Results Information

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Customer ID: Roger Spurr
Project #: P-234
Sample Date Received: 6/20/15
Date of Testing/Analysis: 7/1/15 - 9/17/15
Sample Type: 3 mud fossil samples
Analysis Type: DNA Extraction, PCR, DNA Sequencing and Analysis

Objective:

The objective of this analysis is to perform extraction of genomic and/or mitochondrial DNA from three mud fossil samples. Upon successful DNA extraction from the samples, subsequent analysis will include the amplification of extracted DNA using the PCR technique targeting specific DNA marker sequences and to obtain DNA sequence from the amplified PCR products.

Mud fossil samples analyzed had the following designations:
36 inch tip
Lung
Mud Tip

Procedure:

DNA Extraction

Bacterial DNA was extracted from each of the three submitted mud fossil samples using the Gene Clean Ancient DNA Extract Kit (MP BIO, Solon, OH) according to the manufacturer's instructions. 50 microliters (ul) of proteinase K solution was added to each sample prior to incubation at 56°C and incubation was carried out for 24 hours to ensure complete lysis. A negative control sample was also included in the DNA extraction procedure. The DNA extraction from each sample was collected from the column in two elution steps such that the DNA extraction obtained from each sample consisted of a "first" and "second" elution. Following the DNA extraction procedure, the concentration and optical density of each DNA extraction was measured using the nanodrop spectrophotometer. DNA concentration in nanograms (ng) per microliter (ul) and optical density

of each of the three mud fossil samples is listed in the results section. The DNA extraction from each sample was then used as DNA template for qualitative PCR analysis.

PCR

Qualitative PCR analysis incorporated oligonucleotide primers that target specific marker DNA sequences found in the mitochondrial genome. Three sets of mitochondrial DNA specific primers were selected from the published literature (Alonso et al. 2003) and used in all PCR experiments performed. The three sets of mitochondrial DNA specific primers selected have previously been demonstrated to amplify short amplicons from the mitochondrial genome of approximately 100 base pairs (bp) from ancient DNA samples.

The PCR conditions used for all reactions followed the procedure established in Alonso el al. 2003: 95°C for 10 minutes followed by 36 cycles of 95°C, for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C.

All reactions involved one primer pair per reaction. In some instances, better quality amplification of the target mitochondrial DNA sequences was achieved using DNA from the second elution of the sample DNA extraction.

The negative control DNA extraction sample was included in all PCR experimentation. Following the PCR procedure, the PCR products were run on a 1.5% agarose gel at 90V for 70 minutes, after which the agarose gel containing the PCR products was visualized under UV light and examined for PCR amplification products that represent the amplification of the target DNA markers. The image of the agarose gel containing the PCR products is shown in the results section.

Results:

DNA Extractions

DNA was successfully extracted from each of the three samples (36 inch tip, lung and mud tip). The concentrations and optical densities of the DNA extractions are listed below

Sample Name	Concentration 1st Elution (ng/ul)	Optical Density	Concentration 2nd Elution (ng/ul)	Optical Density
36 Inch Tip	135.1	1.91	43.5	2.55
Lung	299.8	1.62	172.7	1.55
Mud Tip	91.9	2.20	37.7	2.52

PCR

A positive PCR result for a specific mitochondrial DNA marker indicates that the marker DNA sequence was successfully amplified in the sample. A sample testing negative for a specific mitochondrial DNA marker indicates that the marker DNA sequence was not successfully amplified. In each of the samples that tested positive, one single amplicon of the correct size (as measured in base pairs) was amplified. These amplicons appear as one single distinct band on the agarose gel (see figure 1). Amplicons produced from primer pairs A1/A1R and 4F/4R were the most robust amplicons produced and as a result, these amplicons were selected for DNA sequencing.

Results of PCR using mitochondrial DNA primers A1/A1R described in Alonso et al. 2003

Sample	**Result**
36 Inch Tip	positive
Lung	positive
Mud Tip	positive
Negative Control	negative

Results of PCR using mitochondrial DNA primers 2F/2R described in Alonso et al. 2003

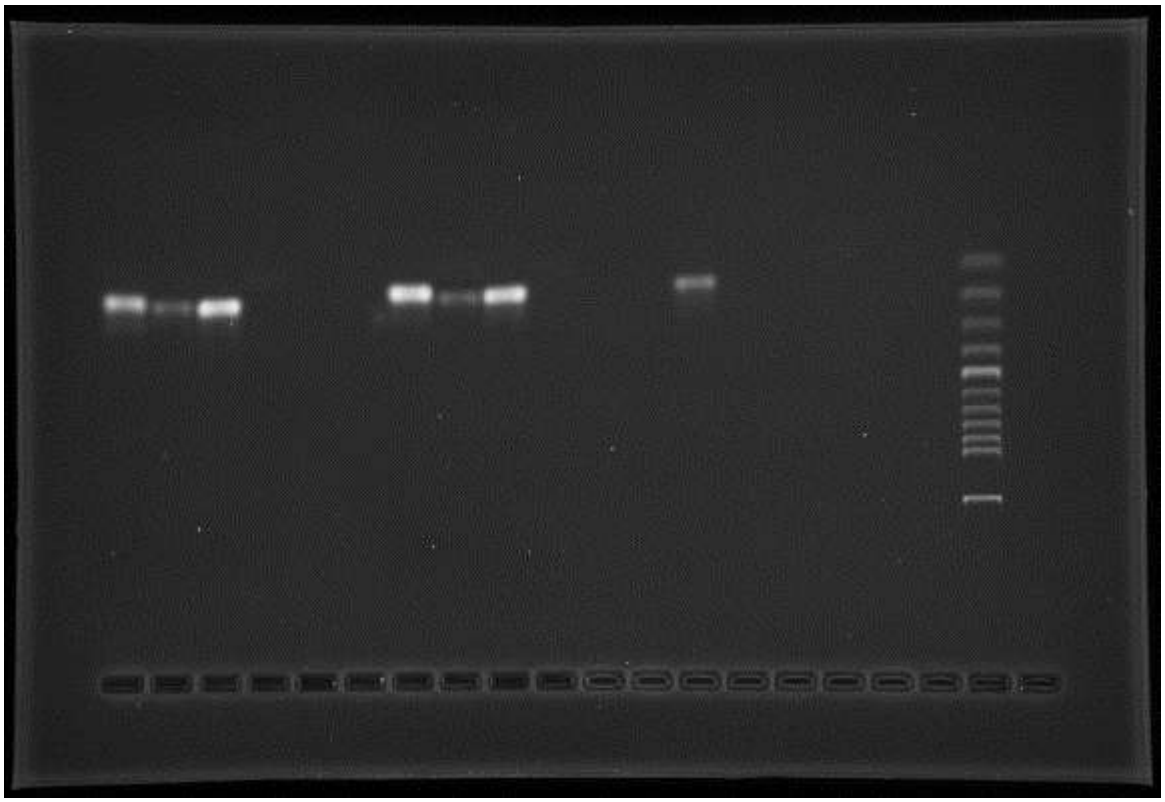
Sample	**Result**
36 Inch Tip	positive
Lung	positive
Mud Tip	negative
Negative Control	negative

Results of PCR using mitochondrial DNA primers 4F/4R described in Alonso et al. 2003

Sample	**Result**
36 Inch Tip	positive
Lung	positive
Mud Tip	negative
Negative Control	negative

Figure 1

Agarose gel containing the products of the PCR amplification:



Lane	Sample	Primers
1	36 Inch Tip	A1/A1R*
2	36 Inch Tip	2F/2R
3	36 Inch Tip	4F/4R*
4	Negative Control	A1/A1R
5	Blank	
6	Blank	
7	Lung	A1/A1R*
8	Lung	2F/2R
9	Lung	4F/4R*
10	Negative Control	2F/2R
11	Blank	
12	Blank	
13	Mud Tip	A1/A1R*
14	Mud Tip	2F/2R
15	Mud Tip	4F/4R
16	Negative Control	4F/4R
17	Blank	
18	Blank	
19	100 Base pair ladder	

"*" indicates PCR products that were used for DNA sequencing

DNA Sequencing

PCR products were submitted to Eton Biosciences Inc. (Union New Jersey) for DNA sequencing. The primer pairs A1/A1R and 4F/4R that were used to generate the PCR products were also used in DNA sequencing. Excellent quality DNA sequence was obtained for the 36 inch tip sample and for the lung sample using primers A1 and 4F (see chromatograms, figures 2 and 3). The quality of DNA sequence obtained for the 36 inch tip sample and for the lung sample was on average less using primers A1R and 4R.

Following the processing and formatting of the DNA sequence data, approximately 80 to 100 base pairs (bp) of DNA sequence generated for the 36 inch tip sample (using primers A1 and 4F) and for the lung sample (using primers A1 and 4F) were submitted individually into a nucleotide "BLAST" search, using the National Center for Biotechnology Information (NCBI) database, whereby each DNA sequence was matched to all DNA sequences contained in the NCBI DNA sequence database.

Results of the BLAST search using DNA sequences generated from the 36 inch tip and lung samples using mitochondrial DNA primers A1 and 4F:

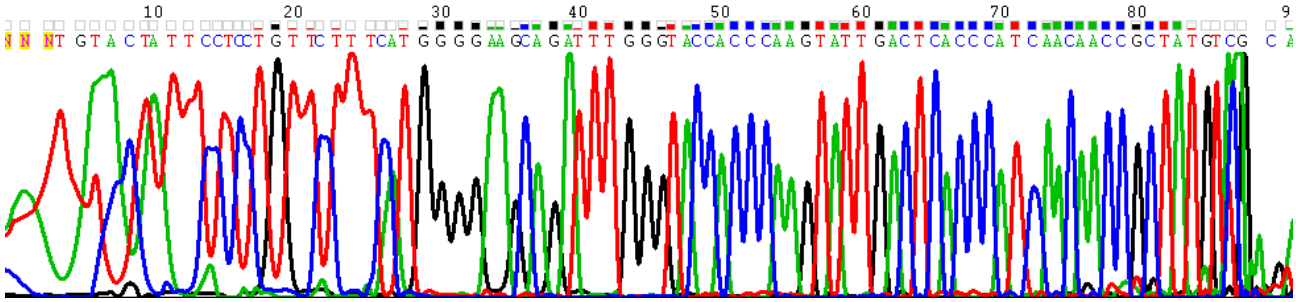
Sample	**Primer**	**Sequence Match**
36 inch tip	A1	Homo sapiens Mitochondrial Cytochrome b Gene
36 inch tip	4F	Homo sapiens Mitochondrial D-Loop Region
Lung	A1	Homo sapiens Mitochondrial Cytochrome b Gene
Lung	4F	Homo sapiens Mitochondrial D-Loop Region

Figure 2

Chromatograms of DNA Sequences Obtained for 36 Inch Tip Sample Using Primers A1 and 4F

Sample: 36 Inch Tip

Primer: A1



Sample: 36 Inch Tip

Primer: 4F

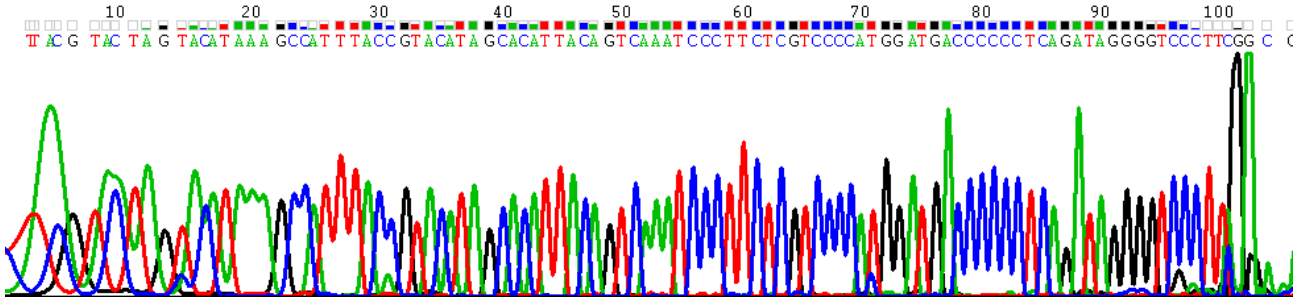
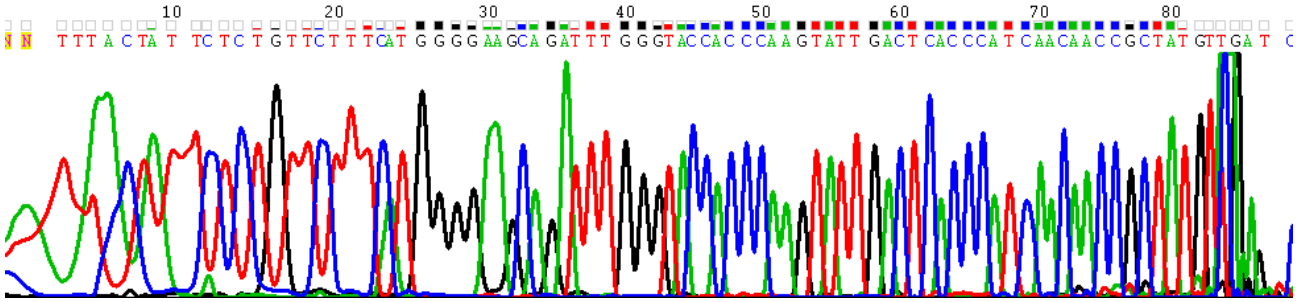


Figure 3

Chromatograms of DNA Sequences Obtained for the Lung Sample Using Primers A1 and 4F

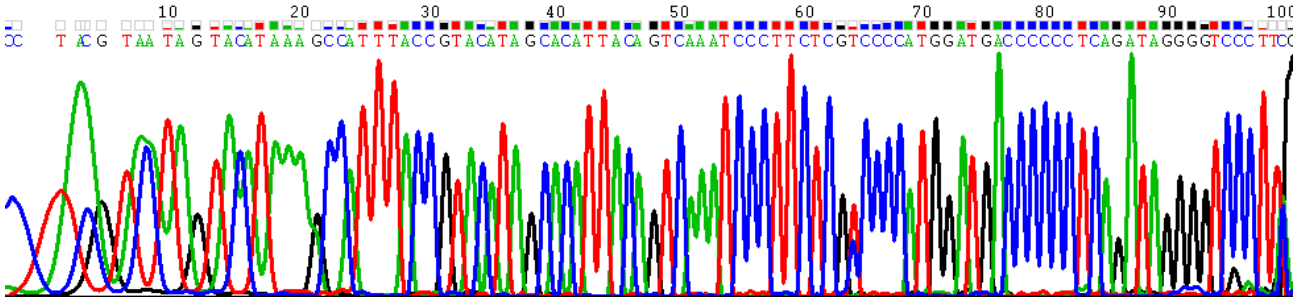
Sample: Lung

Primer: A1



Sample: 36 Inch Tip

Primer: 4F



These results were verified by Tom Prychitko, Laboratory Director for Helix Biological Laboratory.