Report On The DNA Analysis From Skeletal Remains From Two Skulls

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Two sets of remains were received by Trace Genetics and were processed for genetic analyses. The remains consisted of two skulls presented by Mr. Lloyd Pye for DNA analysis.

SAMPLING

Prior to attempts to extract DNA from the remains, the remains were inventoried and taped using a video camera. Video records of the sampling procedure and the initial extraction on all samples were taken and archived by Trace Genetics.

Samples were cut from the left parietal of an abnormally shaped skull, identified as the Starchild skull on February 10, 2003. Equipment used to sample was sterilized using a bleach solution prior to use. Sampling was performed in a room not used for any genetic analyses.

Fragments weighing a total of 0.8g were cut from the parietal using a rotary cutter with a previously unused blade. The fragments were placed in a sterile conical tube labeled SCS-1 and stored for analysis. A second 0.7g fragment adjacent to the sample retained by Trace Genetics was placed in a sterile conical tube labeled SCS-2 and returned to Mr. Pye.

Two teeth were removed from maxilla of a skull presented in association with the Starchild skull on February 10, 2003. The right first molar tooth and root weighing 1.7g was removed and labeled "SA-1." The tooth and root were placed in a sterile conical tube and retained for genetic analysis by Trace Genetics. A portion of the root was fractured in the process and remained in the maxilla. The right premolar and root (sample labeled "SA-2"; total weight 1.0g) were also removed from the maxilla, placed in a sterile conical tube. The SA-2 sample was returned to Mr. Pye.

EXTRACTION AND ANALYSIS OF DNA

SCS-1:

Extraction 1:

A first extraction was performed on a 0.24g fragment of the parietal bone from sample SCS-1. The extraction was performed in a dedicated ancient DNA laboratory beginning on 7 March 2003 and was performed in parallel to an extraction of SA-1 and a reagent blank (negative control). Both surfaces of bone were sanded with a rotary sander to remove any surface

contaminants and lacquer preserves present on the outer surfaces of bone. Subsequent to sanding, the bone was exposed to ultraviolet (UV) irradiation (254nm) for 300 seconds per side.

The bone surface was then cleaned with bleach (2% sodium hypochlorite), rinsed with sterile EDTA and placed in a fresh 15ml conical tube and immersed in approximately 2ml of 0.5M EDTA. The tube was sealed with parafilm and placed on a rocker. After 10 days, the tube was opened and 150μl of 0.1M PTB [1] and 20μl of 100mg/ml proteinase-K was added to the sample and EDTA. The sample was incubated with agitation overnight at 64oC. DNA was extracted from the digested sample using a 3-step phenol/chloroform extraction method. Two extractions with phenol:chloroform:isoamyl (25:24:1) of equal volume to the digested product were followed by an extraction with an equal volume of chloroform:isoamyl (24:1). The extracted DNA solution was concentrated by ammonium-acetate precipitation using two volumes of cold 100% filtered ethanol and 1/2 volume of 5M ammonium acetate. This solution was then stored at -20°C for approximately 4 hours to facilitate precipitation, then centrifuged at high speeds (10,000-12,000rpm) for 15 minutes to pellet the precipitated DNA. The supernatant was discarded and the remaining DNA pellet dried and resuspended in ~300μl sterile ddH2O. To further purify the DNA and remove additional PCR inhibitors co-extracted with the DNA, the DNA solution was purified using the Promegaâ Wizard PCR Preps DNA Purification Kit as directed by the manufacturer. DNA was eluted from Promegaâ columns with 100μl sterile ddH2O, the elutant labeled SCSex1 and stored at (minus) -20°C.

Attempts to amplify segments of mtDNA from extract SCSex1 were performed as described below in METHODS. Single amplifications for fragments containing the diagnostic mutations for Native American haplogroups A, B, C and D[2] did not reveal a known Native American haplogroup, however, the extraction did not amplify consistently. A single amplification of a fragment of the mtDNA first hypervariable segment (HVSI) between np 16210 and np 16328 was sequenced using a cycle sequencing procedure with ABI Big-Dye 3.1 chemistry and analyzed on an ABI automated genetic analyzer. The sequence obtained revealed a transition relative to the Cambridge reference sequence at np16273. This sequence did not match either any personnel with access to the ancient DNA facilities or a sequence obtained from Mr. Pye. Subsequent amplifications of this fragment were not successful and the sequence could not be confirmed. Attempts to amplify fragments of the amelogenin gene located on the X and Y chromosome[3] were uniformly not successful.

Extraction 2:

A second extraction was performed beginning April 21, 2003 on 0.21g of the parietal sample from SCS-1. The extraction was performed as above with the following modifications:

1. The sample was run in parallel with a reagent blank (negative control) but was not processed with any other samples.

2. The bone was exposed to 900 seconds of UV irradiation per side.

3. The bone was completely immersed in 2% sodium hypochlorite for 5 minutes.

4. The sample was left in EDTA with agitation for 22 days prior to digestion with proteinase-K.

5. At digestion, ~50μl of Tween-20 was added with 100μl of PTB.

6. The silica extraction columns (Promega®) were eluted with 80μl of ddH2O and sample labeled "SCSe2."

Attempts to amplify mtDNA for fragments containing the diagnostic mutations for Native American haplogroups A, B, C and D were performed on extract SCSe2. Multiple amplifications indicated that the sample possessed an AluI restriction site at np 13262 indicative of Native American haplogroup C [2]. Sequence obtained for a fragment of the first hypervariable segment of the mtDNA control region from np16210 to np 16367 revealed transitions at np16223, np 16298, np 16325 and np 16327. These mutations are characteristic of haplogroup C in the Americas [4].

Multiple attempts to amplify a segment of the amelogenin gene were unsuccessful using various amounts of SCSe2 extract as template. 30μl of the original extract was concentrated to a final volume of ~10μl using a microcon YM-30 concentrator. Attempts to amplify this concentrated template were not successful.

Extraction 3:

A third extraction was performed beginning on June 4, 2003 as described above for extraction 2 with the following modifications:

1. The extraction was performed on the entire remaining 0.40g of bone.

2. The sample was immersed in ~3.5ml of EDTA.

3. To ensure adequate demineralization of the sample, the sample was left immersed in EDTA with agitation for 30 days.

4. The final elution from the silica spin columns (Promega®) was performed twice, each time with 35μl of ddH2O preheated to 65oC.

Attempts to amplify fragments of mtDNA were performed to test for the presence of diagnostic mutations fo r Native American haplogroups A and C. The sample did not appear to possess the diagnostic HaeIII mutation and np663 indicative of haplogroup A. Multiple amplifications did reveal the presence of the AluI site gain at np13262 indicative of haplogroup C.

A single amplification of a fragment of the amelogenin gene located on the X and Y chromosomes [3] produced a single amplification product 106bp in length. Multiple subsequent amplifications did not reproduce this event, as all subsequent attempts did not produce a PCR product.

SA-1:

Extraction 1:

A first extraction was performed on 0.53g fragment of the molar tooth from sample SA-1 beginning on 7 March 2003 and was performed in parallel to an extraction of SCS-1 (above) and a reagent blank (negative control). The extraction was performed in the manner describe above for extraction 1 of SCS-1 save that the outer surface of the tooth, which had previous to sampling been firmly rooted in the maxilla, was not sanded and the final elution of the silica spin column (Promega®) was eluted to 100μl and labeled SAex1.

Multiple attempts to amplify segments of mtDNA containing amplifications for fragments of mtDNA containing the diagnostic mutations for Native American haplogroup A revealed a HaeIII restriction site at np663 consistent with known Native American haplogroup A [2]. Amplifications for fragments containing the diagnostic sites for haplogroups B, C and D did not show presence of mutations indicative of these haplogroups. A single amplification of a fragment of the mtDNA first hypervariable segment (HVSI) between np 16210 and np 16327 revealed transitions relative to the Cambridge reference sequence at np16223, np16290 and np16319. These mutations are consistent with Native American haplogroup A.

Multiple amplifications of a fragment of the amelogenin gene on the X and Y chromosomes consistently produced a single band 106bp in length when visualized on an electrophoretic gel consistent with DNA from a female [3].

Extraction 2:

A second extraction was performed beginning Ap ril 21, 2003 on 0.42g of the tooth sample from SA-1. The extraction was performed similar to extraction 1 on SA-1 (above) with the following modifications:

1. The sample was not run in parallel to any samples from SCS-1.

2. The sample was immersed in EDTA for 26 days prior to digestion with proteinase-K.

The final elution was labeled SAe2 and stored at –20oC.

Multiple amplifications of a mtDNA fragment indicated the presence of a HaeIII restriction site at np663 indicative of Native American haplogroup A. Amplifications of the extraction did not possess the AluI site gain at np16262. Multiple amplifications of a fragment of the amelogenin gene produced a single band when visualized on an electrophoretic gel consistent with DNA from a female.

DISCUSSION:

MtDNA from virtually all modern, full-blooded Native Americans belongs to one of five mitochondrial lineages or matrilines (designated haplogroups A, B, C, D, and X) marked by the presence or absence of characteristic restriction sites or by the presence of a nine base pair (9-bp) deletion [2, 5]. Analyses of ancient DNA from Native Americans likewise indicates that these haplogroups constitute virtually all prehistoric Native American individuals as well [see: 6].

The sample taken from the Starchild Skull (SCS-1) has mtDNA consistent with Native American haplogroup C, as revealed through two independent extractions performed on fragments of parietal bone. While a single first extraction did not appear to type similarly, this inconsistent result is likely a product of a low level of contamination. This single extraction neither amplified consistently nor was the single sequence of HVSI reproducible. Contamination could have occurred either prior to sampling, introduced in the extraction process, or during PCR amplifications. It is unlikely that contamination could account for the haplogroup C mtDNA as this type is not possessed by any researcher with access to the ancient DNA facilities and the reagent blanks did not indicate systematic contamination in the extractions.

The sample taken from the associated skull (SA-1) has mtDNA consistent with Native American haplogroup A as determined through both extractions. The sample also appeared be from a female individual as evidenced by repeated amelogenin typing. It is unlikely that contamination could account for the haplogroup A mtDNA as this type is not possessed by any researcher with

access to the ancient DNA facilities and the reagent blanks did not indicate systematic contamination in the extractions.

As mtDNA exists in high copy number (upwards of three orders of magnitude relative to any single copy nuclear DNA locus), it can be recovered from prehistoric biological material in sufficient quantities for amplification and analysis using the polymerase chain reaction (PCR) [see: 7, 8]. MtDNA is present in haploid condition with inheritance being passed down exclusively through maternal lines [9]. Thus, that the samples analyzed from SCS-1 and SA-1 possessed markedly different mtDNA types excludes a mother-offspring relationship between the two individuals. As it was possible to type and confirm both to known pre-Columbian mtDNA types found in the Americas, both individuals most appear to have possessed Native American mothers.

While it is possible to obtain nuclear DNA as well from ancient samples, the reduced copy-number at any particular nuclear locus relative to mtDNA makes it less likely that a particular extract will contain sufficient DNA for the analysis of a nuclear genetic locus using presently available PCR methods. The ability to amplify nuclear DNA from the SA-1 extractions but not from the SCS-1 extractions could be a product of any of a number of factors.

In ancient DNA analysis, success rates from teeth are generally higher than from bone [10, 11]. Further, there is some indication that X-Ray exposure damages and degrades DNA, which may have decreased the quantity and quality of DNA available in the bone prior to extraction. The lone amplification using the amelogenin primers on extract SCSe3 could not be confirmed through additional amplifications and likely indicates a sporadic contamination of a single PCR reaction caused either by a female individual in the laboratory or could have been introduced to laboratory disposables (e.g. pipette tips, PCR reaction tubes). Such contamination has been noted elsewhere [12] and consequently, any conclusions drawn from the single un-reproduced PCR reaction should not be taken as any reliable indication as to the DNA present in the sample.

The presence of reliably typed mtDNA from SCS samples does indicate that mtDNA is present in the bone. The inability to analyze nuclear DNA indicates that such DNA is either not present or present in sufficiently low copy number to prevent PCR analysis using methods available at the present time.

AMPLIFICATION METHODS:

All reagents used to extract and amplify were first tested to detect any DNA contamination and ancient DNA facilities were cleaned using bleach to remove possible sources of contamination. Further additional contamination controls and precautions are described below.

PCR amplifications of mtDNA were conducted in 25 μl volumes using 4μl dNTPs (10mM), 2.5μl 10X PCR buffer (Gibco), 1.3μl BSA (20mg/ml), 0.75μl MgCl2, 0.2μl Platinum Taq DNA polymerase (Gibco) 2 to 6 μl of DNA template and sterile ddH20 sufficient to bring reaction volume to 25μl. After an initial 4- minute denaturation step at 94o C to activate the hot start Taq, 40 PCR cycles were performed consisting of a 94oC denaturing step, a 50-55oC annealing step (temperature depending on primers utilized), and a 72oC extension step of 30 seconds each. A final 3- minute extension at 72oC was added after the last cycle. A portion of the amplification product (~5μl) was run on a 6% polyacrylamide gel together with a size standard ladder, stained with ethidium bromide and photographed under UV light using a digital imager (ISO 2000 imaging system, Alpha Innotech, San Leandro, CA). To assess the presence or absence of diagnostic restriction sites, the remaining 20μl were incubated with 10 units of the appropriate restriction enzyme overnight at 37o C, and then subjected to electrophoresis in the manner previously described. Primers used for amplification of these segments and restriction enzymes used are shown in table 1.

Amelogenin amplifications [3] were attempted using 1, 3, and 8 μl of DNA template in 25 μl reaction volumes, adjusting ddH2O amounts to maintain concentrations of other reagents.

Table 1. Primers used to amplify ancient mtDNA

Site	First Primer Coordinate	Second Primer Coordinate	Polymorphism	Reference:
Haplogroup A	591-611	765-743	*HaeIII* np663 "+" = A	[13]
Haplogroup B	8195-8215	8316-8297	9 base pair deletion Deletion = B	[14]
Haplogroup C	13236-13257	13310-13290	*AluI* np13262 "+" = C	[15]
Haplogroup D	5099-5120	5211-5190	*AluI* np 5176 "-" = D	[15, 16]
HVSI	16192-16209	16385-16368	Sequence	[15, 17]
HVSI	16192-16209	16348-16328	Sequence	[15, 17]

CONTAMINATION CONTROLS:

Ancient DNA is typically highly degraded and survives in much lower copy numbers than modern DNA. Consequently, ancient DNA is highly vulnerable to contamination from modern sources and specific precautions against contamination, as summarized by Kelman and Kelman [18], were utilized in this study to both minimize contamination and, importantly, to identify contamination when present so that it does not lead to false inferences.

These measures include: 1. Use of dedicated laboratory space, supplies, reagents and equipment for preparation of ancient DNA samples inside UV irradiated glove boxes; 2. Use of sterile, disposable labware and clothing whenever possible; 3. Use of separate pre- and post-PCR facilities; 4. Periodic UV irradiation and bleaching of all materials used to help eliminate any surface contamination; 5.

Running negative controls at all stages of the extraction and amplification process to identify the presence of contaminants; 6. Confirmation of results by multiple amplifications of multiple extractions. All positive results were confirmed though multiple amplifications of each extraction and multiple extractions performed at different times.

REFERENCES CITED:

1. Poinar, H.N., et al., Molecular coproscopy: Dung and diet of the extinct ground sloth Nothrotheriops shastensis. Science (Washington D C), 1998. 281(5375): p. 402-406.

2. Schurr, T.G., et al., Amerindian mitochondrial DNAs have rare Asian mutations at high frequencies, suggesting they derived from four primary maternal lineages. American Journal of Human Genetics, 1990. 46(3): p. 613-23.

3. Mannucci, A., et al., Forensic Application of a Rapid and Quantitative DNA Sex Test by Amplification of the X-Y Homologous Gene Amelogenin. International Journal of Legal Medicine, 1994. 106(4): p. 190-193.

4. Torroni, A., et al., Asian affinities and continental radiation of the four founding Native American mtDNAs. American Journal of Human Genetics, 1993. 53(3): p. 563-590.

5. Forster, P., et al., Origin and evolution of native American mDNA variation: A reappraisal. American Journal of Human Genetics, 1996. 59(4): p. 935-945.

6. Eshleman, J.A., R.S. Malhi, and D.G. Smith, Mitochondrial DNA Studies of Native Americans: Conceptions and Misconceptions of the Population Prehistory of the Americas. Evolutionary Anthropology, 2003. 12(1): p. 7-18.

7. Kaestle, F.A. and D.G. Smith, Ancient mitochondrial DNA evidence for prehistoric population movement: The Numic expansion. American Journal of Physical Anthropology, 2001. 115(1): p. 1-12.

8. Stone, A.C. and M. Stoneking, mtDNA analysis of a prehistoric Oneota population: Implications for the peopling of the New World. American Journal of Human Genetics, 1998. 62: p. 1153-1170.

9. Giles, R.E., et al., Maternal Inheritance of human mitochondrial DNA. Proceedings of the National Academy of Sciences of the United States of America, 1980. 77(11): p. 6715-6719.

10. Malhi, R.S., Investigating prehistoric population movements in North America with ancient and modern mtDNA, in Anthropology. 2001, University of California: Davis.

11. Hummel, S., Ancient DNA Typing: methods, strategies and applications. 2003, Berlin: Springer-Verlag.

12. Schmidt, T., S. Hummel, and B. Herrmann, Evidence of contamination in PCR laboratory disposables. Naturwissenschaften, 1995. 82(9): p. 423-431.

13. Stone, A.C. and M. Stoneking, Ancient DNA from a pre-Columbian Amerindian population. American Journal of Physical Anthropology, 1993. 92(4): p. 463-471.

14. Wrischnik, L.A., et al., Length mutations in human mitochondrial DNA: direct sequencing of enzymatically amplified DNA. Nucleic Acids Research, 1987. 15: p. 529-542.

15. Handt, O., et al., The retrieval of ancient human DNA sequences. American Journal of Human Genetics, 1996. 59(2): p. 368-376.

16. Parr, R.L., S.W. Carlyle, and D.H. O'Rourke, Ancient DNA analysis of Fremont Amerindians of the Great Salt Lake Wetlands. American Journal of Physical Anthropology, 1996. 99(4): p. 507-518.

17. Eshleman, J.A., Mitochondrial DNA and prehistoric population movements in Western North America, in Anthropology. 2002, University of California, Davis: Davis, CA.

18. Kelman, L.M. and Z. Kelman, The use of ancient DNA in paleontological studies. Journal of Vertebrate Paleontology, 1999. 19(1): p. 8-20.