Characterizing DNA Degradation in Severely Burned Human Skeletal Remains Using STR Genotyping and NGS Targeted Capture Techniques

Matthew V. Emery, Katelyn L. Bolhofner, Suhail Ghafoor, Stevie Winingear, Robert Oldt, Sreetharan Kanthaswamy, Jane E. Buikstra, Laura Fulginiti, and Anne C. Stone

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Background: The Taphonomy of Burned Human Remains

- Burned victims are encountered in:
  - Accidental fires in structures
  - Vehicles
  - Forests and open fields
  - Homicide
  - Suicide
  - Crematoria
  - War zones
  - Mass disaster
  - Terrorist attack
  - Archaeological sites
Project Questions

• To what extent does charring/burning affect our ability to recover DNA?

• Are ancient DNA methods more effective at recovering DNA from thermally altered human bone?

• Can we obtain high quality full and/or partial CODIS STR profiles from severely burned bone?

• What is the extent of fragmentation and damage in DNA recovered from different tissues with different levels of burning?

• How do the different levels of burning impact the quality and quantity of NGS data?
Obtaining DNA From Highly Degraded Ancient and Forensic Remains: Limitations

- Low sample representation
- Low DNA yields
- Inefficient DNA extraction methods
- Short DNA fragments
- 5’ and 3’ terminal deamination
- Inefficient qPCR and multiplex STR assay design
- Allele/loci dropout
- Contamination/sample mixing
Samples were classified into 5 levels of burning (Schwark et al. 2011).

<table>
<thead>
<tr>
<th>Burn Category</th>
<th>Color</th>
<th>Temperature °C (°F)</th>
<th>Sample Size D/L (STR)</th>
<th>Sample Size D/L (NGS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>White, yellow</td>
<td>&lt;200 (&lt;392)</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>II</td>
<td>Yellow, brown</td>
<td>200 – 300 (392 – 572)</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>III</td>
<td>Carbonized black</td>
<td>300 – 350 (572 – 662)</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>IV</td>
<td>Grey, blue</td>
<td>550 – 600 (1022 – 1112)</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>V</td>
<td>Calcined, white</td>
<td>&gt;650 (&gt;1202)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>D/L n=51/48</td>
<td>D/L n= 58/48</td>
</tr>
</tbody>
</table>
Applying Ancient and Forensic DNA Methods to Forensic Burn Cases: Workflow
Methods: DNA Extraction

Loreille et al. 2010

- Bone sampled demineralized in 0.5 M EDTA and digested
- Incubate in hybridization oven (56°C) for 24 hrs
- Transfer buffer solution to Amicon Ultra-15 Centrifugal Column (fitted with Ultracel-PL 30-kDa membrane)
- Resulting solution purified over MinElute columns

Dabney et al. 2013

- Bone demineralized in 0.5 M EDTA and digested
- Resulting DNA bound to silica-membrane (Roche biopurification columns) using 5 M GuHCl-based binding buffer
- Extract collected over two rounds of elution

Burn Category 1 – 3 extracts sheared to 300 bp (Covaris)
Methods: NGS Preparation of Burned Remains

Double-Stranded DNA Library Preparation

- NEBnext (SPRI clean-up)
  - Blunt End
  - Adapter Ligation
  - Unique dual-indexing for multiplex sequencing
- Post-indexing quantification using i5 and i6 universal primers (longamp) for pooling and library QC

Targeted Enrichment Using Synthetic RNA baits

- Library/Hybridization mix, cycled
- Bead clean-up
- Hybridization
- Post-hybridization clean-up
- Re-amplification
- Enrichment round #2
  - Deep capture
Methods: RNA Bait Design (Arbor Biosciences)

**mtDNA Bait Design**
- Global Diversity Panel composed of 197 mtDNAs
- Captures whole mitochondrial genome

**Nuclear SNP (4.5 k) Bait Design**
(designated by Dr. Odile Loreille, FBI)
- 4,500 loci (autosomal, X and Y)
- 20,000 baits (52 nucleotides long)
  - Individual Identification SNPs
  - Phenotype Informative SNPs
  - Ancestry Informative SNPs
  - ABOSNPs (blood type)
Results: Ancient and Forensic DNA STR Genotyping

1. Extraction Protocol
   - Dabney et al. (2013)
   - Loreille et al. (2010)

2. STR Profile by Extraction
   - Full STR Profile (Dabney et al., 2013)
   - Partial STR Profile (Dabney et al., 2013)
   - Full STR Profile (Loreille et al., 2010)

3a) Figures from Emery et al. (2020)
MitoPipe 1.0: Mitochondrial DNA Genome Assembly

- Python script (snakemake) that calls targets for each application in the pipeline
- Demultiplexed using bcl2fastq
- Adapters trimmed and PE reads merged using leeHom (Renaud et al. 2014)
- Mapped using BWA (Li and Durbin 2010)
- Quality filtered using SAM/BAMtools
- Consensus sequence called using Schmutzi with deam5. and deam3. parameters removed; ‘q’ parameter set to 3X (Renaud et al. 2015)
- Haplogroups called using Haplogrep 2/Phylotree17 (Weissensteiner et al. 2016)
- Deamination measured using mapDamage2.0 (Jónsson et al. 2013) and BAM files scanned for multiple haplotypes using haplocheker
mapDamage2.0

- We used mapDamage2.0 to identify the frequency of terminal deamination (i.e., C -> T and G -> A transitions) for the higher burned samples (burn categories IV and V). (1.) Depicts the damage profile from an Iron Age period (Italy) individual (library LIAV 7) showing characteristic terminal deamination (Emery et al. 2018). (2.) Example of a mapDamage profile for NIJ library 22D4 (burn category IV) and (3.) NIJ library 22D5 (burn category V). Damage profiles #s 2/3 show no signs of terminal deamination. Deamination is a consequence of hydrolysis and time, two variable missing in acute fire events.
Fragment Length Distributions: Dabney (top) vs. Loreille (bottom)

1a) Single-end read length distribution
   Library 09D1

1b) Single-end read length distribution
   Library 09L1

2a) Single-end read length distribution
   Library 12D3

2b) Single-end read length distribution
   Library 12L3
Results: Average Number of Mapped Reads and Depth of Coverage (rCRS) Across Burn Categories: Loreille vs. Dabney

1.

2.

Extraction
- Dabney et al. 2013
- Loreille et al. 2010
Results: Average Number of Mapped Reads and Depth of Coverage (rCRS) by Skeletal Element
Haplogroup Diversity

<table>
<thead>
<tr>
<th>Haplogroup</th>
<th>No. of groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>2</td>
</tr>
<tr>
<td>H5</td>
<td>2</td>
</tr>
<tr>
<td>J1</td>
<td>2</td>
</tr>
<tr>
<td>K2</td>
<td>2</td>
</tr>
<tr>
<td>A2</td>
<td>3</td>
</tr>
<tr>
<td>C4</td>
<td>1</td>
</tr>
<tr>
<td>L2</td>
<td>1</td>
</tr>
<tr>
<td>V3</td>
<td>1</td>
</tr>
<tr>
<td>U5</td>
<td>1</td>
</tr>
<tr>
<td>T2</td>
<td>2</td>
</tr>
</tbody>
</table>

N=82 haplogroups out of a total of 106 libraries
77% of libraries successfully enriched for mtDNA
Haplochecker detected contamination in 5 libraries – those libraries were omitted from further analysis
Conclusions

• Both extraction methods effective at generating adequate quantities of DNA from burn categories 1 – 3 (~200 – 350 C°) for STR/NGS profiling

• Ancient DNA protocol (Dabney et al. 2013) is likely more effective at amplifying mini-STR assays due to ultra-short DNA retention

• A systematic decrease in STR profiles and mtDNA coverage/mapped reads is observed in samples subjected to temperatures exceeding 550 C°

• The data gap between burn categories 3 and 4 suggests an acute and rapid point of DNA thermal degradation

• STR and NGS data suggests a higher quantity of ultrashort DNA retention for mini-STR multiplex amplification and PE reads using aDNA protocol

• SNP enriched libraries are currently being sequenced
References

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Slide Notes

Greetings, and welcome to my written presentation about DNA preservation in thermally altered forensic human remains. First, I want to thank the AAPA organizing committee for allowing presentations to appear digitally this year, especially given the extreme circumstances surrounding the Coronavirus pandemic. The following outlines the main talking points associated with each slide as a means to provide a little more background to the bullet points, images, and figures throughout the presentation. Corresponding slide numbers are located on the bottom left corner of each slide, and figure numbers occur directly left of the referenced image. In-text (presentation) references are found on slide 18. If you have any questions, comments, or constructive criticisms about the slides or commentary, please contact me at: memery5@asu.edu.

(Slide 1, title slide) Acute environmental insults, such as fires, reduce the probability of acquiring adequate DNA yields for standard forensic identification. These problems manifest as low qPCR results, incomplete (or partial) STR profiles, and/or low quality NGS data (e.g., whole-mitochondrial genome or genome-wide SNPs). However, the field of ancient DNA is rapidly optimizing laboratory and in-silico protocols to increase DNA yields from poorly preserved ancient skeletal materials, as well as authenticate endogenous DNA molecules and assemble those reads to a reference genome. These applications include identifying DNA damage patterns and DNA fragment lengths (e.g., mapDamage2.0), assembling partial and/or whole genomes using short read aligners (e.g., BWA), and variant calling (e.g., using GATK). In this presentation we document DNA yields, STR profiles, and preliminary NGS data obtained from highly burned forensic skeletal samples via two comparative forensic (Loreille et al. 2010) and ancient DNA (Dabney et al. 2013) extraction protocols.

(Slide 2) Burned human remains are encountered in both forensic and archaeological contexts. Most often, fire-related deaths in modern forensic cases occur in accidental structural fires (e.g., buildings and houses), vehicles, are discovered in forests and/or open fields, and are
found in large-scale tragedies such as in mass disaster scenarios, war zones, and terrorist attacks. Criminal perpetrators will also attempt to conceal their crimes by burning their victims following homicide and, conversely, will commit suicide by burning themselves in an act of protest. In archaeological cases, however, incinerated human remains (cremains) are normally recovered from containers representative of ceremonial offerings, in mortuary contexts, or midden deposits.

(Slide 3) Since few studies have systematically explored the effects of different levels of burning on human bones and teeth, we addressed the following main questions outlined in slide 3 to gain insight into impact of burning/charring on downstream DNA yields, processing, and analysis. (Slide 4) There are a number of limiting factors in both ancient and forensic DNA research that impede DNA quantity, quality, and subsequent STR and/or NGS analysis. These include (but are not limited to) the main contributing factors outlined in slide 4.

(Slide 5) In collaboration with the Maricopa County Office of the Medical Examine (MCOME) in Phoenix, AZ, we received multiple skeletal elements (representing a total of 27 individuals) displaying various degrees of burning/charring. Samples were classified into 5 burn categories (or levels) according to observed discoloration patterns outlined by Schwark et al. (2011). Since multiple skeletal elements displayed more than one level of burning, multiple samples were removed from the same skeletal element for intra-skeletal comparative analysis. Burn category samples sizes, and total sample size by extraction method (i.e., Loreille et al. 2010; Dabney et al. 2013) are shown in slide 5. (Slide 6) DNA was extracted from each subsample according to two established protocols (see above; Slide 7). A portion of each DNA extract was sent to the Kanthaswamy DNA laboratory at ASU West Campus for STR genotyping, and the remaining extract was converted to double-stranded DNA libraries (Slide 8). Libraries were then enriched using two synthetic RNA bait sets (Arbor Biosciences); one to capture whole-mitochondrial genomes, and another to target whole-genome SNPs (Slide 9). Libraries were pooled at equimolar concentration and sequenced on an Illumina MiSeq platform at Arizona State University’s Genomics core facility.

(Slide 10) We quantified and generated STR profiles for both Dabney and Loreille extracted samples using the Quantifiler™ Trio DNA kit and Promega’s PowerPlex® ESX 17 system, respectively. Quantitative PCR results show a systematic decrease in DNA concentration across burn categories I – V (Figure 1; slide 10). Out of a total of 80 samples (combination of Dabney and Loreille extracted samples), we generated 48 complete STR profiles (60% success rate). We observed a dramatic decrease in STR profiles between burn categories III and IV (Figure 2, slide 10). A comparable number of profiles were observed between both sets of extracts across burn categories I – III. However, we generated full STR profiles for two burn category IV samples (one for each extraction procedure), and three partial STR profiles for one burn IV and two burn category V samples (Figure 2, slide 10). Within burn categories IV and V, we also observed higher quality electropherogram peaks (Figure 3a/b: top STR profile generated using the Dabney extraction method, and the bottom STR profile generated using the Loreille extraction method, slide 10), and the preferential amplification of mini-STRs from the Dabney extracted samples (amplicon size <180 bp) (Figure 3b, slide 10).

(Slide 11) Whole-mitochondrial genomes were assembled using a novel pipeline written in Python (snakemake), called MitoPipe1.0. We used a combination of modern and ancient DNA
applications to trim and merge paired-end reads (leeHom), align merged and properly-paired reads to the rCRS (BWA), quality filter and remove duplicates (SAMtools), generate contamination estimates and measure DNA damage (HaploChecker and mapDamage2.0), and call consensus sequences (Schmutzi) for haplotype calling (Haplogrep). MitoPipe1.0's workflow is depicted in Figure 1, slide 11.

(Slide 12) MapDamage2.0 is an ancient DNA authentication application used to identify and determine the frequency of terminal deamination in an NGS library. Figure 1 (slide 12) shows a high frequency of C → T and G → A deamination on the terminal ends of the reads. This pattern is normally observed in ancient DNA libraries (a library constructed from an Iron Age Individual, southern Italy, for example). However, no terminal deamination was observed for the higher burn category samples (see Figure 2 and 3, slide 12). Deamination is a consequence of hydrolysis and time, two variables that are missing in acute fire-related cases. Our mapDamage2.0 analysis across burn category I – V libraries did not reveal terminal deamination.

(Slide 13) Another way to determine DNA damage is to quantify the average read fragment length (fragment length distribution, or FLDs). In our case, however, the fragment length distribution of individual libraries, especially less burned samples, exceeded the length of the paired-end chemistry (2x250 bp read chemistry) for short-read sequencing, so the natural fragment length remains unknown for a large number of libraries. However, we observed an interesting fragment length pattern between the two extraction methods. Dabney extracted samples show shorter fragment length distributions than the Loreille extracted samples. We hypothesize that this discrepancy in fragment length is due to differences in extraction protocol, in that the Dabney extraction method is optimized to retain short DNA fragments, while the Loreille protocol is retaining longer DNA fragments. This pattern is depicted in Figures 1a/b (top Dabney; bottom Loreille) and 2a/b (top Dabney; bottom Loreille), slide 13. Interestingly, we measured a difference in read FLDs between Dabney extracted burn category I and burn category III libraries (Libraries 09D1 and 12D3, Figures 1a and 2a, slide 13). This shift towards shorter reads suggests a degree of higher DNA fragmentation with increasing temperature.

(Slide 14) Similar to DNA yields (see above, Figure 1, slide 10), we observed a systematic decrease in the number of mapped reads and depth of coverage (rCRS) across burn categories I – V (Figures 1 and 2, slide 14). The number of mapped reads and depth of coverage across the mitochondrial genome is comparable between the two extraction protocols for burn categories I – III. However, we measured an increased number of mapped reads and a higher depth of coverage on average for burn category IV Loreille extracted samples. We suspect that small sizes (Loreille burn category IV, n = 4) account for a degree of variance between the two methods. More samples are required to produce statistically significant results. (Slide 15) We also measured the number of mapped reads and depth of coverage (rCRS) across representative skeletal elements. We found comparable results across the two extraction methods but determined a higher number of mitochondrial reads and depth of coverage for the femur, parietal, phalanges, and ulna (Figures 1 and 2, slide 15). A larger sample size is necessary to determine statistically significant patterns of DNA quality across the broad range of skeletal elements in our sample.
(Slide 16) Haplotypes were generated from library consensus sequences to in order to detect potential cross-contamination between samples and authenticate mitochondrial genome assembly. The haplogroup composition of our sample is typical of the maternal history of the American southwest (Figure 1, slide 16). Out of a total of 106 libraries (combined Dabney and Loreille sub-samples), we successfully called the haplogroups for 82 sub-samples (77%), higher than the number of STR profiles generated from autosomal DNA. Our data confirm that it is possible to reconstruct complete mitochondrial genomes from highly incinerated human remains, and at temperatures >550°C. F

(Slide 17) To conclude, our analysis revealed that both the Dabney and Loreille extractions methods are effective at obtaining adequate DNA yields for STR genotyping and whole-mitochondrial genome reconstruction. However, substantial decreases in DNA concentration >350°C lead to a reduction in both complete STR and mitochondrial DNA profiles. The data gap between 350°C – 550°C suggest an acute point of DNA degeneration. Further analysis regarding the systematic and controlled (over particular intervals of time) burning of skeletal materials may narrow this broad temperature range and. Overall, our research suggests adopting ancient DNA extraction methods to complement current forensic procedures, or when traditional applications fail.