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AN EVALUATION OF THE EFFECTIVENESS OF EUKARYOTIC DNA EXTRACTION FROM BURIAL SOIL SAMPLES

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Abstract

DNA is a valuable resource as a unique identifier of an individual's dietary habits, sex, ancestry, and a variety of other characteristics. Due to a variety of factors that contribute to genetic decay, forensic and bioarchaeological investigators have limited outlets in which to extract viable DNA after most of the organic materials have fully decomposed. This preliminary research focused on extracting DNA from the soil surrounding buried *Sus scrofa domesticus* cadavers to confirm the presence of viable and analyzable DNA. After a decomposition period of five months in Montana, soils were collected at incremental distances above the remains and sequenced to identify endogenous DNA. The primers targeted a genetic template specific to *Sus scrofa,* and sequence fragments were confirmed by sequence submission to BLAST. The results of this study indicate basic temporal characteristics and leaching attributes of DNA preservation in soils surrounding burial sites with an interred cadaver. The conclusions of this study promote the collection of soil in crime scene investigation procedures, or catalyze research opportunities across biological and archaeological disciplines.

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INTRODUCTION

The application of DNA analyses in criminal investigations began with an English rape and murder case in 1983 (Dirkmaat 2012). Since then genetic analyses have become fundamental components of crime scene investigations across the globe. In the United States, forensic methods presented as evidence in court recently saw a revitalization in scientific integrity with the 1993 Supreme Court ruling presented in Daubert vs. Merrell Dow Pharmaceuticals. While DNA has proved to be an invaluable resource to identify a multitude of characteristics including dietary habits, sex, phylogenetic analysis, and population movement, this method of identification relies on uncontaminated that contain biological material (Dirkmaat 2012). Reliable biotic sources of human and animal DNA include bodily fluids, hair, coprolites, and tissue (Brown and Brown 2011). However, quantifiable DNA becomes increasingly difficult to extract during the final stages of decomposition when only bone and teeth remain. Thusly, researchers have broadened their scope and focused on other DNA extraction origins, including residual human or animal DNA in soils. With emerging technologies, even partially degraded and fragmented DNA sequences can be amplified and sequenced more accurately and at a faster rate than in previous years. When applied to soil, these analyses can yield information regarding the biodiversity of an area, and have the potential to be used at crime scenes or archaeological sites to gain information from decomposed human or animal remains (Anderson et al. 2012, Young et al. 2014).

The goal of DNA extraction in forensic and anthropological contexts is to extract viable DNA to make assessments about an individual or population. Due to extraneous factors resulting in DNA degradation, this research proposed a new resource for human and animal DNA recovery when other sources are not available. This research focused on the identification

of one specific individual in a soil sample. This research sought to match the genetic fingerprint, a method to identify individuals based on unique genetic differences at the nucleotide sequence level, of a buried organism to the DNA extracted from the soil surrounding the burial site using PCR methods. Using a 212 bp amplicon for eukaryotic mitochondrial DNA fragments, this research analyzed whether the DNA of a specific individual buried cadaver could be extracted from soil above the interred remains.

It was hypothesized that DNA from a buried organism would likely be present in soil samples up to 100mm away from the center of the burial, but no farther than 160mm away. Due to a long decomposition period and limited information on DNA leaching toward the surface, successful DNA extraction is unlikely at incremental distances farther than 100 mm.

Tissue and DNA Decomposition Processes

The biological processes associated with decomposition rate and severity are fundamental to understanding DNA decay and developing potential extraction methodologies from forensic sites. Decomposition, or postmortem changes to the corporeal organism, begins immediately after death (Dent et al. 2012). The earliest stage of decomposition, known as autolysis or "self-digestion", is characterized by the release of carbohydrate, protein, and fat breakdown products initiated by the dead organism's hydrolytic enzymes (Burns 2013, Dent et al. 2003). Autolysis of the cell membranes allows for DNA leakage into the environment (Bogas et al. 2009). The second stage, putrefaction, is primarily associated with the breakdown of soft tissue, proteins, carbohydrates, and fats by bacteria and enzymes produced by the decaying remains or microorganisms present in the soil (Dent et al. 2003). Proteolysis, the act of enzymes breaking down proteins, occurs inconsistently (Dent et al. 2003). Proteins of the neuronal and epithelial

tissues are often the first to be destroyed during decomposition, while the epidermis, collagen, and muscle proteins are more resistant to decay (Dent et al. 2003). These two stages are responsible for a majority of the loss of genetic material during decomposition, but the exact amount is unknown.

At the cellular level, death in vascular animals, including humans, is marked by the cessation of blood perfusion to tissues, resulting in deprivation of oxygen and abrupt reduction in adenosine triphosphate (ATP) (Alaeddini et al. 2009). All cells are negatively affected by the loss of ATP production, but differentiated tissues requiring higher oxygen levels will experience earlier, more profound effects (Alaeddini et al. 2009). Two effects that are determined by the level of ATP present in the tissue are apoptosis and necrosis, or cell death (Alaeddini et al. 2009). Apoptosis occurs when cells are irreversibly damaged or have reached the end of their lifespan (Campbell et al. 2008). Loss of microvilli and nucleic separation are characteristics of apoptosis, and, in living organisms, other cells digest the remaining cell debris (Alaeddini et al. 2009, Campbell et al. 2008). The activation of endogenous endonucleases degrade chromosomal DNA into oligomers approximately 180 bp long, depending on time since death (Alaeddini et al. 2009).

By contrast, necrosis exhibits an increase in cell volume, cytoplasmic organelle swelling, and erosion of chromatins, with ultimate rupture of the cell membranes. This leads to the release of nutritious fluids, which are sought after and metabolized by environmental microorganisms, resulting in further degradation of the dead organism's macromolecules.

During the post-mortem period, the first agents to initiate DNA cleavage are exogenous nucleases released by microorganisms and invertebrates in the surrounding environment (Alaeddini et al. 2009). Over 70% of soil microorganisms contain nuclease enzymes, and under

certain conditions, nuclease enzymes may deactivate before completely breaking down strands of DNA (Alaeddini et al. 2009). Environmental conditions impact the rate at which microbial soil communities decompose organic substances, but little information is available regarding the specific microorganisms in soil that participate in the breakdown of lipid membranes or their role in DNA movement and destruction (Dent et al. 2003, Pezzolla et al. 2014). A better understanding of the molecular processes involved in organismal decomposition may assist researchers studying DNA persistence and recoverability in soil.

The Role of Soil and Environmental Influences on Decomposition

Soil types vary widely and are subject to compositional changes at varying depths and distances (Murray and Tedrow 1992). The dynamic structure of soil harbors prokaryotic and eukaryotic microorganisms (Pezzolla et al. 2014, Skelton 2011). The distribution and profusion of soil microbes is correlated to a complex suite of variables including soil structure and texture, soil pH, and organic carbon content (Dequiedt et al. 2011, Pezzolla et al. 2014). A study by Dequiedt et al. (2011) found that physicochemical characteristics such as soil texture, carbon, and low pH, and land use have the largest effect on the soil microbial biomass. Soil microorganisms use labile and recalcitrant organic compounds as growth substrates, and thus function to degrade organic matter and mineralize nutrients in soil (Pezzolla et al. 2014, Tzeneva et al. 2008). Soil microbial communities contain specific species that metabolize such substrates to promote bacterial turnover, further facilitating these processes (Pezzolla et al. 2014).

Surface changes to shallow of human/animal burial sites have a high probability of occurring over time (Dupras et al. 2012). Such changes include soil depressions over the body, variation in plant growth, and variation in plant composition (Dupras et al. 2012). The intermixing of soil layers during burial alters the levels and distribution of organic nutrients commonly found near

the surface (Dupras et al. 2012). However, little is known about the behaviors of microbial ecosystems since roughly 99% of microbial diversity has not been cultured and their functions are unknown (Sharma et al. 2014, Tzeneva et al. 2008). Our incomplete knowledge of microbial community activities, population densities, and life histories restricts our understanding of the ecology and organisms that participate in the destruction of DNA during decomposition (Sharma et al. 2014).

Numerous other environmental factors contribute to DNA preservation in post-mortem tissues (Katzenberg and Saunders 2008, Schwarz et al. 2009). One such factor that promotes polynucleotide (DNA) breakdown is water content. Water availability supports bacterial growth and weakens the bond that links the base and the nucleotide within the individual strands of the DNA helix (Alaeddini et al. 2009, Brown and Brown 2011). Adenine and guanine residues are almost 20 times more susceptible to polynucleotide breakage than cytosine and thymine residues by a process known as depurination (Brown and Brown 2011, Hansen et al. 2006). Depurination is the severing of the bond connecting a purine base, adenine or guanine, and its corresponding deoxyribose sugar at the glycosidic linkage site (Campbell et al. 2008). Following depurination, cleavage of the phosphodiester backbone occurs more readily and may be the primary chemical reaction responsible for obstructing DNA amplification by polymerase chain reaction (PCR) (Hansen et al. 2006). Other research suggests crosslinking as a factor in DNA diagenesis (Hansen et al. 2006). This creates missing sections in the DNA strand, but methods to fill in such gaps have proved unreliable (Brown and Brown 2011).

There is evidence that the local environmental conditions have a more significant role in DNA preservation than other causes, including time (Alaeddini et al. 2009, Burger et al. 1999). Logic predicts a positive correlation between time and DNA damage, but studies of

fossil remains in the Siberian permafrost have yet to confirm this notion, as researchers have extracted usable DNA from 400,000-year old specimens (Anderson et al. 2012, Hansen et al. 2006). Overall, constant cold and arid environments with permafrost and neutral or slightly alkaline soil pH are optimal conditions for specimen preservation. By contrast, hot, humid, and acidic climates have the worst rates of tissue and DNA preservation (Hansen et al. 2006, Katzenberg and Saunders 2008, Mulligan 2006). Cooler temperatures indeed have a profound effect: a 20˚C decrease in temperature may generate up to a 25-fold reduction in the rate of DNA decomposition (Alaeddini et al. 2009). Rising temperatures lead to the increased activity of DNases, which decreases the half-life time of DNA, which has been calculated to be about 521 years dependent on local conditions (Allentoft et al. 2012, Bogas et al. 2009).

Humic acids and fulvic acids are among the chief components of soils formed as a result of plant and animal decay (Kasu and Shires 2015). Fulvic acids are found in forest environments and do not greatly inhibit PCR (Kasu and Shires 2015). Humic substances are also common constituents in organic soils or waters, resulting lower quality and lower purity of recovered environmental DNA in addition to inhibition of PCR (Kasu and Shires 2015, Sutlović et al. 2005). The concentration of humic acids range from 2 mg/g of soil in arid environments to as high as 25 mg/g in fertilized or organic rich soils (Kasu and Shires 2015). The chemical properties of humic acids are similar to those of double-stranded DNA and are known to inhibit DNA extraction by binding to silica matrices used in DNA recovery protocols, or to the DNA molecules themselves (Kasu and Shires 2015).

Macro soil analysis is very important for crime scene investigation, as it may contain evidence of pollen, fibers and other trace and transfer evidence with the potential to link suspects and victims to certain sites (Skelton 2011, Young et al. 2014, Young et al. 2015). Research

conducted by Bogas et al. (2009) compared DNA preservation of buried blood-stained fabrics in clay, sand, and marsh soils. The marsh soils exhibited the greatest degree of DNA degradation, while sandy environments resulting in the lowest extent of DNA degradation (Bogas et al. 2009).

DNA extraction procedures can be unpredictable and successful recovery is limited by the extent of postmortem decay and various other environmental influences. Even within the same burial or excavation site, differential extraction efficiencies can produce varying degrees of success (Katzenberg and Saunders 2008). It is rarely possible to obtain DNA fragments longer than 300 bp at most ancient sites (Brown and Brown 2011). To combat this issue, technological sensitivity for yielding short DNA products increased with the development of PCR (Alaeddini et al. 2009, Dirkmaat 2012, Mulligan 2006). PCR enables the amplification of a specific region in the genome, producing as many as one billion copies per DNA template (Jones 2010, Mulligan 2006, Schwarz et al. 2009).

PCR has proved invaluable for exploring specific genetic markers in detail especially with damaged or ancient samples (Alaeddini et al. 2009, Mulligan 2006, Schwarz et al. 2009). In addition to animal and human DNA in sediments, this method can apply to pollen-based taxa relevant to vegetative reproduction, productivity, and dispersal for ancient, historic, and forensic applications (Willerslev et al. 2003). Overall, specific detection of organisms of interest from environmental soil samples often relies on PCR analysis (Fitzpatrick et al. 2010).

Despite the implementation of various research facilities throughout the United States that focus on the geographically-influenced taphonomic variables involved in human decomposition, commonly referred to as body farms, there is little research designated to DNA decay or the influence of the activity of microorganisms on DNA recovery. To combat this scarcity in research, the current project investigates the distance to which pig DNA leaches from the

decomposing body into soil at a burial site. For this project, soil samples were taken at various depth intervals above the buried carcass. By evaluating the distance to which DNA from a static organism leaches toward the surface, the results of this exploratory study may suggest a sampling strategy for DNA collection of decomposing remains. The findings provide preliminary data to inform further studies relating to soil microbial communities, and to establish a baseline for temporal longevity as well as the travel patterns of DNA in relevant environments, which is beneficial to forensic and archaeological examinations involving buried individuals.

METHODOLOGY

As a substitute for a human specimen, two sections of a hind leg of *Sus scrofa domesticus* (domesticated pig) were used in this study. Lolo Meat Locker prepared and cut the pig cadaver in accordance to FDA regulations before sale. Both portions of the hind leg were frozen and wrapped in plastic before placement in a cooler during the approximately 2-hr transit to the site.

The burial site selected for this project was located at the Lubrecht Experimental Forest, which contains 8,498 hectares of land managed by the University of Montana College of Forestry and Conservation. The State of Montana Department of Natural Resources manages the remaining 2,832 hectares (www.umt.edu). This research site was approximately 48 kilometers northeast of Missoula, MT in Greenough, MT.

The burial site was directly across from the main entrance to Lubrecht Forest on Montana Highway 200 East. The area was completely surrounded by a barbed wire fence with a locked metal gate to prevent cows from escaping. The fence was locked at all times, and only employees and researchers using the site have access to the gate code. The location of the burial site within Lubrecht Forest was situated in the Northwest corner of the Southwest corner in

Section II (T13 North and R15 West) at 46˚53’34”N, 113˚28’03”W. The area featured a dense, wooded environment with predominantly loamy and mixed Typic Eutroboralfs (www.nrcs.usda.gov).

To ensure DNA leaching, the two cadaver portions were placed in pits in the early afternoon on April 17, 2015. The high temperature for that day was 19 degrees Celsius (67 degrees Fahrenheit) with an overnight low of -0.5˚C (31˚F). The portions of the pig were buried at two separate locations, approximately 3 meters apart. The two burials were oriented in the same direction, the length of the burial was north to south and the width of the burial placed west to east. The orientation of both burials can be found in Figure 1.1. Both sites were completely enclosed by a wire cage for protection beneath the surface to prevent contamination and wildlife scavenging. The cages were 610 mm long by 445 mm across, and 508 mm high. The pits were dug manually using a shovel to accommodate the cages, with a small portion of the cage exposed to the elements. Each burial was adjusted for an average depth of 250 mm by adding the disturbed soil above the base of the cage before the carcass portion placement. This required adding dirt to the bottom of the pit, covering the bottom of the cage to create a more natural burial scenario. Each portion was placed into the middle of the pit in a similar manner: caudal side inferior to the burial and cranial side superior. The burials were covered with soil until they were level with the surrounding undisturbed soil, and enclosed in the cage.

On September 9, 2015, more than 20 weeks after the initial placement, the burials were measured again to account for soil impaction over the summer months. Each burial had a width of approximately 425 mm and was approximately 580 mm in length. Using these measurements as a baseline, the middle of the burial was calculated at 215 mm. The top layer of soil, including new plant growth, was removed from each burial site using hand tools cleaned with bleach. A

trowel was used to skim the surface layer, with the blade moving north to south to avoid transfer of soils.

Each soil sample from each burial site was approximately 10cm in depth and was stored in a small plastic container that had been cleaned with a 10% bleach solution beforehand to prevent cross-contamination of soils collected at different depth intervals. Two soil samples were collected in triplicate at 2 cm increments and twice at 5 cm increments starting at 215 mm. The samples were collected toward the east using two margin trowels cleaned with a 10% bleach solution after every sample. The flattened portion of the margin trowels were placed into the soil at each increment, both blades were pressed together to trap the soil, and then transfer the sample to a labelled plastic container. For each burial there were a total of thirteen samples including a control sample collected 160 mm south of each burial. It was expected that the two control samples would not contain any pig DNA, thus providing a reference point to the maximum distance DNA leaches into soil. All samples remained in breathable containers cleaned with bleach before analysis to inhibit condensation and heat buildup, which commonly result in further DNA degradation. The samples were kept at -20˚C in a freezer located in a locked laboratory before DNA extraction. Additional soil samples could not be collected due to the removal of burial soil by scavenging wildlife.

DNA Extraction from Tissue Samples

DNA extraction of a processed sample of pig muscle was conducted in triplicate using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). The pig muscle samples were used as controls to test the sensitivity of the primers. The kit contained enough material for 10 preps, including binding buffer, digestion buffer, wash buffers 1 and 2, an elution buffer, RNase A, proteinase K, spin columns, and collection tubes. The protocol provided by Invitrogen was

followed without deviation. A dry bath was heated to 55˚C, and triplicate 20 mg samples of pig muscle tissue were placed into three individual microcentrifuge tubes. The samples were treated with 180 µl of the digestion buffer and 20 µl of proteinase K, ensuring that the tissue was completely submerged in the liquids. With occasional vortexing, the samples were incubated overnight to complete cell lysis.

The samples were then centrifuged for 3 min at room temperature before the supernatant was transferred to a new tube. Twenty µl of RNase A solution (in 50 mM Tris-HCl, 10 mM EDTA) was added to the lysates, vortexed, and incubated for 2 min. After the incubation period, 200 µl of the lysis/binding buffer was mixed with each sample, after which 200 µl of 98% ethanol solution was added and vortexed. Next, 600 µl of each lysate was added to an individual spin column and collection tube, then centrifuged at 10,000 x g for 1 min at room temperature. The spin column was next placed into a clean collection tube before 500 µl of wash buffer was added and centrifuged for 1 min. The spin column was then placed into a new collection tube and 500 µl of the second wash buffer was added before centrifuging for 3 min. Finally, the spin column was placed into a clean 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added and incubated for 1 min. After centrifugation for 1 min, another 50 µl of elution buffer was added to each column and tube centrifuged for 1.5 min, which produced approximately 100 µl of purified genomic DNA. The purified tissue DNA samples were quantified three times each using the Qubit 2.0 by Invitrogen, and the average of the three values was used for further dilution calculations for the PCR template.

Table 1.1 Quantification of Pig Tissue Samples

Quantification	Pig Tissue 1 (ug/ µl)	Pig Tissue 2 (ug/ µl)	Pig Tissue 3 (ug/ µl)
1	103	60.8	63.9
2	101	59.9	60.9
3	99.3	60.1	60.7
Average	101.1	60.3	61.8

The concentrations of all 3 pig tissue DNA samples were diluted with emulsion buffer to 15 ng/µl in a total volume of 50 µl. The first dilution is composed of 7.418 µl of stock and 42.582 µl of emulsion buffer. The second pig tissue sample contained 12.44 µl of stock and 37.56 µl of buffer. The third tissue sample had a final concentration as the second tissue sample with 12.14 µl of stock and 37.86 µl of buffer.

DNA Extraction from Soil Samples

All containers and tools were cleaned with a 10% bleach solution and exposed to UV light for about 30 seconds at 300 nm. Gloves were worn at all times when handling soil and extracted DNA samples due to the sensitivity of PCR and the possibility of contamination by human cells. The soil samples were purified using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA). The kit contains 50 PowerBead Tubes, PowerSoil™ Solution C1, C2, C3, C4, C5, and C6. Also included are PowerSoil™ Spin filters and 200 Collection Tubes.

The manufacturer's recommended procedure was employed for DNA extraction and amplification as detailed in the PowerSoil® DNA Isolation Protocol from MO BIO Laboratories and detailed in Supplementary Data. Using 0.25 grams of soil, the sample was vortexed in a

PowerBead Tube provided in the kit. The PowerBead Tubes contain garnet beads and a guanidine thiocyanate buffer that will disperse the soil and dissolve humic acids while preventing DNA degradation. Sixty microliters of solution C1, which contains anionic detergents that break down fatty acids and lipids integral to organismal cell membranes was added to each PowerBead Tube mixture. The samples were vortexed for approximately 10 minutes to ensure that homogenization and cell lysis were complete. After centrifuging at 10,000 x *g* for 30 sec at room temperature, 500 µl of supernatant was transferred to a new 2 ml collection tube. The sample was treated with 250 µl of a aqueous lysis reagent to remove organic and inorganic substances, incubated at 4˚C for 5 min, then centrifuged at 10,000 x *g* for 30 sec at room temperature Six hundred µl of supernatant was transferred to clean 2 ml collection tubes, 200 µl of a second inhibitor removal solution was added to continue the breakdown of non-DNA contaminating materials found in soils and sediments. After the 5 min incubation period at 4˚C and centrifugation, 750 µl of the supernatant was added to a new tube. A small amount, 1.2 ml, of a salt solution was added to encourage DNA binding to the silica matrix of the purification column. For each sample, 675 µl of the supernatant was loaded onto a spin filter, centrifuged for 1 min, and the flow through discarded three times to process the entire volume of each sample through its individual column. The repeated process confirmed that as much DNA was bound to the membrane as possible. An ethanol-based wash was added to remove residual salt and contaminants before centrifugation for 30 seconds at 10,000 x *g*. The wash flow-through was discarded and the spin filter was carefully transferred to a new 2 ml microcentrifuge tube, after which 100µl of sterile elution buffer was added to the filter membrane of the spin filter and the samples were centrifuged for 30 seconds at 10,000 x *g*. The

spin filters were then discarded and the tubes of purified DNA were stored at -20˚C prior to further analysis.

PCR Protocol

A preliminary agarose gel was run to confirm DNA presence in all of the pig tissue and soil samples taken from the burial sites. Following that gel analysis, a PCR protocol was conducted to test the ability of the selected primers to produce amplicons of the expected size. Both the pig tissue and soil DNA samples were subjected to the same PCR protocol: 15.72 µl of H_2O, 0.4 µl dNTP (from a stock solution of 2.5 mM for each of the 4 deoxynucleotides), 0.40 µl Forward Primer with Tm (1M Na+) 82°C and Tm (50mM Na+) 60°C (0.40 µl Reverse Primer with Tm (1M Na+) 67°C and Tm (50mM Na+) 46°C, 2.0 µl of 10X PCR $MgCl_2$ Buffer,1.0 µl $MgCl_2$ (from a 50mM stock), and 0.08 µl of Taq (from a 2 U/rxn stock).

Initially, a different set of primers taken from Campana et al. (2014) were used to amplify a 254 bp sequence of mitochondrial pig DNA. The forward primer was 5'TGG ATG CCC AAG AAG TAG AAA-3' and the reverse primer was 5'ATT GTC TAC TTC TAG TAG TCG-3' (Campana et al. 2014). After the PCR, but prior to the cleanup phase of the project, the agarose gel showed clear bands of the predicted size from all of the samples. This suggested, but did not confirm, that the *Sus scrofa* DNA manifested in all 26 samples. These samples were submitted to the University of Montana Genomics Core for sequencing. All of the samples failed to sequence with that set of primers, and I did not recover any confirmatory DNA sequences. Due to the conflicting results, genomic DNA and high-sensitivity ScreenTapes by Agilent, a highly precise and digitized version of agarose gels, were run for the samples to confirm the original results. The results obtained for the genomic DNA samples and high sensitivity DNA ScreenTapes for both sample types contradicted the gel electrophoreses of the post-PCR product.

ScreenTapes are a more reliable alternative to standard gel electrophoresis. The high sensitivity ScreenTape size range includes bp lengths from 35 to 1000. The upper and lower markers were used as references to establish the average molecular weight of the genomic DNA samples. The results, shown in Figures 2.2 and 2.3, did not reveal any DNA between the 200 and 300 bp mark, indicating that the *Sus scrofa* primers did not produce the desired 254 bp product. Various alterations to the PCR protocol, agarose gel runs, and cleanup protocols suggested that the original primers would not anneal to the sample properly due to their wide melting temperature (Tm) difference (over 20˚C), probably explaining their failure to amplify.

For this reason, the primers described by Pangallo et al. (2010) were used for the final analysis. The Tm difference for the second set of primers were still out of range according to authors' description, but the difference was less substantial and they ultimately were successful.

The second set of primers, CO2susF2 (5' GCCTAAATCTCCCCTCAATGGTA -3') and CO2susR2 (5'AGAAAGAGGCAAATAGATTTTCG-3'), yielded a 212 bp fragment of mitochondrial DNA from *Sus scrofa domesticus* (Pangallo et al. 2010). The annealing temperature of these primers was calculated at 47.1˚C per 60 cycles, but the temperature was raised to 65˚C since the original calculation temperature was too low a temperature to avoid non-specific binding, which would enable the primers to bind at other sites of the template not associated with the specific 212 bp pig sequence. This would result in a false positive or generate no products at all.

After the gel and PCR phase, 5 µl of PCR sample aliquots were treated with 2 µl of ExoSAP-IT. The mixture was incubated at 37˚C for 15 minutes to remove free primers and excess nucleotides, followed by incubation at 80˚C for 15 minutes to deactivate the ExoSAP-IT. To verify the existence of *Sus scrofa* DNA, an agarose gel was run for all 26 samples. If the gel

yielded faint bands, those samples were run a second time with a greater quantity of PCR product to determine the presence of adequate DNA for sequencing. All samples produced the predicted 212 bp bands in the gel when viewed under UV light when compared with the ladder size markers. The samples were incubated at -20˚C for long-term storage.

The soil samples were purified and amplified again before being placed on the 3130x Genetic Analyzer by Applied Biosystems (Foster City, CA) for DNA sequence analysis. Using a 96-well plate, 3 µl of template and 1.1 µl of primer was added to each well. This protocol used a master mix that included 9.45 µl of molecular grade H_20, 1.65 µl of buffer, and 0.65 of Big Dye per 1 sample as indicated in Protocol 3.2. The ingredients were added to the Master Mix in the order shown in Protocol 2.2. The initial sequencing sample included two external burial samples and two samples for each increment away from the burial. The total sample size was n = 26. Each ingredient of the Master Mix was multiplied by 53, mixed, then 12 µl of the Master Mix was added to each well.

The Agilent Genomic DNA ScreenTapes were conducted at the University of Montana Genomics Core for three of the *Sus scrofa* tissue samples prior to PCR. All ScreenTapes were run without deviation from the manufacturer's specifications reported by Agilent Technologies.

All of the DNA sequences obtained were uploaded to Sequencher 5.4 for editing. The primers and ambiguous base pairs were removed from the sequences. The primers were identified within the sequences and also removed in a similar manner. The forward and reverse sequences of each of the three pig samples were combined using the contig command in Sequencher 5.4. All of the finalized sequences were run through Basic Local Alignment Search Tool (BLAST), registered to the U.S. National Library of Medicine and National Center for Biotechnology Information. The nucleotide database was used to search for the sample

nucleotide queries using algorithms that correspond to a sequence based on nearest nucleotide matches.

RESULTS

The preliminary data for the two external burial samples confirmed the presence of total DNA after completion of agarose gel electrophoresis. These samples had not yet been analyzed with PCR, and while the origin of the DNA was unknown it likely belonged to the soil's microbial community. The results of the DNA Screentape analysis using Campana's et al. (2014) primers are shown in Figures 2.2 and 2.3 and the ladder depicted in Figure 2.1. The ScreenTapes, similar to the gel electrophoresis, contained plenty of total soil DNA in both samples with peaks reaching over 19,000 bp. These results confirmed the presence of DNA from these samples. For comparison, Figures 2.4 and 2.5 show the genomic ScreenTape of prePCR pig tissue samples. The latter figure is similar to the postPCR soil ScreenTape, suggesting that Campana's (2004) primers did not anneal or amplify. The agarose gels of the new primers manifested slight bands in all wells, as shown in Figure 3.1. Sanger sequencing revealed clean pig sequences in the tissue samples as depicted in Figure 4.1. Due to the unambiguousness of the samples in BLAST and a strong overlap of the fragments in the same region, only the sequences obtained from the forward sequencing primers were analyzed for the soil samples. All soil samples, including the intended negative control samples, which were collected 160 mm south of each burial, yielded viable sequences. All of the edited sequences queried 100% for pig DNA in BLAST.

DISCUSSION

Variable temperatures and the passage of time creates difficult conditions that inhibit DNA extraction from surrounding soils. This research serves as an introduction to the behavior of microorganisms in the context of buried human/animal remains in northeastern Montana. The analyses described herein provide information regarding the migration of organismal DNA from the remains in burial soil, and the sensitive capacity of Sanger sequencing and optimized PCR to establish the presence of the remains by examining adjacent soil samples. The results suggest that the soil surrounding a buried organism may potentially maintain viable DNA beyond five of the spring and summer months in northeastern Montana if the cadaver remains interred.

With the organism still interred, samples were taken at measurements east of the cage, or burial border, beginning at 215 mm. The remaining soil samples were taken from the following measurements: 235 mm, 255 mm, 275 mm, 315 mm, and 375 mm. The samples were taken above the buried individual, after the surface layer had been removed by skimming with a trowel. All of the samples yielded *Sus scrofa* DNA, which suggests that DNA migrates radially from the origin during decomposition. The microorganisms that facilitate decomposition in Montana's sylvan environments leach DNA away from the body, but also above the body. This project did not confirm the distance at which DNA is no longer present, nor did it determine whether similar results can be found in a burial site in which the body has been removed, but it seems likely that strands of DNA will remain. In forensic contexts, collection of soil around a burial is a suggested procedure, especially in individuals that have been buried for less than six months when much of the genetic material is present.

In this specific instance, the sample organism was treated with common butchering techniques involving removal of the outer layers of skin and hair, and draining the blood. These

practices leave less genetic material to the environment. The pig sample remained frozen until the day of burial, but it is unclear as to how much DNA remains in animals processed for consumption. One hind leg of a domesticated pig was placed in each of two burial pits, further limiting the amount of DNA originally present. However, musculoskeletal cells retain DNA longer during decomposition than other physiological elements, which may contribute to the longevity of DNA in this study (Alaeddini et al. 2009). Due to the varying DNA decomposition rates, it is possible that a complete body is subjected to different leaching patterns. The removal of the outer layer of skins, hairs, and other vital tissues may also have impacted the rate of DNA leaching in the soil or the preservation of DNA.

To examine the effects of extreme temperature changes on DNA survival, the sample was buried during the spring, and samples collected in late summer. Temperatures during the decomposition period ranged from a high of 39˚C (103˚F) to a low of -1.6˚C (29˚F). Studies have found that high temperatures accelerate physiological and genetic decomposition, and thus the summer time frame is the most natural form of acceleration. Even with high temperatures and rain, the DNA remained stable. The limitations of this research did not compare seasons in Montana with DNA viability, but this study confirms that DNA from remains persists in soil after exposure to the summer months, the most hazardous of all the seasons to genetic material.

New waves of genetic technology remain expensive and require specific resources. This project incorporated simplified DNA extraction kits and focused on Sanger sequencing methods. These materials proved to be sufficient for DNA extraction and identification in this sample. The DNA extraction from soil kit yielded a high quantity of DNA, but not necessarily of high quality, and the pig primers amplified a species-specific region as opposed to an individual one. Accurate identification of an individual on a genetic basis would require more specific methods,

especially in forensic investigations involving multiple victims including mass disasters. The methodologies used in this study will benefit institutions with limited resources since this study utilized less expensive sequencing methods and materials.

The implications of the PCR results yielding *Sus scrofa* DNA in both burials are vast and will have uses in both archaeological and forensic contexts. Depending on the location and preservation of DNA, ancient burial soils may have traces of DNA from the buried individual as well as DNA from other organisms. This will prove useful for identification of individuals in mass graves. It can also serve as a method of identifying kinship relationships, which has the potential to describe mortuary practices within families and societies. Evidence of animal eukaryotic DNA at a specific site will also manifest butchering or sacrificial habits of ancient peoples. DNA from soil can provide insight on practices and lives of historic peoples. This method serves as a less invasive alternative to other DNA extraction methods taken directly from biological material, and can be a universal source of DNA that is protected from many of factors that facilitate its decomposition in tissue.

Limitations

This project is subject to certain limitations. One major limitation is the use of *Sus scrofa domesticus* in place of a *Homo sapiens* subject. While this organism is the most anatomically similar to humans, many differences remain. This paper strongly assumes that DNA degradation in *Sus scrofa domesticus* behaves similarly to *Homo sapiens*. Another limitation is the sample size of the project. This research used different leg sections from one individual of *Sus scrofa domesticus*, and each portion was buried in a different location within a 7.62 meter radius. Burials elsewhere in the same environment may yield varying degrees of viable DNA.

During forensic investigations, DNA extractions from soil should be conducted within 24 hours of initial collection (Young et al. 2014). Microorganisms can grow in collected soil samples and fungal species can survive longer in colder temperatures, indicating that refrigeration may not be a storage option unless frozen at -80˚C (Young et al. 2014).

In the course of this project, DNA extractions took place on Sept. 21, 2015, about 12 days after the soil collection. The samples were stored at -20˚C before extraction without any protein inhibitors. They were stored at a low temperature to slow the growth of microorganisms that degrade DNA. However, it is possible that the growth of some fungal species may have impacted the samples and further destroyed *Sus scrofa* DNA. However, if fungal species participated in genetic decay while the soil was undisturbed, the pig DNA would have been difficult to amplify.

At the time this project was carried out, other research projects were in progress in the same area. While most projects are conducted through the College of Forestry, it is unclear as to the exact nature of the projects. These studies represent an unknown factor that potentially impacted the biodiversity of the terrain. Land-use changes have effects on soil carbon, soil texture, and pH (Lauber et al. 2008). Many areas of Western Montana are subjected to controlled fires that may influence transformations in the soil bacteria and fungi, possibly resulting in different rates of DNA preservation. While no such study occurred in the vicinity or the time period of this project, it is possible that previous fire-related projects have occurred in the area and resulted in soil composition disruption. Also, a number of cattle roam the portion of land where this project took place. Little information is available on the influence of organic materials derived from livestock on microbial communities (Pezzolla et al. 2014). This may also impact the rate of DNA degradation as opposed to terrestrial landscapes without cattle.

Future Research

There are many potential options for future research that utilize chemical, geological, genetic, and biological processes to determine DNA leaching and preservation. Differing locations, seasons, temperatures, soil types, amount of humic acid, and weather conditions may also have an impact on DNA preservation in soils. Each of these factors should be investigated for a variety of environments to provide a baseline of the rates of DNA decay and degradation similar to the research conducted for taphonomic processes of organismal decay. Also, burial sites at which the body has been removed for a period of time may yield interesting results that differ from standard burials. While some studies have investigated the biochemical processes surrounding differing burial strategies, DNA degradation rates and factors impacting them were not included in those prior reports.

Studies regarding the microbial diversity of varying environments may reveal additional microorganisms and ecological processes that inhibit or assist DNA preservation. An in-depth analysis of the microorganisms in at a particular location is required for understanding body decomposition and DNA decomposition as well. High-throughput sequencing will facilitate determination of chemical and mineral composition to guide DNA extraction protocols (Young et al. 2014). A year-long study analyzed the soil bacteria from five habitats, which showed monthly fluctuations in bacterial demography (Young et al. 2014). This indicates the dynamic feature of soils, which may have varying influences on DNA decay. An analysis of seasonal soils may manifest higher or lower yields in DNA preservation. Forensic research has begun to incorporate Microbial Community Profiling to identify various microbial species using DNA fragment length differentiation (Young et al. 2014). However, limited research incorporates non-microbial DNA in soil samples. The relatively new techniques used for DNA extraction

from soil are not yet universally used. The contaminants contained in soil, in combination with limited resources, results in the exclusion of soil samples during crime scene collection (Kasu and Shires 2015). These advancements are in their infancy and will require future research and investigation to identify the microbial organisms involved in tissue decay and DNA decomposition.

Other potential research topics include temporal factors. This specifically pertains to archaeological sites and excavations. Eukaryotic DNA may degrade over time, making extraction from soil more difficult and less reliable as a source at older sites but there are contradictory studies. There are multiple research possibilities that will test the extent and limitations of DNA extracted from soils.

CONCLUSIONS

The results of the study confirm that eukaryotic DNA extracted from soil will be available for analysis of buried victims and suspects in forensic settings. This can be applied to forensic cases in which the body has been moved or connected to a secondary burial. It serves as a way to connect victims of violence or mass fatalities to their relatives. In terms of this specific research, the extraction of eukaryotic DNA from Montana soil is also important. The results of this study indicate that the soil from Montana preserves a limited source of DNA from a multicellular organism after remaining in the soil for five of the summer months. Using DNA extracted from soil successfully during PCR analyses will encourage further uses due to the inexpensiveness of the PCR runs as opposed to other methods that involve next generation sequencing. The complex ecosystem of soils and its impact on DNA preservation necessitates further research for future applications in archaeological and forensic circumstances.

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Conflict of Interest: None declared.

APPENDIX

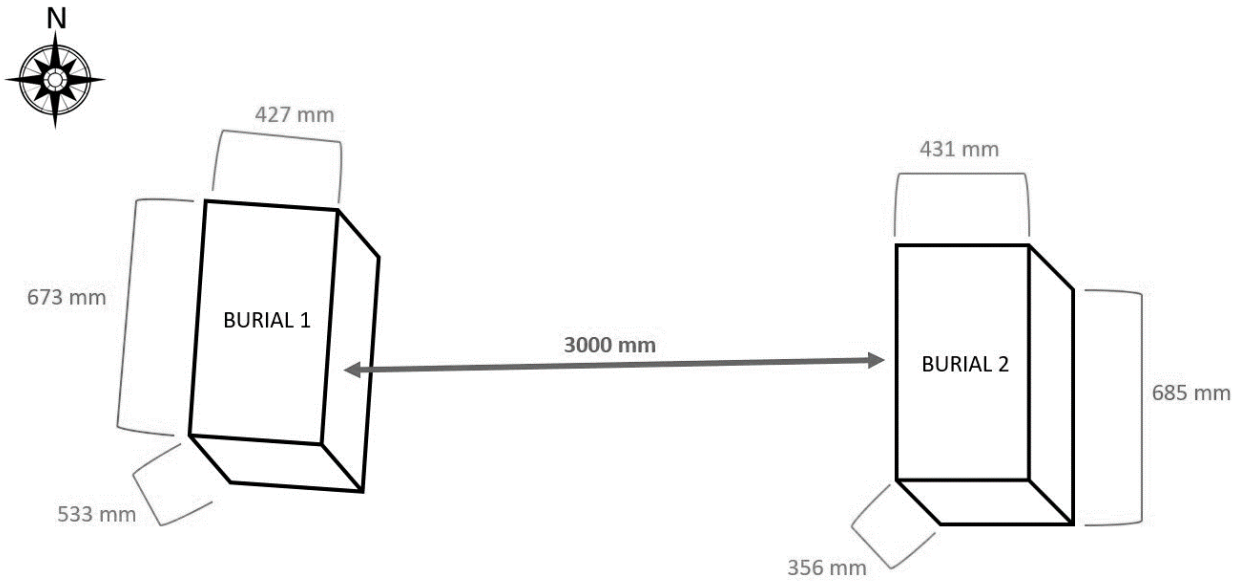

Figure 1.1 Diagram of the orientation and direction of both burials. Samples were taken from west to east, with the first sample collected at the halfway measurement of the length (215 mm) and the final sample was taken at the 375 mm measurement. The control samples were taken 160 mm south of the southern border of the burial.

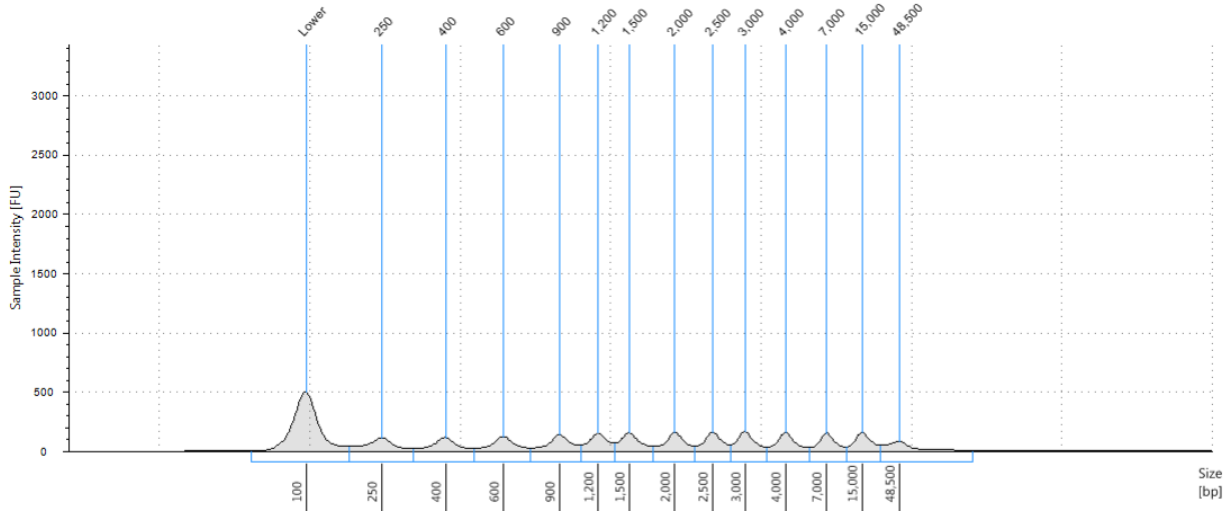

Figure 2.1 Ladder of genomic ScreenTape for postPCR soil samples from both burials.

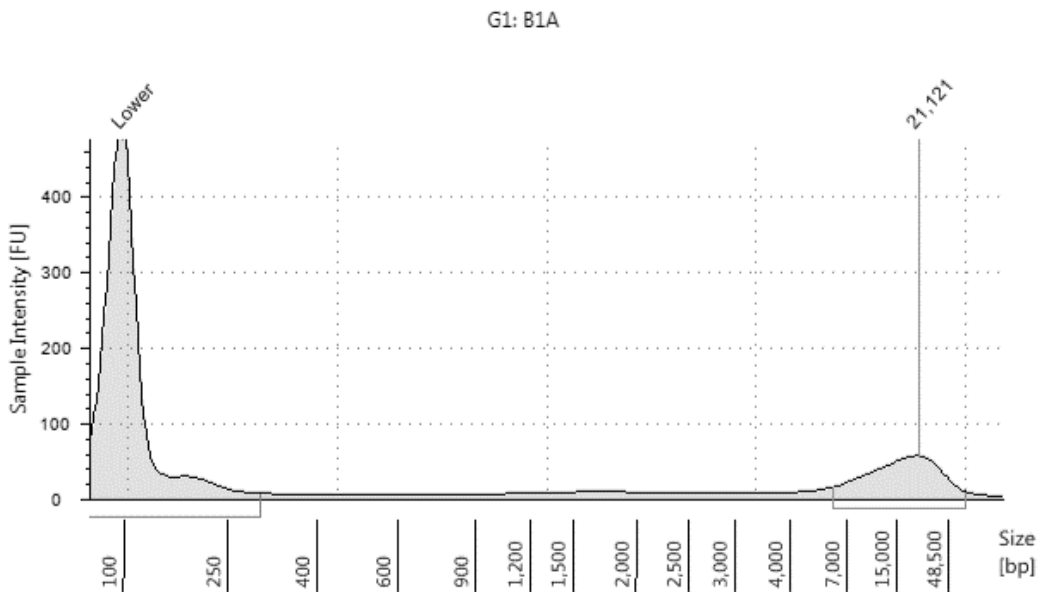

Figure 2.2 Results of the genomic ScreenTape of a postPCR soil sample from burial one taken at 215mm from the edge of the burial. The results represent the PCR protocol using the first set of primers. The vertical axis represents the sample intensity in florescent units (FU), and the horizontal axis represents sequence size in base pairs (bp). The results indicate that the primers failed to amplify the proper sequence.

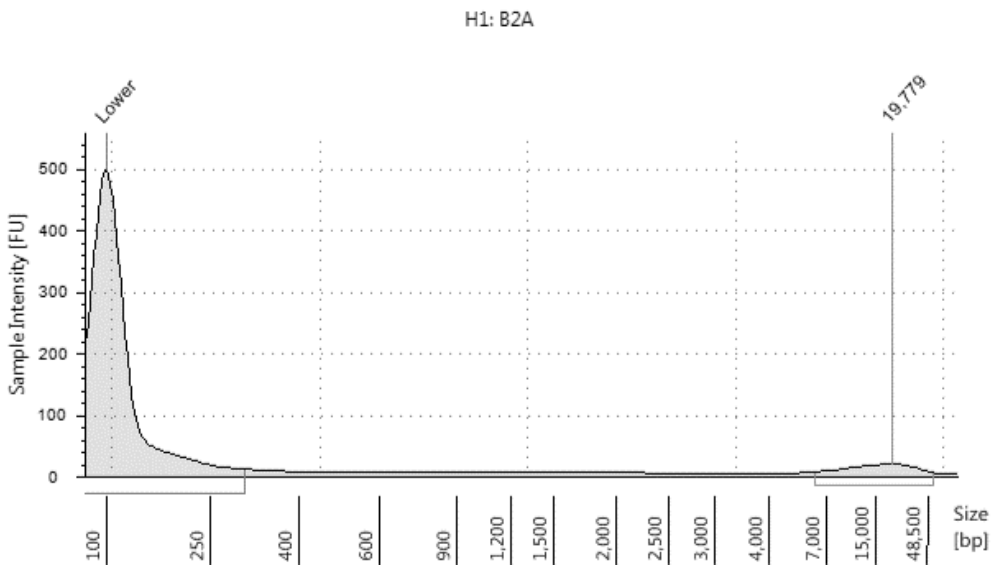

Figure 2.3 Results of the high sensitivity ScreenTape of a postPCR soil sample from burial two at the 215mm using the first set of primers. The vertical axis represents the sample intensity in florescent units (FU), and the horizontal axis represents sequence size in base pairs (bp). The results indicate that the primers failed to amplify the proper sequence.

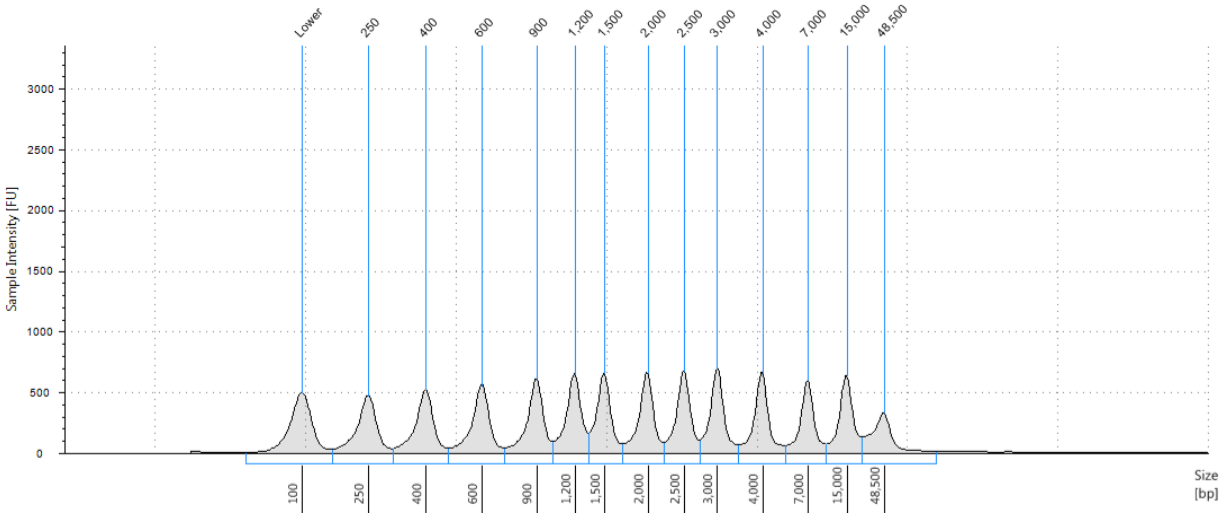

Figure 2.4 Ladder of genomic DNA ScreenTape of the pre-PCR samples taken from processed pig tissue.

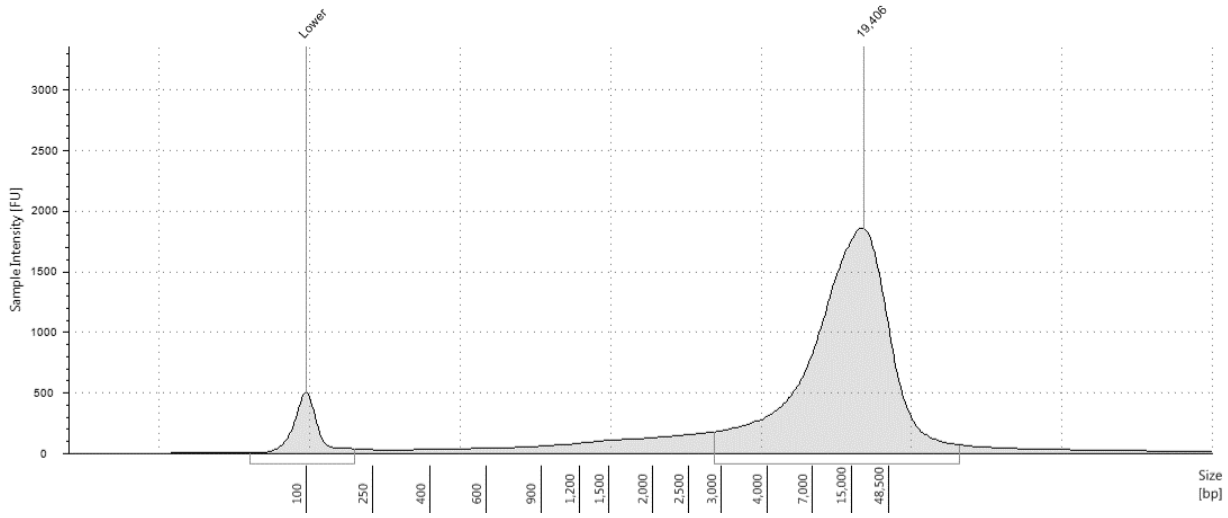

Figure 2.5 Results of genomic DNA ScreenTape of a pre-PCR sample taken from a processed pig. The vertical axis represents the sample intensity in florescent units (FU), and the horizontal axis represents sequence size in base pairs (bp). The results indicate the presence of DNA strands in the sample, which was used to test both sets of primers in the PCR phase.

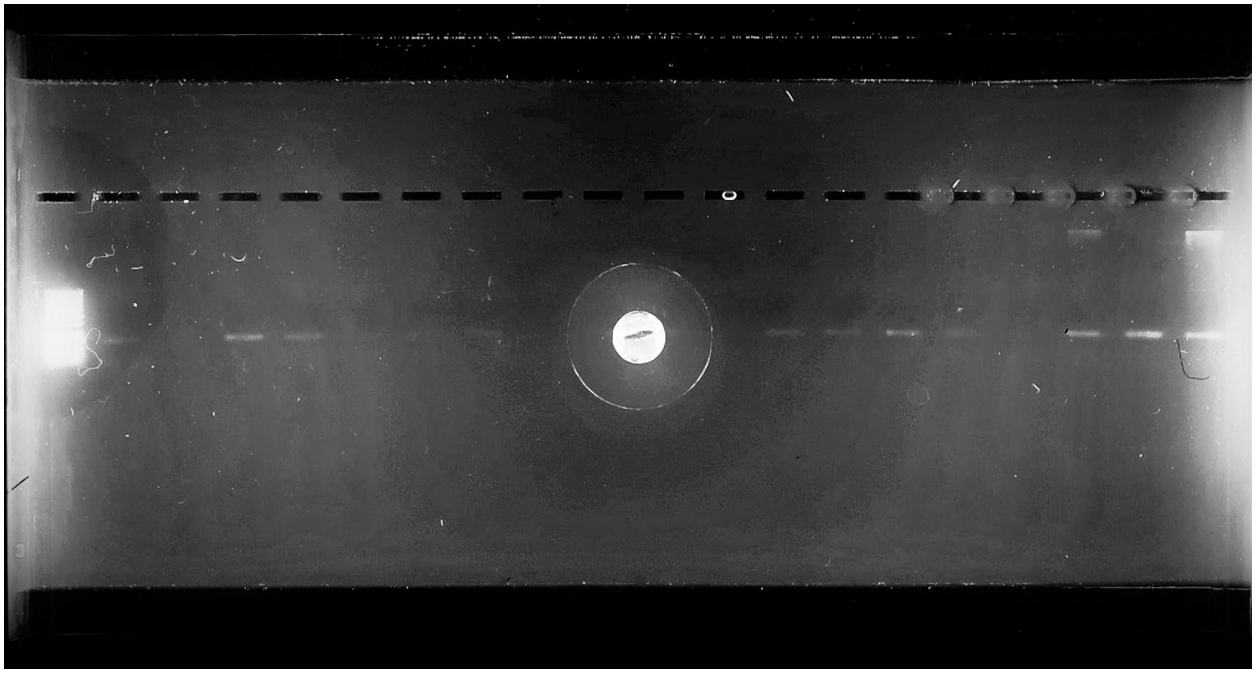

Figure 3.1 Agarose gel for a majority of the samples taken from both burial sites including the two control samples. The samples loaded in the wells from left to right are as follows: ladder, 5-12, 17-24, 13, and 25-26. The gel was run at 109 volts for 10 minutes. The samples migrated down the gel and settled between the 200 bp and 300 bp mark of the ladder indicating PCR success.

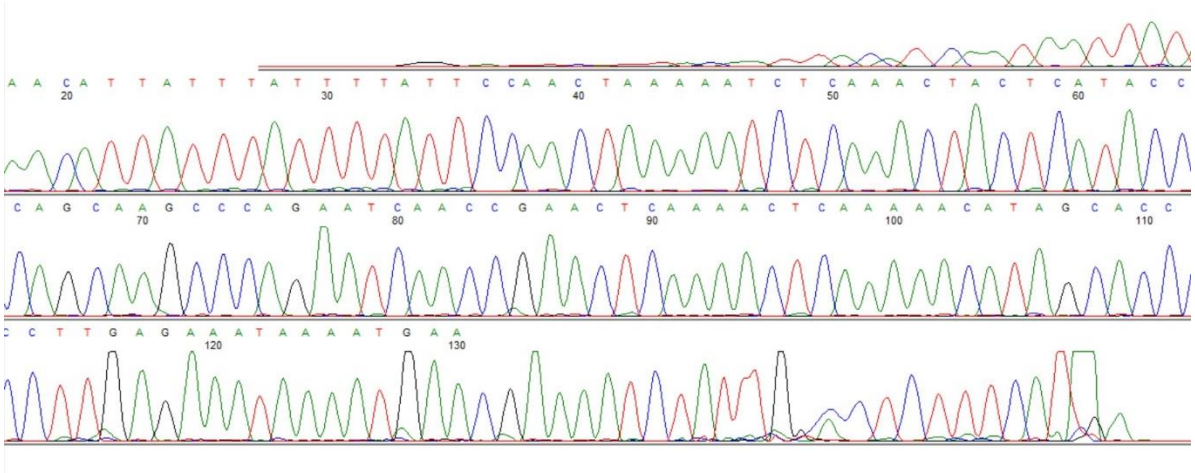

Figure 4.1 Sequencing chromatogram of 375 mm soil sample from burial one. The horizontal axis show the nucleic acid present in the sequence with red coding for thymine (T), black representing guanine (G), green codes for adenine (A), and blue represents cytosine (C). The vertical axis represent the peak height, which corresponds to the relative concentration of that base at that specific location in the sequence. The chromatogram shows minimal noise along the baseline of the peaks, and little overlapping.

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