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SER029-17 Final Report

File #: SER029-17

Date: 04 May 2017

Report of Expert

Expert's Name: Stephen Fratpietro, M.Sc., B.Ed.
Title: Technical Manager, Paleo-DNA Laboratory

I, the undersigned, as requested by Thierry Jamin, Instituto Inkari-Cusco, submit my professional opinion in reference to the following matter: This examination of exhibits is connected to an ancient DNA analysis.

ITEMS EXAMINED:
The following items (see Table 1) were submitted for genetic analysis by Thierry Jamin, Instituto Inkari-Cusco. These samples were designated the following case and sample number by the Paleo-DNA Laboratory (PDL):

PDL Case Designation	PDL Sample Designation	Sample Type	Comments
SER029-17	1	Unknown tissue	Sample of possibly biological material from a cranial brain
SER029-17	2	Bone and Tissue	Sample of bone extracted from a hand (possibly non-human)

Table1. Samples submitted to the Paleo-DNA Laboratory.

EXAMINATION REQUESTED: Ancient DNA Analysis: extraction of DNA, mitochondrial and nuclear DNA feasibility test, universal Identification, and sex identification.

REQUIREMENTS REQUESTED: Determine if any genetic information could be extracted from the sample. Unless otherwise discussed, the industry standard extraction, purification and amplification protocols were to be used and attempted in this case.

The Paleo-DNA Laboratory agreed to work on the project in accordance with high scientific and professional standards, but as we had not been involved with the collection and storage of the sample, nor have we inspected the sample, nor have we assessed the condition of the sample, the Paleo-DNA Laboratory did not promise success in achieving any desired result. The Paleo-DNA Laboratory undertook this project giving no warranty of fitness for a particular purpose, or any other warranty, expressed or implied, on the results of your project or the tests carried out pursuant to

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your project. This includes no guarantee or warranty that the recommended protocol will achieve your desired results.

EXAMINATION METHODOLOGY:

All aDNA samples are prepared pre-amplification in a room dedicated specifically to limited quantity DNA samples. This environment is monitored quarterly for the presence of DNA. This lab has restricted access and requires protective gear to be worn at all times: tyvek suit covering head and feet, gloves, hairnet, facemask. All persons entering this lab have their DNA profiled and kept for future comparison.

Sample Preparation

All aDNA samples are surface sterilized with two washes of sterile water and one wash of 70% ethanol. Each sample is separately milled into a fine powder using a mixer mill.

DNA Extraction

Total Demineralization [Loreille et al, 2007]:

Approximately 1-2g of sample powder is mixed with 9.0mL 0.5M EDTA, 150uL 20% Lauryl Sarcosinate, and 100uL Proteinase K (20mg/mL) in a sterile 15.0mL tube. This reaction is incubated overnight at 56°C with gentle agitation. The resulting supernatant is transferred to next step.

Silica Bead Purification [modified Boom et al, 1990]:

The supernatant is mixed with 18mL 4M Guanidinium Thiocyanate and 15uL silica. This is allowed to sit for 4 hours at 4°C [to allow DNA to bind to silica] after which the supernatant is removed and the remaining silica washed with Working Wash Buffer (10mM Tris-HCl, 50mM NaCl, 1mM EDTA, anhydrous ethanol) and 100% ethanol, then allowed to dry. The silica is resuspended in 55uL sterile water and incubated for 1 hour at 56°C to allow DNA to unbind from silica and dissolve in the water. The resulting supernatant is transferred to the next step.

Size Exclusion Column Purification [Matheson et al, 2009]:

The purified DNA extract is further filtered using Biorad Micro Bio-Spin P30 Chromatography Columns as per manufacturer's instructions.

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It is important to note that an extraction control (negative) is carried through this entire process as a quality control measure.

PCR Amplification

DNA is amplified in 25uL reactions using Quanta Biosciences™ AccuStart™ II PCR Supermix (2X) with 12.5uL of AccuStart II PCR Supermix (2X), 0.25uL of 10uM each primer, 3uL template. Cycling parameters: hot start of 94° for 2 min, and 50 cycles of 94°C for 30s, 60°C for 1 min., 72°C for 2 min.

The amplicon sizes vary in length and are distinguishable from each other. Primers used amplify regions 16s, mt16191-16420, and 12s of the mitochondrial DNA.

Primer Information:

16s6	5'-TTTCGGTTGGGGCGACCTCGGAG-3'	Poinar etal. 2001. PNAS. 98(8): 4317-4322
16sB	5'-CTCCGGTTTGAACTCAGATC-3'	Xiong and Kocher 1991. Genome 34: 306-311
16191F	5'-CCC ATG CTT ACA AGC AAG TA-3'	Kolman etal. 2000. Am. J. Phys. Anthropol. 111(1): 5-23
16420R	5'-TGA TTT CAC GGA GGA TGG TG-3'	Vigilant etal. 1989. PNAS. 86: 9350-9354
12sF	5'-ACTGGGATTAGATACCCCACTATG-3'	Melton T, Holland C. 2007 J. Forensic Sci. 52, 1305–1307
12sO	5'-GTCGATTA AGGACAGGTTCCTCTA-3'	Poinar etal. 1998. Science 281(5375): 402-6

Amplicons and Length for aDNA analysis.
16s6 – 16sB = 270bp
16191F – 16420R = 229bp
12sF – 12sO = 150bp

Each PCR reaction batch includes a positive and negative PCR control as well as the negative extraction control. Each amplicon is amplified at least twice for replication.

Quantification

Nuclear DNA is targeted using Life Technologies Quantifiler™ Human DNA Quantification kit as per manufacturer's instructions run on the Cepheid Smart Cycler® II.

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GEL Electrophoresis

PCR products are mixed with a dye and loaded onto a 6% Polyacrylamide Gel (PAGE) that uses electricity to separate any DNA products produced by the PCR reaction. The gel is stained with ethidium bromide that binds to the DNA in the gel and fluoresces under ultra violet light. A picture is taken for visual verification of amplification products present within the PCR reaction. Each primer region will produce a DNA band of a specific size if DNA is present.

Successful PCR products are purified by mixing 20uL of PCR product with 2uL *Exo I* nuclease [Lucigen] and 4uL of Shrimp Alkaline Phosphatase (SAP) [Thermo Fisher]. The mixture is incubated at 37ºC for 15 minutes, then the enzymes are deactivated at 80ºC for 15 minutes.

Sequencing

Purified PCR products are direct sequenced with the Life Technologies Big Dye Terminator Ready Reaction Kit v3.1 in both the forward and reverse direction. 0.5uL Big Dye Terminator Ready Reaction Mix v3.1, 0.25uL 10uM primer, 2uL 5x Big Dye Terminator Sequencing Buffer, 4.2uL of sterile water, and 3uL purified PCR Product. Cycling parameters: Hot Start of 96°C for 60s; 15 cycles of 96°C for 10s, 50°C for 5s, 60°C for 75s; 5 cycles of 96°C for 10s, 50°C for 5s, 60°C for 90s; and 5 cycles of 96°C for 10s, 50°C for 5s, 60°C for 2 min. Sequencing products are purified with a sodium acetate/ethanol precipitation as per Applied Biosystems Automated DNA Sequencing Chemistry Guide. Sequencing products are resuspended in 15uL Hi-Di Formamide and run on the ABI 3130xl for sequencing analysis.

The DNA sequence is compared to the BLAST (basic local alignment search tool) database for a closest match. The BLAST database is an extensive collection of various DNA sequences. A 100% match means that 100% of the sequence acquired for that sample was a complete match to the one identified in the database. A 95% match means that only 95% of the sequence acquired for that sample was a match to the one identified in the database and that was the closest match that could be found within the database. Where more than one Family or Genus could be a match it means that the sequence acquired for that sample was a match to all identified Families/Genera equally (same confidence level) and that was the closest match that could be found within the database. There is an equal chance that it could belong to any one of these Families/Genera.

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Mitochondrial sequencing data is edited and aligned to the Revised Cambridge Reference Sequence using Gene Codes Sequencher™ Software v4.10.1

Fragment Analysis

The amelogenin (sexing) region is amplified in 25uL reactions using Quanta Biosciences™ AccuStart™ II PCR Supermix (2X) with 12.5uL of AccuStart II PCR Supermix (2X), 0.25uL of 10uM each primer, 3uL template. Cycling parameters: hot start of 94° for 2 min, and 40 cycles of 94°C for 30s, 60°C for 1 min., 72°C for 2 min, and a final 72°C for 80 min. This PCR reaction batch includes a male positive, female positive and negative PCR control. Each locus is amplified at least twice for replication.

Primer Information: Sullivan et al. 1993. Biotechniques. 15(4): 636-641

Amel 1 F	5'-(6FAM) CCC TGG GCT CTG TAA AGA ATA GTG-3'
Amel 1 R	5'-ATC AGA GCT TAA ACT GGG AAG CTG-3'

RESULTS: The results below relate only to the items tested.

Universal ID – 12s Region

A DNA sequence could not be produced for the universal 12S mitochondrial region from both samples.

Universal ID – 16s Region

A DNA sequence was produced from the biological material from the cranial brain (1).

TACGTAGGACTTTAATCGTTGAACAAACGAACCTTTAATAGCGGCTGCACCATCGGGATGTC
CTGATCCAACATCGAGGTCGTAAACCCTATTGTTGATATGGACTCTAGAATAGGATTGCGCT
GTTATCCCTAGGGTAACTTGTTCCGTTGGTCAAGTTATTGGATCAATTGAGTATAGTAGTTCG
CTTTGACTGGTGAAGTCTTAGCATGTACTGCTCGGAGGTTGGGTTCTG

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The closest match of readable 16S sequence, using a genetic database (National Center for Biotechnology Information (NCBI) BLAST, Basic Local Alignment Search Tool, nucleotide database) was identified as a 100% match to *Homo sapiens* (human).

A DNA sequence was produced from the bone extracted from the hand (2).

ACGTAGGACTTTAATCGTTGAACAAACGAACCTTTAATAGCGGCTGCACCATTGGGATGTCC
TGATCCAACATCGAGGTCGTAAACCCTATTGTTGATATGGACTCTAGAATAGGATTGCGCTG
TTATCCCTAGGGTAACTTGTTCCGTTGGTCAAGTTATTGGATCAATTGAGTATAGTAGTTCGC
TTTGACTGGTGAAGTCTTAGCATGTACTGCTCGGAGGTTGGGTTCTG

The closest match of readable 16S sequence, using a genetic database (National Center for Biotechnology Information (NCBI) BLAST, Basic Local Alignment Search Tool, nucleotide database) was identified as a 100% match to *Homo sapiens* (human).

Universal ID – mt16191-16420

A DNA sequence was produced from the biological material from the cranial brain (1).

ACAGCAANCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACT
AGGATACCAACAAACCTACCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACAT
AGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCCCTCAGATAGGNGTCCCT
TGAC

This sequence did contain DNA damage. The closest match of readable sequence, using a genetic database (National Center for Biotechnology Information (NCBI) BLAST, Basic Local Alignment Search Tool, nucleotide database) was identified as a 99% match to *Homo sapiens* (human). The 1% difference is due to DNA damage causing a mismatch.

A DNA sequence was produced for the bone extracted from the hand (2).

TACAGCAATCAACCTTCAACTATCACACATCANCTGCAACTCCAAAGCCACCCCTCACCCAC
TAGGATACCAACAAACCTACCCACCCTTAANAGTACATAGTACATAAAGCCATTTACCGTACA
TAGCACATNACAGTCAAATCCCTTCTCGNCCCCATGGATGACCCCCCTCAGATAGGGGTCC
CTTGA

This sequence did contain DNA damage. The closest match of readable sequence, using a genetic database (National Center for Biotechnology

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Information (NCBI) BLAST, Basic Local Alignment Search Tool, nucleotide database) was identified as a 99% match to *Homo sapiens* (human). The 1% difference is due to DNA damage causing a mismatch.

From this data, the evidence suggests the source of DNA from the biological material from the cranial brain (1) and the bone extracted from the hand (2) belongs to *Homo sapiens* (humans).

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The results for the human nuclear DNA feasibility test were positive. There was sufficient nuclear DNA detected to perform further testing.

Sex Identification
The amelogenin test for sex identification found:

- The biological material from the cranial brain (1) belongs to a **male** individual.
- The bone extracted from a hand (2) belongs to a **male** individual.

The combination of replication, fragment sizes obtained, procedures in place for laboratory sterilization and elimination of Paleo-DNA Laboratory DNA profiles suggest the results are authentic and not contamination. However, no modern comparison samples were submitted with this batch from the archaeologists or any other individual who may have handled the sample and potentially contaminated it. Therefore, we cannot guarantee that these profiles are authentic and not a previous handler.

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NOTES:
Controls were run at every step of the analysis and gave expected results. This analysis complies with the requirements requested by the client. Details of the experimental procedures and analysis of this case are found in the case file of the Paleo-DNA laboratory, case number SER029-17. Your feedback is important to us! Please fill out our customer survey at http://lucas.lakeheadu.ca/customer-survey.

Technical Manager: _____ Date: 11 May 2017
Stephen Fratpietro

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