Differential DNA Preservation of Thermally Altered Tissue and Bone

by

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ABSTRACT

Recovering high-quality deoxyribonucleic acid (DNA) from thermally altered human remains poses a significant challenge for research and law enforcement agencies due to high levels of DNA degradation resulting from exposure to extremely high temperatures (e.g., fire). The current standard practice for the DNA identification of badly burned skeletal remains is to extract DNA from dense cortical bone collected from recovered skeletal elements. Some of the problems associated with this method are that it requires specialized equipment and training, is highly invasive (involving the physical destruction of sample material), time-consuming, and does not reliably guarantee the successful identification of the remains in question. At low-medium levels of thermal exposure, charred tissue is often adhered to these skeletal remains and typically discarded. In cases where burned/charred tissue is recoverable, it has the potential to be a more efficient alternative to the sampling of cortical bone. However, little has been done to test the viability of thermally altered soft tissue in terms of DNA identification to date. Burned/charred tissue was collected from skeletal samples provided by the University of Tennessee Forensic Anthropology Center, as a part of a controlled burn from donor individuals, for downstream laboratory processing and DNA analysis as part of the Stone Lab (Arizona State University, School of Human Evolution and Social Change). DNA from this charred tissue was extracted using the Qiagen DNeasy Blood and Tissue Kit, and resulting yields were quantified via fluorometry using the Qubit Fluorometer 2.0 and Agilent TapeStation 4200 High-Sensitivity D5000 assay. It was found that between the temperatures of ~200-300 ℃ (burn category 2) and ~300-350 ℃ (burn category 3), tissue was the most efficient extraction type, especially from tissue taken from the surface of

the ilium and the rib. As for bone, both the Dabney and the Loreille protocol performed similarly, so choice in extraction type comes down to personal preference, type of equipment on hand, and training. Although, for samples with low input material, the Dabney protocol is optimal.

DEDICATION

To my family who supported me through all the ups and downs, thank you for being there every step of the way. To Boscoe, who gave me a reason to get up in the morning and smile when I was having a bad day. And to Cody, thank you for everything you've done to help me. Thank you for always believing in me even if I didn't believe in myself, I literally could not have done this without you.

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CHAPTER 1

INTRODUCTION

When DNA is exposed to extreme temperatures, there can be significant amounts of DNA degradation, making it challenging for both researchers and law enforcement to identify these individuals using DNA identification techniques (1). This can be a major concern when attempting to identify victims involved in criminal activity (e.g., arson or burning to conceal the identity of the descendent), mass casualty events, or even less criminal instances such as house, car, or forest fires. Currently, the standard practice is to rely on the recovery of DNA from dense tissues such as cortical bone, dentin, or other calcified biological tissues that tend to be more resistant to the extreme morphological degradation caused by exposure to intense heat (2). The sampling of skeletal remains for DNA analysis and identification can require time-consuming, highly specialized protocols that may not be practical at many forensic laboratory facilities. To further complicate this, these tissues often tend to exhibit poor DNA preservation even under ideal conditions (3). However, in many instances, degraded/charred tissue may still be adhered to burned skeletal samples. Because of the apparent extreme degradation, this tissue is often ignored or discarded before proceeding with the processing of the skeletal remains themselves. To date, very little research has been done to determine the viability of charred tissue in the context of forensic DNA identification. This is especially relevant as emerging technologies over the past decades have greatly increased our ability to recover DNA from challenging substrates. This research aims to better understand DNA preservation in severely burned tissue and assess the usage of this potentially valuable substrate in downstream DNA identification processes as an alternative to dense skeletal

remains. This is of particular interest to the forensics community, as DNA extraction from soft tissue is typically done using more commonly available commercial products and kits. This could potentially bypass the need for more costly extraction protocols and require less investment in specialized training.

The aims of this research are: 1) to compare systematically DNA extraction success rates stemming from both burned tissue and the corresponding skeletal elements from which it is recovered, 2) to qualify and quantify the extent of damage to DNA recovered from this tissue and examining how it impacts the quality of downstream analyses, and 3) to determine how varying degrees of thermal exposure affect DNA recovery from soft tissue.

CHAPTER 2

LITERATURE REVIEW

2.1 Degraded DNA

DNA can become degraded from environmental factors such as temperature, pH, and ultraviolet light but ultimately the result is always the same – the length of amplifiable DNA is significantly decreased (4,5). Thermal degradation causes the covalent bonds within each DNA strand to break, leading to DNA fragmentation (6). When DNA is exposed to extremely high temperatures, the double-helix structure undergoes denaturation, also known as DNA melting. Depending on the guanine-cytosine content, length of the molecule, and its salt content, DNA typically melts into single strands at around 50-100℃ and complete DNA degradation can occur above 190℃ (6,7). In forensics cases involving heat and fire, temperatures can get as high as 500℃, leading to significant thermal degradation of DNA. In these cases, obtaining useable DNA from remains is extremely difficult. Since thermally degraded DNA can be similar to ancient DNA in terms of the damage observed in recovered DNA fragments (*e.g.*, severely shortened fragment lengths), there has been a surge of research focused on the use of ancient DNA techniques and protocols in modern forensic samples (8–10), especially skeletal remains. Although skeletal remains are ideal for identification because of their potential for excellent preservation, they can be extremely difficult to work with and tend to yield only trace amounts of host DNA after extraction.

2.2 DNA in Bone

Bone consists of a hard, homogeneous intercellular matrix composed primarily of hydroxyapatite crystals interspaced with living cells such as osteocytes, osteoclasts, and inactive osteoblasts (11). Osteoblasts are the immature bone cells responsible for bone formation and are located on the surface of osteoid seams where they secrete a protein mixture that mineralizes into the osteoid (a mineral essential for the calcification of bone). These osteoblasts then bind to the periosteum, a dense layer of vascular connective tissue that envelops the bone. As the periosteum covers the bone, it traps osteoblasts within small voids throughout the matrix (lacunae), transforming them into osteocytes. These osteocytes are the source of most of the preserved DNA housed within the bone, as it tends to be well-preserved and sheltered from outside contamination (12).

However, because of the mechanism by which DNA is preserved within these layers of calcified tissue, it can be extremely difficult to extract successfully (13). Despite the challenges of being a poor substrate, it is still often the most useful tissue for obtaining DNA from very degraded samples (14). Since the DNA is preserved in the dense bone, it is shielded from many damaging external factors making it ideal for cases where no other biological tissue available.

2.3 DNA Extraction

DNA extraction is the process used to isolate DNA that is both pure and devoid of contaminants such as RNA and proteins, as well as environmental contaminants from the biological material containing it. There are three main techniques: organic, nonorganic, and adsorption methods (15). Depending on which method of DNA extraction is chosen

and the modifications that are made, the reagents and steps can differ but there are always three main steps that stay consistent: lysis, precipitation, and purification (16).

For lysis, a buffer solution that contains both buffering salts (*e.g.,* Tris-HCl) and ionic salts (*e.g.,* NaCl) is used to break open cells. Detergents are also added to help break up membrane structures. Next, in the precipitation step, DNA is separated from any cellular debris using sodium ions to neutralize the negative charges in DNA molecules, making it more stable and less water soluble. Then alcohol, such as isopropanol or ethanol, is added to the aqueous solution, creating a precipitation of DNA because it does not dissolve in alcohol. Finally, the DNA is washed using alcohol so that contaminants such as proteins, lipopolysaccharides, and small RNAs are removed and purified DNA is retained (15,17).

One of the most commonly used DNA extraction method is silica mediated extraction, a type of adsorption method, that utilizes a silica media (e.g., coated beats, silica filtration, etc.) to bind and isolate DNA (18). There are three main steps for a silica-based DNA extraction protocol: bind, elute, and wash. In the presence of chaotropic salts, nucleic acids are absorbed into the silica gel membrane while other molecules such as polysaccharides and proteins remain in the solution (18). Most commercially available kits, such as the one used here for tissue extraction, are designed to allow the user to easily remove these compounds, and other potential contaminants or enzyme inhibitors, without removing the target DNA. After washing away non-nucleic acid materials, pure DNA is then eluted under low or no salt conditions to free the DNA from the silica, resulting in an aqueous solution of pure DNA (18). This method is particularly popular,

as it tends to be less expensive and faster than other extraction techniques. Additionally, silica-mediated DNA extraction does not utilize harsh chemicals such as phenol or chloroform, making it a safer and easier extraction technique.

2.4 DNA Fingerprinting

The advancement of DNA extraction has not only had an impact on our understanding of the human genome, but has also played a very important role in the criminal justice system. In criminal cases, DNA evidence can be used to identify and exonerate individuals that have been accused or convicted of a crime, as well as identify any unknown victims. Dr. Alec Jeffreys at the University of Leicester was the first scientist to identify and utilize DNA markers for the purpose of identifying the human sources of evidentiary material in 1985 (DNA fingerprinting) (19). DNA fingerprinting, or DNA typing, is the analysis of individually unique genetic markers in individuals to generate a “fingerprint” to aid in the qualification of the origin of unknown biological markers. Today, this is most typically done using short-tandem repeat (STR), relatively short stretches of DNA that often repeat in predictable patterns, analysis (20). Because these repetitive stretches are generally not under selective pressure, the exact composition and number of repeats is governed by random mutation and are highly variable between individuals (21). Jeffreys referred to these regions as variable number tandem repeats (VNTRs) and used radioactive probes to identify these repeating sequences reliably. He used this method to recognize specific individuals based on the pattern of observed VNTR representation in their genomes (19).

The first instance in which Jeffrey's DNA fingerprinting was successfully used to identify an individual in a law-enforcement context was the case of an immigrant family from Ghana whose son was denied entry back to their home in the United Kingdom using a forged passport. The family contacted Jeffreys asking whether he could use his DNA techniques to confirm that the boy was related to the rest of the family. DNA samples were subsequently taken from the boy, his mother, and the mother's additional three children. Using DNA fingerprinting, it was confirmed that not only was the individual in question a close biological relation to the mother, but also to the other three children tested (22).

Following this initial success, Jeffreys went on to further assist law enforcement with identifying biological samples connected to a double murder case in 1986. Jeffreys modified his original technique using a multi-loci probe to utilize instead a single-locus probe targeting two DNA segments in an individual, one maternal and one paternal. Using this new and improved technique, Jeffreys produced a DNA profile from a blood specimen collected from the alleged suspect and another from tissue and semen specimens collected from the two victims. It was revealed that the semen from both victims was of identical origin, indicating that a single individual was responsible for the rape and murder of these victims. In addition, the DNA profile generated from these two samples did not originate from the suspect that law enforcement had in custody at the time. Subsequently, this individual was released and cleared of all charges, becoming the first suspect to be cleared based on DNA evidence. DNA profiles from individuals who lived near the crime scene were then collected and compared to the DNA samples from the victims; however no match was found. Six months later, a woman reported a man

named Colin Pitchfork, who had forged the blood sample given to law enforcement in the aforementioned double-murder case to confound investigators using the serological typing methods commonly used at the time. A sample of Pitchfork's blood was taken, and the resulting DNA profile was compared to the semen samples. These were found to be a match, resulting in his conviction for the murders of both individuals. This was the first successful outcome of DNA profiling to be used in a criminal case to convict a killer (22).

DNA fingerprinting allows for not only the convictions of suspected criminals but can also lead to the exoneration of innocent individuals. As of 2018, 133 individuals have been exonerated, based on DNA evidence, of crimes for which they were previously found guilty (23) . DNA fingerprinting also allows for the identifying unknown individuals, like missing persons or in cold cases.

2.5 The Expansion of DNA Fingerprinting and Introduction of Sanger Sequencing

Since this initial usage of DNA profiling, there have been many advancements in the forensic usage of DNA identification including the introduction of PCR, fluorescent labels, and capillary electrophoresis (19). DNA sequencing has also been used in forensic casework. In 1977, Frederick Sanger and colleagues created a DNA sequencing method (Sanger sequencing) that utilized the use of radiolabeled partially digested fragments called the "chain termination method" and quickly became the most widely used sequencing technique for the next three decades (24). It was used to sequence the first ever complete genome of bacteriophage λ (48,502 base pairs) in 1977 and used to sequence the entire human genome (16,569 base pairs) for the first time in 2003 (24). It

can also be used to produce DNA sequence reads of >500 nucleotides and has a very low error rate (99.9%) (25).

In 1983, Kary Mullis used Sanger's DNA sequencing method to create the polymerase chain reaction (PCR), allowing scientists to quickly amplify a very specific sequence of DNA into billions of copies (15). In Sanger sequencing, only a single primer is used for amplification while PCR utilizes two, one for the forward strand and the other for the reverse strand (24). Using two primers allows for a very specifically targeted sequence to be replicated. PCR is especially vital in degraded samples, since very short sequences of DNA are able to be replicated very easily for downstream analyses (26). One way to analyze degraded DNA is to look at the short tandem repeats (STRs), short, repeated fragments of DNA that are highly variable among individuals (21) which are examined using the process of PCR. The largest complication when working with degraded samples, is that this degradation often leads to poor PCR amplification because of the drastic reduction in the yield of whole target fragments (26).

2.6 Large-Scale Advancement and Usage of DNA for Forensic Casework

With these advancements, it has become possible to sequence large numbers of samples reliably, easily, and cost-effectively, making the process an integral part in solving crimes. The Federal Bureau of Investigation (FBI) created a system known as the Combined DNA Index System (CODIS) to further the use of DNA in forensic casework. CODIS, officially started in 1998, allows for the use and exchange of DNA profiles within law enforcement at the local, state, and national levels (27). This main database consists of three major indexes: The Forensic Index, The Offender Index, and the

Missing Persons Index. At the national level, the National DNA Index System (NDIS), an integral part of CODIS, contains DNA profiles contributed by federal, state, and accredited forensic laboratories across the United States. It was designed to compare a target DNA record against DNA profiles within the database for use as evidentiary DNA samples in criminal cases.

The CODIS system utilizes a set of 20 core STR loci in order to make DNA matches, with at least eight core loci and a combined match rarity of at least one in ten million necessary for comparison in criminal inquiries (28). Alternatively, all 20 CODIS core loci must be recovered for missing persons before a match against the NDIS database may be attempted (28). With the use of these databases, biological evidence from a crime scene can be collected and compared to millions of offender profiles within the database, potentially leading to the identification of a suspect and solving of a case.

With the introduction of next generation sequencing (NGS), used to rapidly sequence whole genomes and deeply sequence specific target regions very quickly (29), researchers were given a new tool for use in DNA identification. Compared to Sanger Sequencing, NGS can sequence millions of fragments simultaneously, resulting in lower cost and faster turnaround time using the same amount of input DNA. NGS also has a higher sensitivity to detect low quantity of DNA input while providing a more comprehensive genomic coverage, increasing the ability to identify novel variants (30). This new generation of sequencing allows single nucleotide polymorphism (SNP) markers to be targeted and the ability to assess all or part of the mitochondrial genome very quickly and cheaply which can be very helpful in the use of forensics investigations

(31). With NGS, crime laboratories can significantly increase the scope and fidelity of DNA profiles that they produce by refining and adding incredible detail to the DNA fingerprinting process.

2.7 The Usage of Skeletal Tissue for DNA Identification

Typically, extraction of DNA from bone samples involves the collection of bone powder produced via drilling into the bone but this process results in the destruction or considerable damage to the remains. The efficient use of minimal sample amounts, especially when working with irreplaceable samples such as archaeological or forensic remains, is extremely important, as it is vital to preserve the archaeological record and forensic evidence whenever possible. The Dabney et al. protocol (Dabney) (32) is a DNA technique typically used in ancient remains because it is optimized to efficiently extract highly degraded, short fragment DNA from very little input material (<100 mg). Dabney is the most current and widespread ancient DNA protocol used for archaeological samples. The Loreille et al. protocol (Loreille) (33), is biochemically very similar to the Dabney et al. protocol but it is used in modern forensic samples and optimized for whole genomic DNA extraction from large quantities of bone powder (≥250 mg). Loreille is currently the standard for modern forensic samples in both federal and international organizations such as the Federal Bureau of Investigation (FBI) and the International Commission on Missing Persons (ICMP) (33,34).

Since the standard for use of identification in thermally altered samples is to sample from skeletal tissue, any degraded soft tissue that is still adhered to the skeletal remains is often removed and disposed of (33,35) to minimize potential contamination.

Little research has been done into whether this burned soft tissue could be used as a substitute for the bones itself (36). Since burned tissue is typically discarded, no standard method is commonly used. In this study, burned tissue adhering to burned skeletal material was sampled and DNA extracted using the Qiagen DNeasy Blood and Tissue Kit to compare yields (quantity and quality) against DNA extracted from the corresponding skeletal tissue. This kit was chosen because it is readily available and straightforward to use compared to other extraction methods and requires no additional, specialized training in techniques uncommon outside of most DNA laboratories.

CHAPTER 3

METHODS

3.1 Sampling

Burnt bone and charred tissue adhering to skeletal samples were recovered from the controlled burning of 10 donor cadavers at the University of Tennessee Forensic Anthropology Center (FAC). The donor cadavers were placed on a wood-fired, cinder block and sheet metal pyre that was loaded with approximately the same amount of fuel and fuel type for each instance. A temperature probe was placed directly into the fire, and on the cadaver to monitor burning patterns throughout the burning process (~3 hours per individual in total).

A total of 150 skeletal samples representing 8 individuals were sampled and processed at the Stone Lab Ancient DNA laboratory on the Arizona State University campus in Tempe, Arizona (Table 2). These remains were processed in a dedicated clean room environment specifically designed to minimize the potential exogenous contamination in low-yield, highly degraded sample materials. The remains were photographed and a visual examination was utilized to assess and estimate the level of thermal degradation to each sample using the 1-5 scale introduced in Schwark et al. (2011) (2) with a slight modification consisting of the addition of intermediary categories: 1: well/moderately preserved, 2: yellow or brown (~200−300 °C), 2.5: yellow/brown transitioning to black/smoked (~250-325 °C), 3: black or smoked (~300−550 °C), 3.5: black/smoked transitioning to grey (~350-550 °C), 4: grey (~550−650 °C), 4.5: grey/blue transitioning to white/calcined (~550-600) (Table 1).

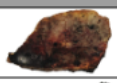
Burn Category		Description	Temperature (C)
2		Yellow; Brown	200-300
2.5		↕	250-325
3		Carbonized; Black	300-350
3.5		↕	350-550
4		Grey; Blue	550-600
4.5		Grey;Blue → White;Calcined	600-650

Table 1. Burnt bone categorization and associated color change according to temperature range, based on visual estimation scale by Schwark et al., 2011.

Element	Dabney	Loreille	Tissue	Total N
Femur	10	10	5	25
Calcaneus	1	1	1	3
Humerus	5	5	5	15
Os Coxa	5	5	6	16
Metacarpal	5	5	4	14
Navicular	3	3	1	7
Phalanx	8	8	7	23
Rib	10	10	7	27
Tibia	2	2	0	4
Vertebra	4	4	2	10

Table 2. The total number of skeletal elements that were extracted per each extraction method. A total of 150 skeletal samples representing 8 individuals are shown.

3.1.1 Sub-sampling

Initially, the burnt/charred tissue was gathered from the skeletal samples by manually scraping using a sterile scalpel. Approximately 1 gram of bone fragments was pulverized into a single, homogenized powder using a Spex model 8000 mixer mill. The removed tissue and bone powder were weighed and transferred to two separate sterile, low DNA binding 2 ml microcentrifuge tubes for downstream DNA extraction.

3.2 DNA Extraction from Tissue Samples

There was a total of 53 burned bones with charred tissue still attached to them. DNA was extracted from 20mg (±1mg) of this recovered charred tissue using the Qiagen DNeasy Blood and Tissue Kit, following a modified version of the manufacturer's suggested protocol. 180ul of Buffer ATL and 20 µl of Proteinase K was added, vortexing between each addition. The mixture was then placed into a thermomixer and incubated at 56 °C until the tissue was completely lysed. After initial testing, the ideal time was found to be about 72 hours of incubation instead of the proposed 24 hours in the protocol. After incubation, 200 µl of buffer AL and 200 µl of ethanol were added, vortexing before and after each step. This mixture was then pipetted into the DNeasy Mini Spin Column, placed in a 2 ml collection tube, and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were subsequently discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl of Buffer AW1 was added. The centrifugation step from above was repeated, with the flow-through and collection tube again being discarded. After replacing the collection tube, 500 µl of Buffer AW2 was added and centrifuged for 3 minutes at 13,200 rpm to dry the DNeasy membrane. Finally,

the DNeasy Mini spin column was placed in a 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was pipetted directly onto the DNeasy membrane. The mixture was then incubated for 1 minute and centrifuged for 1 minute at 8000 rpm to elute the DNA (See Appendix B for full protocol).

3.3 DNA Extraction from Bone Samples

The bone samples were processed for comparison using two methods of DNA extraction as part of a separate study. The two protocols include one devised by Loreille and colleagues and commonly used in forensic analyses of degraded samples. The second protocol uses a modified Dabney and colleagues extraction protocol, commonly used in analyses of ancient DNA. The Dabney protocol (See Appendix 2 for the full protocol) is a single silica-based DNA extraction method optimized to extract short fragment DNA from minimal input material efficiently. First, an extraction buffer consisting of EDTA and proteinase K is used to release DNA from the powdered bone samples. Then, a binding buffer containing sodium acetates, sodium chloride, and guanidine thiocyanate is added to DNA that is bound to silica using a silica spin column. Next, the DNA bound to silica is desalted using an ethanol wash buffer, and finally, the DNA is eluted into a low salt buffer (32).

The Loreille protocol (see Appendix B for the full protocol) is also a silica-based extraction technique but is optimized for whole genomic DNA extraction from large quantities of bone powder and utilizes complete demineralization. Samples first undergo a demineralization and lysis in a buffer containing 10% sodium N-lauroyl sarcosinate, proteinase K, and 0.5 M EDTA and then are incubated overnight. Extracts are then

centrifuged to pellet the remaining bone fragments. The supernatant is concentrated using Amicon columns and then purified using MinElute silica filter columns.

3.4 Quantification

All DNA extractions were quantified for raw concentration, endogenous concentration, and fragment length using the Qubit 4.0 fluorometer, the Quantifiler Trio DNA Quantification Kit, and the Agilent 4200 TapeStation D5000 HS , respectively. 30 µl were then aliquoted for downstream STR analysis (see Appendix B for full protocol). All quantitative data (raw DNA, endogenous DNA, fragment length) was visualized in R (37) using ggplot2 (38), with any categorical data (e.g., thermal exposure level or skeletal element) with sample sizes of $n < 4$ excluded from downstream analyses.

3.5 STRs

The 30 µl aliquots were sent to the Kanthaswamy lab at the Arizona State University School of Mathematical Natural Sciences in Tempe, Arizona for STR profile generation. STR profiles were generated using the PowerPlex ® Fusion 6C STR kit (Promega), and the results were sent back to the Stone lab for analysis. A consensus STR profile was created for each individual by combining all allele calls from each sample within each individual. The profiles recovered from each sample among all individuals were then matched against the reference profiles using MixSTR (39). The resulting comparative data were used to determine the overall quality of the match.

CHAPTER 4

RESULTS

Total, or raw, DNA recovery is the first metric we typically look at to determine whether a sample is a good candidate for potential downstream analysis. At low levels of thermal exposure (~200-300 ℃, burn category 2) we see the highest median recovery of DNA in the tissue samples (2.176 ng/µl), followed by the Dabney protocol extraction (0.884 ng/µl) with the Loreille protocol extractions yielding the least raw DNA (0.420 ng/µl). However, when taking into account low sample sizes of the tissue extractions in respect to the Dabney and Loreille, no significance can be assigned (n =5, n =5, n =2 for the Dabney, Loreille, and tissue protocols respectively) (Figure 1). At estimated thermal exposure of roughly 250-325 ℃ (burn category 2.5), we observe a large drop in the median raw DNA we were able to recover across all extraction types (0.064 ng/µl, 0.155 ng/µl, and 0.035 ng/µl respectively), whereas at ~300-350 ℃ (burn category 3) there is a reversal of this trend with all extraction types recovering higher concentrations of DNA than at the previous level of thermal exposure (0.157 ng/µl, 0.599 ng/µl, and 0.265 ng/µl respectively). At levels of thermal exposure estimated to be > 350 ℃ (burn categories 3.5

– 4.5) we observe an acute decrease in raw DNA recovery (to near zero across all samples) (Figure 1).

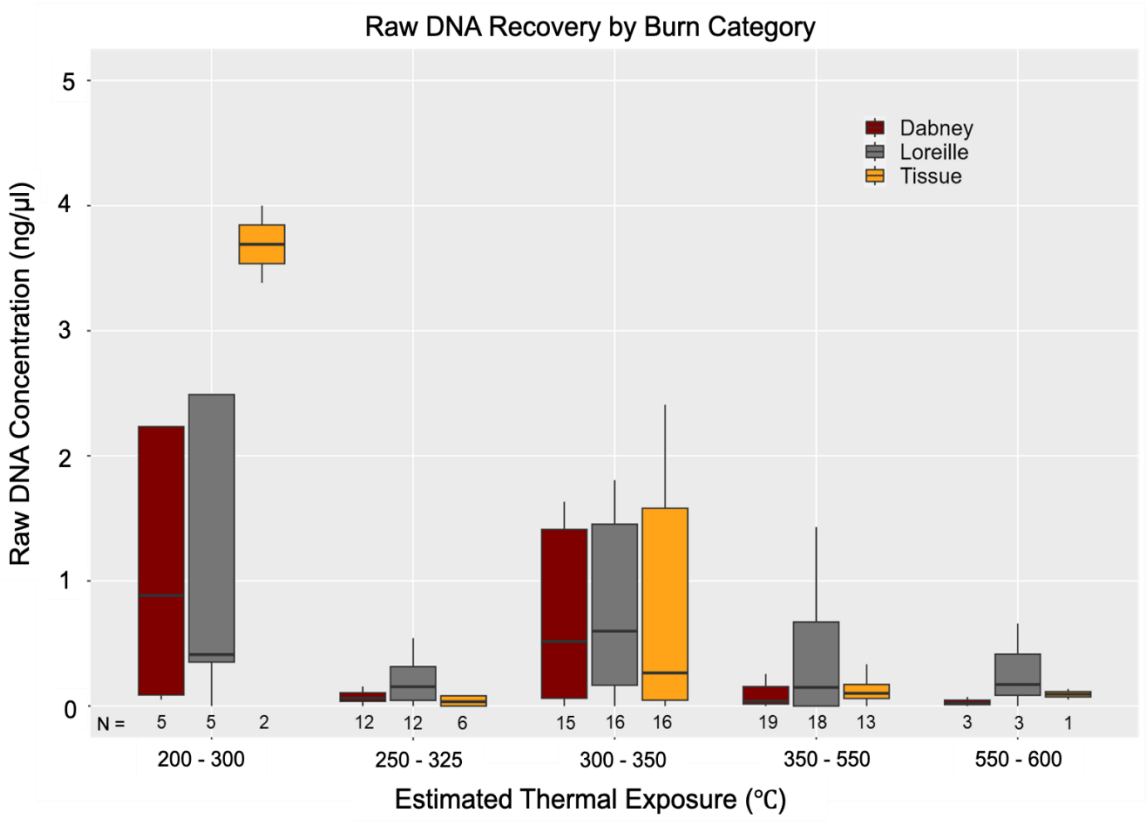


Figure 1. Total, or raw, DNA recovery across estimated thermal exposure.

When looking at overall of efficiency of DNA extraction techniques and substrates across levels of thermal exposure (ng of DNA recovered per mg of input sample), we found that in samples exposed to lower thermal temperatures (~200-300°C, burn category 2), the highest median efficiency (excluding tissue samples as N < 3) is from the Dabney protocol (1.769 ng/mg, N = 5) when compared to the Loreille protocol (0.1648 ng/mg, N = 5). At levels of thermal exposure estimated to be approximately ~250-325 °C (burn category 2.5), we observe the highest efficiency from charred tissue

samples with a median yield of 0.347 ng/mg (N = 6), followed by the Dabney protocol with a yield of 0.127 ng/mg (N = 12), and the Loreille yielding 0.062 ng/mg (N = 12). At estimated thermal exposures of ~300-350 ℃ (burn category 3) the tissue samples performed best (1.473 ng/mg, N = 16), followed by the Dabney (1.033 ng/mg, N = 15) and Loreille (0.240 ng/mg, N = 16) protocols. Similar to the trend we observed in raw DNA yield, at roughly 350-550℃ (burn category 3.5), we observe a large drop in efficiency with tissue yielding 0.5128 ng/mg (N = 13), followed by the Dabney (0.0951 ng/mg, N = 19), and the Loreille (0.0701 ng/mg, N = 18) (Figure 2).

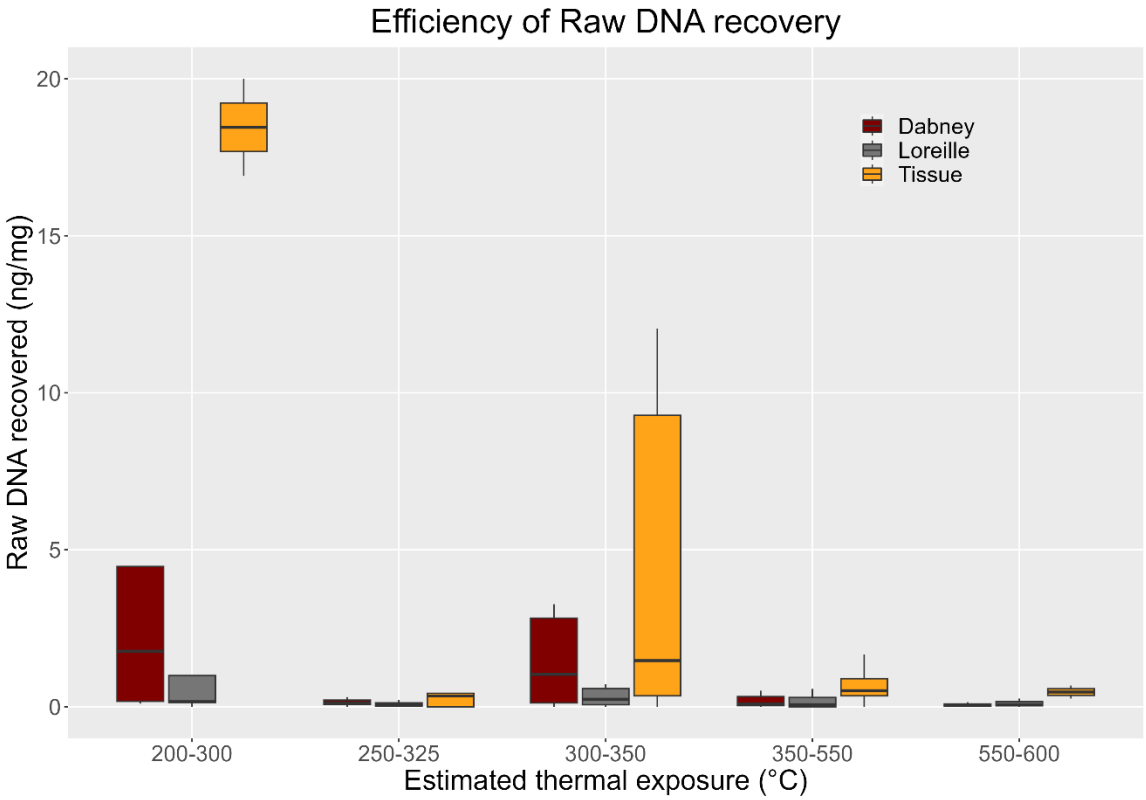


Figure 2. Efficiency of total, or raw, DNA recovery across estimated thermal exposure.

In overall DNA recovery across skeletal elements, we observe highest median yields when isolating DNA from the ribs with charred tissue yielding 1.305 ng/µl (N=7), Dabney extracts yielding 0.724 ng/µl (N = 10), and Loreille extracts yielding 0.325 ng/µl (N=10). The ilium also showed excellent DNA recovery with tissue samples yielding 1.226 ng/ µl (N = 6), Dabney extracts yielding 0.368 ng/µl (N=5), and Loreille extracts yielding 1.337 ng/µl (N=5). In all other skeletal elements, except for the vertebrae (where N < 5 for all extraction types) and Loreille extractions stemming from the femur (median recovery = 0.407 ng/µl, N=10) all extraction performed similarly (> 0.3 ng/µl median recovery) (Figure 3).

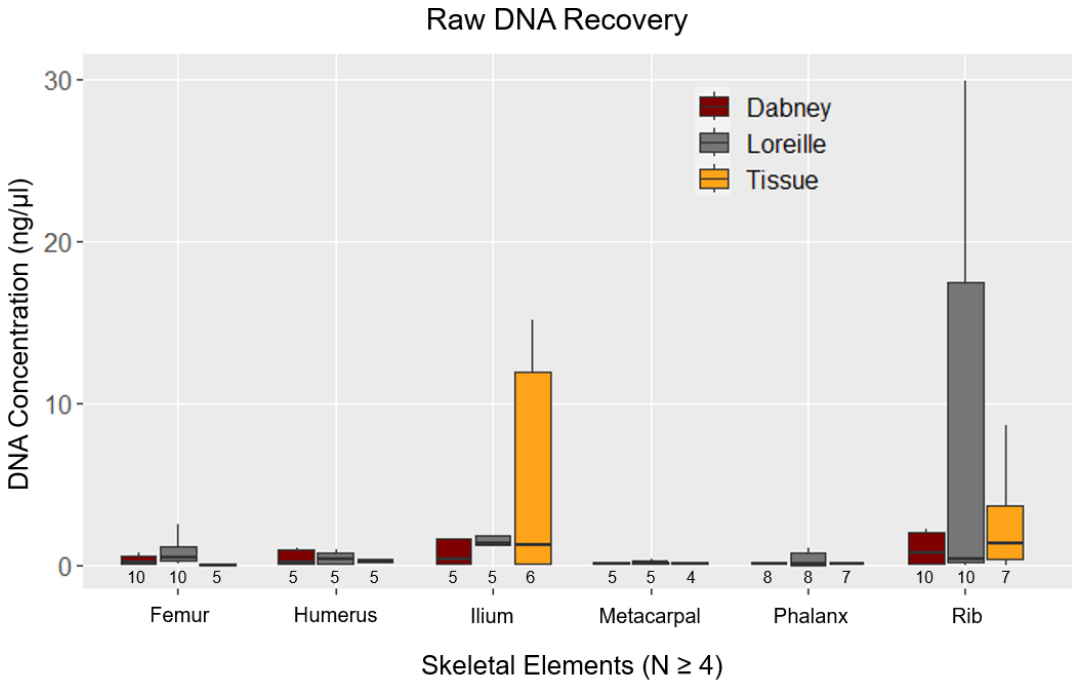


Figure 3. Total, or raw, DNA recovery across all skeletal elements.

In terms of the efficiency of raw DNA recovery across skeletal elements, it was found that extraction stemming from the ilium (N = 16) and rib (N = 27) was most efficient, particularly in samples extracted from recovered tissue (11.089 ng/mg and 6.524 ng/mg, respectively) (Figure 4).

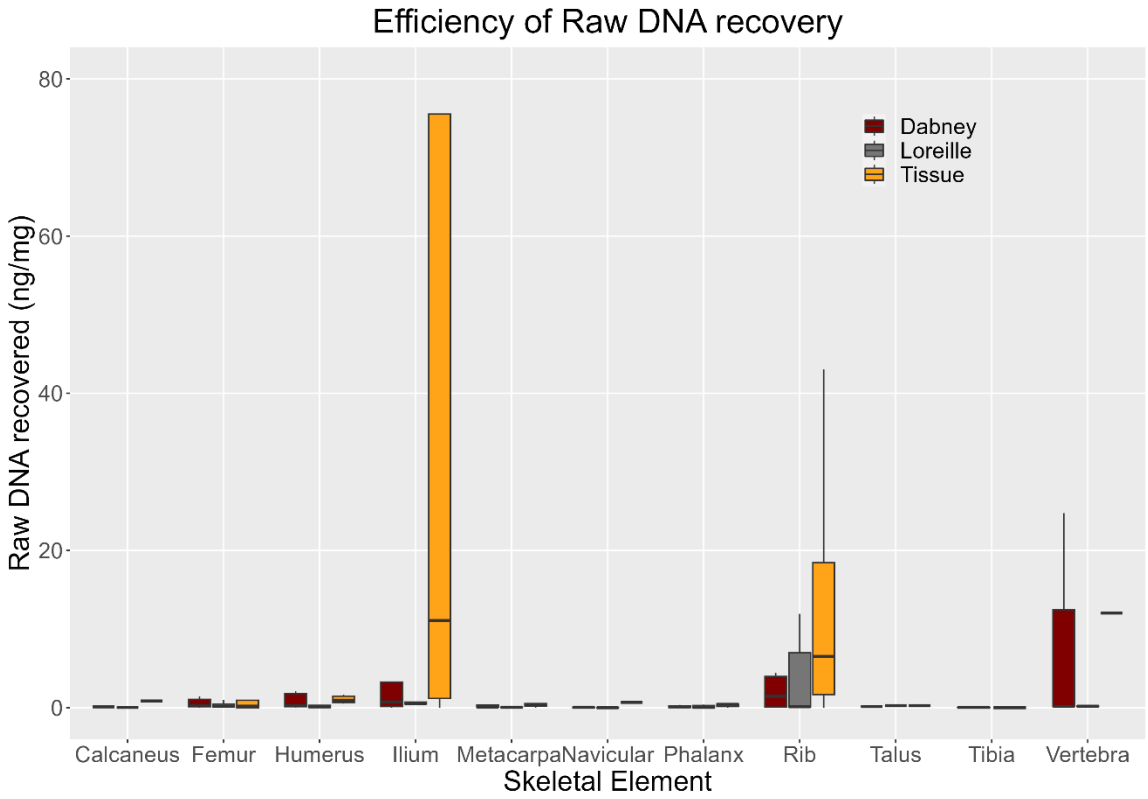


Figure 4. Efficiency of total, or raw, DNA recovery across all skeletal elements.

Beyond total overall yields, assessing the chances of success for each DNA extraction endeavor is of equal importance, as it is often more important to obtain enough input material of DNA consistently for downstream analysis than for the overall DNA yield itself. Successful DNA extraction is defined here as an extraction attempt yielding DNA of sufficient concentration to meet the suggested minimum input parameters of the PowerPlex ® Fusion 6C STR kit (> 250 pg/15 µl). Here we observe no significant

difference in success rate across the different levels of thermal exposure and skeletal elements (ANOVA of mean DNA recovery: $p > 0.05$) regardless of extraction type or substrate. (Figure 5, 6; Appendix C, Column Name).

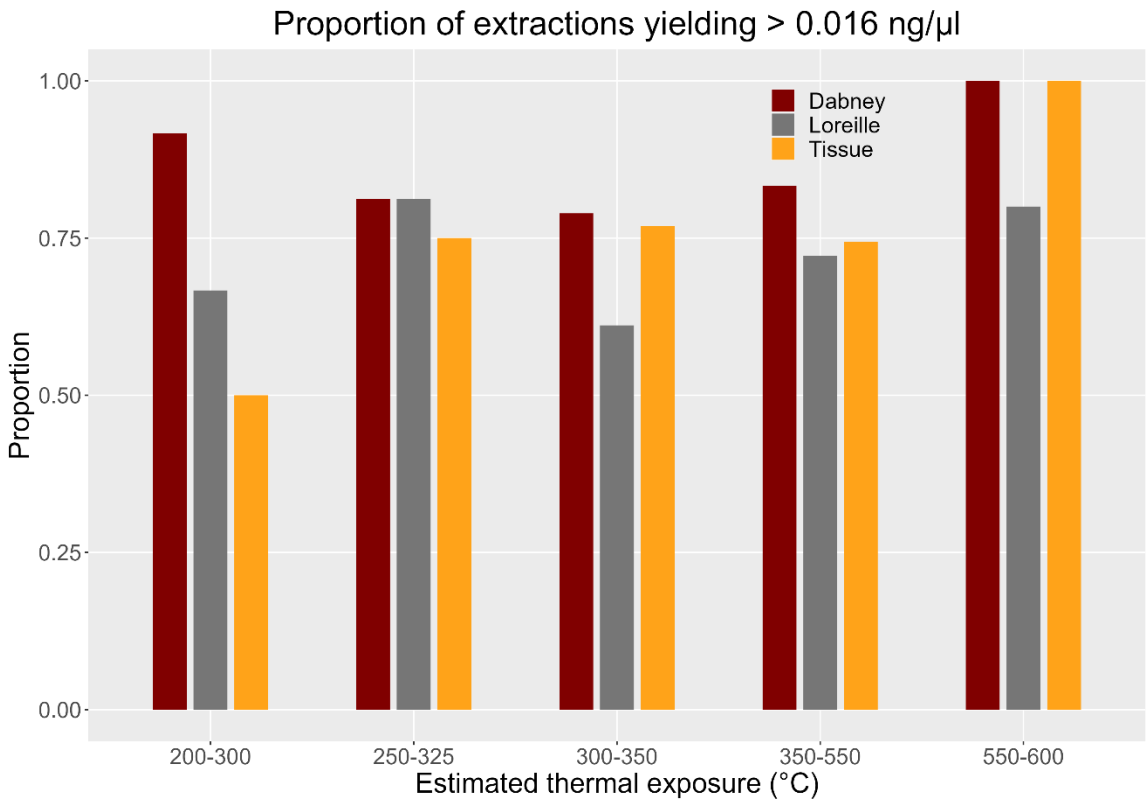


Figure 5. Proportion of extractions that yielded sufficient concentration to meet the suggested minimum input parameters of the PowerPlex ® Fusion 6C STR kit (> 250 pg/15 µl) per estimated level of thermal exposure.

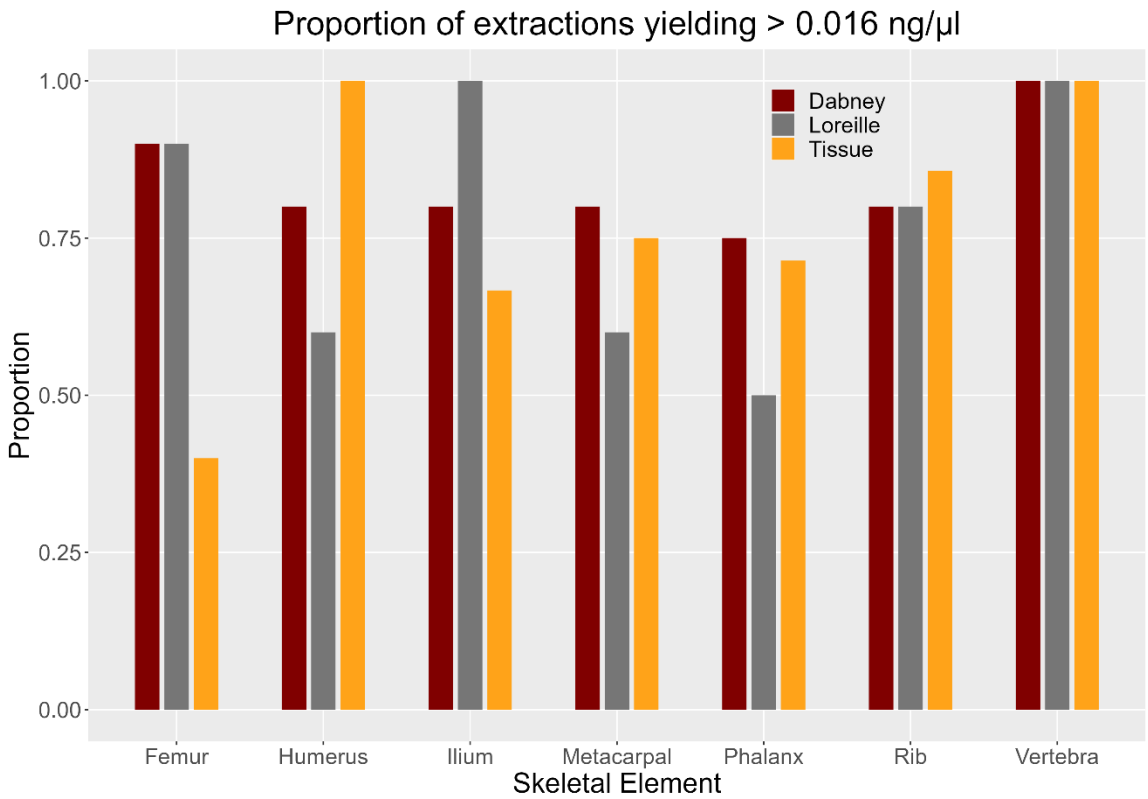


Figure 6. Proportion of extractions that yielded sufficient concentration to meet the suggested minimum input parameters of the PowerPlex ® Fusion 6C STR kit (> 250 pg/15 µl) across skeletal elements.

Both previous metrics are centered around the total amount of DNA recovered from a sample, however there is no guarantee that this recovered DNA stems from the individual in question, or is even of human origin at all (especially in the case of highly degraded samples). Here we find that, in regard to endogenous DNA recovery as obtained using the Quantifiler Trio DNA Quantification Kit, in most cases, we can infer the presence of significant exogenous non-human contamination, the recovery rate of human DNA (Figure 7, 8) is consistent with what we would expect given the patterns observed in overall DNA recovery (Figure 3, 4). We see the highest median of endogenous DNA recovery using the Dabney protocol for samples exposed to lower

levels of thermal alteration (~200-300 ℃, burn category 2) (1.045 ng/µl, N = 5), followed by the Loreille protocol (0.010 ng/µl). For samples exposed to temperatures 250-600 ℃ (burn categories 2.5-4), endogenous DNA recovery was found to be about similar across all extraction types and substrates (> 0.02 ng/µl) (Figure 7).

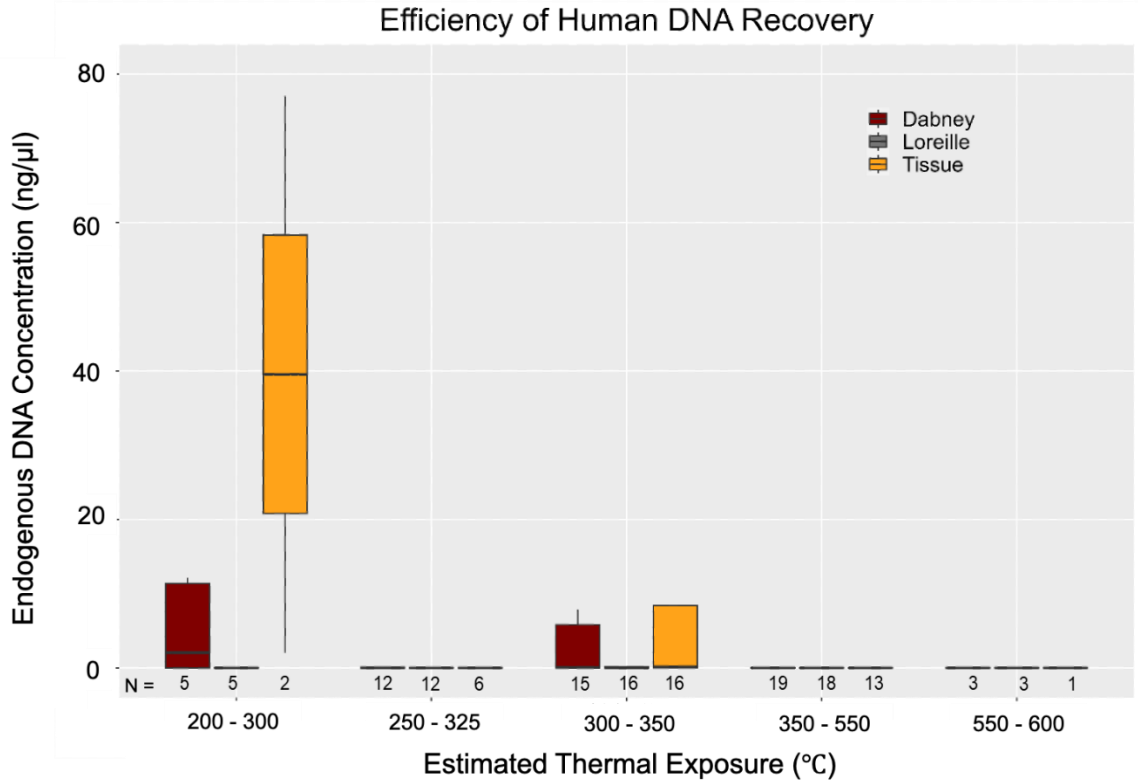


Figure 7 Comparison of endogenous DNA recovery observed in thermally altered bone and charred soft tissue samples after DNA purification and isolation across estimated levels of thermal exposure

As for median efficiency of endogenous DNA recovery, tissue was found to be extremely efficient at lower temperatures (~200-300 ℃, burn category 2) (39.554 ng/mg), followed by Dabney (2.049 ng/mg) and Loreille (0.004 ng/mg). Around 250-325 ℃ and 350-550 ℃, we see similar results (> 0.02 ng/mg) across all extraction types,

while at 300-350 ℃ we see the highest median efficiency from the Dabney protocol (0.640 ng/mg), followed by the charred tissue (0.152 ng/mg) and the Loreille protocol (0.003 ng/mg) (Figure 8).

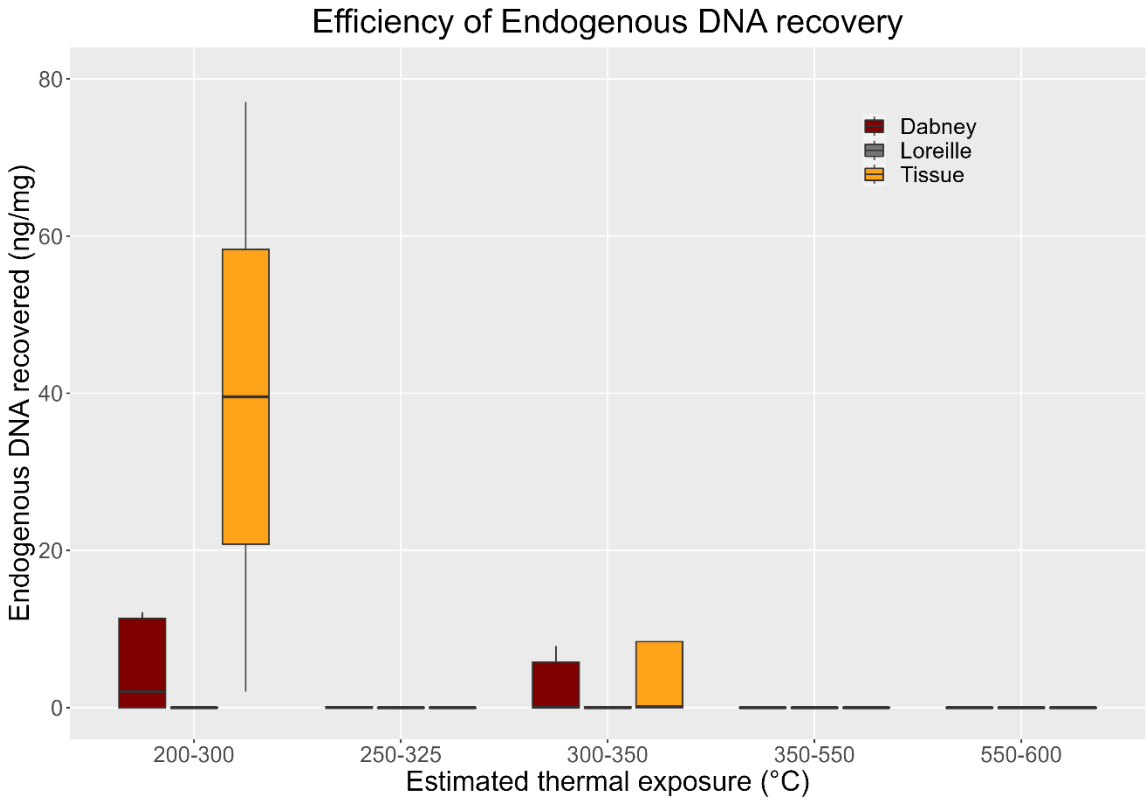


Figure 8. Efficiency of endogenous DNA recovery across estimated thermal exposures

STR profile recovery was assessed using a total of 27 loci. Although it was found that the Loreille protocol yielded very low amounts of endogenous DNA (> 0.013 ng/mg), this seems to have had little impact on the number of STR loci we were able to recover, and this protocol as successful as the other extraction types (Figure 9) in terms of STR profile recovery. We observe the highest number of loci recovered for samples exposed to ~200-300 when using the Loreille protocol (22 loci on average), with Dabney recovering 19 loci, and the burned tissue recovering around 17.5 loci on average.

Consistent with the trends observed in both raw DNA and endogenous DNA, we also see a large decrease in the total STR profiles recovered at ~250-325 ℃ (category 2.5) and < 350 ℃ (category 3.5+) (Figure 9). The highest average number of loci recovered from the ilium (18.8 loci, N =16) and rib (13.5 loci, N = 17), similarly consistent with the trends noted in our earlier assessments (Figure 10).

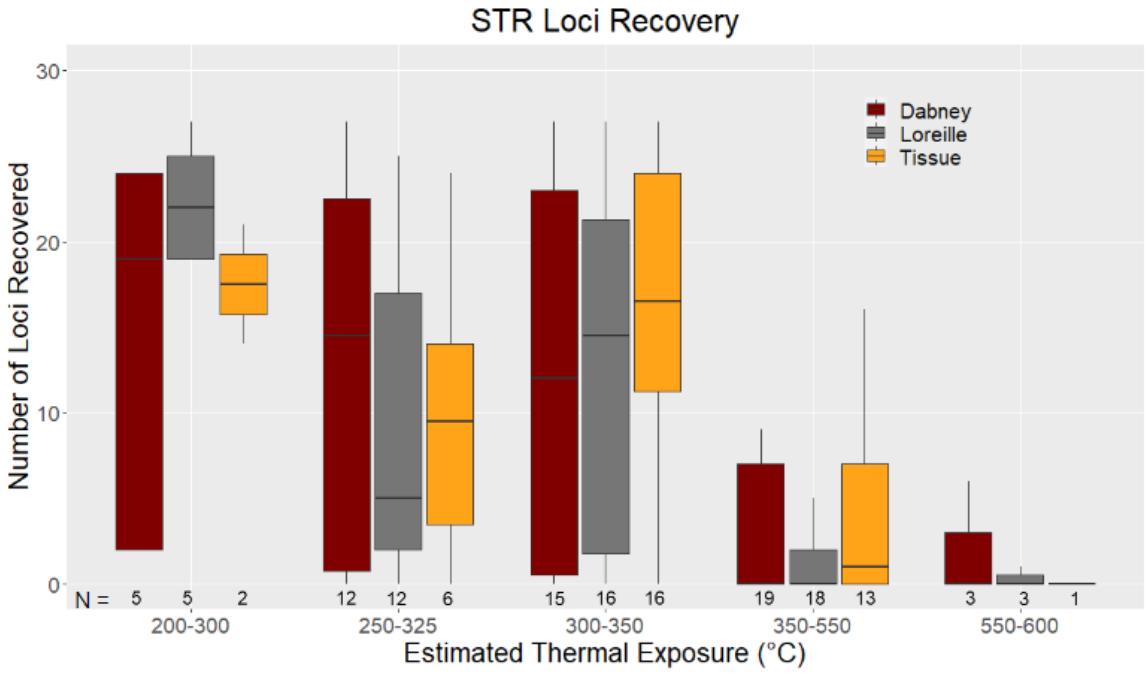


Figure 9. Total number of STR loci recovered across estimated thermal exposure.

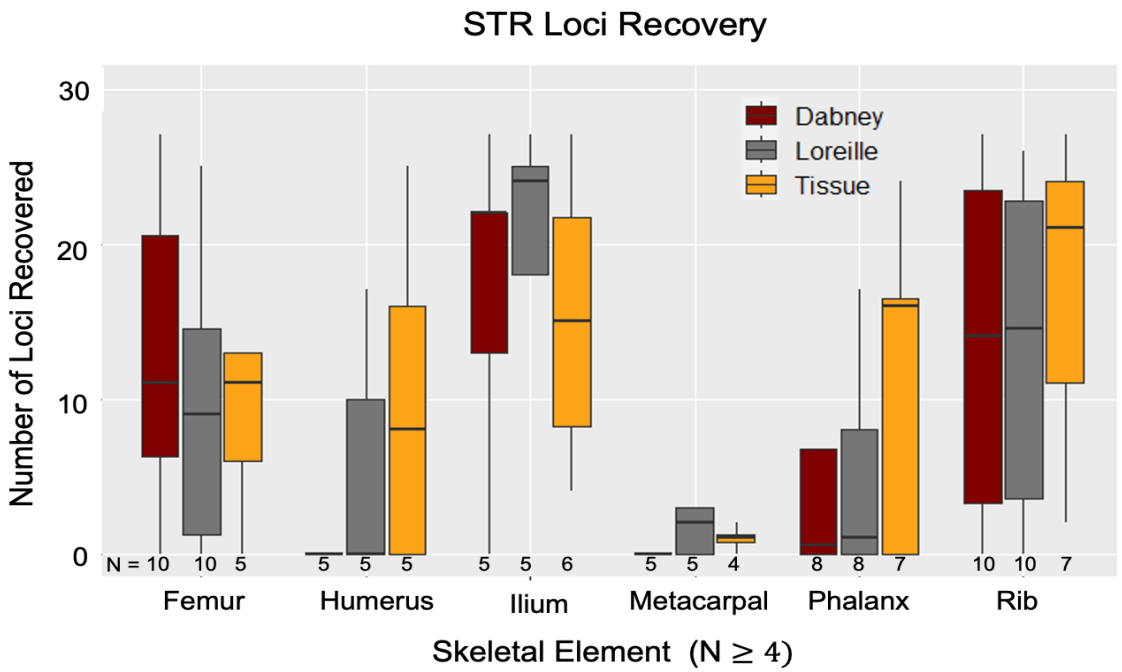


Figure 10. Total number of loci recovered across skeletal element.

As for the quality of STR profiles recovered, a "success" was defined as those profiles consisting of >13 unique loci, as the more loci that are able to be recovered, the more statistically relevant a resulting match is. The Dabney protocol exhibited high variability of STR loci recovery, which resulted in a relatively low proportion of high-quality profiles (>13 STR loci) recovered (37.04%). Keeping in mind low sample sizes, profiles stemming from the charred soft tissue samples were overall more consistently of high quality than either skeletal extraction method (46.15%). Match quality was generally high (< $1x10^{16}$ probability of random match) in all high-quality profiles, with most mismatched alleles due to allelic dropouts and not missing alleles. Although we expected to see a relatively high amount of dropout due to the low quality and quantity of DNA

templated, we observed relatively low dropout rates across all extraction types and substrates (See Appendix C).

CHAPTER 5

DISCUSSION

As mentioned before, raw DNA recovery is often the first metric used to determine downstream analysis. We see very little difference between extraction type in terms of median raw DNA recovery across different levels of thermal exposure, and continue to see the same trend across all analyses. When observing both raw DNA and endogenous DNA yield, we see two acute points of DNA loss, the first one occurring at ~250-325 ℃ (burn category 2.5) and above 350 ℃ (burn category 3.5+) (Figure 1, 7). These trends are consistent with what has been observed in previous research done on the topic (8). This decrease could indicate that there may be something, either chemical or structural, that severely inhibits the amount of DNA being recovered at burn category 2.5 (~250-325 ℃), with the decrease in recovery >350 ℃ most probably due to the thermal destruction of the DNA itself. Though not assessed in this study, it is possible that these inhibitors could be released by the structural and chemical alteration of proteins and mineralized components of bone, which could interfere with the DNA extraction process itself, or in the downstream processes we used to analyze the extracts.

Tissue was found to be extremely efficient in the extraction of endogenous DNA, especially in lower temperatures (Figure 2) and in the ilium and rib (Figure 8). One working theory on why this may be the case is that the placement/positioning of the individuals on the pyre on which they were burned could have significantly impacted the amount of thermal exposure to each skeletal element. Elements that were better protected

or insulated, such as the ribs and the ilium, could, in theory, exhibit higher amounts of endogenous DNA recovered. We mainly see low levels of endogenous DNA in samples extracted using the Loreille protocol. However, despite this, it had no impact on the quantity or quality of STR profile recovery, again suggesting that inhibition of our downstream analyses may be a factor in the trend.

Across all extraction types and substrates, the same trends in raw and endogenous DNA were also seen in STR loci recovery. For example, a similar dip in performance in both the Loreille and soft tissue samples was observed at ~250-325 ℃ (burn category 2.5) and above 350 ℃ (burn category 3.5+) (Figure 9). This could also be a result of inhibitors preventing the PCR reactions involved in STR profiling from occurring at peak efficiency, resulting in lower amounts of STR loci recovered in these ranges of thermal exposure. Our previous alternative hypothesis was that these inhibitors could be causing a decrease in the amount of total DNA, but seems unlikely considering the quality of STR loci recovery was excellent in the Loreille extracted samples, despite not appearing rich in endogenous DNA. Additionally, the ilium and the rib were the most successful in STR loci recovery, which is a surprise considering these skeletal elements are usually considered poor substrates in other degraded samples, such as archaeological remains.

Overall, all of our high-quality STR profiles matched to their respective references (≥ 90% allelic match percentage), exceeding the generally accepted margin for a positive match in most criminal cases The vast majority of our miscalls appear to come from allelic dropouts. Allelic dropouts are extremely common in degraded samples such

as these, but we do not observe a corresponding elevation in the rate of allelic dropouts as thermal exposure increases (Appendix C).

CHAPTER 6

CONCLUSIONS, SUGGESTED GUIDELINES, AND FUTURE WORK

Overall, it was found that when identifying individuals using thermally degraded samples, it is not as simple as finding a one size fits all protocol. Each burned sample has to be evaluated based on a multitude of different factors (i.e., what kind of bone, how long the bone burned, the temperature of the fire, if tissue is available, etc.) and a case-by-case determination made.

Generally, if there is tissue available it should be used since it was found to be the most efficient substrate for samples exposed to lower temperatures (~200-300 ℃, burn category 2) and ~300-350℃ (burn category 3). As for bone, the best skeletal element to use for thermally altered remains was observed to be the ilium and the rib. Both the Dabney and the Loreille protocols performed similarly across all temperatures for STR loci recovery, so preferences between the two come down to the laboratory skills and equipment at hand, as well as what technical training you have. Although, for samples with minimal input material, the Dabney protocol would be the optimal method.

Based on the amount of inhibition we experienced, more work should be done to figure out what precisely these inhibitors are and ways to diminish them without affecting the endogenous DNA present. Additionally, a higher sample size encompassing a broader selection of skeletal elements across the thermal exposures could be tested to see if the observed trends continue.

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APPENDIX A

SAMPLING DATA

[Consult Attached Files]

APPENDIX B

BONE AND TISSUE PROTOCOLS

[Consult Attached Files]

APPENDIX C

RAW DATA

[Consult Attached Files]