Document Title:	**Optimizing the Analysis of DNA from Burned Bone Using Ancient DNA Techniques**
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Document Number:	**309915**
Date Received:	**December 2024**
Award Number:	**2019-DU-BX-0044**

Project Title:
Optimizing the analysis of DNA from burned bone using ancient DNA techniques.

Award Recipient Organization:
Arizona Board of Regents, on behalf of Arizona State University
P.O. Box 876011, Tempe, AZ 85287-6011

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NIJ Award #: 2019-DU-BX-0044

Project Period (Start Date, End Date): 1/1/2020 – 6/30/2024

Award Amount: $ 783,744.00

1. Project Summary:

1.1 Goals and Objectives

The proposed research tests new genomic methodologies to understand and optimize our capacity to generate DNA profiles using extremely low quantities of DNA. The major goals of this project, as stated in the funded application, are to address the following questions:

1. Does the Dabney et al. (2013) ancient DNA extraction method recover more DNA from bones that have undergone high degrees of charring/burning compared to the Loreille et al. (2010) protocol for both traditional (CODIS) and NGS analyses? Our initial results testing burned bone suggest that it does work better but a larger sample size is needed.
2. Does single-stranded DNA library construction improve our ability to increase the proportion of unique molecules from highly burned samples for subsequent mtDNA and nuclear SNP capture, and, is it more effective than current double-stranded DNA library build protocols?
3. What is the extent of fragmentation and damage in DNA recovered from bones and teeth with different levels of burning, and how does this impact CODIS and NGS results after capture using single- and double-stranded DNA library preparation protocols specifically designed for ancient samples? Most samples from our previous work were from hand and foot bones. For this proposed research, we will collect additional samples from cases in Maricopa County, but we will also expand our sample to include a broad range of skeletal elements from five individuals burned under controlled conditions.

To achieve these goals, we sampled skeletal elements from fire death cases in Maricopa County, investigated by the Maricopa Office of the Medical Examiner (MCOME), and from individuals subjected to controlled burning at the University of Tennessee's Forensic Anthropology Center (FAC) over a three-year period. Samples (n =109) were selected from different skeletal tissues showing different levels of burning. The study was designed to augment our previous research where most samples were obtained from hand and foot bones (Emery et al. 2020, 2022, and 2024). In addition, over the course of the project, we initiated a preliminary study to assess whether the burned tissue residue that was being removed from bone surfaces also contained DNA. DNA analyses of extremely burned remains continue to be a significant challenge. In this technical report, we summarize the preliminary findings, and outcomes achieved during this period, as well as discuss progress towards publication of final results.

1.2 Research Questions:

We assembled a team of experts in forensic anthropology, bioarchaeology, evolutionary genetics, forensic genetics, and ancient DNA in order to address the following aims in our original proposal according to a strict workflow:

I. Examine the success rate of two DNA extraction methods for CODIS STR analysis. We will use the newly developed Promega PowerPlex® Fusion System STR kit. This kit includes all expanded CODIS loci (i.e., 23 autosomal STRs plus amelogenin for sex determination), possesses rapid amplification protocols designed for quick cycling capabilities to

reduce STR processing time. Samples will be characterized into 5 levels of burning according to Schwark et al. (2011), then purified over Roche High Pure Biopurification columns.

1. Does the choice in purification column (e.g., MinElute vs. Roche Biopurification columns) affect the total amount of DNA recovered post-extraction? Preliminary results suggest that Roche High Pure Biopurification columns retain more DNA than conventional Zymo columns (see section 2.3 for preliminary data).

2. What is the success rate of the recovered DNA for standard CODIS and mini-STR analysis given these novel extraction procedures to obtain DNA fragments of varying lengths?

II. Investigate how different methods of extraction, double- and single-stranded DNA library preparation, and targeted enrichment affect DNA recovery for different skeletal elements. All extracts will be converted to DNA libraries using two ancient DNA library protocols (i.e., ssDNA and dsDNA libraries) (Meyer and Kircher 2010; Kircher et al., 2012; Gansauge and Meyer 2013; Gansauge et al., 2017). We will capture complete mtGenomes and genome-wide SNPs from both sets of DNA libraries for total quantification and sequencing.

1. How does a new single-stranded method of DNA library construction compare to double-stranded library preparation methods in average yield and unique reads stemming from thermally damaged tissue?

2. Do these methods impact recovered DNA yields and downstream identification analyses from specific skeletal elements or burn categories?

III. Assess read count and unique reads post-sequencing using NGS computational pipelines designed specifically for degraded samples. Raw reads (in FASTQ file format) will be demultiplexed, trimmed, merged, and mapped using ancient DNA specific pipelines. Mapped and filtered reads will be assessed damage patterns, depth of coverage (the number of times a site is sequenced on average) and data quality for mtDNA and SNP reads (i.e., mapDamage 2.0 and SAMtools) (Jonsson et al. 2013; Li et al., 2009).

1. What is the extent of fragmentation and damage in DNA recovered from different skeletal elements, and how do these variables impact NGS sequencing and SNP data quantity and quality?

Over the course of the project, we made a few minor changes to our specific aims. First, after preliminary testing, we realized that the Roche Biopurification column performed best so we used this column for the remainder of the project. Second, as noted above, we did some preliminary testing to assess preservation of DNA extracted from the burned tissue residue that was being removed from bone surfaces. This consisted of using the Qiagen DNeasy Blood and Tissue kit to extract the DNA, and then quantifying the DNA recovered to compare it to our results from the underlying bone DNA extractions. We also generated STR results from these samples, as there were sufficient reagents left over from the kits after testing the bone sample extracts. Third, we decided to use the Claret Bio SRSLY PICO ssDNA library kit to make our single-stranded DNA libraries given their ease of use. Finally, given the availability of new SNP panels for genome-wide SNP capture, we decided to use the FORCE panel (Darcel Arbor

Biosciences). Tillmar et al. (2021) designed the FORCE panel targeting ~5400 SNPs for use in forensic DNA analysis, and initial testing found ~44% of SNPs could be recovered (10x coverage) from historic bones.

1.3 Summary of Project Design and Methods

Over the course of this project, two DNA extraction protocols were tested for burned bone samples: the complete digest DNA extraction protocol developed by Loreille et al. (2010) and the Velsko et al. (2019) protocol which is a modification of the ancient DNA extraction protocol developed by Dabney et al. (2013). DNA extracts were first used for CODIS STR analyses (using Promega's PowerPlex® Fusion 6C System). They were then converted into two different types of DNA libraries for subsequent Next Generation Sequencing analyses: double-stranded DNA (dsDNA) libraries were made using the Meyer and Kircher (2010) method and single-stranded DNA (ssDNA) libraries were constructed using the SRSLY DNA NGS PicoPlus Library Preparation Kit (ClaretBio). Finally, in-solution hybridization enrichment using two bait sets targeting (1) the complete mitochondrial genome sequence (H. sapiens Representative Global Diversity Panel by Daicel Arbor BioSciences) and (2) a set of approximately 5400 forensically relevant genome-wide SNPs (the FORCE v2 panel, Daicel Arbor BioSciences) were coupled with NGS (Illumina sequencing) to recover mitochondrial genomes and nuclear SNPs informative of identity, ancestry, and phenotypes using both libraries (figure 1).

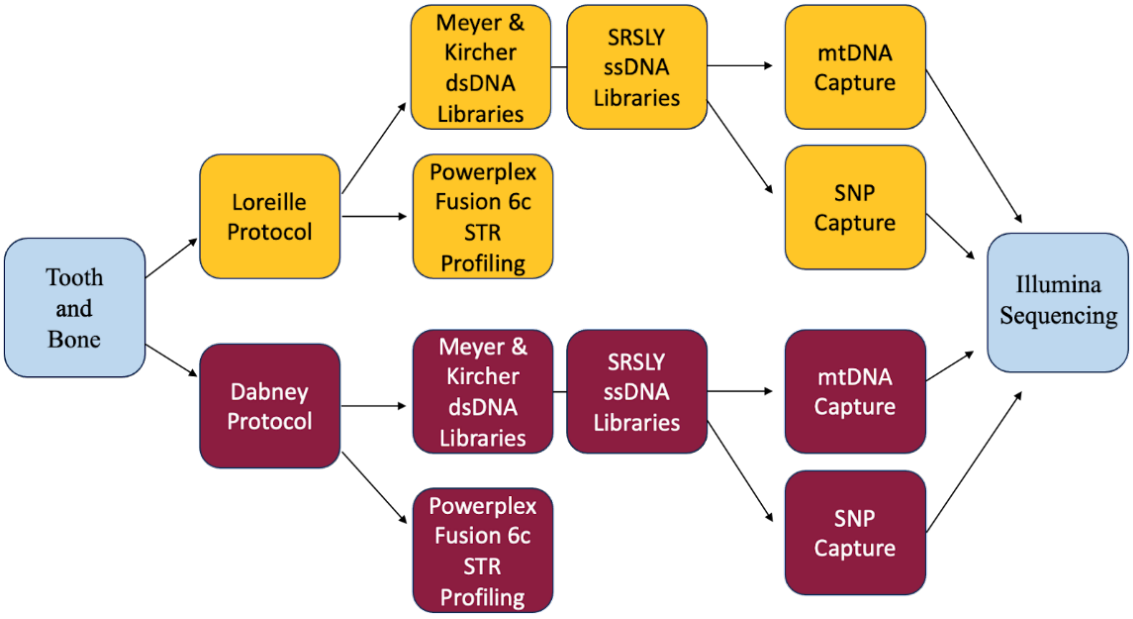

Figure 1: General workflow for each sample.

1.3.1 Samples obtained: The MCOME requested that we contact families to request samples for this research. We agreed upon the protocol for contact and the script for talking to families, and sample collection commenced during the reporting period. However, as the COVID-19 pandemic began soon afterward and fire deaths due to accidents declined substantially, we obtained only a few samples (n =10) from 6 individuals from MCOME. Drs. Giovanna Vidoli

and Joanne Devlin of the University of Tennessee's Forensic Anthropology Center (FAC) supervised the controlled burning of ten donor cadavers, each providing 7-9 distinct skeletal samples from targeted sites across the skeleton (Figure 2, n = 76 distinct skeletal elements total from FAC). Where possible, tissue samples adhering to the bone at or near corresponding cortical bone sampling sites, were also collected, (n = 39). These were used by masters student Amber Coffman to assess the robustness of existing methodological guidelines calling for the removal and discarding of this potential substrate (McKinnon et al. 2021; de Boer et al., 2020; Prinz et al. 2007),. In total, 39 paired bone and tissue samples were analyzed. For the larger study of DNA recovery from bone samples, bone powder from a total of 109 distinct skeletal sampling sites was collected. This resulted in a total of 109 Dabney protocol and 109 Loreille protocol extractions, all of which were processed to generate both single and double-stranded DNA libraries for downstream analysis.

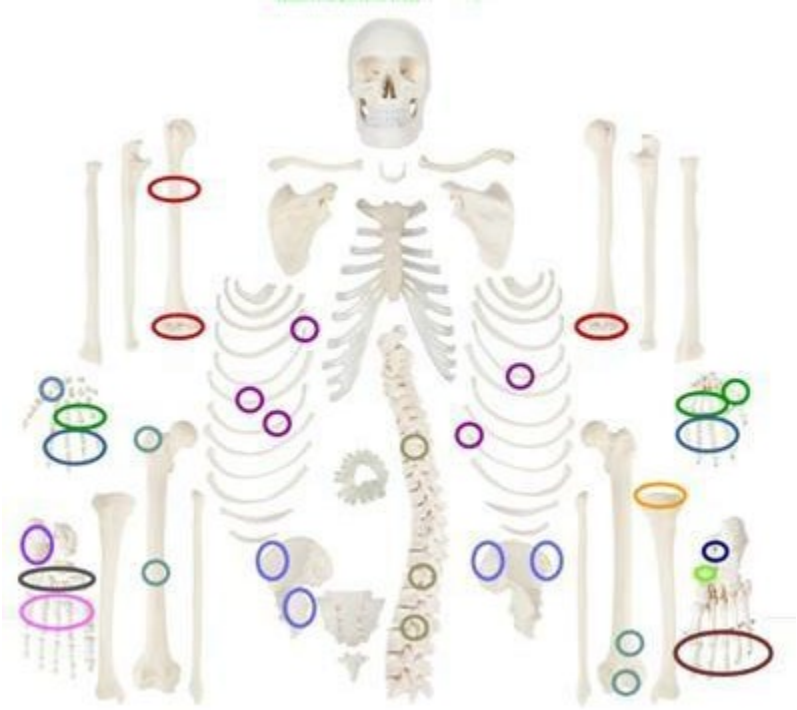

Figure 2. Skeletal elements collected from the controlled burns of 10 donor cadavers. Circles in the diagram indicate approximate locations of sampling from each element.

1.3.2 Controlled burning conditions: At FAC, all donors agreed to participate in post-mortem trauma research, were not autopsied, weighed less than 215 pounds, and were less than 6'1'' in height (See Table 1). All thermal alteration of donor bodies occurs on property owned by the University of Tennessee, Institute of Agriculture. Donor cadavers were placed on a cinder-block pyre with temperature probes placed on the abdomen and in the fire itself (for representative temperature data, see Figure 3) and burned using locally sourced wood for approximately 2-3 hours each until a Glassman-Crow Level 3 charring level is observed (Glassman and Crow 1996) in which the torso is charred but intact and there is limb disarticulation. No accelerant was used on the body and the fire was extinguished by removing the heat source.

Table 1. Donor individual summary data.

Individual	Age	Sex	Weight (pounds)	Burn date	Samples obtained
1	84	Male	152	26-Jan-2021	12
2	56	Male	201	26-Jan-2021	13
3	44	Female	123	2-Mar-2021	10
4	85	Female	163	2-Mar-2021	9
5	73	Female	123	7-May-2021	11
6	74	Female	158	7-May-2021	10
7	67	Male	115	31-May-2022	12
8	75	Female	160	31-March-2022	11
9	79	Male	143	31-May-2022	9
10	75	Male	157	31-March-2022	12

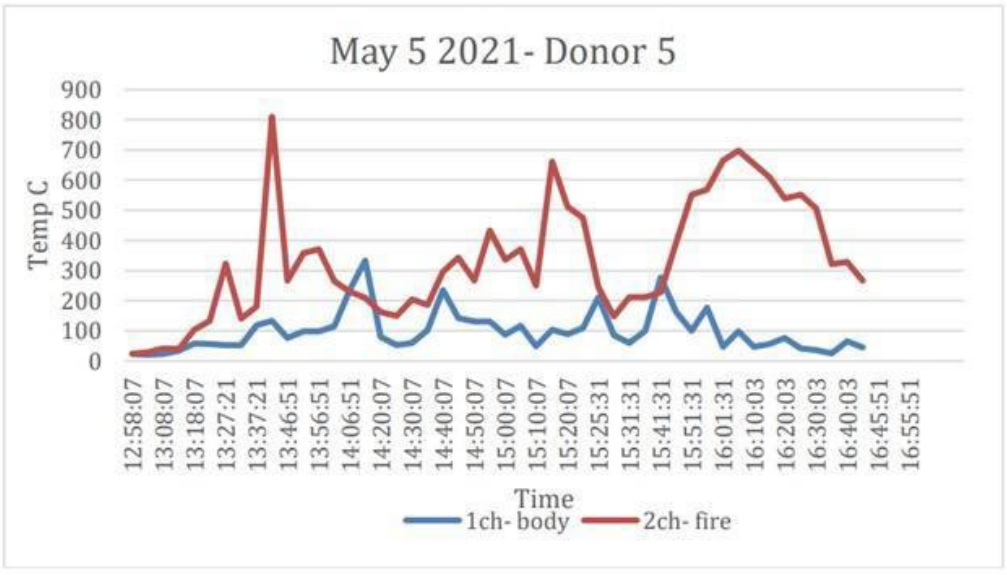

Figure 3. Representative data depicting recorded temperatures from probes within the fire (red) and placed on the donor body (blue) during the thermal alteration of a single donor.

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1.3.3 Burn category determination: A visual examination was used to assess the levels of thermal insult to each sample using the 1-5 scale introduced in Schwark et al., 2011 (level 1 with estimated exposure to temperatures < 200 °C thru level 5 at > 650 °C). In order to maintain consistency, all burn categorization was performed by Dr. Cody Parker (Postdoctoral fellow with the Stone Lab) and Dr. Katelyn Bolhofner (Asst. Professor of Forensic Anthropology, ASU School of Mathematical and Natural Sciences). The burn category of tissue samples was based on the categorization of the bone below it (Table 2).

Table 2: Visual estimation of thermal exposure for sampled locations (n = 109) based on Schwark et al. (2011).

Burn Category	Color	Estimated exposure (˚C)	Example	N
2	Yellow, brown	200 – 300		5
2.5*	↕	250 – 325		17
3	Carbonized black	300 – 350		24
3.5*	↕	350 – 550		40
4	Grey, blue	550 – 600		11
4.5*	↕	600 – 650		11
5	Calcined, white	>650		1
		Total		**109**

1.3.4 Sample preparation and DNA extraction: Each sampling location was first manually de-fleshed before being cleaned using only de-ionized water. In the process of defleshing, a masters' student assisting with this project (Amber Coffman) collected tissue samples for a comparative analysis of DNA preservation in the adhering tissue (n = 39 paired bone and carbonized soft-tissue samples). After cleaning, approximately one gram of bone was converted to a single homogenized bone powder stock by a mixer-mill for use in subsequent DNA extractions (both Dabney and Lorielle protocols) for each sampling location. DNA extractions were performed in the Stone Lab Ancient DNA laboratory on the ASU Tempe campus to minimize the potential contamination with exogenous DNA. From each bone sample powder stock (n =109), DNA extraction was performed using both the standard Lorielle method (the Lorielle et al. 2010 protocol currently in use by federal and international organizations such as the FBI and ICMP), and the most current and widespread ancient DNA protocol that Velsko et al., (2020) modified from the Dabney and colleagues method (Dabney et al., 2013; Dabney et al., 2019). The latter has been optimized to retain highly degraded, ultrashort DNA fragments from poorly preserved archaeological samples. For the Lorielle protocol, 250 ± 10 mg of bone powder was first demineralized in an EDTA-sarcosyl-proteinase K buffer for 16 hours 56 °C, with the resulting lysate then concentrated using Amicon Ultra-15ml centrifugal filter columns. DNA purification and isolation were then achieved using the Qiagen MinElute DNA purification kit for each sample. For the Modified Dabney protocol, 50 ± 2 mg of bone powder was demineralized in an EDTA-proteinase K buffer for 36 hours at 25 °C. DNA purification and isolation from the resulting lysate are then performed using a modified version of the Roche High-Pure biopurification kit. DNA isolation from comparative tissue samples was performed using the

standard Qiagen DNEasy Blood and Tissue Kit protocols. All extracts were then quantified using the Qubit 4.0 fluorometer and the Agilent 4200 TapeStation.

1.3.5 STR analyses: For each sample, 30 uL of forensic and ancient DNA extract was sent to the Kanthaswamy DNA Laboratory at ASU's West Campus for Quantifiler Trio DNA Quantification (Applied Biosystems) and STR analysis in July and October, 2020. STR analysis of the Dabney and Loreille DNA extracts was conducted for a subset of the samples using Promega's PowerPlex® Fusion 6C Systems. This STR kit analyzes 27 markers, including the sex-typing locus. Our analytical thresholds are in accordance with NIST's recommendations which are a minimum detection threshold of 50 relative fluorescence units (RFUs), a peak height ratio (PHR) of 60%, and a stutter threshold of 15%. STR analyses were completed for 36 samples by the Kanthaswamy laboratory. Unfortunately, the laboratory did not complete the analysis of the remaining samples, and these were retrieved from Dr. Kanthraswamy's laboratory on October 27, 2023. The sample retrieval and removal of Dr. Kanthaswamy as a Co-PI on this project was recommended by the ASU Office of Research Integrity and Assurance after he refused to return the raw data files from previous analyses. After subsequent discussion, we have now received those data (as of June, 2024). We then collaborated with Dr. Mitchell Holland, Pennsylvania State University who completed the STR analyses for this project. For the remaining samples, we used the Quantifiler Trio DNA Quantification Kit (Applied Biosystems) to assess extraction DNA quantity and quality. The remaining samples were then sent to PSU for STR analysis using Promega's PowerPlex® Fusion 6C System which was completed in March 2024.

1.3.6. DNA Library Preparation, Enrichment, and Sequencing: Double-stranded DNA library construction used a modified Meyer and Kircher (2010) method. These were indexed using unique primer combinations synthesized by Integrated DNA Technologies (IDT). Single-stranded DNA library construction was performed using the SRSLY DNA NGS Library Preparation Kit from ClaretBio. Library enrichment for mtDNA genome sequences used the mitochondrial RNA baits synthesized from the H. sapiens Representative Global Diversity Panel (197 mtDNA sequences), and enrichment for genome-wide SNPs used the FORensic Capture Enrichment (FORCE) panel (V2), which targets 5402 forensically relevant/diagnostic SNPs (Arbor Biosciences, Ann Arbor, MI) in accordance with myBaits Manual v.4.01 guidelines. Enriched libraries were measured on a 7900HT Fast Real-Time PCR System using IS5_longamp and IS6_longamp primers and quantified against a PhiX standard serially diluted from 100 pM to 62.5 fM. All libraries were pooled at equimolar concentrations. These enriched libraries were then sequenced at Admera Health using the Illumina Novaseq platform.

1.3.7. Bioinformatic Analysis: The mtDNA sequence data were processed using the nf-core/eager bioinformatics pipeline (Fellows Yates et al. 2021) for quality control, read mapping statistics and DNA damage analysis. Mitochondrial haplogroup assignment was done using a combination of Haplogrep (Kloss-Brandstatter et al. 2011) and Empop (Parson et al. 2014). SNP profiling and variant calling are being queried using NCBI's dbSNP database together with their chromosome positions, and REF/ALT alleles. These data are subsequently being used to build 3 reference VCF, BED, and eigenstrat files for SNP enriched library filtering via the nf-core/eager pipeline (Fellows Yates et al. 2021) and bcftools (Danecek et al. 2021). These bioinformatic analyses are still in progress.

1.4 Summary of Results

1.4.1: Quantification results: In preliminary analyses of samples from eight donor individuals burned at the FAC, we investigated the overall efficiency of DNA extraction techniques and substrates across levels of thermal exposure (ng of DNA recovered per mg of input material). We observed higher overall extraction efficiency from charred tissue samples followed by that from bone extracted using the Dabney protocol (Figure 4). We also observed a significant increase in efficiency for DNA recovery from those samples in category 3 (i.e., exposed to temperatures estimated between 300-350 °C) compared with samples in category 2.5. When examining extraction efficiency across skeletal elements with paired sample sizes of $n > 3$, we see DNA extraction efficiency greatest in Dabney extracted bone samples and corresponding soft tissue samples stemming from the ribs (Figure 5). Several caveats should be noted about these preliminary quantification analyses: First higher burn categories (categories 4-5) were not included in downstream analyses of paired bone and tissue since tissue was generally not preserved at these levels of thermal exposure and was only recovered in 3 cases where the burn category ≥ 4. In addition, these analyses only included 5 paired bone and tissue samples (15 extractions) that fell into category 2.5.

Fragment size analysis of the different DNA extractions (from all ten individuals) using the TapeStation 4200 does not show a clear trend across burn categories suggesting that the DNA doesn't fragment steadily as temperature increases, but instead degrades acutely as temperatures rise to $\gtrsim$ 550-600ºC, resulting in very poor DNA preservation above burn category 3.5 (Figure 6). Additionally, analyses of potential fragment length differences between skeletal elements and/or burn category show no statistical trend ($p < 0.05$, data not shown).

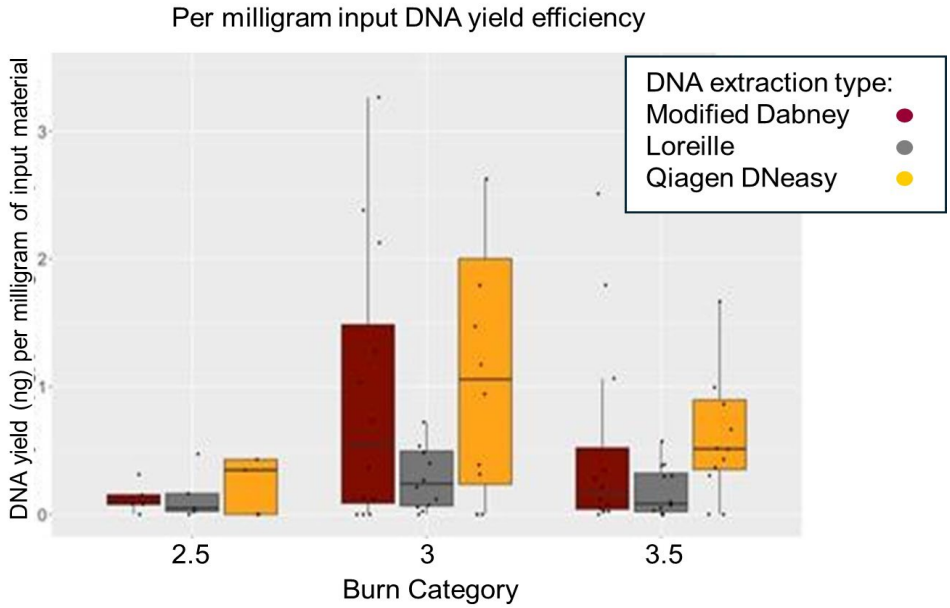

Figure 4: Comparison of DNA extraction efficiency observed in burned bone (extracted using Dabney and Loreille protocols) and corresponding charred soft tissue samples across estimated levels of thermal exposure (with paired sample size of $n > 3$). All samples were quantified using a Qubit 4 fluorometer.

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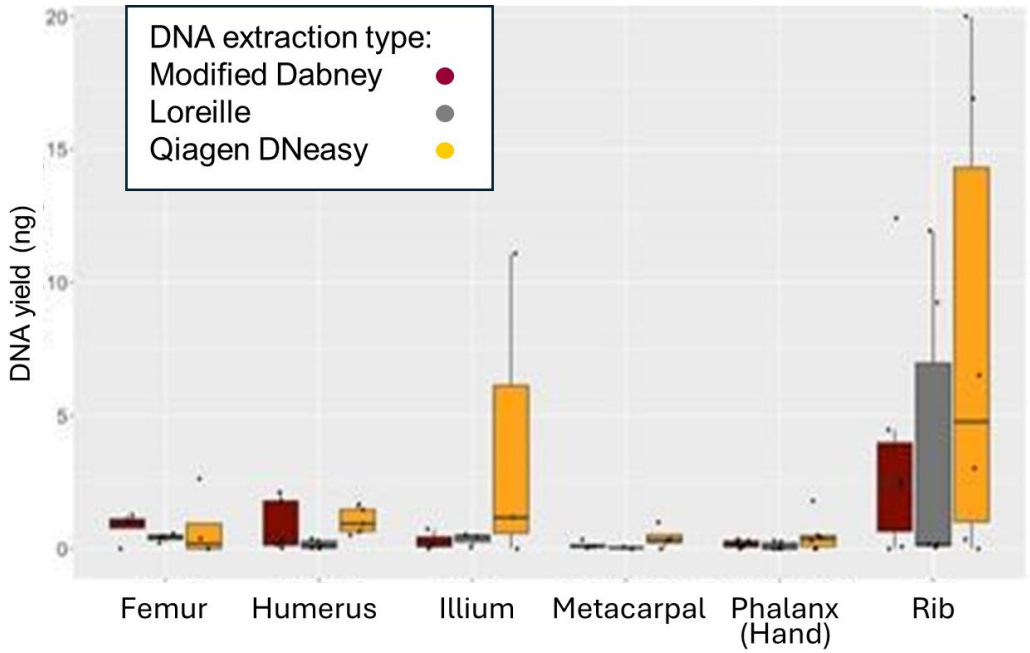

Figure 5: Comparison of DNA extraction efficiency observed in burned bone (extracted using Dabney and Loreille protocols) and corresponding charred soft tissue samples across all recovered skeletal elements with sample sizes of N > 3 as reported using a Qubit 4 fluorometer.

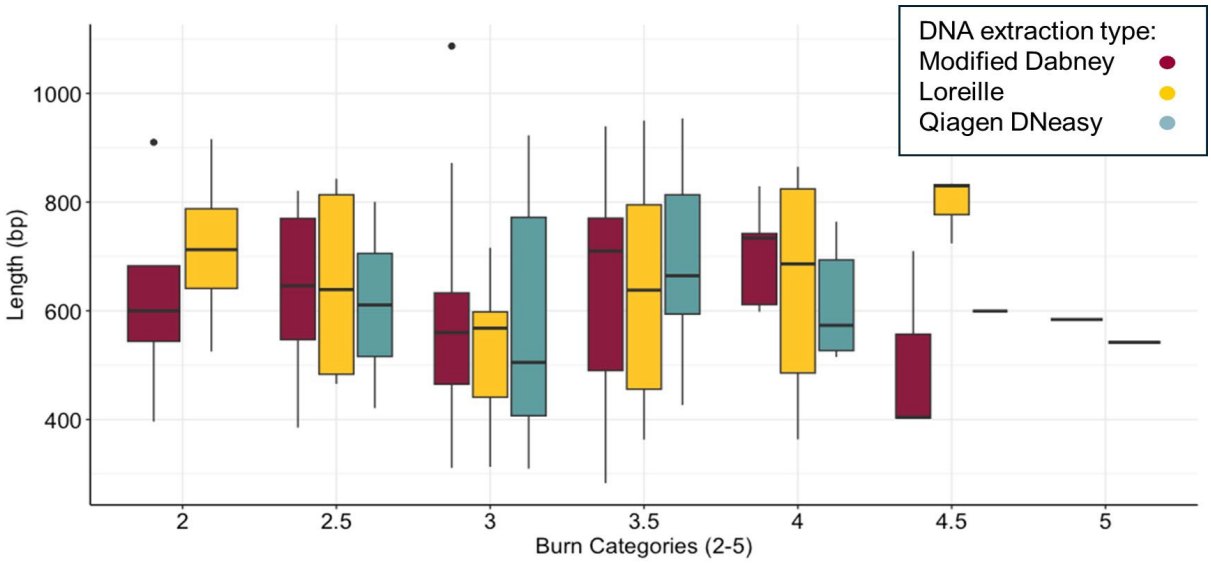

Figure 6: Fragment length results produced using the TapeStation 4200 by extraction type and burn category.

A subset of samples was assayed using the NanoDrop spectrophotometer and showed indications of high levels of contamination with organic compounds (absorption ratio at 260/280 ≤ 1.8, Figure 7) and salts and phenolic compounds (absorption ratio 260/230 < 2, Figure 8). While this inhibition affected our DNA quantification assays of extracts, the effects on STR recovery was

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less evident (see STR results below). Further research is needed to assess what types of compounds are produced during the burning process that may affect DNA recovery.

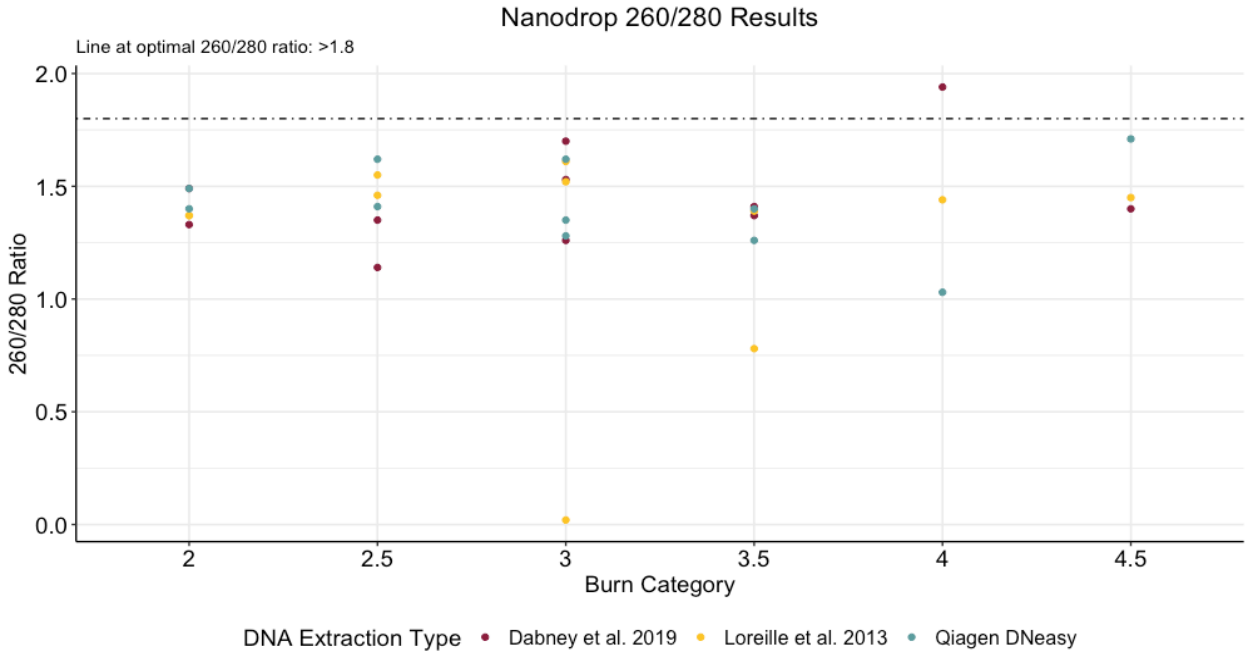

Figure 7: NanoDrop spectrophotometer results showing high levels of contamination with organic compounds (absorption ratio at 260/280 ≤ 1.8).

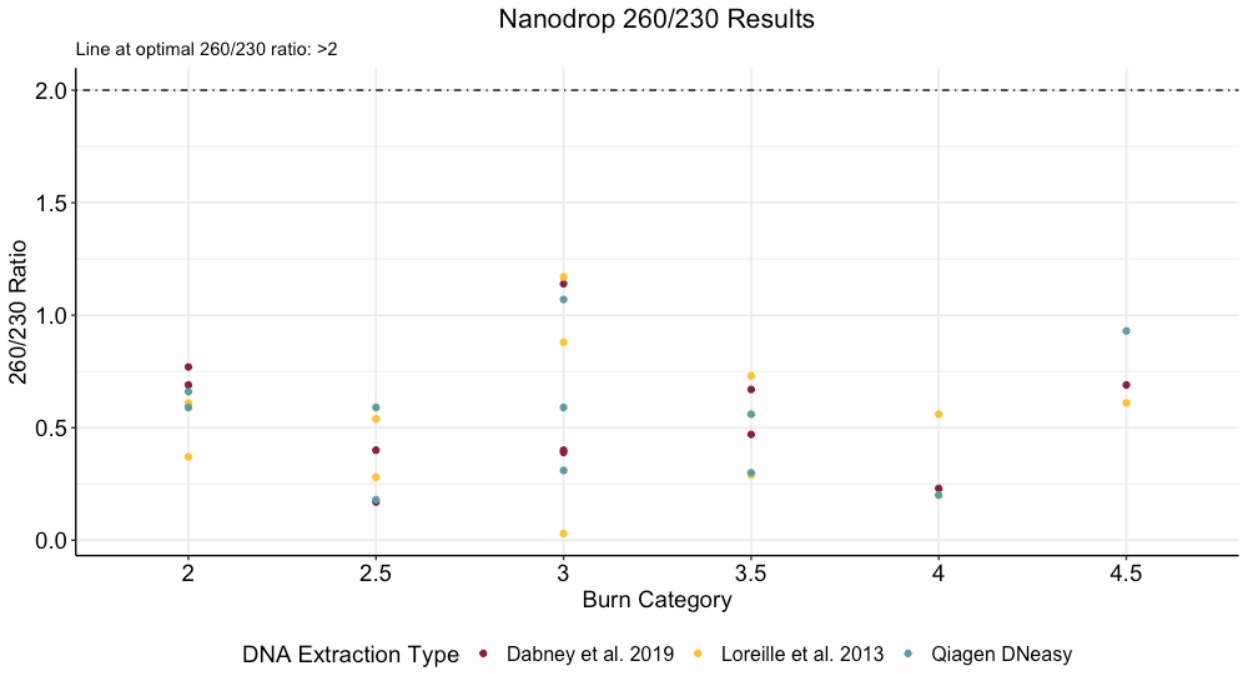

Figure 8: NanoDrop spectrophotometer results showing high levels of contamination with salts and phenolic compounds (absorption ratio 260/230 < 2).

Our preliminary results from 47 tissue samples and the corresponding (i.e., underlying) bone samples from all ten individuals also suggest that, when burned tissue is available, the amount of endogenous DNA recovered (as determined using the Quanifiler Trio) is equal to or greater than that recovered from bone (Figure 9) across burn categories. Note that while all burn categories are shown, tissue samples were not recovered from burn category 5. We also observe a significantly higher average DNA recovery in those samples from category 3 exposed to temperatures of approximately 300-350 °C, regardless of extraction type or substrate compared with burn category 2.5. We hypothesize that this may be the result of organic compounds that are produced during the burning process that inhibit DNA recovery and/or quantification in category 2.5, and that these compounds have burned off from samples in burn category 3. We also found that flat and irregular bones and overlying tissue produced the highest amounts of endogenous DNA (Figure 10).

In statistical tests assessing donor characteristics and DNA recovery characteristics (fragment length and endogenous DNA), we find that both the amount of endogenous DNA and the average fragment length recovered are significantly associated with the weight of the individual ($p = 0.004743$ and 0.008116, respectively), but not with sex or age (Table 3).

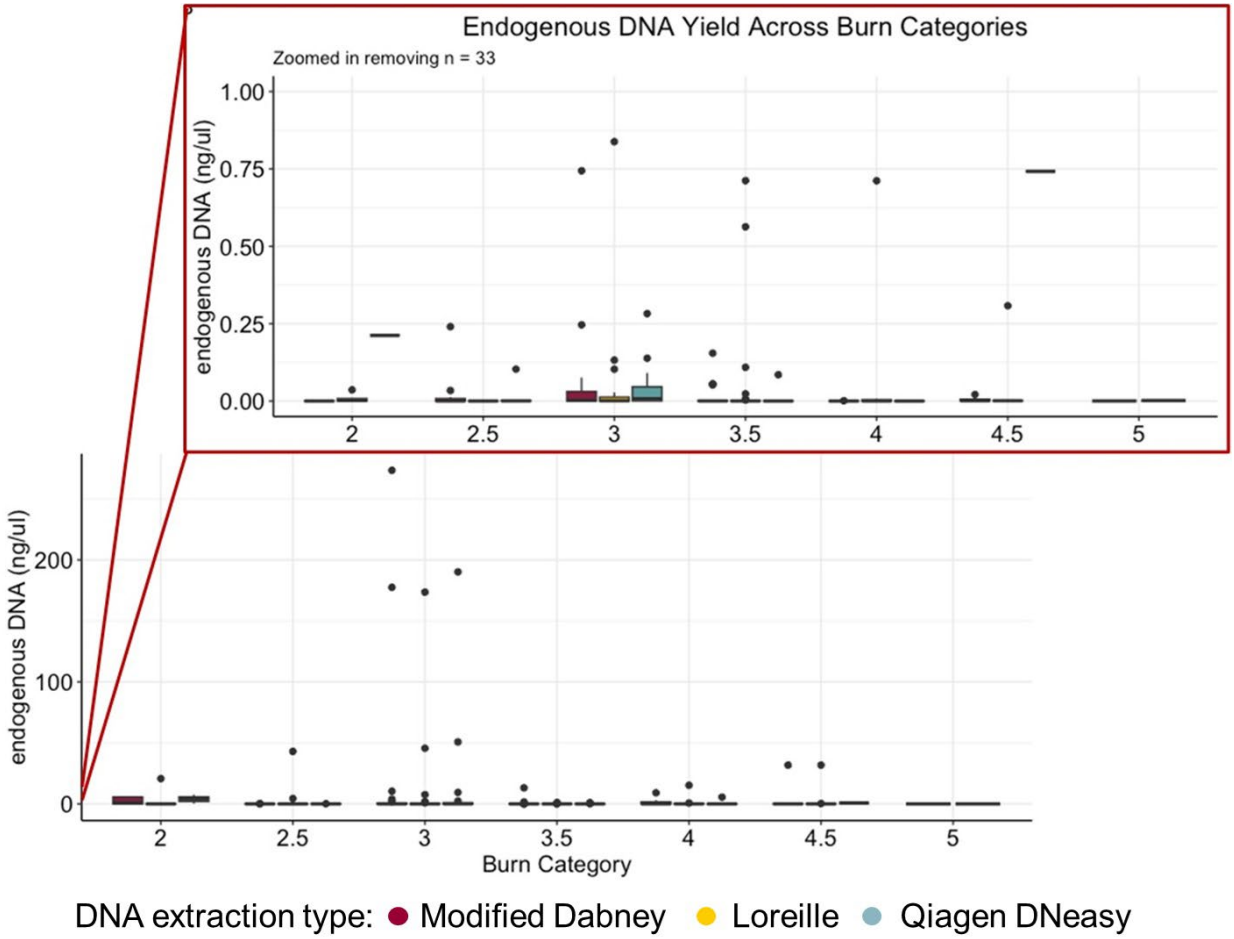

Figure 9: Comparison of DNA recovery observed from thermally altered bone (extracted using the Dabney and Loreille protocols) and corresponding charred soft tissue samples (extracted using the Qiagen DNEasy blood and tissue kit protocol) across burn categories using the

Quantifiler Trio kit. All levels of thermal exposure had a paired sample size of n > 3, except that there were no tissue samples from burn category 5.

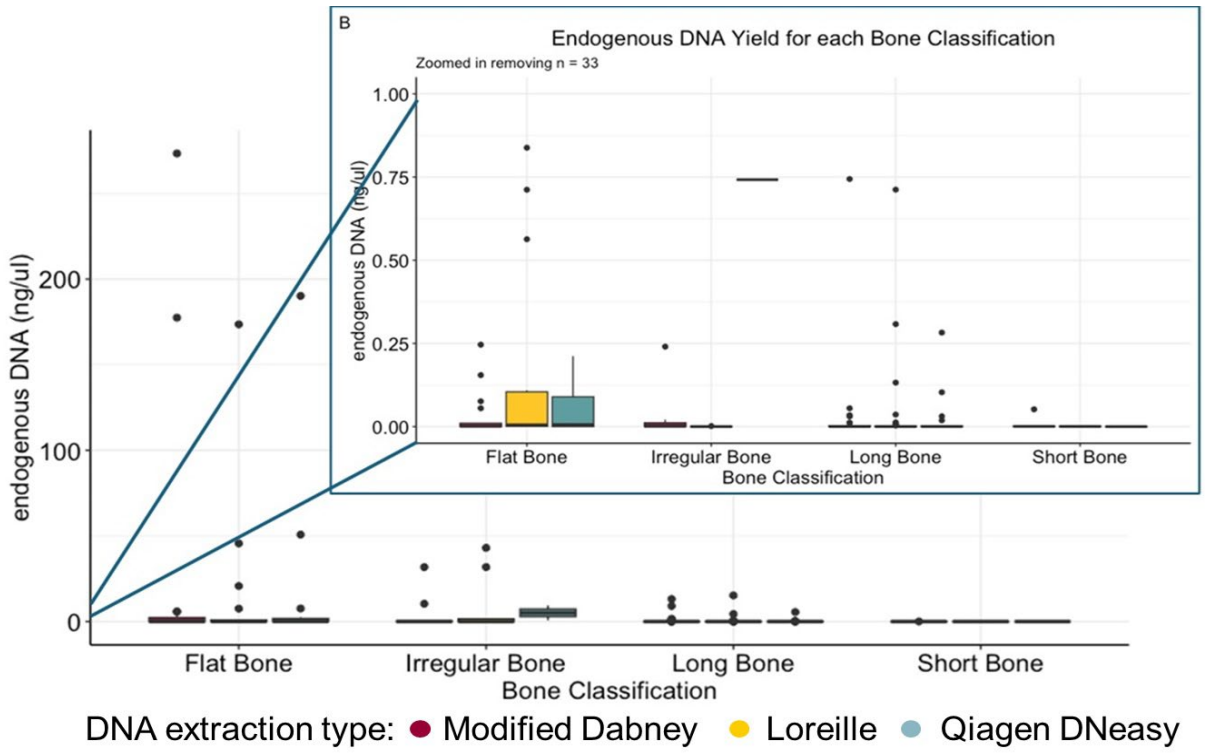

Figure 10. Per extraction type (Dabney and Lorielle protocols for bone and Qiagen DNEasy blood and tissue kit for corresponding charred soft-tissue) endogenous DNA yields across skeletal elements as reported by the Quantifiler Trio kit.

Table 3: Statistical assessments of demographic variables of donors and endogenous DNA recovery or DNA fragment length. Weight and age were assessed using a Spearman correlation text while biological sex was assessed with a two-sample T test.

Response Variable	Demographic Variable	Test	Correlation	p-value
Endogenous DNA	Sex	Two-sample t-test	0.0811	0.4119
Endogenous DNA	Age	Spearman correlation	0.0794	0.4923
Endogenous DNA	Weight	Spearman correlation	0.3186	*0.004743*
Fragment length	Sex	Two-sample t-test	0.0657	0.6069
Fragment length	Age	Spearman correlation	-0.1003	0.4265
Fragment length	Weight	Spearman correlation	-0.3257	*0.008116*

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1.4.2: STR results: DNA extraction success is often defined as those extractions yielding sufficient DNA concentrations to meet the suggested minimum input parameters of the PowerPlex Fusion 6C STR kit (> 250pg/ul) and resulting in STR profiles. We did not observe a clear linear correlation between Quantifiler Trio results and STR recovery from these samples (Figures 11 and 12), possibly due to the aforementioned presence of co-extracted inhibitors. We did, however, observe a strong positive correlation between the observed recovery of any DNA (regardless of quantification method) and number of STR loci recovered, further reinforcing that co-extracted inhibitors are impacting our assays.

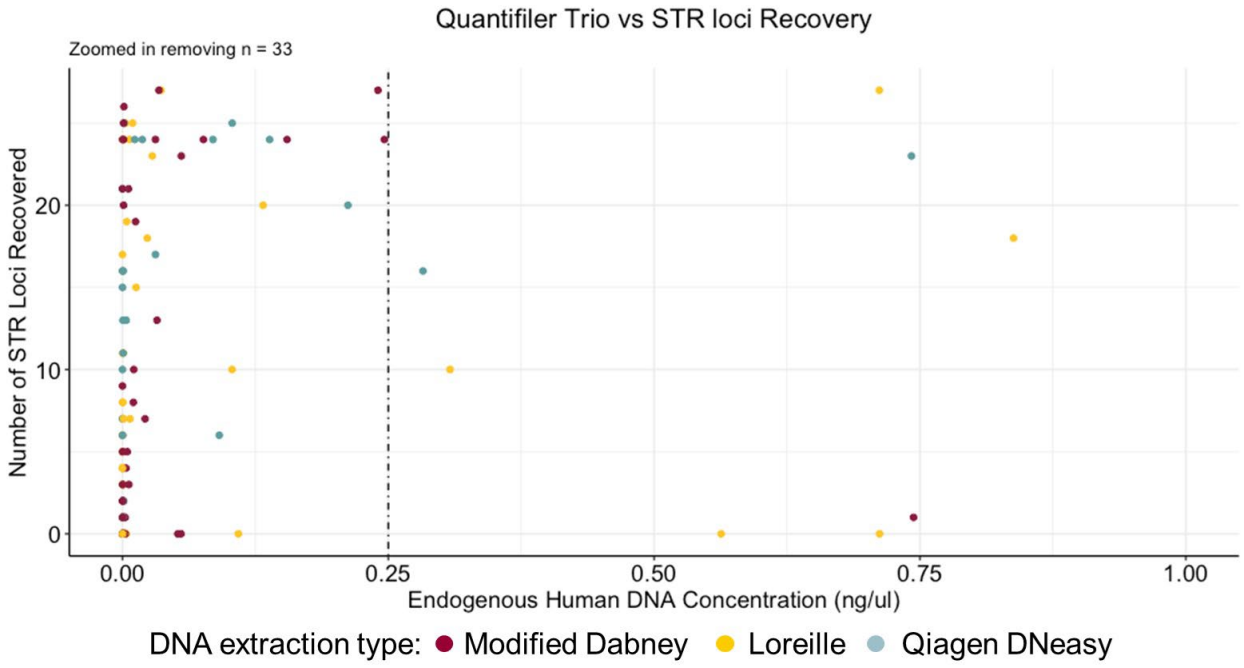

Figure 11: Comparison of the endogenous DNA concentration for each extract with the number of STR loci recovered for samples with more than 1 ng/ul of DNA (n = 186).

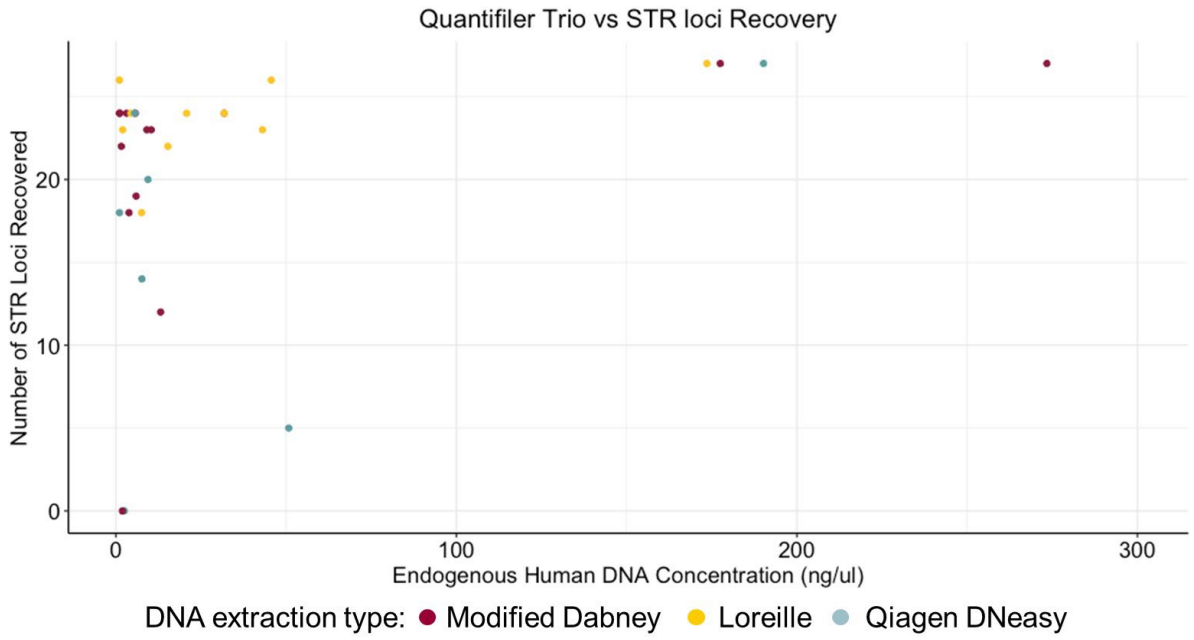

Figure 12: Comparison of the endogenous DNA concentration for each extract with the number of STR loci recovered for samples with more than 1 ng/ul of DNA (n = 33). The dotted line shows the recommended minimum concentration for the PowePlex Fusion 6C STR analysis.

In our research, we find that we were able to recover high-quality STR profiles (profiles where at least 13 core CODIS loci are recovered) from charred tissue samples more reliably than the corresponding bone tissues in all levels of estimated thermal exposure. However, this result was not statistically significant (STR recovery mean: Dabney = 8.40, Loreille = 7.85, and Tissue = 10.47 loci). We also see a general decline in recovery with increasing burn class except in category 2.5 (estimated exposure of 250-325°C), where, as previously noted, we suspect high levels of co-extracted inhibitors in our DNA extracts (Figure 13). We also find that these charred tissue samples perform best from irregular bone elements though the sample size is limited (n =2). Overall we find that extractions from long and flat classes (6.9 and 12.9 loci recovered on average, respectively) were significantly better than those from the short bone class (1.4 loci recovered on average) (Figure 14).

STR locus recovery varied substantially across individuals (Figure 15) and was correlated with body mass (Table 3). When examining the success rate for the recovery of individual STR loci, like other investigators, we note the pattern of greater success with shorter loci regardless of DNA extraction type or substrate (Figure 16). From these data, we can confidently state that current guidelines calling for the removal of charred tissue from severely burned samples should be re-evaluated, as these samples have proved to be highly informative in this study.

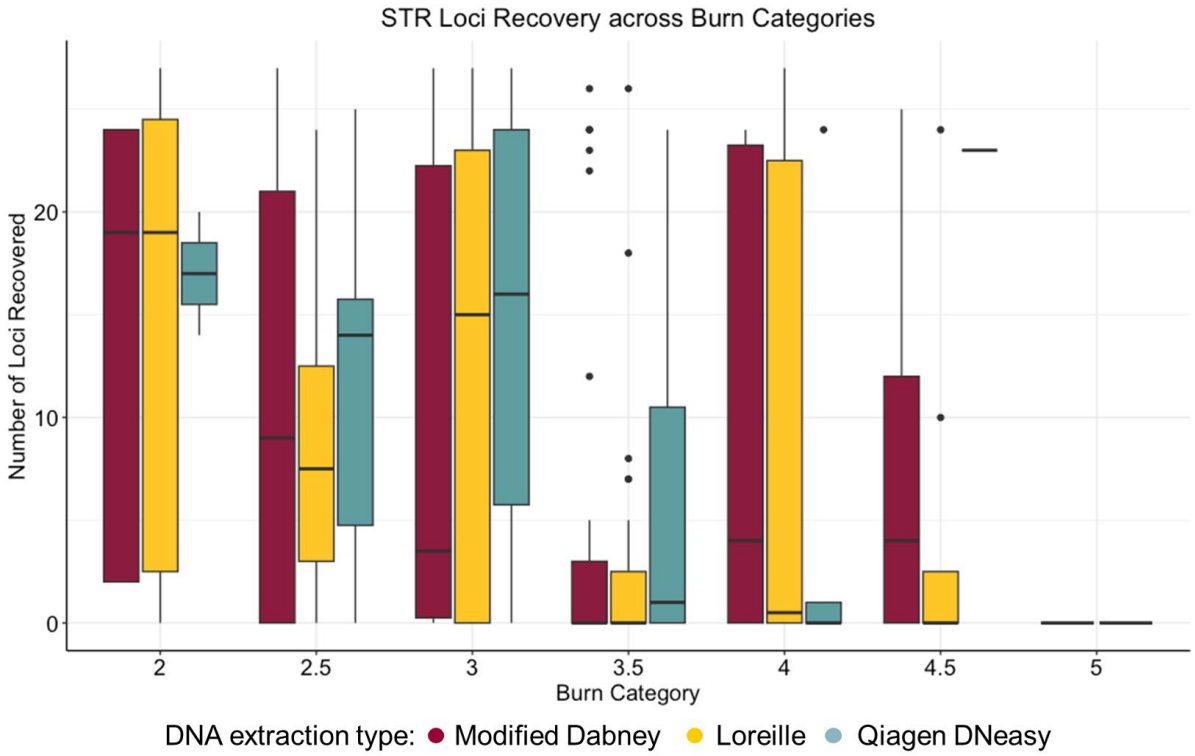

Figure 13: Comparison of the number of PowePlex Fusion 6C STR loci recovered by extraction type across burn categories.

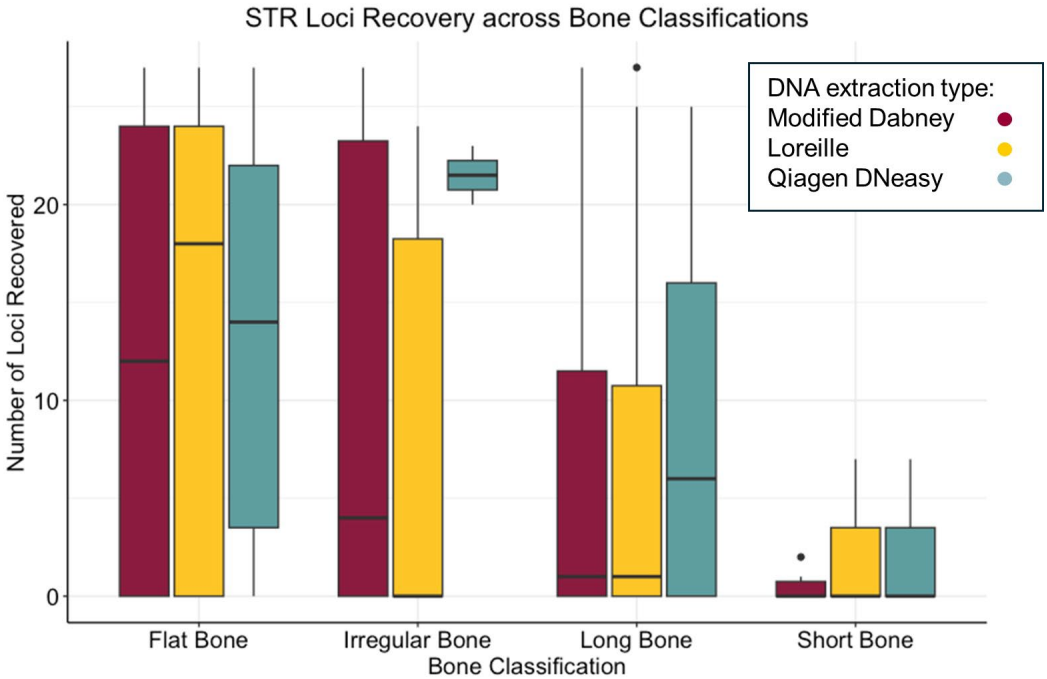

Figure 14: Comparison of the number of PowerPlex Fusion 6C STR loci recovered by extraction type across skeletal element class.

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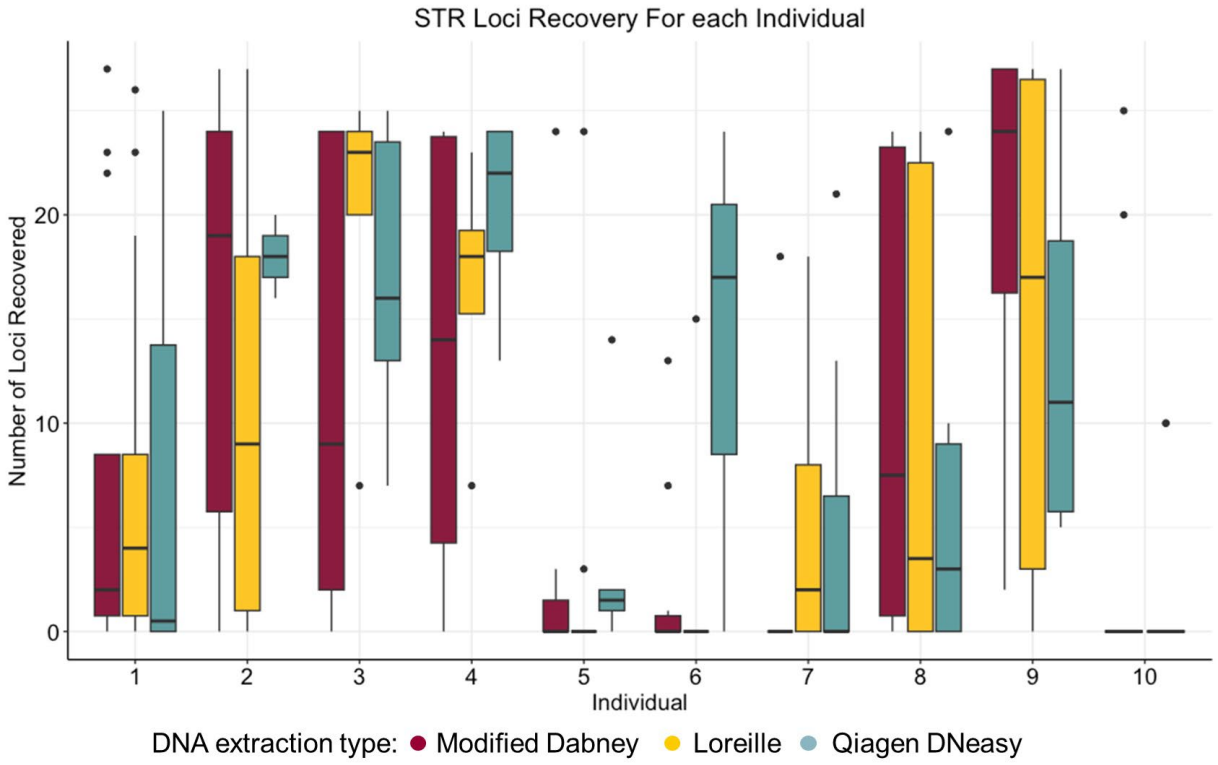

Figure 15: Number of PowePlex Fusion 6C STR loci recovered from skeletal elements and tissues by extraction type per individual.

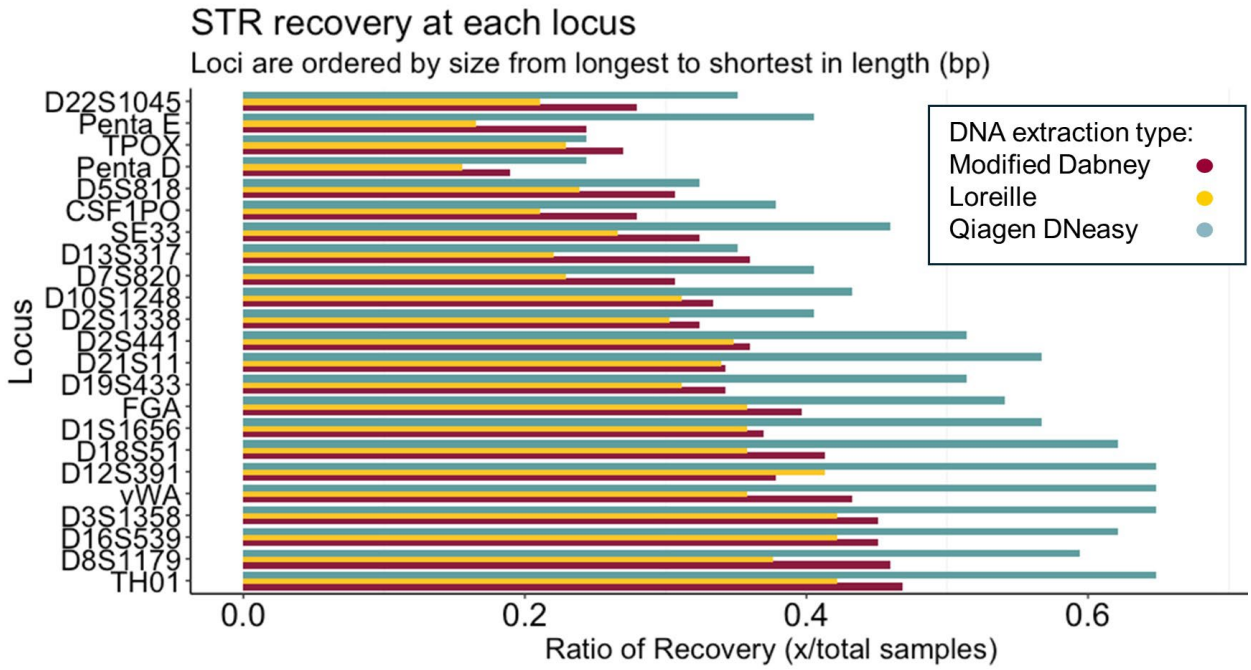

Figure 16: Proportion of Powerplex Fusion 6C autosomal loci recovered by extraction type with loci ordered by length (longest in bp at the top to shortest in bp at the bottom).

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1.4.3: Library comparisons and enrichment results: Bioinformatic and statistical analyses of the mtDNA and SNP enrichment sequence data as well as of the comparisons between ssDNA and dsDNA library methods are currently underway. This work was slowed by the COVID global pandemic and lab construction delays. We were unable to work in the lab and experienced a materials shortage (e.g., long back-orders of pipette tips and microcentrifuge tubes) for an extended period at the height of the pandemic. Following that, the building our lab was housed in went under construction, causing us to relocate, and this kept us out of the lab for almost 1 year. As a result, though all wet lab work is complete, data analyses are ongoing.

1.5 Applicability to Criminal Justice

The DNA identification of remains which have been heavily degraded by exposure to high temperature (e.g., fire), such as found in the contexts of disaster (natural or otherwise, e.g., forest fire), house or car fires, and attempts to conceal criminal activity can be very challenging. This research seeks to help quantify the effects of fire on DNA degradation, DNA recovery, and potential methodological advances which may help mitigate challenges associated with the resulting generation of genetic profiles suitable for forensic identification. Our findings show that, when available, charred tissue yields sufficient DNA for identification at levels comparable to or better than skeletal tissue. At higher temperatures or where tissue is not available, skeletal elements that have higher mass of tissue surrounding them are likely to have better molecular preservation. We also find that there is no clear linear correlation between DNA quantification results and recovery of STRs, and thus, detection of any DNA appears to justify the continued processing of even poorly quantifying samples in the attempt to retrieve STR or other genetic data successfully. Finally, we note that severe DNA inhibition affects assessment of DNA yield from extracts, particularly at burn category 2.5, and that there is a significant decrease in data recovery after burn level 3. Future research should work to analyze and identify these inhibitors in order to develop methods to counter them. Finally, preliminary analyses of average fragment length distribution suggest that, as overall DNA fragmentation does not appear to be one of the underlying challenges associated with DNA identification of severely burned remains, shearing techniques may be useful prior to library construction to maximize the effectiveness of NGS library construction. In sum, while burned forensic contexts are challenging for DNA identification, our results show that with continuing research, we can optimize our capacity to generate useful genetic information from these severely degraded samples.

2. Products:

2.1 List of all scholarly products

2.1.1 Publications: Because of the COVID pandemic and laboratory move, our results are not yet published. A manuscript reporting the results of STR and quantification analyses performed for this project is in preparation for submission by October 1, 2024 to the *Journal of Forensic Science*. Subsequently, the manuscript(s) reporting the results of mtDNA and SNP analyses as well as the single- vs. double-stranded library comparisons will be undertaken.

2.1.2. Datasets generated: The data generated over the course of this project are from donor individuals either from the FAC or from the MCOME, and thus, the raw data are the property of those organizations. Any requests for data will be evaluated by them.

2.2 List of all dissemination activities

2.2.1.Conference presentations:

Parker C, Coffman A, Rawls E, Rohrlach A.B., Bolhofner KL, Vidoli G, Devlin J, Stone AC. (2024) The recovery and analysis of DNA from burned bone and tissue. Poster presentation at the International Society of Forensic Genetics conference, Santiago de Compostela, Spain.

Parker C, Coffman A, Rawls E, Rohrlach A.B., Bolhofner KL, Vidoli G, Devlin J, and Stone AC. (2024) The recovery of DNA from burned forensic contexts. Presentation at the Society for Molecular Biology and Evolution Meetings, Puerto Vallarta, Mexico.

Parker C, Rawls E, Coffman A, Bolhofner KL, Fulginiti L, Vidoli G, Devlin J, Oldt R, Kanthaswamy S, and Stone AC. (2024) The Application of Ancient DNA Methodologies to Badly Burned Forensic Samples and Their Potential to Aid in the Identification and Analysis of Difficult Samples. Presentation at the American Academy of Forensic Sciences Meeting, Denver, CO.

Parker C, Ralls E, Bolhofner KL, Fulginiti L, Vidoli G, Devlin J, Kanthaswamy S, and Stone AC. (2023) Application of ancient DNA methodologies to badly burned forensic samples and their potential to aid in the identification and analyses of difficult samples. Presentation at the American Academy of Forensic Sciences conference, Orlando, FL.

Parker C, Rawls E, Coffman A, Emery M, Bolhofner KL, Fulginiti L, Oldt R, Kanthaswamy S, Vidoli G, Devlin J, and Stone AC. (2023) Adapting laboratory techniques developed for the extraction and analyses of ancient DNA for use in the identification of burned forensic remains. Poster presentation at the American Association of Biological Anthropologists Conference, Reno, NV.

Parker C, Emery M, Bolhofner KL, Ghafoor S, Wissler A, Rawls E, Winingear S, Oldt R, Kanthaswamy S, Buikstra JE, Vidoli G, Devlin J, Fulginiti L, and Stone AC. (2022) Evaluating the use of ancient DNA laboratory protocols in the downstream DNA identification of burned forensic remains. Presentation at the International Society of Forensic Genetics conference, Washington DC.

Parker C, Rawls E, Bolhofner KL, Fulginiti L, Vidoli G, Devlin J, Kanthaswamy S, and Stone AC. (2022) Application of ancient DNA methodologies to badly burned forensic samples and their potential to aid in the identification and analyses of difficult samples. Presentation at the American Academy of Forensic Sciences meetings, Seattle, WA.

Parker C, Emery MV, Bolhofner KL, Ghafoor S, Rawls E, Winingear S, Oldt R, Kanthaswamy S, Buikstra JE, Vidoli G., Devlin J., Fulginiti L, and Stone AC. (2022) Evaluating the use of ancient DNA laboratory protocols in the downstream DNA identification of burned forensically-

derived samples, Poster presentation at the International Society of Applied Biology, Dubrovnik, Croatia.

2.2.2 Webinars, workshops:

Workshop: W23: The impact of burning on skeletal and DNA evidence at the American Academy of Forensic Sciences Meeting, Denver, CO. Presentations by Parker C, Rawls E, Bolhofner KL, Fulginiti L, Vidoli G, Devlin J, and Stone AC. We had approximately 105 attendees in the workshop which lasted from 1:00-4:30 pm on Tuesday, February 20, 2024. The Program Description stated: In this workshop, attendees will learn how to characterize levels of burning in bone. In addition, they will gain a deeper understanding of laboratory methods that can optimize DNA recovery. The educational objectives of the workshops were: After attending this workshop, attendees should be able to: (1) describe methods used to identify the extent of burning in bone; (2) understand the different extraction methods applied to burned and highly degraded skeletal tissues, and (3) evaluate which downstream analyses are likely to produce useful data for case identification.

2.2.3. General press, podcasts, and other media:

Essay for the Conversation:
Stone AC (2023) Identifying fire victims through DNA analysis can be challenging − a geneticist explains what forensics is learning from archaeology. The Conversation, August 18, 2023, https://theconversation.com/identifying-fire-victims-through-dna-analysis-can-be-challenging-a-geneticist-explains-what-forensics-is-learning-from-archaeology-211589

3.0 References

Dabney J, Knapp M, Glocke I, Gansauge MT, Weihmann A, Nickel B, Valdiosera C, Garcia N, Paabo S, Arsuaga JL et al. . 2013. Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proc Natl Acad Sci U S A 110(39):15758-15763.

Dabney J, and Meyer M. 2019. Extraction of Highly Degraded DNA from Ancient Bones and Teeth. Methods in molecular biology 1963:25-29.

Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A, Keane T, McCarthy SA, Davies RM, Li H. Twelve years of SAMtools and BCFtools. Gigascience. 2021 Feb 16;10(2):giab008. doi: 10.1093/gigascience/giab008.

de Boer HH, Maat GJR, Kadarmo DA, Widodo PT, Kloosterman AD, and Kal AJ. 2018. DNA identification of human remains in Disaster Victim Identification (DVI): An efficient sampling method for muscle, bone, bone marrow and teeth. Forensic science international 289:253-259.

Emery MV, Bolhofner K, Spake L, Ghafoor S, Versoza CJ, Rawls E, Winingear S, Buikstra JE, Loreille O, Fulginiti LC, and Stone AC. 2024. Targeted enrichment of whole-genome SNPs from

highly burned skeletal remains. Journal of Forensic Sciences. Feb. 28, doi: 10.1111/1556-4029.15482

Emery MV, Bolhofner K, Ghafoor S, Winingear S, Buikstra JE, Fulginiti LC, and Stone AC. 2022. Whole mitochondrial genomes assembled from thermally altered forensic bones and teeth. Forensic science international Genetics 56:102610.

Emery MV, Bolhofner K, Winingear S, Oldt R, Montes M, Kanthaswamy S, Buikstra JE, Fulginiti LC, and Stone AC. 2020. Reconstructing full and partial STR profiles from severely burned human remains using comparative ancient and forensic DNA extraction techniques. Forensic science international Genetics 46:102272.

Fellows Yates JA, Velsko IM, Aron F, Posth C, Hofman CA, Austin RM, Parker CE, Mann AE, Nägele K, Arthur KW, Arthur JW, Bauer CC, Crevecoeur I, Cupillard C, Curtis MC, Dalén L, Díaz-Zorita Bonilla M, Díez Fernández-Lomana JC, Drucker DG, Escribano Escrivá E, Francken M, Gibbon VE, González Morales MR, Grande Mateu A, Harvati K, Henry AG, Humphrey L, Menéndez M, Mihailović D, Peresani M, Rodríguez Moroder S, Roksandic M, Rougier H, Sázelová S, Stock JT, Straus LG, Svoboda J, Teßmann B, Walker MJ, Power RC, Lewis CM, Sankaranarayanan K, Guschanski K, Wrangham RW, Dewhirst FE, Salazar-García DC, Krause J, Herbig A, Warinner C. The evolution and changing ecology of the African hominid oral microbiome. Proc Natl Acad Sci U S A. 2021 May 18;118(20):e2021655118. doi: 10.1073/pnas.2021655118. PMID: 33972424; PMCID: PMC8157933.

Gansauge MT, Gerber T, Glocke I, Korlevic P, Lippik L, Nagel S, Riehl LM, Schmidt A, and Meyer M. 2017. Single-stranded DNA library preparation from highly degraded DNA using T4 DNA ligase. Nucleic Acids Res 45(10):e79.

Gansauge MT, and Meyer M. 2013. Single-stranded DNA library preparation for the sequencing of ancient or damaged DNA. Nat Protoc 8(4):737-748.

Glassman DM, Crow RM. Standardization model for describing the extent of burn injury to human remains. J Forensic Sci. 1996 Jan;41(1):152-4.

Jonsson H, Ginolhac A, Schubert M, Johnson PL, and Orlando L. 2013. mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29(13):1682-1684.

Kircher M, Sawyer S, and Meyer M. 2012. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res 40(1):e3.

Kloss-Brandstatter A, Pacher D, Schonherr S, Weissensteiner H, Binna R, Specht G, and Kronenberg F. 2011. HaploGrep: a fast and reliable algorithm for automatic classification of mitochondrial DNA haplogroups. Human mutation 32(1):25-32.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, and Durbin R. 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25(16):2078-2079.

Loreille OM, Parr RL, McGregor KA, Fitzpatrick CM, Lyon C, Yang DY, Speller CF, Grimm MR, Grimm MJ, Irwin JA et al. . 2010. Integrated DNA and fingerprint analyses in the identification of 60-year-old mummified human remains discovered in an Alaskan glacier. Journal of forensic sciences 55(3):813-818.

Mckinnon M, Henneberg M, Higgins D. 2021. A review of the current understanding of burned bone as a source of DNA for human identification, Science & Justice, 61(4): 332-338

Meyer M, and Kircher M. 2010. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harbor protocols 2010(6):pdb prot5448.

Parson W, Gusmão L, Hares DR, Irwin JA, Mayr WR, Morling N, Pokorak E, Prinz M, Salas A, Schneider PM, Parsons TJ; DNA Commission of the International Society for Forensic Genetics. DNA Commission of the International Society for Forensic Genetics: revised and extended guidelines for mitochondrial DNA typing. Forensic Sci Int Genet. 2014 Nov;13:134-42. doi: 10.1016/j.fsigen.2014.07.010. Epub 2014 Jul 29.

Prinz M, Carracedo A, Mayr WR, Morling N, Parsons TJ, Sajantila A, Scheithauer R, Schmitter H, Schneider PM, and International Society for Forensic G. 2007. DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI). Forensic science international Genetics 1(1):3-12.

Schwark T, Heinrich A, Preusse-Prange A, and von Wurmb-Schwark N. 2011. Reliable genetic identification of burnt human remains. Forensic science international Genetics 5(5):393-399.

Tillmar A, Sturk-Andreaggi K, Daniels-Higginbotham J, Thomas JT, and Marshall C. 2021. The FORCE Panel: An All-in-One SNP Marker Set for Confirming Investigative Genetic Genealogy Leads and for General Forensic Applications. Genes 12(12).

Velsko I, Skourtanioti E, and Brandt G 2020. Ancient DNA Extraction from Skeletal Material. protocols.io