MtDNA ANALYSIS OF AN EARLY LATE WOODLAND INDIVIDUAL IN EAST CENTRAL INDIANA

AN UNDERGRADUATE HONORS THESIS
SUBMITTED TO THE HONORS COLLEGE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE BACHELORS OF SCIENCE IN BIOLOGY

BY

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MUNCIE, IN

MAY 2006
ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Carolyn Vann for providing me with the opportunity to conduct research on a topic that I am so interested in. Her support and guidance were essential for undertaking this project. This project would not have made it this far without her enthusiasm and determination to see it happen.

I would also like to acknowledge Dr. Evelyn Bowers for her support in the project. She was always available to discuss ideas with and proved to be a valuable resource on where to look next when we might encounter a problem. I would like to acknowledge Beth McCord and Don Cochran for allowing access to the remains used in the research. Their assistance was often necessary, and greatly appreciated, whenever any of the various roadblocks would occur.

I would like to acknowledge my fellow researchers Brandon Rapier and Tam Dang. They were both infinitely helpful, whether it was finding yet another paper or optimizing a protocol. They were also willing to help any time that I would have any questions and that was really appreciated.

I would like to acknowledge the Dr. Neil Lambert for his assistance with the teeth. His cooperation was important for the success of this project.
I would like to acknowledge the committees of the HGABEL grant through the Biology department and the Troyer grant through the Anthropology department at Ball State University. The money received from these grants made it possible to do this research. Finally, I would like to acknowledge the Archaeological Resources Management Service, Ball State University for their generous donation that allowed for the final step of the research to be completed.
The purpose of this project was to determine reliable methods for successfully obtaining mitochondrial DNA sequences from an ancient individual. This is made difficult by the DNA’s highly degraded nature, as well as its high likelihood for being contaminated. Strict contamination protocols were laid out which each individual working in the lab was required to follow. The methods used were first optimized using a modern DNA sample. These same methods were used on the ancient DNA samples;
however more optimization was required to obtain the best results possible from our samples.

The GENECLEAN kit for aDNA isolation was used to isolate the DNA from the dentin samples. Approximately 35 μg/mL were obtained from 1.100 g of dentin from the intrusive individual. The next step, PCR, required the most optimization. Multiple runs at varying temperatures and reagents were done to determine which recipe was the most effective for amplifying the isolated DNA. PCR revealed that the isolation was successful and that no contamination had occurred. The TA Topo cloning method required some optimization to determine the best way to grow the colonies and then isolate them for sequencing. The clones were successfully grown. The clones were sent to Davis Sequencing for sequencing and were analyzed using the program Vector NTI. Sequences were obtained from Davis for half of the clones sent. The other half displayed an unexplained primer overlay at one end of the sequence.

The samples used in this study were obtained from the Windsor Mound site located in Randolph County, Indiana. The human remains were excavated and curated by the Archaeological Resources Management Service. The Anthropology Department granted permission for the use of the remains for this project, which is in compliance with the Native American Graves Protection and Repatriation Act.
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INTRODUCTION

The purpose of this project was to determine methods that provided consistent results for isolating and analyzing ancient mitochondrial DNA. The results of this research can be used to further study the relationships between the individuals of the site used in this study and, in general, the mtDNA from any other ancient remains. The Archaeological Resources Management Service of the Anthropology department at Ball State University provided the remains for this research, which were recovered from Windsor Mound, located in Randolph County, Indiana. In this particular paper, the remains of the intrusive burial excavated from the mound were studied.

The research was carried out in a clean room obtained by Dr. Carolyn Vann from the Biology department. Contamination was the primary concern of this research so the acquisition of a clean room was important in having control over as many of the potential contaminating factors as possible. The isolation steps were carried out in this clean room. PCR set-up was completed in a dedicated hood and all post-PCR steps were completed in the general laboratory.

This study first used sample mtDNA from a modern individual to test and optimize the methods and protocols that were going to be used in the research. The
sample used was a baby tooth, donated by Tam Dang. Once the protocols were worked out, research began on the ancient individuals. Further optimization of the protocols occurred to compensate for the degraded and complex nature of the aDNA.

The isolation was completed using the GENECLEAN kit from Qbiogene. There were three potential combinations of the dehyb solutions that could be utilized by the kit. Each of the students participating in this research used one of the combinations and all were able to isolate aDNA. Two protocols for PCR were initially attempted to determine which had a higher yield. In the end, a modified protocol that included the addition of various reagents was used on the ancient samples. After amplification, the samples were put on a low-melt electrophoresis gel to isolate the DNA for insertion into a vector which would then be incorporated into competent cells for cloning. The TA TOPO cloning kit was used for this step. The cells were grown on ampicillin plates to select only those colonies that contained the desired DNA sequence. The colonies were then selected and the DNA was isolated from the cells using the GeneJET™ Plasmid Miniprep kit.

Once the DNA was isolated, it was sent to Davis Sequencing (davissequencing.com) for sequencing. Sequence analysis was completed using the Vector NTI program from Invitrogen. The results of the sequences for the intrusive individual were compared and a consensus sequence was determined. There are four major mutations present in Native American populations. Each mutation corresponds to a particular haplogroup and each haplogroup corresponds to a particular wave of migration into the Americas. The determination of a haplogroup for this individual can be used in future research to determine any relatedness between the intrusive individual and other native populations.
LITERATURE REVIEW AND SITE BACKGROUND

Windsor Mound

Windsor Mound, located in Randolph County, is the largest prehistoric earth mound in east central Indiana. It was documented in 1988 as being slightly elliptical, with measurements of 35 m by 47 m by 4.5 m in height. A radiocarbon date obtained from the lower portion of the mound dates the mound to approximately 70 BC ± 70 (McCord 1996:46). Windsor Mound was initially explored in 1986 by local amateur excavators. The Archaeological Resources Management Service of Ball State University and the Upper White River Archaeological Society collaborated to conduct a limited formal excavation in 1992 and 1993 (McCord 1996:1).

A minimum of 44 individuals were recovered from the mound. A majority of the individuals consisted of crania only. The crania exhibited hyperbrachycephalic as a result of cradleboard deformation. Between 73% and 78% of the remains recovered were identified as males (McCord 1996:25). An intrusive burial was recovered in a burial pit located on the top of the mound (Fig 1). The intrusive individual was determined to be a
Figure 1. Location of the intrusive burial in Windsor Mound.
female in her early 20s. Based on the associated lithics, the intrusive burial is assigned to the Early Late Woodland time period (Figs 2 and 3).

Numerous artifacts associated with the site were collected and curated at the ARMS. There were 40 lithic artifacts recovered, with the majority of them being flakes (McCord 1996: 39). Pottery sherds (50) were also recovered from the mound. The rim forms were similar to those found at Mounds 4 and 7 at the New Castle site and can be related to the Adena Plain and the McGraw Plain (McCord 1996:42). Other artifacts recovered primarily from the rock mound include shell disk beads, faunal bone fragments, fire cracked rock, charcoal, burned clay and red ochre (McCord 1996:45).

Previous studies have been completed on the skeletal remains of Windsor Mound. Michele Greenan (1999) completed a paleopathology study on three sites in east central Indiana that included Windsor Mound. She re-examined the remains to determine the sex and age of the individuals, as well as the significance of any pathologies found on the remains (malnutrition, disease, etc). Rebecca Sick (2000) completed a nonmetric analysis of four east central Indiana sites that included the remains from the mound in this study. She examined various nonmetric traits of the remains to determine if there is a significant difference between any of the sites. No DNA research has been completed on Windsor Mound and this study hopes to be able to document reliable sequences for an individual located at the mound.

Mitochondrial DNA

Once an individual has died, a series of enzymatic processes, nonenzymatic hydrolytic cleavage of phosphodiester bonds in the DNA backbone, and hydrolytic
Figure 2. Excavation photo of the intrusive burial at Windsor Mound.

Figure 3. Photo of artifacts associated with the intrusive burial at Windsor Mound.
cleavage of glycosidic bonds between bases results in the degradation and fragmentation of the DNA in a cell. Nuclear DNA is only present in the cell in two copies, one from each parent. In studies involving ancient remains, nuclear DNA is almost never used because of the low copy number present in cells. Mitochondrial DNA is a circular molecule approximately 16,500 bases in length that is present in hundreds of copies in the each cell. The mtDNA is often degraded into fragments 100-500 bases in length. However due to the large copy number, it is possible to obtain a consensus sequence for the DNA.

mtDNA is maternally inherited, does not recombine, and has a high mutation rate; therefore it is possible to use the sequence to determine relationships between a mother and her children (Katzenberg and Saunders 2000:354). This could be used for any cemetery site to determine familial relationships of the individuals located there. There are two noncoding hypervariable regions located in a region called the D-loop on the mtDNA. Specific mutations located in these regions are used to classify four lineages referred to as haplogroups. Haplogroup A is characterized by the addition of HaeIII site, haplogroup B by a 9bp repeat, haplogroup C by the addition of an AluI site and haplogroup D by the loss of an AluI site. There has been mention of a fifth haplogroup called X; it is characterized by the loss of a Ddel site and the addition of a HaeIII site (Mulligan et al 2004:298). It is basically a catch-all group for mutations that do not fall into the four major haplogroups (Figure 4 and Table 1).
Figure 4. Mitochondrial DNA showing haplogroup markers.

Table 1. Table showing the typical haplogroups and their characteristic markers.

<table>
<thead>
<tr>
<th>Haplogroup</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+ HaeIII</td>
</tr>
<tr>
<td>B</td>
<td>9bp repeat</td>
</tr>
<tr>
<td>C</td>
<td>+ Alul</td>
</tr>
<tr>
<td>D</td>
<td>- Alul</td>
</tr>
</tbody>
</table>
Previous Studies

Using DNA for analysis in an archaeological setting is a fairly recent approach. There have been numerous studies completed with varying degrees of success. The first human DNA analyzed from an ancient individual was done in 1985 from a 2400 year old mummy of a child. Isolation and sequencing were both successfully used in this case. mtDNA sequences have also been successfully obtained from the brain of an 8000 year old individual and from the remains of mummies found in Peru and Egypt in 1986 (Katzenberg and Saunders 2000:354).

In 1988 PCR was utilized for the first time in mtDNA research on a 7000 year old brain found in Florida. mtDNA was first isolated from bone samples ranging in age from 300 – 5000 years B.P. in 1989. This study revealed that the degree of presence of DNA was directly affected by the preservation of the bone and not the age of the sample. In 1990, DNA was successfully extracted using the dentin found in teeth (Katzenberg and Saunders 2000:355).

Since the occurrence of these hallmarks, the use of DNA to study remains has been widely used and is becoming more and more accepted as a viable means to analyze remains beyond their physical characteristics. DNA can be used in an archaeological context to aid in the determination of the sex of an individual. It can also be used to determine any potential pathologies in the individual. On a larger scale, the use of DNA can be useful in determining migration patterns in populations and any potential relationships between individuals of a particular site.
MATERIALS AND METHODS

Samples

The modern sample used for the first practice run of the procedures was a baby tooth donated by an undergraduate student, Tam Dang. The ancient samples were obtained from the curation facilities of the Archaeological Resources Management Service of Ball State University.

The teeth were initially cleaned in the dedicated clean room. The researchers wore lab coats, gloves, goggles and face masks to protect the samples from contamination. The teeth were thoroughly cleaned using a 15% bleach solution and then rinsed with pure millipore water. The teeth were irradiated with a UV light for 5 – 10 min. The teeth were placed in individual bags to be taken to the dentist.

The dentist, Dr. Neal Lambert, was given sterile petri dishes to place the extracted dentin in. He used separate virgin drill bits on each tooth to prevent contamination. The petri dishes were sealed and not re-opened until the samples were back in the clean room.
Isolation

The DNA isolation was done using the GENECLEAN® for Ancient DNA kit from Qbiogene (Irvine, CA). Three dehybemation solutions were included with the kit. Dehyb solution A is a guanidine based solution and dehyb solution B is an EDTA based solution. Dehyb solution A2 is mixed to dehyb solution A to potentially enhance its performance. Each individual involved in the research used a different dehyb solution. The combination of A and A2 was used in this particular experiment. The dehybemation solutions are used to prepare the DNA for extraction from the cells.

After soaking in the dehyb solution, glassmilk was added to the sample. The glassmilk contains small glass beads which the DNA will adhere to. The sample containing the glassmilk is put into a spin filter column. The sample is washed first with Salton wash #1 and then Salton wash #2. The washes are important for cleaning the mtDNA. Next, the mtDNA is washed with the Ancient DNA alcohol wash, which ensures that all of the Salton wash solution is clear of the DNA. At this point the mtDNA is bound to the filter in the column. An elution solution is used to remove the mtDNA from the filter and into a tube. Two sequential elutions of the filter were done. Once the elutions were made, the sample was placed into a spectrophotometer to determine the concentration.

Polymerase Chain Reaction

For the modern sample, two protocols were used to determine which had a better yield. The first was used by a graduate student at Ball State University (Reed 2001). Two reactions were set up, with one using 0.4 μM concentration for the primers and the other
using 0.2 μM. Both reactions had a hot start at 94°C for 5 min. The sample was denatured at 94°C for 30 s, annealed at 54°C for 1 min and elongated at 72°C for 2 min. These three steps ran for 40 cycles. The final elongation was at 72°C for 7 min.

The second protocol was obtained from a paper on the analysis of DNA from museum specimens (Cooper 1994). This protocol was set up the same way, with two concentrations of primers at 0.2 and 0.4 μM. This protocol also called for a hot start period of 94°C for 5 min. The sample was denatured at 92°C for 30 s, annealed at 50°C for 1 min and elongated at 72°C for 1 min. The final elongation was at 72°C for 7 min.

For the ancient sample, a modified protocol was used. In addition to the standard reagents used in PCR, MgCl₂ and BSA were added to the reaction tubes. A different polymerase, the Diamond DNA polymerase by Bioline (Randolph, MA), was also used in this protocol. This protocol had a hot start period at 95°C for 5 min. The denaturation was at 94°C for 30 s, annealing was at 55°C for 30 s and the elongation was at 72°C for 30 s. These three steps ran for 45 cycles. The final elongation took place at 72°C for 9 min.

**Gel Electrophoresis**

The modern sample was run on a 1.5% agarose gel in 1X TE buffer. It was run twice; the first was a standard ethidium bromide gel and the other was soaked in a solution called GelStar® Nucleic Acid stain from Cambrex (East Rutherford, NJ). The two gels were compared to determine which stain had a greater sensitivity for the mtDNA.

The ancient sample was also run on a 1.5% agarose gel to determine the presence of mtDNA. It was run with an extraction negative sample, a PCR negative control, a
series of positive control dilutions and the DNA ladder. Once this presence was established, the sample was run on a 1.5% low melting agarose gel for DNA extraction for the cloning step.

Cloning

The sample was cut out of the low melting agarose gel and then melted in a small amount of TE buffer. Cloning was carried out using the TOPO® TA cloning kit from Invitrogen (Carlsbad, CA). The vector provided for ligating the amplified PCR sequence into was the pCR®-TOPO® vector which contains both ampicillin and kanamycin resistant genes. The recombinant molecules were then transformed into competent E.Coli cells and grown on agar plates containing ampicillin. Any colonies that grew on the plates contained the vector encoding the resistance to the ampicillin plus the aDNA insert since the vector was linearized and unable to ligate itself. Ten colonies growing on the plates were selected to be grown for sequencing analysis. Once these colonies had grown, were recombinant molecules was isolated from the cells by using GeneJET™ Plasmid Miniprep Kit from Fermentas (Hanover, MD).

Sequencing Analysis

The samples were sent to Davis Sequencing for sequencing. Some of the samples were not at a high enough concentration so they were spun down in a speed vac and reconstituted at a higher concentration. The T3 and T7 forward and reverse primers provided by Davis Sequencing were used for the sequences. The sequences were provided with phred values by the Davis. Phred values between 20 and 50 were
considered acceptable sequences. The Vector NTI® software by Invitrogen was used in the analysis of the sequences. The sequence from the ancient individual was compared to that of the Cambridge reference sequence for human mtDNA.
RESULTS AND DISCUSSION

Fig 5 and 6 show 5 μL of the modern mtDNA on gels stained using two different methods. A concentration of 955 μg/mL was obtained from the isolation. Fig 5 was stained using the GelStar® nucleic acid stain and Fig 6 was stained using ethidium bromide.

These gel results show the ethidium bromide staining to be the most sensitive. The GelStar® was expected to be more sensitive, but given the age of the product, it is likely that a new sample of GelStar® will provide more sensitive staining. The lack of a band for the 0.2 μM primer concentration under the Reed conditions and the presence of it in the Cooper conditions indicates that the Cooper conditions yield more amplified DNA.

Fig 7 shows the results of the ancient DNA isolation. A concentration of 35 μg/mL was obtained from 1.100 g of dentin. The gel was stained using ethidium bromide.

The absence of bands in lanes 2 and 7 indicate that there has been no contamination of the reagents in both the isolation and the PCR steps. The presence of bands in lanes 3 – 5 indicate that all of the reagents are reacting properly. The presence of
Figure 5. Gel electrophoresis for the modern sample using the GelStar ® stain. Lane 1 is Reed 0.4 μM primers. Lane 2 is Reed 0.2 μM primers. Lane 3 is Cooper 0.4 μM. Lane 4 is Cooper 0.2 μM primers.
Figure 6. Gel electrophoresis with the modern sample using the ethidium bromide stain. Lane 1 is Reed 0.4 μM primers. Lane 2 is Reed 0.2 μM primers. Lane 3 is Cooper 0.4 μM. Lane 4 is Cooper 0.2 μM primers.
Figure 7. Gel electrophoresis on the ancient sample.
a band in lane 6 indicates that the extraction and isolation of the mtDNA from the ancient
tooth was successful.

Ten clone colonies were sent to Davis Sequencing for sequencing. The T3 and T7
primers provided by Davis were used for obtaining forward and reverse sequences for the
desired mtDNA sequence. Of the ten clones sent, five had useable sequences. These five
were aligned using the Vector NTI software and contained identical sequences (Fig 8).
The five sequences that were not useable had a multiple primer sequence overlay at one
end of the sequence. It is not sure why this overlay occurred.

The sequences could not be used to determine a definite haplogroup for the
individual because the sequence isolated by the primers used was not long enough to
include the region containing the B haplogroup. A longer sequence was not isolated due
to the fragmented nature of the mtDNA. More PCR and sequencing will be needed to
accurately determine the haplogroup of the individual.
Figure 8. mtDNA sequence obtained from a clone.
CONCLUSION

This study was able to show that accurate mtDNA sequences could be obtained from ancient remains in east central Indiana. The next sequence downstream on the mtDNA will need to be sequenced next to determine a haplogroup for the individual. The sequence found in this study can be used in the future as a continuation of the research.

The research completed by this study is just a small portion of the work that can be done in this field. There are numerous other individuals from which mtDNA can be obtained, not just from the Windsor Mound site, but from various other sites excavated in Indiana and the Midwest in general. Once sequences have been obtained from more individuals, more archaeological conclusions can be drawn. These conclusions include, but are not limited to, haplogroup association, familial lineages, sexing, and pathologies. A continuation of molecular research on ancient remains could provide more information for understanding the Native American people who once lived in this area.
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