ANALYSIS OF MITOCHONDRIAL DNA
FROM
AN ANCIENT MIAMI INDIAN

AN UNDERGRADUATE HONORS THESIS
SUBMITTED TO THE UNDERGRADUATE
SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE
BACHELORS OF SCIENCE IN BIOLOGY
BY
CHRISTOPHER M CUMMINGS

CHAIRPERSON
DR. CAROLYN N. VANN
BALL STATE UNIVERSITY
MUNCIE, INDIANA
JULY 2002

[Signature]
CAROLYN N. VANN 7/26/02
ABSTRACT

Thesis: Analysis of Mitochondrial DNA from an Ancient Miami Indian
Student: Chris Cummings
Degree: Bachelor of Science
College: College of Science and Humanities
Department: Biology
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The purpose of this research study was to analyze the mitochondrial DNA of an ancient Miami Indian. The results from this study may be used by other researchers to make comparisons to other individuals of the Miami Indian population, ancient and modern. The Miami people lost federal recognition in the mid-1800's. If a close genetic relationship between a modern and known ancient population can be proven, a strong case may be available for regaining the recognition of these people by the federal government.

For this analysis, a tooth was provided from a burial site that was excavated in Henry County, IN. The tooth has been stored in the Anthropology department's museum and has been donated by Don Cochran for use in this project. The Ancient DNA was
extracted from the dentin of the tooth that was drilled by Dr. Neal Lambert DDS. The DNA was cut with a blunt-ended restriction enzyme, *Hae* III. Double-stranded DNA adaptors were ligated to the blunt ends. A single primer was used to amplify the resulting fragments using PCR. Using this library, the DNA was readily reamplified using a small amount of the PCR product. The individual’s haplotype was determined by amplifying specific regions of its mtDNA. An agarose gel was created to visualize any existing amplification. Upon successful amplification, sequencing of the individual’s D-loop may be possible to categorize the individual into a haplogroup.

Amplification was evident based on observations from the agarose gel, but contamination from DNA other than this individual’s was present. Several amplifications and agarose gels were created to seek evidence to determine this individual’s haplotype, but were ultimately unsuccessful. Sequencing may be an option after future procedures are undertaken to create a successful amplification and agarose gel. Contamination would need to be eliminated in order to sequence the DNA, most likely achieved by a more cautious application of methods.
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I would also like to give thanks to Don Cochran of the Anthropology Department for donated the tooth from the department’s museum. Dr. Neal Lambert DDS was extremely helpful by drilling the dentin from the tooth.

In addition to this, Wes Marchione, Kandeh Kamara, and Vernon Pritchard have been very helpful in the laboratory by given me insight on concepts and methods. I have much gratitude for them for taking the time to distill their knowledge. Amanda Landis has also been of help by working with me in the beginning works of this project.
Lastly, I would like to give thanks to April Reed and Heather Ramsey for the knowledge I have gained from the material of their theses, which without would make this project impossible. Thank you to everyone once again.
INTRODUCTION

The purpose of this study was to determine the specific haplogroup of the Miami Indian, whose tooth has been supplied. Future research may be used to determine any maternal genetic relatedness between an ancient and modern Miami Indian population by means of using mitochondrial DNA (mtDNA). To support this project, Don Cochran of the Anthropology department of Ball State University has donated an ancient Miami Indian tooth stored in the department’s museum. The tooth is from an excavated site in Henry County, IN. The Biology department through Dr. Carolyn Vann, has provided much support and guidance as well as access to lab equipment and supplies.

It has become an important issue over the past few years for several groups of Miami Indians to determine any degree of genetic relatedness among themselves and what is thought to be ancestors found at several burial sites. If genetic relatedness can be proven these tribes will not only gain rights for reburial of excavated remains, but will be granted federal recognition. Federal recognition was lost in the late 1800’s, so using various techniques used to study and prove relatedness among populations; the Miami Indians may build a strong case to regain what was lost a long time ago (Rafert 1996).

To determine relatedness within and between populations, the analysis of mtDNA was used, which was to establish any relatedness through a maternal line. Several mtDNA haplotypes characteristic of Native Americans are used to establish kinship (Stone and Stoneking, 1992). These haplotypes are a categorization that has allowed
researchers to narrow major Native American populations to one of four groups, believed to represent 4 major migrations into the Americas across the Bering Straits. These haplogroups are typically unique to each group and there is generally very little overlap between groups (Lorenz and Smith 1996, Schurr et al. 1990, Torroni et al. 1993a). Each group is represented by specific sequences present or absent at 4 variable locations in mitochondrial DNA.

Because ancient DNA is so fragile and difficult to isolate in sufficient amounts, several methods of isolation and purification are used to produce amplifiable DNA. Primers are used for PCR amplification that are specific to the regions of the mtDNA that are known to vary within the four Native American lineages (Stone and Stoneking, 1992; Bailliet et al. 1994; Torroni et al., 1994). In this study, the mtDNA was further examined in a highly variable region called the D-loop. The D-loop was amplified by PCR using the primers specific to this region of mtDNA. Upon successful amplification, sequencing (Davissequencing.com) of this D-loop is possible to locate this individual within a particular haplotype, and may be used to compare to other individuals analyzed in future research projects.
REVIEW OF LITERATURE

Miami Indian’s Search for Recognition

Native Americans such as the Miami have struggled to maintain their identity in America as other ethnic groups have arrived and expanded throughout Indian Territory. They have fought, often through warfare, to stay together, keep their land, and to keep their rights as an Indian Tribe. Today, the Miami are found in 2 disjunct populations, one federally recognized group in Kansas and another unrecognized group in Indiana.

Much of the conflict began in the mid-1600’s when the Iroquois war parties drove many Miami groups from Indiana to Wisconsin. This pressure was temporary and as threats were lessened, the Miami began to migrate back into their old territory, bringing the Miami into close contact with the French and the British. Pressures mounted throughout the fifty-year Indian Civil War, but after war concluded, the Miami Indians were a stronger and more complex tribe (Rafert 1996, 1-23).

Tension continued to increase between the British and the Miami as the Miami struggled to protect their lands from raids by the settlers at the frontiers. This continued until the British were defeated in the Revolutionary War (1783) (Rafert 1996, 25-37).

The defeat of the British by the Americans subsequently caused many problems to the Miami Indians. The Miami were exposed to many diseases brought by the Europeans and lost a large number of members. This forced new leadership that was not ready for
new acts brought by the new American government, which was trying to force the Indians to concede to the new American ways (Rafert 1996, 45-50).

The new American government hoped Miami would cede a large majority of their land. To accomplish this, laws were passed that made it difficult for the trade and sale of land. When the Miami Indians did not cooperate, President Washington called for the destruction of villages, and forcibly took the land from them. The Miami fought back under the leadership of Little Turtle and defeated the American armies (under guidance of LaBalme (1780), Harmar (1790), and St. Clair (1791)). Later in 1795, the Miami were defeated at Fallen Timbers (Rafert 1996, 45-53).

The Miami negotiated with the American government for the first time at the signing of The Treaty of Greenville in 1795. The treaty formally recognized the Native Americans’ rights to their lands, but the government still refused Indian land purchases (Kappler 1972).

The Treaty of St. Mary’s in 1818 was the beginning of increased debt and the breakup of Miami lands. A large accumulated debt from purchases of goods on credit forced the cession of large areas of land for repayment. This increased dependence on the government (Rafert 1996, 80-83).

The American government continued to incorporate the land of the Indians into the American system throughout the nineteenth century. Debt left the Indians defenseless and the American government reduced the Miami landholdings from 900,000 acres to less than 1% of that by 1840. The Miami population was the only remaining Native American group residing in Indiana by 1838. Cession of most of the land known as the Miami National Reserve was due to increasing debt in 1840. Most of the Miami were
forced to relocate to a reservation in Kansas over the next five years. By 1847 all but six
groups of Miami Indians left Indiana (Rafert 1996, 97-113).

The New Indian Policies (1849) was the final effort to "civilize" Indian groups
and take away the rest of their land. This put an end to permanent reservations and to
tribal governments in Kansas. The Miami Indians left in Indiana were not subjected to
the enforcement of the terms of the New Indian Policies and began to rebuild their
community and culture. However, they did not have federal recognition or any

In the beginning of the twentieth century, the Miami were poor and many were
homeless due to debt. The culture began to fade as some due to discrimination dropped
Indian identity and as families moved to cities and towns in order to survive (Rafert 1996,
180-84). To this day, the Miami still fight to regain government recognition. Claims
have been filed to gain compensation for lands unfairly lost, unfair taxes, and money
promised in treaties that they never received (Rafert 1996, 189-244).

**Searching for an Origin**

The hypothesized entrance of humans into the Americas occurred during periods
of glaciations when a land bridge connected Siberia and Alaska. The time and frequency
of these migrations is still in question. Much support has been given to the hypothesis
that ancestors of modern day Native Americans first entered the Americas approximately
30,000 years ago. Still, much further research is needed in the area of ancient and
modern DNA to support these claims (Torroini et. al., 1994).

To gain information about the genetic relationships among Native Americans,
current research analyzing human DNA analyzes mitochondrial DNA (mtDNA)
sequences. The circular structure of mtDNA is comprised of approximately 16,000 bp. There are also very few noncoding bases between adjacent genes, except in the D-loop region (Anderson et al., 1981). Mitochondrial DNA accounts for less than 1% of total cellular DNA, but each cell contains hundreds of mitochondria with several copies of mtDNA each. Each cell contains 1,000 to 10,000 copies of mtDNA. Ancient samples of bone fragments and/or hair that have been properly preserved and stored usually contain enough mtDNA for analysis, whereas the nuclear DNA may be too degraded to analyze (Biosystems Reporter No. 23, Oct. 1994).

The mtDNA is maternally inherited and does not undergo recombination. Any changes of mtDNA between mother and offspring are results of mutations. Mutations accumulate much faster in mtDNA than in nuclear DNA, so it is possible to obtain a greater diversity in the human gene pool. Further research shows that the D-loop region contains two hypervariable (HV) regions, which will exhibit sequence differences in unrelated individuals but will be identical in maternally related individuals (Biosystems No. 23, Oct. 1994). Applying this information may be used to determine whether individuals are maternally related.

**Categorization by Haplogroups**

Researchers have been able to narrow major Native American population groups to only a small number of mtDNA haplogroups. These haplogroups are characterized by the presence or absence of polymorphisms at specific locations within mtDNA genes, which place individuals within haplogroups A, B, C, D or X (see Table 2). Haplogroup A can be characterized by the gain of the *Hae* III site (Lorenz and Smith 1996, Schurr et al. 1990, Torroni et al. 1993a). Haplogroup B is characterized by the presence of a 9-bp

Primer pairs have been designed to amplify these specific regions (Table 1) and RFLP (restriction fragment length polymorphism) analyses may be used to place an individual within one haplotype (Lorenz and Smith 1996, Schurr et al. 1990, Torroni et al. 1993). RFLP relies on digestion of amplified DNA with restriction enzymes, which cut if specific DNA sequences are present. Whether or not the DNA is cut can be assessed by visualization of the DNA on agarose gels.

The D-Loop: the most variable region of mtDNA

Another region of mtDNA, the D-loop, may be examined to indicate any relatedness amongst individuals. The D-loop is a 440 bp noncoding region of the mtDNA where the initiation of DNA replication occurs (Kiechle 1999). The D-loop is also referred to as the hypervariable region (HV1) (Stone and Stoneking 1999). Because there are no functioning genes within the D-loop it is the most variable region of the mtDNA in both its sequence and its length (Greenberg et al. 1983). (See Figure 1 for these variable regions). Mutations leading to sequence changes in the D-loop are used to help define haplogroups and to more specifically show genetic relationships between individuals within a specific haplogroup. From this information it is obvious that
sequencing of the D-loop is highly informative and provides valuable information on the origins of an individual. This review of the literature on Native American haplotypes and D-loop sequencing was extracted from the Masters Thesis of April Reed (MS, 2001) and serves as the basis for this project.
Figure 1: Diagram of Native American mtDNA Polymorphism Locations. Four arrows indicate the approximate locations delineating contemporary Native American lineages. The variable D-loop region can also be seen.
Table 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primers Sequences (5'–3')</th>
<th>Deletion Absent (bp)</th>
<th>Deletion Present (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hae III-663</td>
<td>L611 ACCTCCCTAAAGCAATACACTG</td>
<td>176</td>
<td>*101/175</td>
</tr>
<tr>
<td>(lineage A)</td>
<td>H743 GTGCTTGATGCTTTCTTCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-bp deletion</td>
<td>L8215 ACAGTTTCTGCCCACCTGTC</td>
<td>121</td>
<td>*112</td>
</tr>
<tr>
<td>(lineage B)</td>
<td>H8297 ATGCTAAGTCAGCTTTACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hinc II-13259</td>
<td>L13232 CGCCCTTACAAACATGACATCAA</td>
<td>*211</td>
<td>158/53</td>
</tr>
<tr>
<td>(lineage C)</td>
<td>H13393 TCCTATTTTTCGAATATCTTTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alu I-5176</td>
<td>L5120 TAACTACTACCGATTCCCTA</td>
<td>*149</td>
<td>77/72</td>
</tr>
<tr>
<td>(lineage D)</td>
<td>H5230 AAAGCCGGTATGCAGGGGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Indicates lineage characteristic

L refers to the light strand of the human mtDNA genome. H refers to the heavy strand of the human mtDNA genome. The number is the nucleotide position of the 3' nucleotide of the primer, numbered according to the reference sequence. The deletion absent refers to the loss of the corresponding restriction site within the designated nucleotide sequence. The deletion present refers to the gain of the corresponding restriction site within the designated nucleotide sequence (Stone and Stoneking, 1992).

Table 2: Polymorphisms which Characterize Each Native American mtDNA Haplogroup

<table>
<thead>
<tr>
<th>MtdNA Lineage (Haplogroup)</th>
<th>9-bp Deletion</th>
<th>Haell-663</th>
<th>AluI-5176</th>
<th>HincII-13259</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>X</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

(Tables taken from the MS thesis of April Reed, 2001)
MATERIALS AND METHODS

SAMPLE

Before any work was done on ancient, irrepeable DNA, a practice tooth was used which was donated by Caitlyn Vann, age 12. After practicing the methods with Caitlyn’s tooth, the methods were applied to the Miami Indian tooth that was donated by Don Cochran of the Anthropology Department at Ball State University. The tooth was excavated from a site in Henry County, IN.

Specific precautions were taken when handling this sample to avoid contamination by other human DNA. In doing this, the tooth was surface sterilized by washing it in bleach, RNase-free and DNase-free water, and irradiating it with UV light. I am the only individual that has handled this sample from beginning to end (except for powdering the dentin performed under sterile conditions by Dr. Neal Lambert, DDS). All reagents such as molecular grade water and PCR 10X Buffer were irradiated with UV light (260 nm) for 15 min, as were the pipettemen, pipette tips, and all tubes used. Gloves and a lab coat were worn at all times to prevent transfer of foreign DNA. Pipette tips equipped with filters were also employed to avoid transfer of DNA in aerosols during pipetting.

PRE-TREATMENT

The tooth was rid of surface contaminants by first washing it in 10% bleach.
solution for 2 min. The tooth was rinsed in RNase-free and DNase-free water for 3 min. The outside of the tooth was then irradiated with UV light (260 nm) for 7 min to degrade any surface DNA (Ribeiro-Dos-Santos et al. 1996).

Dr. Neal Lambert, DDS powdered the dentin of the tooth. He washed the burr used for the drilling in alcohol and then autoclaved it. Dr. Lambert wore powder-free gloves, a long-sleeved lab coat, and a facemask while drilling the tooth. The powdered dentin was placed in a glass petri dish and was parafilmed to seal. The dentin was frozen until the next use in order to preserve the DNA within the dentin.

**DNA EXTRACTION**

**Overnight Soaking Solution**

The powdered dentin was then placed in an overnight soaking solution of Proteinase K (20 mg/ml) (25 μM Tris (pH 7.5), 5 μM calcium acetate, 40% glycerol). Two hundred μl of this proteinase K solution was combined with 200 μl 10% SDS and 5 ml 0.5 M EDTA. The powdered dentin was then added (half of the dentin drilled from the tooth was used in case of a mistake) and the mixture was rotated in an incubator at 37°C for 12-15 h. Following incubation, the solution was placed in a 65°C water bath for 15 min. to inactivate the proteinase K.

**GENECLEAN Kit for Ancient DNA**

The DNA was extracted from the dentin using the GENECLEAN Kit for Ancient DNA (Bio 101, Vista, CA). One mL of DeHybernation Solution A from the GENECLEAN Kit for Ancient DNA was added to the tooth/proteinase K solution that was rotated for 2.25 h. at 60°C. The sample was aliquoted into several microcentrifuge tubes and centrifuged to pellet particulate material. Following the centrifugation, the
individual supernatants were combined in a clean tube and 1.2 ml of Ancient DNA GLASSMILK and 3.0 ml of DeHybernation Solution A were added. The sample was rotated for 2 h at 37°C and then centrifuged at 4,000 x g to pellet the DNA bound to the Ancient DNA GLASSMILK. The supernatant was discarded. To wash the pellet, 0.5 ml of Salton Wash No. 1 was added and the wash and glass beads were transferred to a SPIN Filter, and briefly centrifuged. Then, 0.5 ml of Salton Wash No. 2 was added and centrifuged at 14,000 x g to ensure the cleansing of the GLASSMILK/DNA complex. Another wash of 0.5 ml of the Ancient DNA Alcohol Wash was added and the filter and catch tubes were centrifuged to rinse the beads and DNA. The addition of the Alcohol Wash and centrifugation was carried out two times to ensure washing was successful. The catch tube was emptied and centrifuged for 2 min in order to dry the GLASSMILK pellet in the SPIN Filter.

The filter was then placed into a DNA-free Elution Catch Tube. One hundred μl of DNA-free Elution Solution was added to the pellet in the SPIN Filter. The pellet was washed by briefly vortexing for 2 s. This was centrifuged for 1 min to release the DNA to the catch tube. A second elution was carried out to maximize release of DNA. The SPIN Filter was removed and discarded and the eluted sample was frozen for future use.

LIBRARY CREATION

The creation of a library provides a continuing source of DNA from which numerous amplifications and studies may be performed.

The library creation was described in Weiss et. al. 1994 as follows:

Digestion

Digestion of the extracted DNA from the sample was carried out in order to create
blunt-ended DNA for ligation with the blunt-ended adaptors. Since I was interested in analyzing the D-loop of the mtDNA, the restriction enzyme *Hae* III was used for digestion of the DNA. One hundred μl of the eluate was mixed with 1.5 μl of 10 U/μl *Hae* III (15 total units) and 11.3 μl of the 10X buffer provided with the *Hae* III. The sample was digested at 37°C overnight. The *Hae* III was then inactivated in an 80°C water bath for 20 min.

**Ligation**

Two single-stranded, complementary oligonucleotides, LLSal2A and LLSal2B (5'-pTCGAGTCGACTATATGTACC-3' AND 5'pGGTACATATAGTCGACT-3', respectively) described by Weiss *et al*. 1994 were purchased (Integrated DNA Technologies, Coralville, IA) that, when annealed, became double stranded blunt-end adaptors with a three nucleotide overhang on one side. Using oligonucleotides with a phosphorylated 5' end blocks the ligation of the adaptors to themselves. Also, phosphorylation aids in the ligation of the blunt-ended adapters to the blunt-ended fragments.

In order to anneal the oligonucleotides to make adaptors, the two single stranded oligonucleotides were added in equal portions (10 μl each of 2 μM final concentration) to a microcentrifuge tube. They were placed in an 80°C water bath for 5 min. After the removal from the water bath, they were allowed to slowly cool to room temperature, to allow the single-stranded oligonucleotides to anneal to each other to form double-stranded adaptors with the phosphorylated 5' end sticking out and a blunt end for ligation with the digested, blunt-ended DNA.

In order to ligate the blunt-ended DNA with the blunt-ended adaptors using T4
DNA Ligase, a ligation reaction was performed. One hundred μl of the *Hae* III-digested DNA was placed in a microcentrifuge tube with 4 μl of adaptors, 12 μl of 10X buffer with ATP (provided with the T4 DNA Ligase) and 4 μl of the 400 U/μl T4 DNA Ligase (1600 U total). Ligation was performed at room temperature for 15 min, at 15°C for 2.5 h, and then at 4°C overnight to ensure the maximized ligation of the adaptors.

**Library Amplification**

Amplification of the entire library of DNA fragments with adaptors ligated to them was possible using the shorter single-stranded oligonucleotide, LLSal2B as a primer. This library amplification was done prior to haplotyping. A parallel procedure was started on the negative controls, which had all other components but lacked DNA. The 100 μL PCR reaction mixture contained 20 μL of the ligated DNA (or water in the negative control), 10 μL of the 10 X PCR buffer provided by the manufacturer, 10 μL of 25 mM MgCl₂, 2 μM final concentration of LLSal2B, 2 μL of 12.5 mM dNTP master mix, 1.0 μL of 100 X BSA, 4 U of Sigma Red Taq, and autoclaved molecular grade water to volume. Master mixes were made in order to ensure consistency between samples. The first master mix (excluding the polymerase and nucleotides) was aliquoted into PCR tubes and DNA or water (for control) was added. After an initial hotstart of 5 min at 94°C (Perkin Elmer 2400 Thermal Cycler), a second master mix containing the heat-sensitive polymerase and nucleotides was added. PCR conditions were as follows: 30 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 2 min and, finally, 72°C for 7 min.

**Marker-Specific Amplification**

Due to time constraints the D-loop is the only portion of the entire library that was specifically amplified. This 440 bp portion of the D-loop was reacted under optimized
conditions according to April Reed (2001). The only difference from April Reed’s optimization was the amount of DNA used. A greater volume of DNA was used because of the fragility of Ancient DNA. The final, 100 μL PCR reaction for the amplification of the mtDNA markers was divided into two master mixes. The first master mix contained 25 μL library template, 10 μL 10 X Buffer supplied with the polymerase, 10 μL 25 mM MgCl₂, and 1 μL 10 mg/ml BSA (100 X). The second master mix contained 2 μL of 50 X dNTP Master Mix (250 mM final concentration), 0.4 μM each primer, and 4 Units Sigma Red Taq polymerase. Each master mix was filled with autoclaved, molecular grade water, so that the final PCR reaction was 100 μL. Substituting the same volume of molecular grade water for the DNA continued a negative control. The first master mix was put through an initial hotstart using a Perkin Elmer 2400 Thermal Cycler at 94°C for 5 min. After the initial hotstart, the second master mix was added to the first and was put through the following conditions: 40 cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 2 min, and finally 72°C for 7 min.

Gel Analysis of PCR Products

The PCR products of the D-loop were electrophoresed on a 3.5% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME) in 1 X TBE. Fifteen μL of each PCR product was mixed with 3 μL of 6 X sample buffer and loaded in the gel. A 100 bp ladder (0.5 μg) (Invitrogen, Carlsbad, CA) was used as a molecular weight and concentration reference. The gel was electrophoresed in 1 X TBE [40 mM tris-borate, 1mM EDTA] at 50 V until the blue tracking dye was three-quarters of the way down the gel.

The gel was post-stained in 60 mL of 1 X TBE and 6.0 μL of 10,000 X GelStar
(FMC Bioproducts, Rockland, ME) for 20 min. The gel was rinsed in tap water. A photograph of the gel was taken using a transilluminator and UV light at 260 nm.

Similarities between the negative and the positive control lanes seen in the photograph of the first gel, suggested contamination of the samples. Because of this, a second gel was created, with minor variations from the first. The NuSieve 3:1 is a high-resolution gel suggested for analysis of low molecular weight products, and the manufacturer suggested a 3.5% gel for products in the 100 bp range. Since the D-loop is 440 bp, the second gel was run using 1% NuSieve 3:1 agarose gel. This allowed the D-loop marker to move through the gel with more ease. Also, on the second gel, 20 μL of each PCR product was used without using the 6 X sample buffer. Since Sigma Red Taq was used in amplification, no additional dye was needed to visualize the bands when taking the photograph of the gel. Instead of running one band of each control, 3 positives and 2 negatives were run side by side to compare multiple wells of each control. Doing this, comparisons are made to determine if a mistake has been made when running the lanes on the gel. If each control was consistent, then a mistake was not made, although this doesn’t give further evidence about contamination.

**Gel Extraction**

Extracting the DNA from the agarose gel and re-amplifying the product was used to provide more material for subsequent sequencing and allowed closer examination of this hypervariable region. A kit purchased from QIAquick (Vendor) was used to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. First, the DNA fragment (the D-loop of 440 bp) was cut out of the agarose gel with a razor blade. The slices cut out were weighed (gel volume) in a standard 1.5 ml
microcentrifuge tube. Three times the volume of Buffer QG was added for every 1 gel volume (100 mg ≈ 100 μl). This was incubated at 50°C in a heat block for 10 min, with periodic vortexing. This allows the gel slice(s) to completely dissolve in the buffer. It is important here that the agarose is completely solubilized. One gel volume of isopropanol was added to the sample and mixed. Adding isopropanol increased the yield of DNA fragments. The DNA was bound by applying the sample to the QIAquick spin column in a 2 ml collection tube, and centrifuging for 1 min. The flow-through was discarded and the QIAquick column was placed back into the same collection tube. To remove all traces of agarose, 0.5 ml of Buffer QG was added to the column and centrifuged for 1 min. The DNA complex was washed by adding 0.75 ml of Buffer PE to the column and allowing the wash to stand for 2.5 min before centrifuging for 1 min. The flow-through was once again discarded before centrifuging for 1 more min to completely remove the residual ethanol from Buffer PE. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube, where the elute was stored for future amplification. To elute the DNA, 30 μl of Buffer EB was added in the center of the QIAquick membrane, allowing the column to stand for 1 min, and then centrifuged for 1 min. The DNA was amplified with the same procedures as previously listed. Both the general library amplification and the marker-specific amplification were performed before a new 1% gel was made for the gel extracted DNA.
RESULTS AND DISCUSSION

POWDERING OF THE DENTIN

We were able to obtain a substantial amount of powdered dentin from the Miami Indian tooth. From the amount of dentin that was salvaged, we used half in the procedures discussed in methods and materials. Half was saved for purposes of analyzing different specific regions of mtDNA in the future.

GEL ANALYSIS OF MARKER-SPECIFIC AMPLIFICATION

After the marker-specific library was amplified for the D-loop, the PCR products were run on a 3.5% agarose gel in 1 X TBE with 40 mM tris-borate, and 1 mM EDTA in order to determine if the library had been sufficiently amplified (Fig 2). Upon analyzing the photograph of the gel, there was evidence of contamination, possibly from DNA not belonging to this Miami individual. There are similar bands present in the negative and the positive controls in lanes 1 and 2 between 400 and 500 bp. Because of this, a second gel was run on a 1% agarose gel in 1 X TBE with 40 mM tris-borate, and 1 mM EDTA. Three positive and 2 negative controls were run on this gel to distinguish if any contamination was actually present and to get more product for sequencing. As seen in Fig 3, there is still apparent contamination amongst the positive and the negative controls. There are similar bands present in 4, 5 and 6 as compared to 1 and 2 between 400 and 500 bp. This contamination may be due to the introduction of DNA from myself or someone else in the lab, which is a big risk when working with Ancient DNA.
Figure 2: Gel Analysis of Marker-Specific Amplification. A 3.5% TBE gel was run. Lane 1 contains 15 µl of a 100 µl PCR reaction amplifying the Hae III library. Lane 2 contains 0.5 µg of 100 bp DNA Molecular Weight Marker. Lane 3 contains 15 µl of the negative control.
Figure 3: **Gel Analysis of Marker-Specific Amplification.** A 1% TBE gel was run. Lanes 4, 5 and 6 contain 20 μl of a 100 μl PCR reaction amplifying the Hae III library. Lane 3 contains 0.5 μg of 100 bp DNA Molecular Weight Marker. Lanes 1 and 2 contain 20 μl of the negative control.
GEL EXTRACTION AND RE-AMPLIFICATION

A kit from QIAquick was used to extract the DNA from the second gel that was created (Fig 3), so that further amplification of the D-loop was possible. This was done for two reasons. The first was to permit further evidence of any possible contamination. The second was, if contamination was not present, to allow for possible sequencing of this D-loop region in the mtDNA. Simultaneously, re-amplification of previously saved and stored ligated DNA was undertaken. This was to provide even further evidence of the possibility of contamination. A completely new negative control was created, and these three products were put through PCR for the amplification of the general library and the marker-specific library. Upon successful amplification, these products were run on a 1% agarose gel according to the methods previously used. Figure 4 illustrates the results of this new agarose gel.

From observations of the agarose gel, higher molecular weight products are visible. The observable smear suggests that some DNA is present, but no single DNA bp size is distinguishable. Although, the negative control is without threat of contamination, having no bands in the positive controls remains a problem.

Even though this re-amplification/gel extraction was unsuccessful, an additional re-amplification of the previously ligated DNA may be undertaken in the future for another attempt to obtain a desirable positive band. In the hope of this success, the bands may be sent off for sequencing, and may subsequently determine the haplotype of this Miami Indian individual.
Figure 4: Gel Extraction and Re-Amplification. A 1% TBE gel was run. Lane 2 contains 20 µl of a 100 µl PCR reaction performed on the re-amplified stored ligated material. Lane 4 contains 20 µl of a 100 µl PCR reaction of the gel extraction. Lane 3 contains 0.5 µg of 100 bp DNA Molecular Weight Marker. Lane 1 contains 20 µl of the negative control.
CONCLUSIONS

The purpose of this project was to determine the mtDNA haplogroup of the Miami Indian individual for which we had been supplied with one of it’s preserved teeth. Assumptions determined that this individual would belong to one of the Native American mtDNA haplogroups. With the knowledge of this categorization, future research on similar individuals may be used for comparisons so that further conclusion may be made about the origins and relatedness of these people.

Purification of this individual’s tooth was done to eliminate contaminants that would interfere with PCR amplification. DNA was extracted from the dentin so that it may be directly used in amplification. With an amplified library, ample ancient DNA was available for optimization of reactions without exhausting the supply of irreplaceable DNA. Upon, gel electrophoresis analysis, it is possible to decide if bands of DNA present are usable in sequencing or if they are a result of contamination.

Because my project was unable to obtain a satisfactorily band of DNA suitable for sequencing, it has been unsuccessful in terms of determining the haplogroup in which this individual belonged to. This project did make important contributions to the methods of isolating and amplifying ancient DNA from degraded samples. Future research in this area should use more care when administering the procedures for working with ancient DNA.
Successful categorization of individuals in the future may be able to relate ancient and modern Miami Indians. Since the Miami Indians have struggled to gain recognition as an American Indian Tribe by the Federal Government, studies showing that they belong to a Native American Haplogroup may have important repercussions for them. This would not only give them recognition as an Indian tribe, but with Federal recognition, they would gain rights and opportunities to preserve their unique heritage.

Future plans are to go back and re-amplify previously ligated DNA in another attempt to obtain a desirable band of DNA that may be used for sequencing. If successful, future researchers may use this information as a comparison to their work and a possibility of further conclusions. Despite this outcome, any future research must use extreme caution when working with the fragility of ancient DNA. Much information is packed within this preserved material if appropriate precautions are taken in addition with meticulous procedures.
References


