell death” (2007: 38).

Based on the high amount of enzymatic activity associated with normal mechanisms of cell death, it is reasonable to think that the large majority of DNA leached during cadaveric decomposition exists in an extracellular state. In fact, recent research has demonstrated that extracellular DNA may be present in the soil in drastically larger amounts than intracellular DNA (Corinaldes et al., 2005). Corinaldes et al. (2005) discovered that extracellular DNA in marine sediments persisted at a level that was 10 to 70 times higher than the intracellular component.

Extracellular Processes and the Role of Microorganisms

Much of decomposition is mediated by microbial organisms originating from both the cadaver itself and the surrounding soil environment (Parkinson et al., 2009). Decomposer organisms in the soil, such as microbial and fungal saprophytes, utilize the organic material and nutrients released into the soil (e.g., carbon, nitrogen, phosphorous, etc.) during cadaver decomposition as potential sources for energy (Hopkins, 2008). There are two important ecological strategies by which microbial communities in the soil can be differentiated: zymogenous or autochthonous. Zymogenous populations include r-selected organisms, or those that rapidly respond to nutrient off-loading, while autochthonous refers more to those organisms which take advantage of persisting/stable organic material in the surrounding soil. Regardless of the strategy taken, these

organisms play a substantial role in soil carbon turnover and carbon cycling and are thus important components in the decomposition process (Hopkins, 2008).

Over the years there have been many attempts to identify the microbial communities associated with human decomposition. The fluids released by the cadaver alter the soil composition, which encourages growth among some microbial communities, while inhibiting the growth of others (Parkinson et al., 2009). Early studies found that a large number of species (possibly hundreds) are involved in human decomposition (Vass, 2001). More recently, researchers have focused on the identification of these microbial species as possible biomarkers for time since death estimation (Parkinson et al., 2009).

The presence of large amounts of microbial species is important to the fate of extracellular DNA released into the soil from human cells during decomposition. One of the most important factors impacting the persistence of extracellular DNA in the soil is the presence of bacterial DNases, enzymes specific to the breakdown of DNA (Blum et al., 1997; Levy-Booth et al., 2007; Nielsen et al., 2007). DNases of microbial origin, particularly from prokaryotic organisms, are thought to account for the majority of DNases found in soil (Blum et al., 1997). These DNases break down DNA into its constituent parts (i.e., deoxyribose, inorganic orthophosphate, and nitrogenous bases), which can then be exploited by microbial communities as a source of carbon, nitrogen, and phosphorus. Certain bacterial species, such as *Escherichia coli* and *Serratia marcescens*, have even been known to use DNA constituents as their sole source of carbon (Nielsen et al., 2007). Moreover, bacteria can use extracellular DNA for other functions beyond nutrient uptake; exogenous/extracellular DNA may also be used for transformation or DNA repair (Jorgensen and Jacobsen, 1996).

Thus, once in the soil environment, DNA must not only contest with numerous classes of nucleases (i.e., exodeoxyribonucleases, endo-deoxyribonucleases, restriction endonucleases, endonucleases, and exonucleases) (Nielsen et al., 2007), but also must escape chemical/physical fragmentation and the possibility of bacterial uptake during genetic transformation (horizontal gene transfer among bacteria). Increased temperatures, exposure to mutagens (i.e., pollutants), and other environmental damage could cause DNA fragmentation, especially in the absence of DNA repair mechanisms (Nielsen et al., 2007). Natural transformation, in which bacteria incorporate exogenous DNA into their own genomes can also occur, and research involving plants have shown that even organellar DNA, such as mitochondrial DNA, is not exempt from bacterial uptake in the form of horizontal gene transfer (Bergthorsson et al., 2003).

DNA Preservation and Persistence

As previously discussed, DNA degradation during decomposition can be extensive. Cadaveric DNA is vulnerable to both cellular enzymes from the cadaver itself and extracellular enzymes from increased microbial activity. In addition, non-enzymatic processes such as hydrolysis and oxidation play a role in DNA degradation, though at a much slower rate (Alaeddini et al., 2010).

Numerous studies have demonstrated that plant and microbial DNA can remain in the soil, though many of these studies use artificially designed systems/environments

called “microcosms” (Nielsen et al, 2007); whether these artificial systems are representative of the natural environment is questionable. Additionally, there is an existing gap in the literature on studies focused on the retrieval of mammalian DNA from environmental sources such as soil, and most of those that do exist tend to focus on the presence/absence of species in a particular environment, whether that environment be ancient or contemporary (see Table 2).

For example, Haile et al. (2009) demonstrated through ancient DNA analysis of sediments that the woolly mammoth persisted thousands of years later than originally thought. Similarly, Willerslev et al. (2003) revealed the presence of approximately 19 plant taxa and several taxa of extinct megafauna from ancient organellar DNA (from mitochondria and chloroplasts) from permafrost cores in Siberia. More recently, environmental DNA has been particularly useful in meta-barcoding and species biodiversity analyses. Andersen et al. (2012) purified environmental DNA from soil samples collected from various safari parks, zoological gardens, and farms in an effort to assess vertebrate diversity using 16S mtDNA markers.

It is important to highlight that each of these studies looked at mitochondrial or other organellar markers, such that the persistence of mammalian nDNA is open to question. There is no reason to suspect that nDNA cannot be retrieved from the soil, as nDNA from plant species has been successfully purified from soil (Nielsen et al., 2007). Nevertheless, nDNA is known to be far less protected than its mitochondrial counterpart. The circular nature of mtDNA, similar to that of plasmids, likely offers some protection, however minimal, by limiting access to invading nucleases when compared with linear strands of nuclear DNA (Butler, 2005; Nielsen et al., 2007). Additionally, mtDNA is found in the mitochondria of the cell, which is protected by its own double membrane, providing additional layers of protection from endonucleases.

The only way to ensure survival of DNA over time is to gain some kind of protection from environmental pathways of degradation (i.e., microbial absorption [through bacterial transformation or horizontal gene transfer], nuclease degradation, and general mechanical shearing) by somehow blocking these processes from occurring (Nielsen et al., 2007). Similar to the way in which DNA is thought to adsorb, or bind, to the mineral component (i.e., hydroxyapatite) of bone (Götherström et al., 2002), DNA can also adsorb to the soil matrix of certain soils such as sand and clay, as well as to humic acids (Levy-Booth et al., 2007). By binding to soil matrix, DNA gains protection from DNases as well as other enzymes present in the soil environment. Soil mineralogy, which includes the number of binding sites, concentration of cations and humic substances in the soil, as well as soil pH, can all influence DNA adsorption. Clay, silt, and sand have each demonstrated different degrees of DNA binding capabilities, with clay binding DNA the best, followed by silt, and then sand. The presence of cations, particularly divalent cations, enhances DNA adsorption capabilities (Levy-Booth et al., 2007). DNA is a negatively charged molecule, which under adequate conditions, will adsorb to positively charged surfaces of clays and other mineral components and compounds (Crecchio et al., 2005).

Table 2: Summary of mammalian environmental DNA studies

Reference	Purpose of Study	Location/Site	Substrate	DNA target	Findings
Willerslev et al., 2003	Determine taxonomic diversity using sedaDNA	Siberia	Permafrost (frozen sediment)	rbcl (plant marker) and fragments from 12S, 16S and cytb (vertebrate mtDNA markers)	A number of plant species and megafunal species discovered via DNA analysis
Haile et al., 2007	Looked at DNA leaching and aDNA in nonfrozen sediment	Two Cave Sites in New Zealand	Non-frozen sediment	88bp and 60bp mtDNA control regions: moa and sheep/ chloroplast DNA/ avian 12S mtDNA	Saw vertical migration of DNA. Able to amplify Moa DNA (extinct 500 years ago)
Haile et al., 2009	Using sedaDNA to reveal the presence of species (mammoth) absent from the macrofossil record	Alaska	Permafrost (frozen sediment)	mtDNA	Recovered mtDNA of ancient horse and mammoth despite absence of macrofossils
Andersen et al., 2012	looked at the utility of eDNA as an "indicator of vertebrate diversity"	Sampled Safari parks, zoological gardens, and farms	Non-frozen sediment	mtDNA	Short mtDNA fragments recovered

In addition, differences in soil pH can influence DNA adsorption to the soil matrix; adsorption generally decreases with increasing pH (Levy-Booth et al, 2007). For example, using herring sperm, Ogram et al. (1988) demonstrated that both the amount of montmorillonite clay and the pH of the soil significantly contributed to the degree of DNA adsorption in five soil types, a lake sediment sample, and an acid-washed sand; a low pH combined with a high component of montmorillonite increased overall DNA adsorption. Low pH values influence the protonation of purines and pyrimidines, creating a net positively charged DNA molecule, thereby allowing greater binding with a net negatively charged montmorillonite composite (Crecchio et al., 2005).

Though humic acids have also been known to bind extracellular DNA and offer protection from environmental DNases (Crecchio and Stotzky, 1998), these substances are considered PCR inhibitors, meaning they pose a problem for DNA amplification using polymerase chain reaction (PCR). Humic acids are naturally produced during decomposition and can “oxidize to form quinones, which covalently bind to and inactivate DNA polymerase” (Alaeddini et al., 2012: 298). PCR inhibition is a common problem when using environmental samples, and humic acids are just one of many sources of inhibition.

Modified protocols are available to detect and overcome PCR inhibition. For example, DNA extracts suspected of containing potential inhibitors can be spiked with a known amplifiable standard, and subsequent PCRs can be monitored for amplification success or failure. In such cases, PCR failure is used as an indication of PCR inhibition. Additionally, quantitative real-time PCR (qPCR) can be used for the detection of inhibitors by both assessing PCR efficiency and by the incorporation of an internal positive control (IPC); an IPC is an internal standard that should amplify in a multiplex reaction in the absence of inhibition (Swango et al., 2006; Alaeddini et al., 2012). Once detected, various methods can be used to eliminate inhibition from extracts. For example, Kemp et al. (2006) used a modified protocol from the Wizard® PCR Preps DNA Purification System to re-purify DNA extracts, thus removing existing inhibitors.

Human Identification

The type of DNA purified from a specific sample type, whether from soil, blood, or other source, is important for the methods used in human identification. Within a human cell there are two possible sources of DNA that may be exploited for the purpose of human identification: (1) chromosomal DNA or nDNA within the cell's nucleus and (2) mitochondrial DNA or mtDNA within the mitochondria of the cell (Figure 2). There are 46 chromosomes, comprised of two pairs of 22 autosomes and two sex chromosomes within the nucleus of each cell. Additionally, each cell is packed with hundreds of mitochondria, and each mitochondria can contain multiple copies of mtDNA; therefore, each cell contains hundreds to thousands of mtDNA copies. Unlike nuclear DNA, which consists of approximately 3 billion base pairs (bp) linearly arranged in chromosomes, mtDNA is comprised of only ~16569 bp arranged in a circular molecule (Butler, 2005).

DNA analysis has played an important role in forensic identification and criminal prosecution over the last several decades. Though DNA has been used in forensic

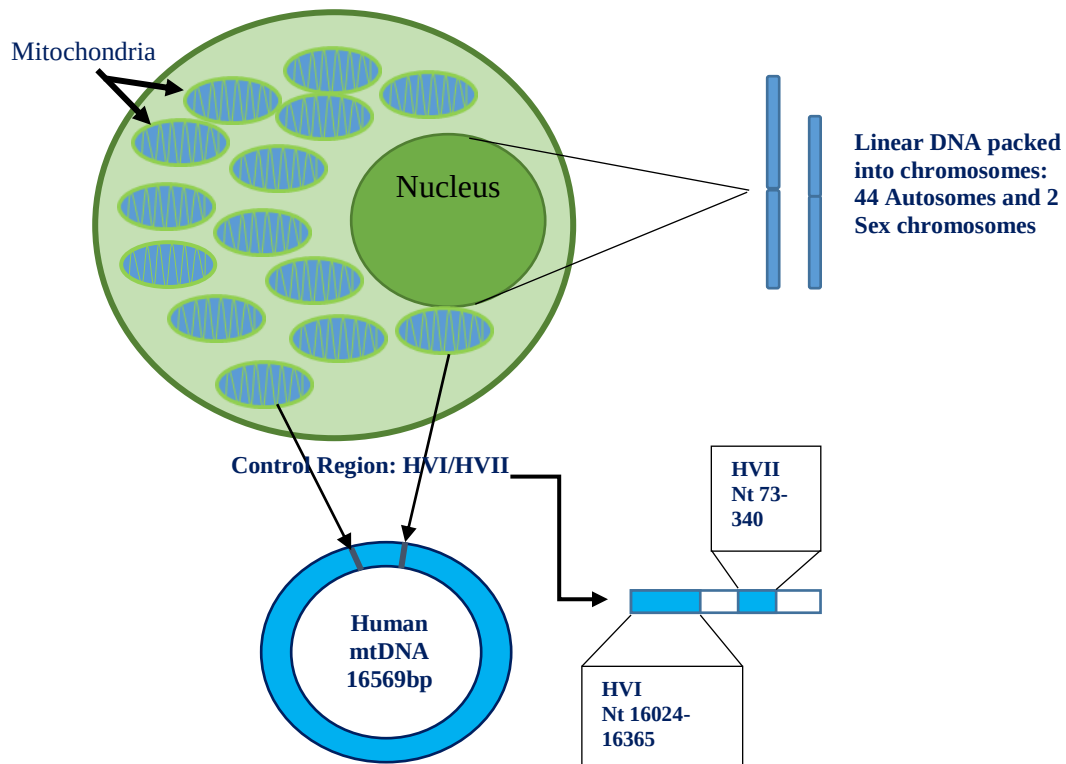


Figure 2: Nuclear (nDNA) and Mitochondrial DNA (mtDNA) Sources for Forensic Identification (adapted from Jobling and Gill, 2004). In every human cell (except red blood cells), there are two copies of nDNA and hundreds of copies of mtDNA. STRs, single tandem repeats, are markers only found in nDNA and are the most commonly used source for forensic identification. MtDNA analysis is usually performed when STR results are incomplete or nonexistent, and involves sequencing and comparing HVI and/or HVII to the revised Cambridge Reference Sequence (rCRS), a reference genome (Jobling and Gill, 2004; Butler, 2005).

casework since the early 1980s, originally using single-locus probe (SLP) technology, it has evolved into an indispensable source of information with the development of PCR-based methods and the validation of short tandem repeats (STRs) for human identification purposes (Jobling and Gill, 2004). STRs are a class of microsatellites representing areas in the nuclear genome that contain repeat units 2-7 bp in length; generally, STR regions total in length from 100-400 bp (Butler, 2005; Butler, 2007).

STR markers vary greatly in the number of repeats between individuals, making them useful for human differentiation and forensic identification. There are thousands of such STRs in the human genome but only a small subset are needed for human identification purposes (Butler, 2007). The suite of STRs used in human identification have been ascertained to adhere to the following criteria: high discriminating power, unlinked, demonstrate typing consistency and reproducibility, low stutter, low mutation rate, and are small in fragment size (< 400 bp) (Butler, 2005). Additionally, such markers are non-coding, which allows them to be highly variable within a population. STR markers are standardized across the forensic community and are multiplexed (amplified together in a single reaction) to increase the overall discriminating power. In 1997, the United States began using 13 specific STR loci (CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11), known as CODIS STRs, after the Combined DNA Index System (CODIS), a national database established by the FBI. Together, these markers allow for a match probability of one in one trillion. Additionally, by multiplexing amelogenin, a sex-specific marker, with these loci, investigators are also able to determine the chromosomal sex of the source of any given DNA sample (Butler, 2005).

When DNA is highly degraded problems such as allelic dropout, in which smaller alleles are preferentially amplified over larger alleles, may arise in STR analysis; such problems lead to erroneous results and incorrect identifications. In such events, other methods of analysis are often required. Within the last decade, many researchers have developed miniSTR multiplexes for use with degraded DNA (e.g., Butler et al., 2003; Grubwieser et al., 2006; Asamura et al., 2007; Parsons et al., 2007, etc.). Miniplex systems take advantage of the smaller sized DNA fragments available for amplification when degradation is present. These systems shorten the amplicon length targeted during PCR amplification but retain the information present in commonly targeted forensic STRs. Miniplexes have proven useful in cases where traditional STRs fail to produce meaningful results (Parsons et al., 2007).

In cases where DNA is so degraded that the amplification of nDNA is unsuccessful, mtDNA can be used for identification purposes. MtDNA is more prevalent in the human cell; therefore, due to its high copy number, the probability that mtDNA can be extracted from a very old/degraded sample is much higher than nDNA (Butler, 2005). The mitochondrial control region is non-coding, meaning more polymorphisms can and do accumulate in this area over the course of generations without deleterious side effects. Because of the greater amount of variability in the control region, this region is often targeted for forensic analysis. The areas most commonly assessed for the purpose of identification are two hypervariable regions, HVI and HVII, within the control region. The revised Cambridge Reference Sequence (rCRS; Andrews et al., 1999) is used for

sequence comparison and is generally used to describe polymorphisms present in a forensic sample. Therefore, mtDNA analysis involves PCR amplification of regions from HVI and HVII followed up with sequencing and sequence comparison with the rCRS.

MtDNA is maternally inherited, meaning it is passed down from mother to offspring with no paternal input. Because mtDNA is strictly maternally inherited, its sequence is not specific to any one individual; siblings and maternal relatives usually contain equivalent, or near-equivalent, sequences. This is why it is insufficient for positive human identification in comparison to an nDNA-based analysis such as STR analysis, which is effectively unique to any given individual when several markers are multiplexed, with the exception of monozygotic twins. Additionally, mtDNA control regions have low discriminatory power because they are only 1-2% variable between individuals, making it a less than ideal source for identification (Butler, 2005). However, if nDNA is not available, the information provided through mtDNA analysis is nevertheless useful, as it can be used to exclude potential matches.

Summary

Currently, to my knowledge, there is no research available on the preservation and persistence of human DNA within soil. As reviewed here, there have been many studies conducted on the survival of DNA of microbial and botanical origin and the mechanisms behind DNA persistence, including research on specific soil types conducive to DNA preservation. However, how these studies relate to the persistence of human DNA is unclear. This study addresses this gap in the research and examines the recovery of human DNA from the soil for the purpose of human identification. Both the quality and quantity of recoverable DNA is of interest.

Chapter III

Research Design

Research Objectives and Hypotheses

This study investigates the extent to which human DNA persists in grave soil throughout decomposition as well as after the cessation of decomposition, with the purpose of assessing whether both quality and quantity of recovered DNA is high enough to establish a forensic profile. In this study, a “successful profile” consists of one of two possibilities: (1) 13 CODIS STRs in the context of nDNA or (2) complete sequence information from HVI and HVII in the context of mtDNA.

This study tests for the presence of human nuclear and mitochondrial DNA from soil underlying four different human cadavers, at different stages of decomposition (as described in Chapter 2). To authenticate any DNA recovered as endogenous to the decomposing human cadaver used for the purposes of this study, rather than exogenous, amplified sequences of HVI/HVII were compared between samples from cadaver blood cards and samples from the soil.

Providing that endogenous nDNA, mtDNA, or both, are recovered, the following hypotheses will be tested:

Hypothesis 1:

The *quantity* of human DNA extracted from the soil will relate both to time and stage of decomposition. Quantity of DNA, measured as copy number, will be highest during Active Decay and Advanced Decay stages and will decrease over the remaining stages of decomposition, continuing following decomposition cessation.

Hypothesis 2:

The *quality* of human DNA will decrease over the course of decomposition, meaning the DNA will become more fragmented over time due to the numerous degradation processes the DNA is exposed to following death.

Materials

Sample Location and Collection

This study uses grave soil samples from four cadavers, two males and two females, collected from May to September, 2012, as the focus of a project assessing microbial changes associated with human decomposition at the University of Tennessee, Knoxville’s (UTK) Anthropology Research Facility (ARF) (Table 3; Cobaugh, 2013). The ARF was established in 1980 by Dr. William M. Bass to enhance understanding of human decomposition. It is located behind the UT Medical Center, across the river from the UTK main campus (Figure 3). A description of the main soil type at the ARF has



Figure 3: Approximate location of the Anthropology Research Facility (ARF), shown within the red-lined rectangle. It is located behind the UT Medical Center, across the river from the UTK campus (adapted from Google Earth).

Table 3: Subject/cadaver information (Cobaugh, 2013).

Cadaver Code	Sex	Cause of Death	Weight (lbs.)	Date of Placement
36-12D	Female	“Natural”	180	5/21/2012
47-12D	Female	Unknown	130	6/27/2012
67-12D	Male	Unknown	165	9/19/2012
50-12D	Male	Colon Cancer	150	7/06/2012

been previously described by Damann (2010) as a Coghill-Corryton Complex. This soil type is characterized as “deep, well-drained, clayey soils that formed in residuum derived from interbedded, leached, calcareous sandstone and shale” (Damann, 2010: 33).

According to Cobaugh (2013), when the cadavers were sited at the ARF, they were placed in areas presumed to be previously unoccupied by human cadavers, as per ARF records. In 2012, this was done to control for the presence of human-specific microbes at each location, but in the context of this project, this condition was necessary to control for human DNA mixtures. Figure 4 provides details of cadaver placement from 2001 to 2005 within the ARF, with the 2012 placement of the cadavers shown with filled red circles. Cobaugh and associated researchers collected soil samples at random locations from depths of 0-3cm beneath the torso region of each cadaver during different stages of decomposition (i.e., Bloat, Active Decay, Advanced Decay, Dry/Remains), totaling twelve to fourteen soil samples per cadaver. Soil samples unused in Cobaugh (2013) were stored at -20°C, pending further experimental analysis.

For the purposes of this study, each cadaver is associated with two sample types: (1) blood samples, which were collected on Fitzco Classic Cards (Fitzco) during routine cadaver intake procedures (see Blood Card Sampling and Extraction, below), and (2) soil samples taken over the course of decomposition from directly below each cadaver, as per above.

Laboratory methods

Controlling for Contamination from Exogenous DNA Sources

Sample contamination from exogenous human DNA sources is a common problem when analyzing highly degraded samples such as those encountered in forensic or archaeological circumstances (Gilbert et al, 2005; Gilbert et al, 2006; von Wurmb-Schwark et al, 2008). DNA derived from living individuals, such as from sample collectors or laboratory researchers, is typically of better quality (i.e., larger fragment sizes of undamaged DNA strands) and will tend to amplify over low quality DNA (a.k.a., degraded DNA or low copy number DNA) because PCR amplification reagents will bind preferentially to the more abundant, higher quality DNA templates. This can create incidences of false positives and lead to inconsistent or inaccurate results. Sample collection in the field or in the lab and DNA extraction and amplification in the laboratory all provide opportunities for the introduction of exogenous contaminants, and so extreme care must be taken to avoid and detect contamination at all costs. The Molecular Anthropology Laboratories at UTK (MAL-UTK) house a cleanroom laboratory for the sole purpose of analyzing degraded material as well as a separate laboratory for DNA extraction and amplification via polymerase chain reaction (PCR) from high quality templates derived from living sources.

In the present study, extractions and amplifications were performed in independent laboratories, exploiting the use of MAL-UTK’s standard and cleanroom DNA laboratory. The cleanroom laboratory provided a contaminant free zone dedicated to the analysis of degraded samples. Workflow was always initiated in the clean labs and

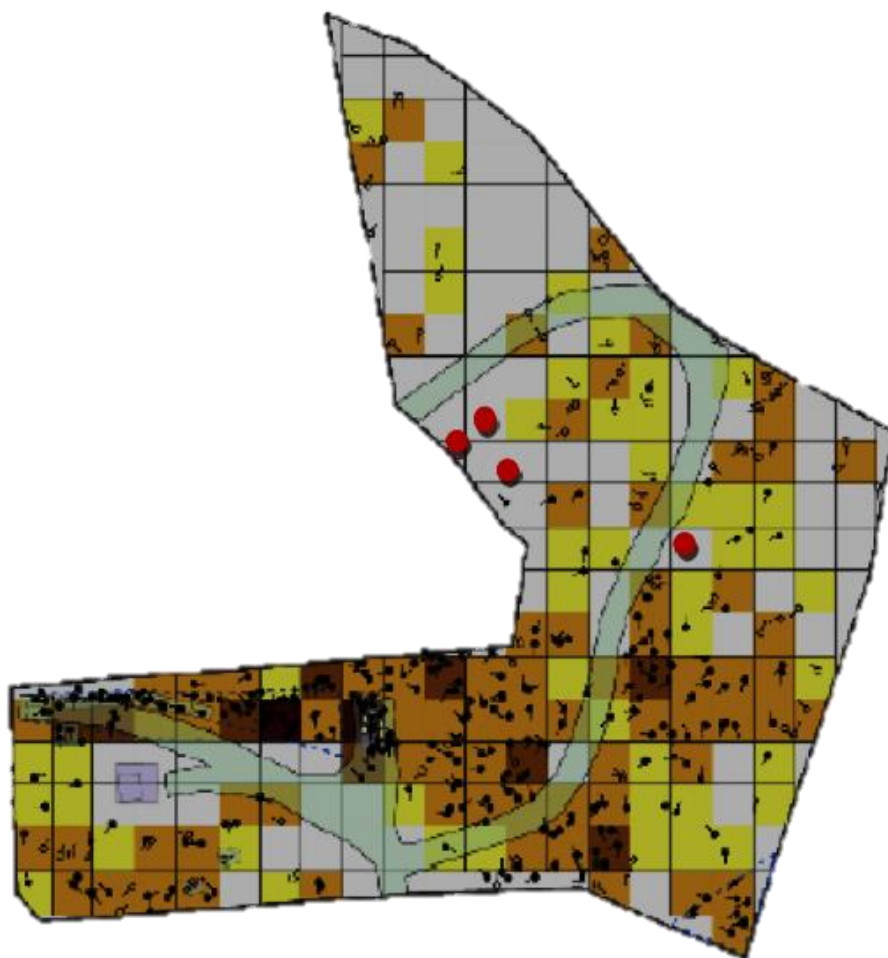


Figure 4: Density of past cadaver placements from 2001-2005 at the ARF. Placement of cadavers from the current study is marked in red; ♀/♂ symbols represent male and female placements (original image from Damann, 2010 and adapted in Cobaugh, 2013).

concluded in the modern lab. In addition to the use of independent labs, ‘personal protective equipment’ (PPE) was used at all times, and negative extraction or no template controls (NTCs) and PCR controls (both negative (NTCs) and positive controls) were monitored throughout experimental testing. Positive controls were stored and introduced to various experimental tests within the modern laboratory only.

Blood Card Sampling and DNA Extraction

For authentication purposes, one circle of blood was sampled from each of the Fitzco Classic Cards associated with the following cadavers: 67-12D, 36-12D, 50-12D, and 47-12D. The collection of blood cards takes place in the Forensic Anthropology Center’s (FAC) William M. Bass Building, located adjacent to ARF, as a normal component of cadaver intake standard operating procedure. Blood is retrieved from the human jugular using a syringe and applied evenly to the blood cards; if possible, two blood cards are collected per cadaver. The integrity of the samples is at the discretion of the collector, usually a volunteer/graduate student; whether these procedures provide a potential point of insert for contaminants has not yet been addressed. The collection of blood cards occurs regardless of the implementation of any experiment or study, meaning there is a blood card for every cadaver processed in the FAC since 2007 regardless of the cadaver’s involvement in research, unless blood is irretrievable. Blood cards are stored in a desiccator chamber until vacuum-sealed in a temperature- and humidity-controlled environment in one of the MAL-UTK’s clean rooms for all future uses.

A total of three 3 mm diameter pieces of the filter card was used per extraction, per cadaver. These pieces were obtained using laboratory scissors, which were soaked for 5-10 minutes in 30% household bleach followed by cleansing with 70% ethanol prior to sampling each individual card. DNA was extracted from the Fitzco card pieces using the QIAamp DNA Investigator kit (Qiagen), a silica-based extraction method. As per manufacturer’s instructions, the protocol “Isolation of Total DNA from FTA and Guthrie Cards” was used. To extract samples, 3mm card pieces were vortexed vigorously and incubated at 56°C with shaking for one hour following the addition of 280 µL of solution ATL, a proprietary lysis solution, and 20 µL of proteinase K. Following incubation, samples were centrifuged and 300 µL of Qiagen Buffer AL, which is likely some form of salt solution to improve DNA precipitation, was added. Tubes were then incubated a second time at 70°C with shaking for approximately 10 minutes. Then, 150 µL of 99.9% ethanol was added and solutions were mixed by pulse vortexing for 15 seconds. Samples were then transferred to a QIAamp MinElute column and centrifuged at 6,000 x g for one minute. The flow-through was discarded and the columns transferred to a new collection tube. This process was repeated after adding 500 µL of Qiagen Buffer AW1, again after adding 700 µL of Qiagen Buffer AW2, and a final time after adding 700 µL of 99.9% ethanol. Buffers AW1 and AW2 were incorporated in order to thoroughly wash the column.

Following washing, the columns were centrifuged at 20,000 x g for three minutes to remove any remaining ethanol. Again, the flow-through was discarded. The columns were then incubated with the lids open at room temperature for 10 minutes. Lastly, 70

μL of Qiagen Buffer ATE, a proprietary elution solution, was added directly to the center of each column and the columns were incubated for approximately five minutes at room temperature prior to centrifugation at 20,000 x g for one minute. The final flow-through/DNA eluent was stored at -20°C to await further analysis.

Soil DNA Extraction

DNA was extracted from soils using the Powerlyzer PowerSoil® DNA Isolation Kit (MOBIO). This extraction kit was selected based on its ability to remove potential inhibitory substances that are typically present in environmental samples. Soil extractions were carried out per the manufacturer's instructions, with modifications in the initial seven steps of the protocol. Rather than using the glass bead tubes and the bead beating method for cell lysis, proteinase K, the "bead solution" and Solution C1 were added to each sample and incubated for 45 minutes at 65°C. 0.25 g of wet weight soil was used for each extraction. The logic behind this modification was to increase the ratio of human to bacterial DNA (or decrease the ratio of bacterial to human DNA). By reducing the amount of microbial DNA present in the final DNA eluent, downstream processes such as human DNA amplification should proceed much more efficiently. Following lysis, no changes were made to the manufacturer's protocol; the extraction proceeded with five additional phases: protein precipitation, inhibitor removal, DNA binding, washing, and elution.

Following incubation, the supernatant of each sample was transferred to a new test tube and Solution C2 was added. The samples were then vortexed and incubated at 4°C for five minutes. The tubes were centrifuged and the supernatant was again transferred to a new test tube. This process was repeated with the addition of Solution C3. Solutions C2 and C3 were integral for the removal of inhibitory substances; both are patented "Inhibitor Removal Technology," and will further be referred to as IRT solutions (MOBIO). Following the removal of inhibitors, the supernatant was transferred to a new test tube, and Solution C4, a highly concentrated salt solution, was added to facilitate binding to the silica spin columns. The samples were then vortexed, and the supernatant was transferred to the silica spin filters in three sequential steps. During each step, the solution was added to the spin filter and centrifuged. Once the supernatant had been added to the spin filter in its entirety and the flow-through properly discarded, the DNA from each sample was bound to the silica membrane.

Samples were then washed using 500 μL of Solution C5 (a patented MOBIO wash solution) and the spin filters transferred to a new collection tube. Once transferred the samples were centrifuged for one minute at 10,000 x g to remove any remaining wash solution. 100 μL of Solution C6 was then applied to the center of the membrane for the final phase of DNA elution. Samples were centrifuged at 10,000 x g for 30 seconds to ensure complete DNA elution.

Negative controls were incorporated along with each extraction to assess the integrity of the DNA extraction, ensuring that laboratory technique and/or reagents were not inserting contaminants into extraction steps. Such controls underwent the entire extraction process without the addition of soil. Only those soil samples associated with

the same cadaver were processed during any given extraction event. Generally, samples were divided into two purification events per cadaver to minimize the amount of samples processed simultaneously, thereby reducing the chance for possible error and contamination.

End-point PCR Assays

In an effort to reduce both time and cost, preliminary end-point PCRs targeting human-specific amplicons of both nDNA and mtDNA were conducted prior to qPCR. A single 67 bp human specific nDNA amplicon of the c-fms proto-oncogene for the CSF-1 receptor gene on chromosome 5A was assayed as per Swango et al. (2006) to assess the presence of nuclear DNA in each samples. A 113 bp amplicon within HVI (Alonso et al., 2004) was amplified to gauge the presence of mtDNA in the absence of positive amplification of nuclear targets.

All end-point PCR amplifications were performed on an Applied Biosystems Veriti 96-well Thermal Cycler using the following cycle conditions: 95°C for 1 min., 35 cycles of 94°C for 30 sec./55-62°C for 30 sec./72°C for 1min., and 72°C for 1 min. All end-point PCRs were performed in 50µL reactions containing Platinum® Taq DNA Polymerase (Invitrogen™), 10 x Platinum Taq PCR buffer, 10 mM dNTPs, 50 mM MgCl₂, 20 µM forward/reverse primers, ddH₂O, and 1-2 µL of DNA extract. The annealing temperature was optimized to the primer set used (Table 4). A touchdown (TD) method was used in some cases, in which 0.1°C is subtracted from the annealing temperature every cycle. Annealing temperatures for all primer sets were optimized by first calculating the melting temperature (T_m) and by then varying temperatures through gradient PCR to determine which temperature provided optimal annealing conditions.

Both positive and negative (no template) controls or NTCs were incorporated into each PCR run to minimize contamination from exogenous DNA sources and assess the overall success of extraction in the event that preliminary PCRs yielded no amplifiable DNA. NTCs consisted of water rather than an unknown DNA source, while positive controls involved the addition of a human genomic DNA standard (Bioline). Positive control templates were added to PCR reaction tubes in the modern DNA laboratory only.

Tests for PCR Inhibition

PCR inhibition is often a problem when dealing with soil; PCR inhibitors such as humic acids are widespread in different soil types and are commonly co-purified with DNA (Alaeddini et al., 2012). Therefore, in the event of negative amplification, meaning the target failed to amplify, yet the positive control (human genomic DNA standard) demonstrated positive amplification, tests for PCR inhibition were subsequently conducted. Samples with suspected inhibition were “spiked” with 1 µL of DNA of similar quality (i.e., thought to share a similar state of degradation) and were re-amplified for the 113 bp mitochondrial target using end-point PCR. Samples were spiked using a DNA extract from Cadaver 36-12D, which was associated with Day 16 following

Table 4: Primers and Probes used for End-Point PCR and qPCR assays. Those used in qPCR are highlighted in yellow. TD refers to a touchdown method, in which 0.1°C was subtracted from the annealing temperature after each cycle.

Primer	Sequence (5' to 3')	Annealing Temp.	Target	Reference
nuCSF-F	GGGCAGTGTCCAACCTGAG	60°C	nDNA	Swango et al., 2006
nuCSF-R	GAAAACTGAGACACAGGGTGGTTA	60°C	nDNA	Swango et al., 2006
15997-F	CACCATTAGCACCCAAAGCT	60°C	113 bp mtDNA (HVI)	Alonso et al., 2004
16071-R	ACATAGCGGTTGTTGATGGG	60°C	113 bp mtDNA (HVI)	Alonso et al., 2004
15913-F	CAGTCTTGTAACCGGAGATG	58°C (TD)	687 bp mtDNA (HVI)	Kemp, 2006
00031-R	GAGTGGTTAATAGGGTGATAG	58°C (TD)	687 bp mtDNA (HVI)	Kemp, 2006
16442-F	CTCTCCTCGCTCCGGGCCCCAT	64°C (TD)	556 bp mtDNA (HVII)	Kemp, 2006
00429-R	CTGTAAAAAGTGCATACCGCCA	64°C (TD)	556 bp mtDNA (HVII)	Kemp, 2006
Probe	Sequence (5' to 3')	Reference		
MtDNA 113bp target	[5HEX] GAAGCAGATTTGGGTAC[BHQ1A]	Alonso et al., 2004		

placement. This sample demonstrated positive amplification with bright bands during gel electrophoresis and should show positive amplification in the absence of inhibition.

Repeat Silica Extraction

Given that most soil extractions showed signs of inhibition, all extractions underwent an additional treatment designed to reduce inhibition. Soil extractions were “re-extracted” using a protocol developed by Kemp et al. (2006), in which the Wizard® PCR Preps DNA Purification System protocol was modified for the purpose of re-purifying DNA extracts to remove existing inhibitors. One minicolumn was prepared for each sample by attaching the syringe barrel to the Luer-Lok extension of each minicolumn. One mL of resin was added to each sample, and the samples were vortexed briefly three times over a minute time period. The DNA/resin mix was then added to each syringe barrel, and the solution was slowly pushed through the minicolumn. Two mL of 80% isopropanol was then added to each syringe barrel and pushed through the appropriate minicolumn. Minicolumns were transferred to 1.5mL microcentrifuge tubes and were centrifuged for two minutes at 10,000 x g. Minicolumns were again transferred to new 1.5mL microcentrifuge tubes and 100µL of ddH₂O was applied to the center of the membrane. Samples were incubated at room temperature for approximately 1 minute and were then centrifuged for 30 seconds at 10,000 x g. The minicolumns were discarded and the flow-through was stored at -20°C for future use.

NanoDrop Protocol

Total DNA was quantified prior to conducting any repeat silica extractions using a NanoDrop 3300 Fluorospectrometer (ThermoScientific) using the AccuBlue Broad Range dsDNA Quantitation Kit (Biotium). This kit provides a sensitive detection of double-stranded DNA (dsDNA) in a linear range from 2-2000 ng, even in the presence of certain contaminants (RNA, proteins, detergents, salts, etc.). DNA extracts (1 µL) were incubated for 15 minutes in black tubes containing 20 µL of 98% AccuBlue Broad Range Buffer Solution, 1% AccuBlue Enhancer, and 1% AccuBlue Dye. Following incubation, samples were measured in triplicate by applying 2 µL of solution to the bottom pedestal of the fluorospectrometer.

MtDNA Sequencing and Source Authentication

Because contamination can occur at any exploratory step, additional testing was required to authenticate the source of the extracted DNA. As discussed earlier, mtDNA can provide a quick, easy, and sensitive evaluation of contamination/DNA mixtures when less than ideal forensic profiles are expected. Therefore, end-point PCRs of mtDNA HVI and HVII regions were conducted as previously described for a subset of the soil samples from each cadaver and for the cadaver blood cards. Sample subsets were chosen based on the ability to amplify full regions of HVI/HVII without using multiple primer sets; the rationale behind this was to reduce sequencing costs, which can be substantial when

multiple regions require sequencing per sample. If subsets, which varied from 6-8 samples per cadaver, were free of contamination, all soil samples associated with the cadaver were included in further analyses.

Following amplification, PCR amplicons were purified using an ExoSap PCR clean-up protocol (Exonuclease I (Thermofisher), Shrimp Alkaline Phosphatase (Affymetrix), and a Dilution Buffer (Affymetrix)) to remove unincorporated primers and dNTPs. Purified PCR products were sent for sequencing at UTK's Molecular Biology Resource Facility (MBRF). Forward and reverse sequences were obtained to resolve potential ambiguities. Such ambiguities were particularly common in sequencing HVII in these individuals, in which a poly 'C' region spanning nucleotide positions 303-315, caused a slip in the polymerase during sequencing which was then present throughout the entire sequence read.

The programs 4 Peaks (Nucleobytes) and Seqman Pro (DNASTAR Lasergene 11) were used for editing and aligning sequences. Cadaver blood card sequences were compared with rCRS (GenBank: [NC_012920](#)) to locate variants specific to each cadaver. Cadaver haplogroup lineages were then predicted using MITOMASTER (mitomap.org). Soil sample sequences from each cadaver were aligned and compared with the sequences obtained from their associated blood card.

qPCR Standard

Following Alonso et al. (2004), a qPCR standard was generated by amplifying a 687 bp target from the human mtDNA HVI using end-point PCR as described above; primer information is located in Table 4. The subsequent PCR product was purified using the ExoSap PCR Clean-up protocol and quantified using the NanoDrop 3300 Fluorospectrometer. PCR product for qPCR standard generation was made daily. Copy number was determined using the Thermo Scientific DNA copy number calculation tool (thermoscientificbio.com/webtools/copynumber). Ten-fold serial dilutions from 10^8 to 10^2 were prepared to build the standard curve for incorporation into subsequent qPCR runs. Serial dilutions were also made fresh daily.

Mitochondrial DNA qPCR

A singleplex assay developed by Alonso and colleagues (2004) was conducted to assess the quantity of human mtDNA copy number using a 113 bp target. In this singleplex assay, the amplifiable target was monitored in real-time to estimate its copy number, consequently providing information regarding the absolute quantity of mtDNA in each sample (Alonso et al., 2004). In qPCR, the number of copies present in a sample is related to the accumulation of fluorescence signal over time; the cycle at which samples accumulate enough fluorescence to extend beyond background levels is known as the cycle threshold or C_q. Samples with large amounts of a specific target will have smaller C_q values compared with samples with small amounts of target. By comparing C_q values between standards of known quantities, absolute copy numbers can be determined for unknown samples.

Reactions were performed using Taqman chemistry in 20 μ L containing 1X DyNAmo Probe Master Mix (Thermo Scientific), 300 nM forward and reverse primers, 250 nM probes, and 2-4 μ L of DNA extract. The cycling protocol consisted of a two-step method with the following cycling conditions: 95°C for 15 min followed by 40 cycles of 95°C for 15 sec. and 60°C for 60 sec. See Table 4 for the primer oligonucleotide sequences. The probe was labeled with a HEX fluorophore; the probe sequence is as follows: [5HEX] GAAGCAGATTTGGGTAC [BHQ1a]. Samples were run in triplicate, standards in duplicate, and at least three no template controls (NTCs) were included in each plate per run. All runs were conducted using the Bio-Rad CFX Connect™ Real-Time PCR Detection System and were subsequently analyzed using both the CFX Manager™ Software as well as Microsoft Excel 2013. Data were reported as copies per 100 μ L extract as well as copies per gram of dry weight sediment (gdw). Gdw was determined gravimetrically and was previously reported by Cobaugh (2013).

Analytical and Statistical Methods

The quantity of human DNA extracted from the soil was expressed as mitochondrial DNA copy number per 100 μ L extract as well as mitochondrial DNA copy number per gram of dry weight sediment (gdw). Copy number data from all cadavers were combined and analyzed by decomposition stage using Microsoft Excel 2013 and IBM SPSS for Windows, Version 22. Both untransformed and transformed ($\log_{10}X$) data were assessed. One-sample Kolmogorov-Smirnov tests and Levene's Test for Homogeneity of Variance were used to determine adherence to parametric statistical assumptions of normality and homogeneity of the variance. A Kruskal-Wallis test was used to assess overall group differences, and Mann-Whitney U tests (using a Bonferroni correction) were conducted as post hoc procedures to the Kruskal-Wallis. An adjusted significance level of 5% was used to determine statistical significance.

Human DNA quality, or the degree of fragmentation, was assessed qualitatively by comparing fragment size of amplicons generated from varying primer sets specific to HVI/HVII. For example, if the 113 bp target from HVI failed to amplify for a specific soil DNA extract, that sample was thought to 1) either contain no human DNA or 2) contain highly fragmented DNA. If the larger human DNA target sets, such as the 687 bp target from HVI and the 556 bp target from HVII showed positive amplification for a specific soil DNA extract, such samples were said to be of high enough quality to provide a successful mitochondrial DNA profile, while samples that failed to show positive amplification of these targets were of lower quality.

Chapter IV

Results

This study was conducted using soil samples previously collected as the focus of another project assessing changes in microbial diversity in the CDI during cadaveric decomposition (see Cobaugh, 2013). Cobaugh (2013) placed four human cadavers at the University of Tennessee's Anthropological Research Facility (ARF) in sites believed to have been unused, meaning sites where cadavers had not been previously placed. Samples were collected from beneath the torso region of each cadaver during various stages of decomposition: Bloat, Active, Advanced, and Dry/Remains. An initial soil sample was collected prior to the placement of each cadaver; no Fresh samples and few Dry/Remains samples were obtained. Stages were determined subjectively by Cobaugh and others using the characteristics outlined by Payne (1965). For the purpose of this project, DNA was extracted from samples associated with each cadaver (including blood card and soil samples) and underwent preliminary PCR, total dsDNA (double stranded) quantitation, sequencing, and qPCR analysis. The results are as follows:

End-point PCR Assays

All no template controls (NTCs) were blank, and all positive controls yielded bands when visualized following gel electrophoresis during preliminary or end-point PCRs. Preliminary PCRs revealed an absence of the 67 bp nDNA target from the nuCSF region in the majority of soil DNA extracts associated with each cadaver (Tables 5-8). A small number of samples showed positive amplification for the nDNA target; there was no obvious pattern related to stage of decomposition or total amount of DNA extracted. While samples that showed positive nDNA amplification generally had large amounts of total dsDNA, a few of the samples that PCR-amplified, such as 50-12A and 50-6A, had total dsDNA quantities below the detection limit (2 ng/ μ L) of the AccuBlue Broad Range Quantitation Kit. Total dsDNA results can be found in Appendix A1.

The 113 bp mtDNA target amplified in most of the samples, especially following repeat silica extractions (Tables 5-8). Nine out of eleven samples associated with cadaver 36-12D showed positive amplification of the 113 bp target. Nine of ten samples were amplified in soil samples accompanying cadaver 67-12D, and twelve of thirteen samples demonstrated positive amplification for cadaver 47-12D. Nine of twelve samples showed amplification for cadaver 50-12D. Additionally, the 113 bp target exhibited positive amplification in a control sample, 50-12C, which was taken approximately two meters away from the cadaver. Following sequencing, this sequence was shown to be consistent with the mitochondrial profile of cadaver 50-12D.

MtDNA Sequencing and Authentication

Sequences of mtDNA HVI/HVII amplified and purified from the DNA extracts from the cadaver blood cards were compared to the rCRS to determine existing

Table 5: Positive/Negative Amplification of Mitochondrial and Nuclear Targets for Cadaver 36-12D before and after Repeat Silica Extractions. *Not a stage from Payne (1965).

Payne (1965) Stages	Stages Defined by Cobaugh (2013)	Sample Code	Before Repeat Silica Ext.		Inhibition (Present/Absent)	After Repeat Silica Ext.	
			67 bp nuCSF	113 bp HVI		67 bp nuCSF	113 bp HVI
Initial*	Initial	36-1	-	+	Absent	-	-
Bloat	Bloat	36-2	-	-	Absent	-	-
		36-4	-	+	Absent	-	+
Active	Bloat-Active	36-6	-	+	Absent	-	+
		36-8	-	-	Absent	-	+
Advanced Decay	Active	36-12	-	+	Absent	-	+
	Initial Advanced Decay	36-14	-	+	Absent	-	+
		36-16	-	+	Absent	-	+
	Advanced Decay I	36-23	-	+	Absent	-	+
Dry/Remains	Advanced Decay II	36-46	-	+	Absent	+	+
	Advanced Decay III	36-87	-	+	Absent	-	+

Table 6: Positive/Negative Amplification of Mitochondrial and Nuclear Targets for Cadaver 67-12D before and after Repeat Silica Extractions. *Not a stage from Payne (1965).

Payne (1965) Stages	Stages Defined by Cobaugh (2013)	Sample Code	Before Repeat Silica Ext.		Inhibition (Present/Absent)	After Repeat Silica Ext.	
			67 bp nuCSF	113 bp HVI		67 bp nuCSF	113 bp HVI
Initial*	Initial	67-1	-	+	Absent	-	N/A*
Bloat	Bloat	67-4	-	+	Absent	-	N/A*
		67-6	-	+	Absent	-	+
		67-8	-	+	Absent	-	+
	Bloat-Active	67-10	-	+	Present	-	+
Active	Active	67-13	-	+	Absent	-	N/A*
		67-17	-	+	Absent	-	+
Advanced Decay	Initial Advanced Decay	67-29	-	+	Absent	-	N/A*
	Advanced Decay I	67-72	-	+	Absent	+	+
Dry/Remains	Advanced Decay II	67-114	-	-	Absent	-	-
N/A*Sample was not tested.							

Table 7: Positive/Negative Amplification of Mitochondrial and Nuclear Targets for Cadaver 47-12D before and after Repeat Silica Extractions. *Not a stage from Payne (1965).

Payne (1965) Stages	Stages Defined by Cobaugh (2013)	Sample Code	Before Repeat Silica Ext.		Inhibition (Present/Absent)	After Repeat Silica Ext.	
			67 bp nuCSF	113 bp HVI		67 bp nuCSF	113 bp HVI
Initial*	Initial	47-1	-	-	Absent	-	-
Bloat	Bloat	47-3	-	-	Present	-	+
		47-4	-	+	Present	-	+
	Bloat-Active	47-5	-	+	Present	-	+
Active	Active	47-6	-	+	Present	-	+
		47-7	-	-	Present	-	+
		47-8	-	+	Present	-	+
Advanced Decay	Initial Advanced Decay	47-9	-	+	Present	-	+
	Advanced Decay I	47-10	-	+	Absent	-	+
		47-13	-	+	Present	-	+
	Advanced Decay II	47-25	-	+	Present	-	+
Dry/Remains	Advanced Decay III	47-48	-	+	Present	-	+
		47-198	-	+	Present	-	+

Table 8: Positive/Negative Amplification of Mitochondrial and Nuclear Targets for Cadaver 50-12D before and after Repeat Silica Extractions. An asterisk is used to indicate that a listed stage is not originally associated with Payne (1965).

Payne (1965) Stages	Stages Defined by Cobaugh (2013)	Sample Code	Before Repeat Silica Ext.		Inhibition (Present/Absent)	After Repeat Silica Ext.	
			67 bp nuCSF	113 bp HVI		67 bp nuCSF	113 bp HVI
Initial*	Initial	50-1	-	+	Absent	-	-
Bloat	Bloat	50-4	+	+	Absent	+	+
Active	Bloat-Active	50-5	-	-	Present	-	+
		50-6	-	+	Absent	+	+
	Active	50-8	-	-	Present	-	+
Advanced Decay	Initial Advanced Decay	50-10	-	-	Present	-	+
	Advanced Decay I	50-12	-	+	Present	+	+
		50-16	-	+	Absent	-	+
	Advanced Decay II	50-28	-	-	Present	-	-
		50-39	-	-	Present	-	+
Drv/Remains	Advanced Decay III	50-83	-	+	Absent	-	+

polymorphisms useful for human identification; the polymorphisms present in HVI/HVII for each cadaver and the primary researcher on this project can be found in Table 10. Polymorphisms were used to match cadaver and soil samples during authentication processes. They were also used for haplogroup prediction using MITOMASTER; haplogroup assignments are listed below (Table 9). Cadavers 67-12D and 36-12D were assigned to haplogroup H5, which is also shared by the primary researcher; while cadaver 50-12D belonged to haplogroup U5b, and cadaver 47-12D belonged to HV0.

Amplified mtDNA targets from a subset of soil samples associated with each cadaver were also sequenced. Sequences were assessed for the polymorphisms specific to each cadaver (Table 10). Eight of fourteen samples from cadaver 47-12D were sent for sequencing, and no contamination was observed. Seven of thirteen samples paired with cadaver 36-12D were sent for sequencing. Though contamination was observed in 36-1A, only minor contamination (the presence of an unknown peak) was seen throughout the remaining samples in the subset; such minor contamination was evident in 36-12A and 36-14A. Six of twelve samples amplified from soils associated with cadaver 50-12D were sent for sequencing, and of these, one sample, 50-1A, demonstrated significant contamination, meaning it contained a complete yet alternative mitochondrial profile from the cadaver with which it was paired. However, following repeat silica extractions, mitochondrial targets HVI/HVII were no longer amplifiable for this sample. Three samples of six showed minor contamination, meaning a single underlying peak of a different base pair was observed in both forward and reverse sequences; the real peak containing the single base pair that matches 50-12D was present and much larger than any underlying peaks in all samples.

Six of eleven samples of 67-12D were sent for sequencing, and one sample showed considerable contamination, sample 67-12A. Additionally, sequences from sample 67-72A exhibited more than one position of an ambiguous base mixture. Rather than showing a low level peak beneath the known base of the cadaver, there were equal sized peaks present, one from cadaver 67-12D and one of unknown origin.

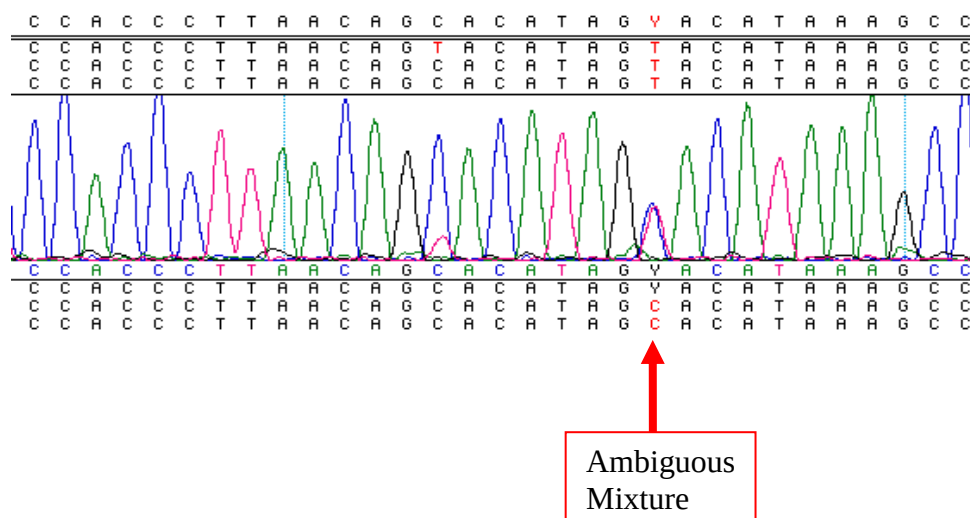
Table 9: Cadaver Haplogroup Predictions

Individual	Predicted Haplogroup
50-12D	U5b
67-12D	H5
36-12D	H5
47-12D	HV0
Researcher	H5

Table 10: Cadaver and Researcher (AL Emmons) mtDNA Polymorphisms

HVI				
Researcher	50-12D	67-12D	36-12D	47-12D
	16270 C>T			
				16298 T>C
16304 T>C	16304 T>C	16304 T>C	16304 T>C	
	16311 T>C			
		16519 T>C		
HVII				
50-12D	67-12D	47-12D	36-12D	Researcher
		00072 T>C		
00073 A>G				
00150 C>T				
				00152 T>C
		00195 T>C		
00228 G>A				
00263 A>G	00263 A>G	00263 A>G	00263 A>G	00263 A>G
		00309 C>CCCT		
	00310 T>TTC	00310 T>C	00310 T>TTC	
00315 C>CC				00315 C>CC

Figure 5: Example of a mixture present in sample 76-72A.



MtDNA qPCR Analysis

MtDNA copy number was estimated by amplifying a 113bp mtDNA HVI target. Cadaver 67-12D was excluded from qPCR analysis due to the large degree of contamination present in samples. Two to three NTCs were run per plate. Five of eight NTCs showed no amplification. However, three NTCs provided high Ct values, one included in the qPCR run with samples from cadaver 47-12D and two from the qPCR run included with samples from cadaver 36-12D. The high Ct values for these NTCs are likely due to primer-dimer formation and occurred more than 6 cycles after the Ct values for the majority of the samples. Samples with Ct values having occurred within six cycles of the NTC Ct values include 36-1A, 36-2A, 50-28A, 50-16A, 50-8A, and 50-39A.

A total of three runs were conducted, one per each cadaver. The standard curves for each run were evaluated based on efficiency (E) and R^2 values (Table 11). The efficiency of the standards averaged 92.5% over 3 runs, and all values of efficiency were greater than 90%. Additionally, the R^2 values of the standards for each run were above 0.99, with three runs averaging 0.995 (Table 11; Figure 6). The means, standard deviations, and standard errors were calculated for each standard; standard deviations and standard errors were consistently low (< 1) for all standards (Table 12). Standards were run in duplicate for each plate. Means and standard deviations were also calculated for each unknown, which were run in triplicate (Table A2). The sensitivity of the qPCR assay or detection limit was approximately 100 copies per 0.008 g of dry weight sediment. Inhibition was not monitored; however, repeat silica extractions, which were conducted for the removal of inhibitors, were performed prior to all qPCR runs to decrease the effect of inhibition on qPCR results.

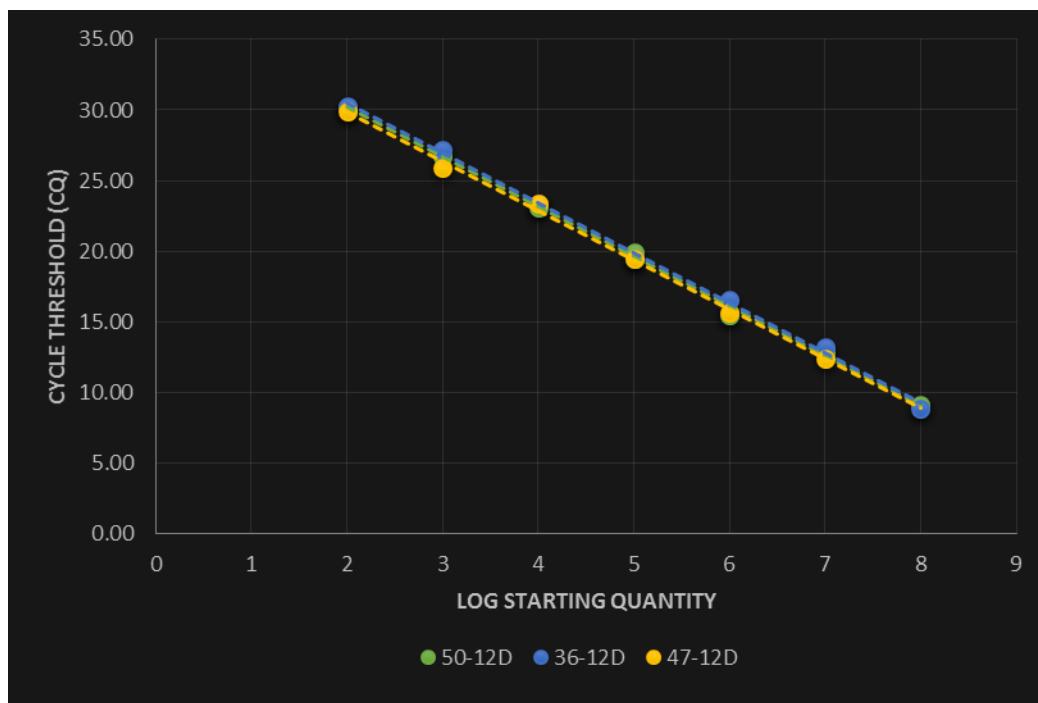


Figure 6: Standard Curve Reproducibility for mtDNA 113bp Target

Table 11: Descriptive Statistics (Means and Standard deviations) for the R^2 , Slope, Y-intercept, and Efficiency across qPCR runs (n=3).

	50-12D	36-12D	47-12D	Mean	SD
R^2	0.997	0.996	0.993	0.995	0.00199
Slope	-3.520	-3.537	-3.491	-3.516	0.02303
Y-intercept	37.224	37.528	36.809	37.187	0.36075
Efficiency (%)	92.34	91.76	93.39	92.50	0.82767

Table 12: Descriptive Statistics (Means, Standard deviations, Confidence intervals, and Standard errors) for each Standard across qPCR runs (n=3).

	Copy Number	Mean Ct	Standard Deviation	95% CI (Lower Bound)	95% CI (Upper Bound)	Standard Error
STD1	10 ⁸	8.9825	0.73582	7.8117	10.1533	0.36791
STD2	10 ⁷	13.0200	0.39774	12.3871	13.6529	0.19887
STD3	10 ⁶	16.0200	0.79381	14.7569	17.2831	0.39690
STD4	10 ⁵	19.7025	0.35556	19.1367	20.2683	0.17778
STD5	10 ⁴	23.23	0.193	22.93	23.54	0.097
STD6	10 ³	26.97	0.239	19.1367	20.2683	0.119
STD7	10 ²	30.2125	0.09912	30.0548	30.3702	0.04956

In order to determine the appropriate route of statistical analysis necessary to compare quantities of mtDNA copy number between sample groups or decomposition stages, the data were tested for concordance with the assumptions of parametric methods of analysis including tests for normality and homogeneity of variance. Two variables (both log transformed ($\log_{10}X$) and untransformed) were tested for normality of data using One-sample Kolmogorov-Smirnov tests: copy number per 100uL extract, copy number per gram of dry weight soil (gdw), log (copy number/100uL extract), and log (copy number/gdw). Tests for normality were conducted among all samples associated with an individual cadaver and among combined samples associated with different decomposition stages (combined data from all cadavers). Test statistics and significance levels are presented in Tables 13 and 14.

Table 13: One-Sample Kolmogorov-Smirnov Tests among soil samples associated with a single cadaver: Significance is indicated by an asterisk ($\alpha=0.05$).

	Copy #/100 μ L		Copy #/gdw		Log (Copy #/100 μ L)		Log/gdw	
Cadaver ID	Test Statistic	P-Value	Test Statistic	P-Value	Test Statistic	P-Value	Test Statistic	P-Value
47-12D	0.126	0.135	0.144	0.048*	0.191	0.001*	0.160	0.016*
50-12D	0.276	0.000*	0.270	0.000*	0.276	0.000*	0.099	0.200
36-12D	0.266	0.000*	0.277	0.000*	0.180	0.003*	0.111	0.200

Table 14: One-Sample Kolmogorov-Smirnov Tests among samples within each decomposition stage. Samples associated with different cadavers were combined based on shared decomposition stage. (1=Initial; 2=Bloat; 3=Active; 4=Advanced Decay; 5=Dry/Remains)

	Copy # / 100µL		Copy # / gdw		Log (Copy # / 100µL)		Log (Copy #)/gdw	
Stage	Test Statistic	P-Value	Test Statistic	P-Value	Test Statistic	P-Value	Test Statistic	P-Value
1	0.240	0.197	0.217	0.200	0.158	0.200	0.145	0.200
2	0.322	0.000*	0.325	0.000*	0.226	0.001*	0.125	0.200
3	0.323	0.000*	0.365	0.000*	0.176	0.090	0.121	0.200
4	0.193	0.000*	0.190	0.000*	0.157	0.005*	0.101	0.200
5	0.340	0.003*	0.336	0.004*	0.264	0.070	0.200	0.200

Samples from a single cadaver did not meet the assumption of normality for any of the four variables tested. However, the variable, log transformed copy number per gram of dry weight soil, consistently demonstrated normality within each decomposition stage when samples were pooled together from each cadaver. However, all variables of interest failed to meet the second assumption of homogeneity of variance; test statistics and significance levels are reported in Table 15.

Table 15: Levene's Test for Homogeneity of Variance

Variable Tested	Levene's Test Statistic	Significance (p-value)
Copy # / 100 uL	8.554	0.000*
Log / gdw	2.957	0.000*
Copy # / gdw	8.787	0.000*
log Copy # / 100 ul	8.204	0.000*

Because the assumptions for parametric statistical analyses were not met, non-parametric tests were conducted. The Kruskal-Wallis test, a non-parametric version of the One-way ANOVA was used to test for significant differences between mtDNA copy number extracted during the different stages of decomposition; test statistics and values of statistical significance (p-values) are located in Table 16. Total copy number per 100 µL extract both log-transformed and untransformed were statistically different between stages of decomposition (p-value = 0.000). Additionally, the total copy number per gram of dry weight soil both log-transformed and untransformed was statistically significant between decomposition groups (p-value = 0.000).

Subsequent pairwise comparisons using Mann-Whitney U (with the Bonferroni correction) were performed on Copy #/gdw. Samples taken at day one significantly differed from all other decomposition stages, excluding stage 5 (Dry/Remains; adjusted p-value = 1.000). MtDNA copy number was not significantly different between Active

and Advanced stages of decomposition (adjusted p-value = 1.000) nor between Active and Bloated stages of decomposition (adjusted p-value = 0.089). However, samples associated with Active Decay were significantly different from samples from Initial and Dry/Remains stages (adjusted p-value = 0.000; 0.032). Copy number did not significantly differ between Bloated and Dry/Remains samples (adjusted p-value = 1.000) nor Bloated and Advanced Decay samples (adjusted p-value = 0.307). These differences are easily visualized in a box plot of the ranked medians of copy number per gram of dry weight sediment produced during the Kruskal-Wallis test (Figure 7). Though the Active Decay ranked median was greater than all other stages; the difference was only statistically significantly different between Active Decay and Initial and Dry/Remains stages.

Nevertheless, a glance at the descriptive statistics for each group is highly informative (Table 17). Copy number per gram of sediment increased as decomposition progressed and seemed to peak during the Active stage of cadaveric decomposition. The quantity of mtDNA purified from the soil then decreased in the Advanced Decay and then again in the Dry/Remains stages of decomposition (Figure 8-9).

Table 16: Independent-Samples Kruskal-Wallis Test. A non-parametric One-Way ANOVA test to distinguish group differences. Significance level of $\alpha < 0.05$ indicated by an asterisk.

	Copy #/100uL	Copy#/gdw	log Copy#/100ul	log/gdw
N	92	92	92	92
Chi-Square	31.064	31.431	31.064	31.431
DF	4	4	4	4
Asymptotic Sig.	0.000*	0.000*	0.000*	0.000*

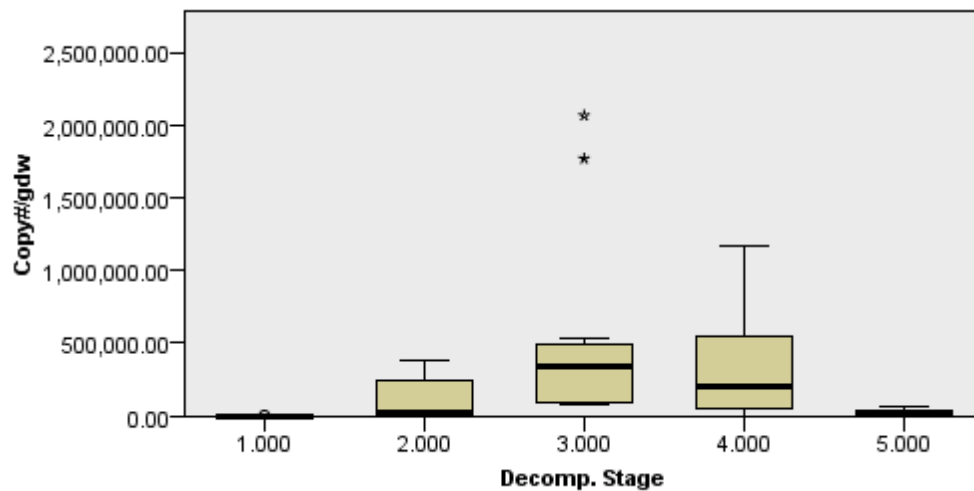


Figure 7: Box-Plots of Independent Samples Kruskal-Wallis Test of Copy Number per Gram of Dry Weight Sediment. Decomposition stages are labeled 1-5 (1=Initial, 2=Bloated, 3=Active Decay, 4=Advanced Decay, 5=Dry/Remains).

Table 17: Means and standard errors of mtDNA copy number per gram of dry weight soil and the log quantity per gram of dry weight soil. (Initial n=7, Bloat n=21, Active n=17, Advanced n=38, Dry/Remains n=7).

Variable	Decomposition Stages				
	Initial	Bloat	Active	Advanced	Dry/Remains
Copy #/gdw	866.133 ± 249.670	135533.228 ± 31357.059	464986.178 ± 140128.472	325175.081 ± 49385.969	21588.437 ± 8196.311
log/gdw	2.855 ± 0.101	4.563 ± 0.195	5.416 ± 0.117	5.059 ± 0.152	4.151 ± 0.161

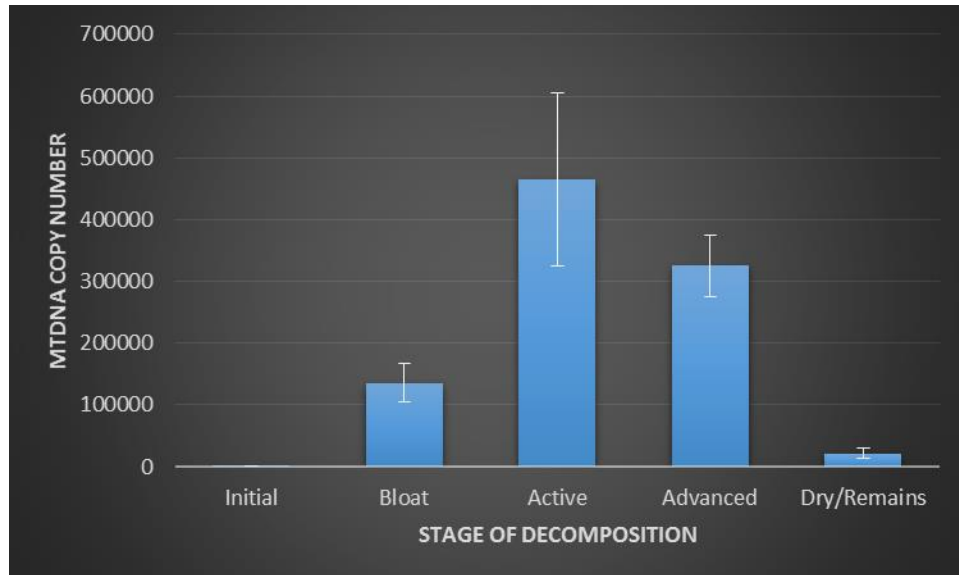


Figure 8: Mean mtDNA copy number per gram of dry weight soil during decomposition (n=92). Error bars indicate the standard error associated with each sample.

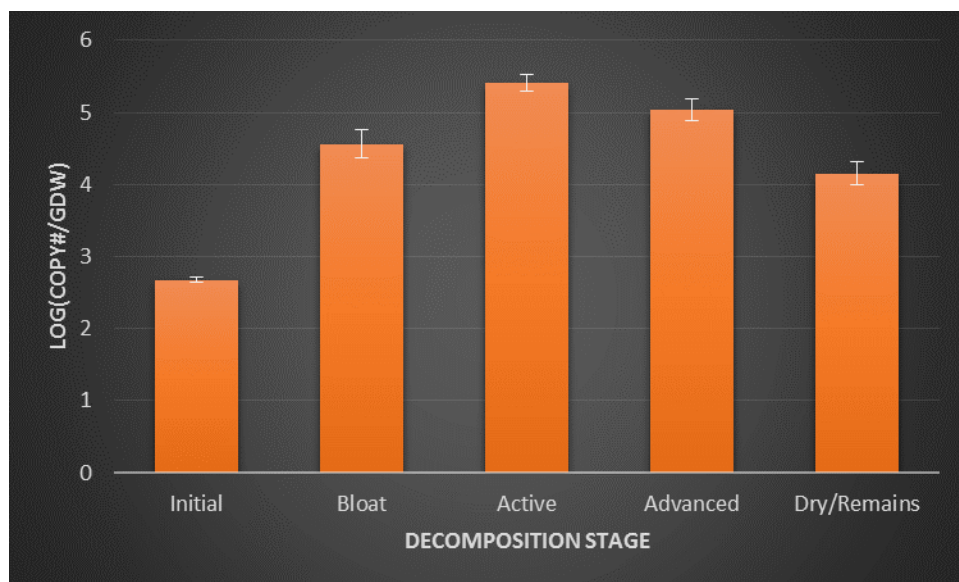


Figure 9: Log transformed mean mtDNA copy number per gram of dry weight soil during decomposition (n=92). Error bars indicate the standard error associated with each sample.

Chapter V

Discussion, Conclusions, and Recommendations

This study investigates the release and persistence of human DNA in the soil during decomposition with the purpose of enhancing human identification efforts to detect primary burial sites, especially when bodies are relocated to a secondary site. This study assessed the presence or absence of two human DNA sources, nDNA and mtDNA, and evaluated the quantity and quality of recovered DNA for the purposes of forensic identification. Additionally, this project looked at the pattern of persisting DNA throughout decomposition by comparing DNA copy number among soil samples obtained during the different stages of decomposition. By characterizing the persistence of DNA throughout the decomposition process, this project contributes to a larger ongoing dialogue regarding the taphonomic processes that occur in the soil environment concomitantly with decomposition.

To address these objectives, initial endpoint PCRs were conducted to evaluate the presence/absence of a human DNA source (i.e., mtDNA, nDNA, or both). Moreover, once the presence of a particular source was established, real-time quantitative PCR (qPCR), which simultaneously amplifies and quantifies a specific target sequence within the genome, was used to assess the quality and quantity of human DNA extracted from the soil environment during human decomposition.

Though no inferences were made at the beginning of this study regarding the source of human DNA recovered, based on endpoint PCR assays, it was evident that nuclear DNA was not easily recoverable in this project, while mtDNA was recoverable throughout all stages of decomposition. There are a number of reasons why this may have been the case. As previously mentioned, the structure and location of mtDNA within the human cell contribute to its ability to escape nuclease degradation (Butler, 2005; Nielsen et al., 2007); being more protected from nucleases, mtDNA has a better chance of survival. Additionally, due to the large number of mitochondrial DNA copies per human cell, statistically, the recovery of mtDNA over nDNA is more likely.

This study tested the hypothesis that the quantity of any persisting human DNA in grave soil would be highest during the Active Decay and Advanced Decay stages of decomposition. This hypothesis was supported for mtDNA. The quantity of mtDNA copy number was relatively low in Bloat and Dry/Remains stages of decomposition, and it was greatest in the Active Decay stage. Following Active Decay, the quantity of mtDNA steadily decreased.

This hypothesis was based on the known dynamics of the decomposition process. Though there is an initial purging of fluids during the Bloated stage of cadaveric decomposition, this release of fluids is much more extensive during the Active stage, in which a single CDI begins to coalesce and form (Carter et al., 2007); therefore, because there are more fluids being released into the soil, logically, the amount of DNA released into the soil would also increase. However, because the CDI is generally more enriched in the Advanced Decay stage (Carter et al., 2007), it was initially thought that the quantity of DNA would remain equally as high in this stage. This is not what was

observed. In fact, there was a decrease in mtDNA copy number as decomposition progressed from Active to Advanced Decay. Though not statistically significant, a downward trend may be related to the presence of high amounts of saprophytic microbes in the soil.

Cobaugh (2013) saw an increase in human-associated bacteroides beginning intermediate to the Bloat and Active Decay stages; these bacteroides remained at increased levels throughout decomposition. Additionally, Cobaugh (2013) saw an increased presence of microbial species throughout all stages of decomposition using 16S gene copies; the average log/gdw increased during Advanced Decay, while a decrease in microbial levels was observed during Active Decay (Cobaugh, 2013). This apparent decrease in microbial populations followed by a subsequent increase in the Advanced Decay stage may be related to the trend observed in this project. Though mtDNA persisted throughout all stages tested in this thesis, without an expansion of this study, spanning years, it is difficult to determine how long mtDNA will persist in the soil. As demonstrated by Haile et al. (2007), in the right conditions, DNA may persist for thousands of years in ancient sediment samples.

As a caveat to the results presented in this project, it is necessary to mention that mtDNA copy number results may be slightly inflated due to the formation of primer-dimers. Primer-dimers are due to non-specific binding and elongation, and lead to the presence of non-specific amplicons; the probability of primer-dimer formation increases with increased cycle number during qPCR. Primer-dimer formation is suspected due to the high critical threshold or Ct values associated with three out of eight NTCs. Amplified samples with high Ct values within six cycles of the NTC Ct values (Samples 36-1A, 36-2A, 47-1A, 50-28A, 50-16A, 50-8A, and 50-39A) likely amplified due to non-specific binding; authentic template amplification for these samples may be minimal. Not surprisingly, these samples, excluding 50-8A, 50-16A, and 50-39A, failed to amplify during preliminary end-point PCR assays, and 50-8A, 50-16A, and 50-39A, only demonstrated amplification following repeat silica extractions. These samples (36-1A, 36-2A, 47-1A, 50-28A, 50-16A, 50-8A, and 50-39A) were included in further analyses only because there is likely a small amount of copy number present in every sample due to primer-dimers, which means the average copy number in all samples is likely slightly inflated.

The second objective of this project was to assess the quality of human DNA over the course of human decomposition. The intended procedure was to compare the quantities of different sized mtDNA targets within HVI of the mitochondrial control region to create a ratio indicative of degradation, as per Alonso et al. (2004). However, due to unforeseen problems arising with the standards used in this project, a great deal of time, energy, and qPCR master mix was invested in optimizing qPCR standards to increase overall reaction efficiency. Because of this, there was not enough DyNamo probe master mix to assess the larger sized mtDNA target, and due to the discontinuation of this product by ThermoScientific, there was no way to purchase additional mastermix. Therefore, to avoid comparing two targets amplified under different conditions, all intended analyses involving the larger 287bp target were eliminated from this project.

However, some general inferences about mtDNA quality can be formulated on the

basis of mitochondrial sequencing attempts. A subset of samples from each cadaver were sequenced, and this subset was largely determined by the ability to sequence HVI and HVII with the smallest amount of primer sets, meaning sequenced samples demonstrated positive amplification across a 687 bp region encompassing HVI and across a 556 bp region encompassing HVII. These samples were then authenticated via comparisons with the cadaver blood cards. A full profile was not possible for samples associated with the Dry/Remains stage of decomposition (excluding sample 67-72A, which was highly contaminated) by amplifying the entire regions associated with HVI and HVII. This does not negate the fact that these samples could ultimately yield full profiles by overlapping several small primer sets and sequencing small fragments. Because 113 bp fragments were amplifiable in all Dry/Remains samples, it is likely that small primer sets could be used to gain a full mitochondrial profile. Small primer sets were not used in this project to minimize overall sequencing costs.

Additionally, samples 36-23A and 36-46A only provided adequate profiles from HVI. However, sample 47-48A provided full sequences of both HVI and HVII. Therefore, mtDNA was of high enough quality to provide full, authentic profiles during the advanced stages of decomposition. More information is needed to fully assess the state of DNA degradation during decomposition, but it appears that DNA quality does decrease as decomposition progresses.

Limitations

There are distinct limitations associated with mtDNA and mtDNA profiles that need to be discussed prior to concluding statements. As previously mentioned, mtDNA does not yield information specific to a particular individual, and thus several individuals could share the same mitochondrial profile. Therefore, it is important to state that it is not beyond the realm of possibility that a contaminated source could match the cadaver profiles.

Additionally, heteroplasmy, in which multiple mtDNA types may be present in a single individual is a particular nuisance in mtDNA sequence comparison and interpretation. Heteroplasmy is not only found between tissue types, but can also be found within the same tissue type and even within the same cell (Butler, 2005). Researchers have reported the frequency of heteroplasmy at different levels. Calloway et al. (2000) reported a heteroplasmy frequency of 11.6% in areas across HVII when comparing five tissue types across 43 individuals, while others such as Tully et al. (2000) have reported slightly higher frequencies. Using a denaturing gradient-gel electrophoresis system, Tully et al. (2000) reported a frequency of low-level heteroplasmy in HVI at 13.8%. Based on this information, it is difficult to determine when a single point mutation or single point mixture represents contamination or heteroplasmy.

As a general rule, according to Butler (2005: 276) “if three or more sites within the 610 bases evaluated across HVI and HVII are found to possess multiple nucleotides at a position (i.e., sequence heteroplasmy), then the sample can usually be considered a mixture – either by contamination or from the original material,” and such samples are

generally excluded from analysis. For example, cadaver 67-12D encompassed multiple samples with contamination occurring at more than a single nucleotide position, and thus all samples associated with this cadaver were excluded from further analyses due to the presence of large amounts of contamination. However, samples associated with cadavers' 36-12D and 50-12D exhibited evidence of mixture at single sites only, and given the fact that DNA from a number of tissue types is likely represented in the soil samples, heteroplasmy cannot be ruled out. Therefore, samples from cadaver 67-12D were excluded from qPCR analysis due to contamination, as mixtures were thought to be beyond the degree generally observed in heteroplasmic individuals. In contrast, samples from 36-12D and 50-12D underwent qPCR analysis.

If instead the single-base double peaks seen during sequence authentication were the result of contamination, mtDNA copy number for these samples would be inflated, as it would include both the source of the DNA contaminant and the DNA from the cadaver. Soil samples used in this project were collected primarily for use in another project assessing microbial changes during decomposition (i.e., Cobaugh, 2013). It is difficult to make assumptions regarding the precautions taken by other researchers during the collection process, and even with the proper precautions, contamination can be fairly ubiquitous and its origins ultimately unknown. It is imperative that all future studies take the necessary precautions to control for contamination during all steps of the research, from collection to sequencing. All supplies used for sample storage and manipulation as well as the surrounding environment need to be as sterile as possible; the use of a clean room laboratory is optimal for maintaining such an environment.

Future Directions

This study would benefit from a more established means of assessing overall DNA quality; if possible, the protocol from Alonso et al. (2004), which compares different amplicon sizes from the same region should be implemented. While it appears that DNA quality decreases as decomposition progresses, additional testing is needed to tease out patterns of DNA degradation. Additionally, exactly what is occurring as human DNA enters the soil is still unknown. Is the majority of this DNA extracellular or intracellular? This question is imperative to understanding how DNA persists, as it is likely that intracellular and extracellular DNA interact with the soil environment in different ways, which likely affects DNA survival in the extracellular environment.

Lastly, the method of extraction used can drastically affect downstream processes. The extraction protocol plays a large role in the outcome of recoverable DNA. Robe et al. (2003) noted that a high degree of variability currently exists concerning the amount of recoverable DNA extracted from the soil. DNA extraction is a critical component of nucleic acid research, and if conditions or methods are not ideal, all subsequent downstream processes can and likely will be affected. Taking this into consideration, it is recommended that any further extensions of this project test multiple extraction methodologies. The method of DNA extraction may be imperative to the recovery of nDNA from the soil. Additionally, as has been previously emphasized, care must be taken to control for contamination at all research steps.

Summary

This study looked at the preservation and persistence of human DNA in the soil during decomposition and assessed the potential of this DNA for use in human identification. Nuclear DNA was recovered in few soil samples in this study, and the pattern of recoverability remains unclear. MtDNA was present during all stages of decomposition, even after 198 days following cadaver placement in samples associated with cadaver 47-12D. Levels of mtDNA were highest during the Active Decay and Advanced Decay stages of decomposition, thus supporting Hypothesis 1. More information is needed to adequately address Hypothesis 2; however, mtDNA quality seemed to decrease as decomposition progressed.

Based on the outcome of this project, in the event that a cadaver was removed from its original location or DNA was not recoverable from a discovered set of remains, mtDNA could potentially be extracted from the soil and used for human identification purposes, especially to exclude potential matches. Though contamination from exogenous sources is a possibility, the cadaver profile may still be identifiable. Though originally thought to be useful in a mass graves context, the ease at which contaminants enter the soil and the difficulty of interpreting mixtures would cause problems in such a situation. However, the presence of large quantities of mtDNA, could still be used to indicate that a particular site did, at some point, contain human cadavers.

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Appendix

Table A1: Fluorospectrophotometer Results of Total dsDNA Prior to Conducting Repeat Silica Extractions

Sample ID	Concentration (ng/ μ L)	Avg. Concentration	RFU	Avg. Total DNA in a 100 μ L extract (ng)
36-1A	2.05	1.93	29.3	192.5
36-1A	1.8		27.3	
36-2A	3.54	3.39	41.3	339
36-2A	3.24		38.9	
36-4A	2.08	2.46	29.5	246
36-4A	2.84		35.7	
36-6A	4.43	4.2	48.6	419.5
36-6A	3.96		44.8	
36-6A	2.61	2.61	33.8	260.5
36-6A	2.6		33.7	
36-8A	5.04	4.88	53.5	488
36-8A	4.72		50.9	
36-12A	0.16	0.21	14	21
36-12A	0.26		14.8	
36-14A	1.27	1.34	22.9	133.5
36-14A	1.4		24	
36-14A	0.93	0.86	20.1	86
36-14A	0.79		19.1	
36-16A	0.24	0.36	14.6	35.5
36-16A	0.47		16.5	
36-23A	4.6	4.64	50	464
36-23A	4.68		50.6	
36-46A	17.92	18.28	157.9	1828
36-46A	18.64		163.7	
36-87A	0	0	8	0
36-87A	0		9.6	
47-A-1	2.19	2.15	30.4	214.5
47-A-1	2.1		29.7	
47-A-3	3.11	3	37.9	300
47-A-3	2.89		36.1	
47-4A	0.11	0.09	13.6	9
47-4A	0.07		13.2	
47-5A	2.34	2.42	31.6	242
47-5A	2.5		32.9	

Table A1: Continued.

Sample ID	Concentration (ng/ μ L)	Avg. Concentration	RFU	Avg. Total DNA in a 100 μ L extract (ng)
47-6A	1.28	1.2	23.1	120
47-6A	1.12		21.7	
47-7A	2.48	2.56	32.7	255.5
47-7A	2.63		33.9	
47-8A	1.05	1.14	21.2	113.5
47-8A	1.22		22.5	
47-9A	0.62	0.62	17.7	62
47-9A	0.62		17.7	
47-10A	1.97	2.25	28.6	225
47-10A	2.53		33.2	
47-13A	0.5	0.45	16.7	45
47-13A	0.4		15.9	
47-25A	3.16	3.13	38.3	312.5
47-25A	3.09		37.7	
47-48A	0.18	0.09	14.1	9
47-48A	0		11.4	
47-198	2.58	2.75	33.5	275
47-198	2.92		36.3	
50-1-A	0.48	0.24	16.6	24
50-1-A	0		12.5	
50-4A	1.13	1.1	21.8	110
50-4A	1.07		21.3	
50-6A	0	0	5.5	0
50-6A	0		7.7	
50-8-A	1.44	1.435	24.3	143.5
50-8-A	1.43		24.3	
50-10A	2.77	2.74	35.1	274
50-10A	2.71		34.6	
50-12C	1.63	1.55	25.9	155
50-12C	1.47		24.6	
50-12A	0	0	7.4	0
50-12A	0		8.5	
50-16A	0	0	7.9	0
50-16A	0		8	
50-28A	0	0	10.3	0

Table A1: Continued.

Sample ID	Concentration (ng/ μ L)	Avg. Concentration	RFU	Avg. Total DNA in a 100 μ L extract (ng)
50-39A	2.56	2.6	33.4	260
50-39A	2.64		34	
50-54	3.74	3.775	42.9	377.5
50-54	3.81		43.5	
67-1A	1.26	1.23	22.9	122.5
67-1A	1.19		22.3	
67-4A	1.96	1.89	28.5	189
67-4A	1.82		27.4	
67-6A	2.03	1.96	29.1	196
67-6A	1.89		28	
67-8A	2.28	2.33	31.2	233
67-8A	2.38		31.9	
67-10A	0	0.01	10.8	0.5
67-10A	0.01		12.8	
67-13A	0.79		19.1	
67-17A	0	0	7.8	0
67-17A	0		6.5	
67-29A	0.59	0.47	17.4	46.5
67-29A	0.34		15.4	
67-72A	0.77	0.94	18.9	93.5
67-72A	1.1		21.6	
67-114A	0	0	5.9	0
67-114A	0		4.4	

Table A2: Descriptive Statistics for the 113 bp target within the mtDNA Control Region

Sample	Avg. (Target copies / g per dry weight (gdw))	Std. Dev.	Avg. (log / gdw)	Std. Dev.
50-1	1384.69917	771.081	3.09789	0.235338
50-4	340457.7755	63545.5	5.528248	0.081535
50-5	235905.4878	12443.2	5.372327	0.023248
50-6	65464.33865	18435	4.802707	0.136238
50-8	66948.79781	10601	4.821844	0.072513

Table A2: Continued.

Sample	Avg. (Target copies / g per dry weight (gdw))	Std. Dev.	Avg. (log / gdw)	Std. Dev.
50-12C	41725.04487	7013.49	4.04643	0.168344
50-16	11715.11879	4703.26	2.804055	0.082728
50-28	644.409801	117.601	3.341467	0.180098
50-39	2325.772741	974.862	3.776319	0.083057
50-83	6048.380697	1164.91	4.616398	0.071766
36-1	634.0267783	312.178	2.768556	0.206172
36-2	1738.428263	817.676	3.211156	0.188556
36-4	20944.50207	5380.16	4.312058	0.106662
36-6	5622.203644	1273.38	3.742947	0.093568
36-6	6946.124236	2540.61	3.822164	0.16013
36-8	377971.4793	135690	5.55954	0.151073
36-12	2245463.128	587187	6.34182	0.109902
36-14	691864.4437	50169.7	5.839262	0.031434
36-14	572311.0462	80893.1	5.754871	0.059254
36-16	481456.4325	54522	5.680645	0.050279
36-23	272162.179	86763.1	5.421065	0.131442
36-46	55445.52642	4300.55	4.742983	0.034052
36-87	11995.91833	3394.47	4.065662	0.136495
47-1	495.9412024	75.9887	2.692866	0.066805
47-3	9693.354242	810.611	3.985487	0.035636
47-4	300130.4886	85517.1	5.463788	0.137189
47-5	223784.0657	15442.1	5.349134	0.030146
47-6	99338.39728	4497.66	4.996825	0.019428
47-7	489416.4633	35439.8	5.688909	0.031776
47-8	466850.4388	59520.3	5.666817	0.055497
47-9	179581.8183	25617	5.25125	0.063004
47-10	972432.939	187797	5.982513	0.083254
47-13	480889.8828	73153.8	5.678747	0.065301
47-25	582707.6261	58972.8	5.764013	0.042934
47-48	194935.4226	88756	5.261753	0.187803
47-198	59892.57059	13861.5	4.769652	0.100183

Vita

Alexandra Leah Emmons was born in Mount Holly, New Jersey on April 10, 1990 to parents Alan and Brenda Emmons. One of two children, she moved to Brunswick, Georgia following her parents separation in 1994, where she eventually graduated Salutatorian from Brunswick High School in 2008. Following graduation, Alexandra relocated to Athens, Georgia, where she began attendance at the University of Georgia. Under the guidance of Dr. Maria Teresa Tersigni-Tarrant, a prominent forensic anthropologist, she received a Bachelor of Arts in Anthropology and a Bachelor of Science in Biology. In the spring of 2012, she was formally accepted to the University of Tennessee to pursue a Master of Arts degree in Anthropology with a concentration in Biological Anthropology under the leadership of Dr. Graciela Cabana.