Analyzing Ancient Mitochondrial DNA from Fontabelle Emergency Excavation Site Remains

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Analyzing Ancient Mitochondrial DNA from Fontabelle Emergency Excavation Site Remains

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from the College of William and Mary

by

Katherine Hoptay

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“...Here we are born, and here are the graves of our fathers. Can we say to their bones, arise and go with us into a foreign land?”

~ Slaves in Barbados, upon being told they were being transported elsewhere (Beckles, 1987)
Abstract

During the first decade of settlement in Barbados, slaves were not the dominant labor force; rather, indentured servitude was the most common source of labor. After the failure of tobacco and cotton crops, however, the successful implementation of sugar crops necessitated more and cheaper labor, and Barbados plantation owners turned to the Atlantic slave trade. Slaves imported to Barbados from Africa belonged to as many as nineteen tribes, originating from all across Africa, but concentrated in the West African coast. The overall objective of this thesis was to use molecular archaeology techniques to determine the origin of the remains discovered during an emergency excavation in Fontabelle, St. Michael, Barbados.

To discover the origin of the unidentified remains, mitochondrial DNA (mtDNA) was extracted in a separate facility to reduce the risk of contamination by modern DNA sources. Because of its maternal inheritance, mtDNA is used to establish lineages across generations. The mitochondrial genome is separated into a coding region and non-coding region; the non-coding region which has a mutation rate higher than nuclear DNA, particularly in two regions, hypervariable regions I and II (HV I and HV II). Certain mutations in these hypervariable regions are more likely to be found in certain ethnic groups, to the extent that ethnic groups can be identified by their specific set of mtDNA mutations, or haplogroups.

Haplogroups have been compiled into a comprehensive phylogenetic tree representing human migration patterns and tracing back to the first maternal ancestor.

After extraction, HV I of each bone sample was amplified. However, due to post-mortem damage sustained by the DNA, sequences were obtained from only 3 of the 5 bones analyzed, only one of which was reproducible. MtDNA from the cheek cells of laboratory personnel interacting with the bones was sequenced as a positive contamination control. All sample sequences were compared to the revised Cambridge Reference Sequence (rCRS), the first complete mitochondrial genome sequence, to determine their haplogroup.

Laboratory personnel were haplotyped as belonging to haplogroups T2b, H and J1b1, all haplogroups of European origin. The ancient sample with reproducible sequencing results was haplotyped as haplogroup C1, a Native American haplogroup of North and South America, which suggests this sample belongs to the descendant of one of forty Amerindians from South America enslaved by the settlers of Barbados. Establishing the origin of the other two samples was less conclusive as there was no additional sequence for determining which mutations were true and which were the result of sequencing errors. The majority of the mutations from both samples, however, when compared with worldwide frequencies of mtDNA mutations, showed the highest match probabilities with Kenyan and African populations, suggesting the samples belong to the remains of African slaves.

Ideally, at least three separate extractions, each producing amplified mtDNA and mtDNA sequences, is obtained from each ancient sample in order to identify and authenticate haplogroup results. Despite attempts at optimizing the extraction protocol—by reducing then increasing the mass of each sample, by increasing incubation time for bone dissolution and cell lysis, by performing repeat extractions to remove PCR inhibitors, and by utilizing mtDNA amplification kits—most extractions did not yield DNA suitable for extraction. This suggests DNA in the bone samples either exists in such low concentrations or has sustained so much damage that it cannot act as the DNA template in PCR reactions. It is unlikely further attempts at extraction with these remains will produce positive results.
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I. Introduction

a. Overview

Modern molecular biology techniques allow for the analysis of ancient DNA and afford archaeologists and anthropologists information regarding kinship patterns, genealogies and migration patterns not available through studying the fossil record and preserved physical evidence of human populations. The focus of molecular archaeology is not genomic DNA but mitochondrial DNA. Any DNA present in ancient samples is highly degraded; successfully extracting any part of one of the two copies of nuclear DNA in each cell is extremely difficult (Budowle et al., 2010). MtDNA has a much higher copy number: multiple copies of the mitochondrial genome are housed in each of the hundreds of mitochondria in each cell (Budowle et al., 2003). Chances of successful DNA typing are further increased by the haploid nature of mtDNA, which simplifies DNA sequencing. Because of the matrilineality of mtDNA, mtDNA sequences are used in modern forensic studies to establish identity and in ancient DNA studies, to establish lineages across generations.

The mitochondrial genome is separated into a coding region and non-coding region. The coding region is heavily conserved, while the non-coding region has a mutation rate much higher than nuclear DNA, particularly in two regions, hypervariable regions I and II (Budowle et al., 2003, 2010). Certain mutations in these hypervariable regions are more likely to be found in certain ethnic groups, to the extent that each ethnic group can be identified by its specific set of mtDNA mutations, or haplogroup.
Haplogroups are compiled into a comprehensive phlyogenetic tree representing human migration patterns and tracing back to the first maternal ancestor (Richards et al., 1998; van Oven and Kayser, 2009). In this way, haplogroups can be used to determine the origin of ancient human remains.

The primary objective of this thesis was to use mtDNA haplotyping to determine the origin of the human remains that were discovered in an unmarked burial site during an emergency excavation at Fontabelle, St. Michael in Barbados. The samples were dated back to the mid-17th to mid-18th century, and due to the largely abandoned nature of the burial site, are hypothesized to be slave remains. Without any documentary, oral or physical evidence to indicate the identity of these remains, historians at the Barbados Museum & Historical Society released a sample of the remains in the hopes that the use of molecular archaeology might be able to provide more information.

b. Barbados

i. Early History

Unlike most of its colonial analogues, the island of Barbados was uninhabited before it was colonized. Three Amerindian tribes had settled on the island previously: the Barrancoids, the Arawaks and the Caribs (Hoyos, 1978). The Barrancoids, usually a nomadic tribe, landed on the island in 800 B.C., where they lived for 1400 years before migrating south to Trinidad and Venezuela. The Arawaks lay claim to the abandoned island in 800 A.D., 200 years after the Barrancoids left. The Arawaks were conquered by
the Caribs, an aggressive, dominant tribe, just 400 years later, but the Caribs’ reign would be short lived. The tribe disappeared 300 years later, around the turn of the sixteenth century, leaving little behind, most likely conquered and enslaved by Spanish and Portuguese sailors on their way to South America. Interestingly, while the Spanish and Portuguese clearly were aware of its existence, making several temporary visits to the island, neither country attempted to settle Barbados.

The British crewmates aboard The Olive were the first Europeans to claim Barbados after they landed on the island by accident on May 14, 1625, during a return trip from Brazil to England (Hoyos, 1978). The ship landed on the western coast of Barbados, at what now is called Holetown, then St. James’s town, named for King James I of England. An expedition of 80 settlers and 10 slaves — a “prize” captured out at sea — aboard the William and John, under the command of Henry Powell, settled on the island on February 17, 1627. Powell shortly traveled to Guyana, where he visited the Dutch governor who gave him seeds for tobacco crops and 40 Amerindians to help the British adjust to Caribbean life. The Amerindians were brought to Barbados as free men but were later enslaved.

Despite the presence of slavery from the time of the colony’s inception, slaves were not the primary labor force during the first decade of settlement. The Barbadian economy relied heavily on white indentured servitude (Hoyos, 1978). Indentured servants agreed to work on a plantation without pay for a number of years, provided they were given free passage to the New World, in this case Barbados, as well as a sum of money and piece of land once they had fulfilled their term of service. Indentured servants, however, did not last as a viable labor force: The land on the island was divided quickly
and controlled by a few large plantations, and interest in indentured servitude waned as potential indentured servants became aware of the shortage of land.

By the 1640s, the Barbadian economy was floundering. Virginian tobacco was both less expensive and higher quality (Hoyos, 1978). An attempt to switch to cotton crops failed: The shallow soil in the interior of Barbados was not conducive to cotton growth. The Barbados settlers again turned to the Dutch, who introduced them to the Brazilian sugar cane crop. When the settlers needed a new labor force to work their new crop, the Dutch introduced them to the Atlantic slave trade.

At this time, purchasing slaves became more cost effective for plantation owners than indentured servitude. In the early years of slavery, plantation owners were able to get the same number of working years out of slaves as indentured servants: Slaves lived, on average, seven to nine years after they arrived in Barbados (Beckles, 1987). Indentured servants worked for six to ten years (Hoyos, 1978). The cost of purchasing a slave and providing passage to Barbados was less expensive than the total cost of an indentured servant, factoring in passage to Barbados from England and compensation after their term of service, including money and land lost from the plantation (Beckles, 1987). In this way, slavery was primarily an economic decision: “Whites enslaved Blacks because they discovered this sort of labor system worked well. Economic exploitation [seemed] … the prime motivation; racism conveniently justified and bolstered the used of forced black labor” (Beckles, 1987; p.12).
ii. Atlantic Slave Trade

Before 1630, there were fewer than 800 slaves in Barbados (Beckles, 1987). Between 1640 and 1700, estimates suggest Barbados received 134,500 slaves. Unfortunately, it is difficult to trace the origins of slaves brought to Barbados accurately because slave traders indicated the port of departure in their reports, not the region of Africa from which the slaves were captured. Additionally, slave traders were known to simplify ethnic diversity in their reports to make their work easier.

The majority of slaves sold in Barbados between 1640 and 1700 came from Dutch merchants, who took slaves primarily from West Africa (Beckles, 1987). During the period of most intensive trade to Barbados, this included the areas now known as Angola, Ghana, Togo, Sierra Leone, Nigeria, Guinea, Senegal, Gambia, the Ivory Coast and the Cameroons (Figure 1) (Beckles, 1987; Hoyos 1978).

Slave traders from the Royal African Company, which supplied most of the slaves to Barbados after 1672, specifically said they supplied mostly “Coromantine,” “Whydah” and Gold Coast Africans (Beckles, 1987). These three groups correspond to as many as nineteen tribes, including the Dahomey, Gun, Ga, Eboes, Edo, Ibo, Popo, Pawpaws, Mocoes, Nagoes, Angolas, Congoes, Mandingoes, Ashanti, Fanti, Ewe, Adangme, Bini and Yoruba tribes (Beckles, 1987; Hoyos, 1978).
Figure 1—Possible Origins of African Slaves

Slave traders did not keep records detailing where slaves specifically were captured in Africa. Dutch slave merchants, the primary source of slaves for Barbados in the second half of the seventeenth century, took slaves from Western Africa, in the regions now known as Angola, Ghana, Togo, Sierra Leone, Nigeria, Guinea, Senegal, Gambia, the Ivory Coast and the Cameroons (outlined in red) (Beckles, 1989; Hoyos, 1978).
iii. Mortality and Burials

During the time of slavery, which in Barbados, lasted until 1834, hundreds of thousands of slaves died and were buried on the island of Barbados (Handler, 1989). Planters imported as many as 3,000 slaves per year to account for the 30 percent mortality rate (Hoyos, 1978; Beckles, 1987). The only slaves not buried on the island were slave rebels, whose remains were disposed of at sea so other slaves could not honor their death with any kind of burial ceremony (Beckles, 1987).

Despite the lack of any documentary, oral or physical evidence indicating the specific location of slave burials, knowledge of the time and process of elimination provides a general idea of where slaves were buried, as well as their burial rituals.

Because they were not baptized, slaves were not buried on church or consecrated grounds; even baptized slaves did not always receive this honor (Handler, 1989). Instead, slaves usually were buried on the plantation where they worked, in a separate location of the plantation specifically for this purpose. According to the rector of St. Michael, “slaves [were] always interred in places set apart for that purpose on each plantation” (Handler, 1989; p. 14). These “slave cemeteries” most likely were located close to the slave village or actually within the slave villages. William Dickson, who lived in Barbados in the late eighteenth century and served as private secretary to the governor at the time, noted that the slaves “love their native spots and venerate the dust of their fathers, which they inter either close to their huts, or within them, under their beds” (Beckles, 1987).

As there is no documentary or oral evidence indicating the specific location of slave cemeteries within slave villages on plantations, nor any records to suggest
plantations across the island held similar practices for handling their deceased slaves, hypotheses regarding the whereabouts of slave burials would best be made using physical evidence. Unfortunately, there are no physical markings of slave cemeteries to use: “Slave graveyards lacked fences, gravestones, or other readily observable features” (Handler, 1989; p. 15).

Even if slaves had marked their gravesites, it is unlikely any physical evidence would have survived the past four centuries: Due to the size of the island, land was an essential commodity for plantation owners. Only soil of the poorest quality, unfit for crop growth, was spared for slave villages and, by extension, slave cemeteries (Handler, 1989). In Barbados, poor soil was shallow soil; slaves most likely were buried in shallow soil unsuitable for protecting the integrity of the burial. The nature of the soil in which slaves most likely were interred can explain not only the lack of physical evidence of slave cemeteries, but also may explain the poor quality of ancient remains unearthed on the island.

iv. St. Michael, Barbados

Barbados is divided into eleven parishes: Christ Church, St. James, St. Thomas, St. Peter, St. Lucy, St. George, St. Philip, St. John, St. Andrew and St. Michael (Figure 2) (Schomburgk, 1848). Parishes were an English tradition. Each parish had its own church and a minister who performed the church services.

St. Michael, while not the largest parish, was consistently the most populated, largely due to the port city Bridgetown, located within its boundaries (Schomburgk,
The island of Barbados is divided into eleven parishes (Harlow, 1926). The samples in this study were discovered in Fontabelle (underlined in orange), in the parish of St. Michael (outlined in red). St. Michael was the most populated parish due to the large port city of Bridgetown (underlined in green), located in Carlisle Bay (underlined in blue), which provided an ideal location for sea trade (Schomburgk, 1848). St. Michael consistently held the largest slave population on the island throughout the seventeenth and eighteenth centuries (Handler, 1989).
1848). St. Michael was not a salubrious part of the island. Water accumulated in the valley after heavy rains, and this — combined with the year-round warm weather (temperatures rarely were recorded below 72°F in any month of the year) — turned the parish into a swamp. The parish was settled not for its health benefits but for its convenience: Bridgetown, located in the Carlisle Bay, was an ideal location for docking and shipping goods.

Unlike the other ten parishes, St. Michael was both an urban and a rural parish. Outside the merchant city of Bridgetown, where tradesmen housed their shops and seamen sold their goods, the rest of St. Michael was divided into plantations. More slaves were required to sustain these two ways of life: Slaves were needed to work on the docks and in the merchant district, as well as in the fields on the plantations. According to census reports, St. Michael consistently held the largest slave population of any of the parishes throughout the seventeenth and eighteenth centuries (Table 1) (Beckles, 1987).

c. Mitochondrial DNA

Mitochondria, subcellular organelles that produce ATP through oxidative phosphorylation, contain their own genetic material that encodes the proteins necessary for this process (van Oven and Kayser, 2009). Each organelle may carry anywhere between 2 and 10 copies of its genetic material, and cells may hold as many as 1,000 mitochondria (Budowle et al., 2003). In any given cell, the copy number of mitochondrial DNA could reach as high as 10,000.
### Slave Populations in Barbados, 1679-1748

<table>
<thead>
<tr>
<th>Parish</th>
<th>1679</th>
<th>1680</th>
<th>1683-1684</th>
<th>1712</th>
<th>1748</th>
</tr>
</thead>
<tbody>
<tr>
<td>St. Michael</td>
<td>5,195</td>
<td>5,185</td>
<td>5,663</td>
<td>9,458</td>
<td>9,906</td>
</tr>
<tr>
<td>Christ Church</td>
<td>4,789</td>
<td>4,723</td>
<td>5,606</td>
<td>6,979</td>
<td>7,120</td>
</tr>
<tr>
<td>St. Andrew</td>
<td>2,248</td>
<td>2,248</td>
<td>3,374</td>
<td>3,666</td>
<td>2,556</td>
</tr>
<tr>
<td>St. George</td>
<td>4,316</td>
<td>4,316</td>
<td>5,222</td>
<td>5,508</td>
<td>3,830</td>
</tr>
<tr>
<td>St. James</td>
<td>2,895</td>
<td>2,895</td>
<td>3,582</td>
<td>2,600</td>
<td>4,416</td>
</tr>
<tr>
<td>St. John</td>
<td>3,303</td>
<td>3,303</td>
<td>3,710</td>
<td>3,937</td>
<td>4,074</td>
</tr>
<tr>
<td>St. Joseph</td>
<td>2,070</td>
<td>2,072</td>
<td>3,460</td>
<td>2,450</td>
<td>2,216</td>
</tr>
<tr>
<td>St. Lucy</td>
<td>1,985</td>
<td>1,965</td>
<td>2,536</td>
<td>2,918</td>
<td>4,781</td>
</tr>
<tr>
<td>St. Peter</td>
<td>2,977</td>
<td>3,977</td>
<td>4,199</td>
<td>3,784</td>
<td>3,369</td>
</tr>
<tr>
<td>St. Philip</td>
<td>4,407</td>
<td>4,702</td>
<td>5,181</td>
<td>6,339</td>
<td>1,900</td>
</tr>
<tr>
<td>St. Thomas</td>
<td>3,386</td>
<td>3,396</td>
<td>4,070</td>
<td>4,227</td>
<td>2,857</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td>37,571</td>
<td>36,782</td>
<td>46,602</td>
<td>51,866</td>
<td>47,025</td>
</tr>
</tbody>
</table>

**Table 1—Slave Populations in Barbados, 1679-1748**
Select census data from 1679-1748. Throughout the sixteenth and seventeenth centuries, St. Michael consistently held the largest slave populations of any parish (Beckles, 1987).
The mitochondrial genome is a double stranded, circular piece of DNA, composed of a purine-rich heavy strand and a pyrimidine-rich light strand (Budowle et al., 2003). Replication of the heavy strand begins at position 1 of the 16,569 base pair genome, which is separated into two regions: the coding region and the non-coding region. The non-coding, control region runs 1,100 base pairs in length; the coding region comprises the rest of the genome (Figure 3) (van Oven and Kayser, 2009).

Due to the low fidelity of mtDNA polymerase and the lack of mtDNA repair mechanisms, the mutation rate of mtDNA is much higher than that of nuclear DNA, anywhere from 5 to 10 times higher (Budowle et al., 2010). Within the mitochondrial genome, the mutation rate of the non-coding region is greater than 10 times higher than the mutation rate of the coding region (van Oven and Kayser, 2009). Evolution rules out deleterious, functional mutations and selects for nondeleterious mutations. Unlike the coding region, the role of the non-coding region lies in replication, not function; consequently, nondeleterious mutations accumulate in this region. The majority of these mutations are concentrated in two specific segments of the non-coding region, thus named hypervariable (HV) regions I and II (Budowle et al., 2003). HV I spans base pairs 16,024 through 16,365; HV II bases 73 to 340.

Because it is extranuclear, mitochondrial DNA is both maternally inherited and nonrecombinant: all daughter cells and their organelles, including mitochondria, are derived from the parent egg cell; sperm contribute only chromosomal DNA (Budowle et al., 2010). Barring any individual mutations, all siblings and maternal relatives, even those separated by generations, have identical mitochondrial genomes (Budowle et al., 2003).
The double-stranded, circular mitochondrial genome is separated into two regions: the coding region and the non-coding region (Budowle et al., 2003). The mitochondrial genome is 16,569 bp in length. The non-coding region takes up 1,100 bp; the coding region comprises the rest of the genome (van Oven and Kayser, 2009). The non-coding region has a mutation rate 5 to 10 times higher than nuclear DNA and 10 times higher than the coding region (Budowle et al., 2010; van Oven and Kayser, 2009). Mutations in the non-coding region accumulate in two regions, hypervariable regions I and II (HV I and HV II) (Budowle et al., 2003). HV I runs from position 16,024 to 16,365; HV II, 73 to 340.
The properties of mtDNA make it extremely useful in identification studies. The haploid nature of the genome simplifies the sequencing process (Budowle et al., 2003). The small size of HV I and HV II are ideal for amplification by the polymerase chain reaction (PCR). The rapid rate of mutation in these two regions and the monoclonal nature of the entire genome correspond to a high probability of exclusion if mtDNA sequences from samples do not match those of the individual in question. Most famously, mtDNA analysis was used to identify and authenticate the remains of Tsar Nicholas II of Russia and his family, as well as the Italian poet Francesco Petrarca (Gill et al., 1994; Ivanov et al., 1996; Caramelli et al., 2007).

d. Haplogroups

Due to its matrilineal heredity, mtDNA can be used to trace human evolutionary history. Variations in mtDNA sequences represent the sequential accumulation of mutations along maternal lineages (van Oven and Kayser, 2009). By comparing mtDNA mutations of individuals from different geographic regions, mtDNA also can be used to reconstruct human migration patterns. This phylogeographic approach led to the establishment of a human phylogenetic tree, which traces human maternal lineages all the way back to the common maternal ancestor in Africa (Figure 4) (Richards et al., 1998; van Oven and Kayser, 2009). The tree, which separates populations with unique mutations into haplogroups, was constructed using the minimum number of branches and the minimum number of “intercontinental migrations to account for the geographic distribution of mtDNA types” (Cann et al., 1987; Richards et al., 1998).
Haplogroups are compiled into a phylogenetic tree tracing back to the common maternal ancestor, represented here by ***. All haplogroups are denoted using capital letters (van Oven and Kayser, 2009). The L haplogroups (boxed in yellow) represent haplogroups of African origin. The haplogroups boxed in purple denote the haplogroups that delineated as a result of the out-of-Africa exodus. All haplogroups outside of Africa evolved from principle haplogroups M, N and R, which itself is a sub-haplogroup of N. M haplogroups (boxed in blue) largely correspond with Asia, while N and R account primarily for Europe (boxed in red). The four Native American haplogroups (bold), which evolved from M, N and R, were the first to be discovered and therefore designated A, B, C and D. The rCRS belongs to haplogroup H (italics) (Andrews et al., 1999).

Figure 4—Phylogenetic Tree
Haplogroups are established by comparing sample mtDNA to the revised Cambridge Reference Sequence (van Oven and Kayser, 2009). The Cambridge Reference Sequence (CRS), first published by Anderson et al. in 1981, was the first complete mitochondrial genome sequence. In the following years, however, uncertainties arose as to the validity of the CRS (Andrews et al., 1999). The CRS contained contaminant HeLa and bovine mtDNA sequences, but the original mtDNA sample came from an individual of European descent. Several sites of the CRS differed from other mtDNA samples, and it was unknown whether these discrepancies were due to polymorphisms between the sequences or errors from the original mtDNA analysis. In 1999, Andrews et al. published a revised CRS (rCRS) after completely resequencing the original mtDNA sample. The rCRS established those discrepancies that were true polymorphisms and corrected all errors, contaminant or otherwise, without changing the original base pair numbering.

MtDNA sequences receive haplogroup status only once three complete, individual sequences show the same set of stable mutations (van Oven and Kayser, 2009). For each haplogroup, only the nucleotides that differ from the rCRS are noted (Budowle et al., 2003). All other positions are understood to be the same as the rCRS.

Most mutations in mtDNA are substitutions (van Oven and Kayser, 2009). Substitution polymorphisms are designated by the position of the substitution, followed by the substituted base (Budowle et al., 2003). Some phylogenetic trees only denote the substituted base if the polymorphism is a transversion; in these cases, transitions are designated only by position number (van Oven and Kayser, 2009).

Haplogroups are denoted using the capital letters of the alphabet except the letter O (van Oven and Kayser, 2009). Subclades are denoted using a series of numbers and
lowercase letters following the capital letter of the principle haplogroup. The first haplogroups characterized were of Native American origin and were classified A, B, C and D. All other haplogroups were assigned arbitrarily. The L haplogroups represent mtDNA of African origin, from which all other mutations and haplogroups arose, supporting the out-of-Africa exodus observed in other genetic and archaeological data. Haplogroups M, N and R, subgroups of haplogroup L3, account for all other mtDNA mutations outside of Africa. M haplogroups belong to Asia, while N and R, itself a subclade of N, account for Europe. The rCRS itself is classified as haplogroup H; individuals of haplogroup H show no mutations from the rCRS (Andrews et al., 1999).

e. Ancient DNA and Contamination Controls

The features of mtDNA that make it useful in modern identification and forensic studies also make it an ideal candidate for studying ancient remains. DNA in ancient samples usually is degraded into pieces 50 to 200 base pairs in length, the result of postmortem damage (Paabo et al., 1988). HV I and HV II are approximately 300 base pairs in length; sequencing the majority, if not all, of each region, is feasible. The haploid nature of the mitochondrial genome and the lack of histones help simplify the already difficult task of amplifying and sequencing ancient DNA (Budowle et al., 2003).

Analyzing ancient DNA was not a practical pursuit until the advent of PCR, which is able to detect small amounts of low molecular weight fragments (Yang et al., 2003). Despite the advantages of PCR, however, the degraded nature of ancient DNA still presents problems for this technique. For one, some ancient DNA samples will have
sustained enough damage as to render the DNA incapable of acting as a DNA template for PCR. Additionally, impurities associated with ancient DNA can act as PCR inhibitors.

Aside from the ways in which the degraded nature of ancient DNA affects the quality of PCR reactions, it also puts ancient DNA at high risk for contamination, especially by modern DNA sources. In ancient DNA studies of human remains, contaminant DNA refers to modern human DNA that may be mistaken for DNA extracted from the samples (Yang and Watt, 2005). It is likely only a few thousand copies of ancient mtDNA exist in an entire sample. Any modern DNA present during PCR will out-number and out-compete ancient DNA as the PCR template easily. Possible sources of contaminant DNA in ancient DNA studies include: DNA from any individual who handles the bone, from excavation to extraction; DNA from individuals who manufacture laboratory reagents, hardware and instruments; and PCR products from previous PCR amplifications conducted in the same laboratory space.

Due to the high risk of contamination and the multiple possible sources of contaminant DNA, a separate laboratory dedicated to ancient DNA is required for bone preparation, DNA extraction and PCR setup (Yang and Watt, 2005). A separate ancient DNA facility helps minimize the risk of contamination by samples simultaneously or previously processed in the lab, as well as the risk of contamination by simultaneously or previously amplified PCR products; all PCR must be conducted in a laboratory space physically separate from the ancient DNA facility. Establishing a dress code for any laboratory personnel who enter the ancient DNA facility helps prevent modern contamination through DNA shed by laboratory personnel.
Cross-sample contamination does not present the same threat in ancient DNA studies as modern DNA studies since there is so little and such degraded DNA in ancient samples to begin with; however, precautions to prevent cross-sample contamination should be implemented (Yang and Watt, 2005). Reducing the risk of cross-sample contamination also reduces the risk of modern DNA contamination from spreading between samples. Changing gloves between samples and wiping down bench and instrument surfaces after use can help reduce this risk.

Unfortunately, no amount of in-laboratory contamination controls can eliminate the risk of contamination, nor can it undo any contamination that may have occurred in the field (Yang and Watt, 2005). For this reason, two additional controls are implemented in ancient DNA studies: mtDNA from all laboratory personnel is sequenced and haplotyped; and all samples are subjected to a decontamination process prior to extraction.

Because contamination presents such a risk to ancient DNA studies, it is necessary to prove any and all results are authentic. Reproducibility of results, therefore, is of utmost importance. Ideally, at least three consistent sequences are obtained through PCR amplification of each sample, with at least two of those sequences resulting from two isolated extractions (Edwards et al., 2004). Sequencing the mtDNA and determining the haplogroup of laboratory personnel, as mentioned above, also helps prove authenticity: if repeated sequencing of ancient DNA shows none of the same mutations as personnel DNA, false positive results can be ruled out with some confidence.
f. Specific Aims for Research

The primary objective of this thesis was to determine the origin of the human remains discovered during the emergency excavation of burial grounds in Fontabelle, St. Michael, Barbados. Specifically, three goals were pursued in order to achieve this main objective:

1. To establish a DNA-free facility specifically for the study of ancient DNA, including bone preparation, DNA extraction and PCR setup.
2. To extract, amplify, sequence and haplotype mtDNA from all laboratory personnel interacting with the samples to act as a positive control.
3. To extract, amplify, sequence and haplotype mtDNA from each of the ancient bone samples.

II. Materials and Methods

a. Ancient DNA Facility

DNA extraction and PCR preparation were carried out in a separate facility in order to minimize the risk of contamination with modern DNA. A vacant lab in Millington Hall was converted into a DNA-free facility for this purpose.

After emptying the room of all removable furnishings, the floor was stripped and re-waxed. The walls were washed and re-painted. After trying several multi-purpose household cleaners, all lab benches, cabinets, sinks and the hood were scrubbed with
LA’s Totally Awesome All-Purpose Cleaner. LA’s Totally Awesome, which does not contain acid, ammonia or bleach, is a spot remover designed to remove grease and stains from any surface without surface damage (LA’s Totally Awesome). LA’s Totally Awesome proved best able to remove residue from lab benches and sink countertops. Sink drains were cleared by pouring Clorox bleach (6% sodium hypochlorite) down the drain until waste deposits were broken up visibly in glass sink traps.

To prevent contamination of the DNA-free facility, all laboratory personnel were required to dress in disposable lab coats, booties, hair nets, face masks and gloves before entering the lab. A second pair of gloves, donned once inside the DNA-free facility, was worn during all following procedures to prevent personal contamination; in case of damage or contamination, the outer pair of gloves could be removed without risking contamination.

An ante-chamber for dressing prior to entry into the DNA-free lab was constructed by partitioning off a small space just inside the entrance to the lab. A second door with its own lock separated the ante-chamber from the DNA-free part of the lab. After construction and painting of the anti-chamber, all floors were mopped with LA’s Totally Awesome cleaner to remove any dirt tracked into the lab during the course of the decontamination process. At this point, the lab was closed to the public; only laboratory personnel were allowed beyond the anti-chamber.

The lab, while at this point clean, was not necessarily DNA-free. All surfaces in the lab were wiped down with a 2:1 water:bleach solution (2% sodium hypochlorite) three separate times to remove any DNA contamination from the facility.
b. Ancient DNA Extraction

DNA from ancient samples was extracted according to a protocol developed by Brian Kemp and coworkers at Washington State University (personal communication, 2011).

i. Samples

The samples in this study were discovered during an emergency excavation of burial grounds in Fontabelle, St. Michael, Barbados. The samples were released to Dr. Lizabeth Allison via Christopher Crain, a Ph.D. student in the Anthropology Department, with the permission of Kevin Farmer of the Barbados Museum & Historical Society. The samples consist of 8-10 teeth (molars), 4 long bone fragments (humerus, tibia and femur) and 4-6 cranial fragments previously dated back to the mid 17th to 18th century.

All bone samples were sealed in separate plastic bags and kept in a dry dark space; in the case of this lab, in a decontaminated filing cabinet drawer.

ii. Surface Decontamination

Bone fragments from each sample measuring 50-250 mg were massed in disposable weighing boats. Enough undiluted Clorox bleach (6% sodium hypochlorite) to cover the bone fragments was poured into the weighing boat. Bones were soaked in sodium hypochlorite for four minutes to remove any surface contamination (Kemp and
Smith, 2005). Weighing boats were shaken lightly to ensure all surfaces of the samples were washed with bleach.

After four minutes, the bleach was poured off into the sink, and enough molecular grade water to cover the bone fragments was added to the weighing boat. Bone fragments were rinsed twice for 30 seconds with light agitation to remove any residual bleach from the surface of the bones and then were moved to 15 mL Falcon tubes labeled with sample name, date, and personnel initials. An empty 15 mL Falcon tube was used as the negative control. To prevent cross contamination between negative controls and samples, all of the following procedures were performed first on the negative control, then on samples. Only one sample tube was ever open at one time.

iii. Dissolution of Bone Samples

4 mL of EDTA was added to both negative control and sample Falcon tubes. Bones were submerged in EDTA and rocked on the Rotator Genie (Scientific Industries, Inc.) for any length of time between one week and five months to dissolve as much bone as possible. After EDTA incubation, 3 mg of proteinase K (equivalent to 90 μL) per 2 mL of EDTA was added to each Falcon tube. Samples were incubated overnight on a heating block set to 65°C. If bone fragments were still visible after the first round of proteinase K, each sample received a second round of proteinase K and was incubated again at 65°C overnight, in an attempt to further dissolve the remaining bone fragments.
iv. Alcohol Extraction

At this point in the extraction process, samples were separated. 2 mL of each sample (containing dissolved bone, EDTA and proteinase K) were moved to a second set of Falcon tubes for alcohol extraction. The remaining samples were rocked on the Rotator Genie until silica extraction.

Equal volume (2 mL) phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. Samples were inverted then rocked on the Rotator Genie for five minutes, followed by a five minute centrifugation at 3,500 rpm. The upper aqueous phase was removed and subjected to a second extraction with phenol:chloroform:isoamyl alcohol as described above. The upper aqueous phase was removed and this time extracted with equal volume (2 mL) chloroform:isoamyl alcohol (24:1). Again, samples were inverted then rocked on the Rotator Genie for five minutes, followed by a five minute centrifugation at 3,500 rpm. The top aqueous phase was removed and moved to a final Falcon tube, to which one-half volume (1 mL) ammonium acetate was added. Equal volume (with ammonium acetate, 3 mL) 100% isoamyl alcohol was added. Each sample was inverted several times and incubated at room temperature overnight.

Before beginning the next day, an aliquot of molecular grade water was placed on the heating block (set to 65°C) for later resuspension of DNA. Samples were centrifuged for 30 minutes at 3,500 rpm. The entire contents were poured down the sink (followed by copious amounts of water) without disturbing the DNA pellet, which was not always visible. One mL of 80% isopropanol was added to each sample, which were then vortexed to dislodge the DNA pellet. Samples again were centrifuged for 30 minutes at
3,500 rpm. After pouring off the isopropanol, sample tubes were inverted in a tube rack and air dried over Kimwipes for 15 minutes. DNA was resuspended in 100 μL of 65°C molecular grade water and moved to labeled eppendorf tubes. Samples were stored in a sample box at 4°C until silica-based column extraction.

v. Silica Extraction

Both alcohol extracted samples and non-alcohol extracted samples were silica extracted using the Wizard PCR Preps DNA Purification System (Promega) in conjunction with the Vac-Man Laboratory Vacuum Manifold (Promega). Before beginning silica extraction, an aliquot of molecular grade water was placed on the heating block (set to 65°C) for later elution of DNA.

The Vac-Man vacuum includes 20 individual luer-lok stopcocks for the extraction of up to 20 samples at one time. To prepare the Vac-Man vacuum, caps were unscrewed from the luer-lok stopcocks to be used for extraction. Labeled Wizard Minicolumns (Promega) were placed in the open stopcocks, and 3 cc syringe barrels were screwed on top of the Wizard Minicolumns. Each column was washed with 3 mL of molecular grade water. During all filtrations and washes, columns in use were opened before turning on the vacuum. Individual stopcocks were closed as columns completed filtration; once all columns completed filtration, the vacuum was turned off and vented.

After washing with molecular grade water, 750 μL of 2% guanidine chlorite resin (courtesy of Brian Kemp, Washington State University) and 250 μL of guanidine thiocyanate were added to each column, followed by direct addition of the sample, either
the 2 mL non-alcohol extracted sample directly from the Rotator Genie, or the 100 μL alcohol extracted sample. Samples were filtered through the column and each column was washed with 3 mL of 80% isopropanol using the method described above.

Syringe barrels were removed from the Wizard Minicolumns and discarded. Wizard Minicolumns were moved from the Vac-Man vacuum to labeled eppendorf tubes, then spun at 10,000 rpm for two minutes. At this time, the Wizard Minicolumns were moved to a final set of labeled eppendorf tubes, and the first set was discarded. To elute the DNA from the column, 50 μL of molecular grade water preheated to 65°C was added to each column, directly over the filter. The columns were incubated on the bench for 3 minutes before they were spun at 10,000 rpm for 30 seconds. A second 50 μL round of preheated molecular grade water was added to each column, followed by an additional 3-minute incubation on the bench. The columns were spun again at 10,000 rpm for 30 seconds. The columns then were discarded and the eppendorf tubes containing the eluted DNA stored in the fridge.

c. Modern DNA Extraction

The source of DNA for the purpose of sequencing and haplotyping the mtDNA of laboratory personnel working in the ancient DNA facility was a sample of cheek cells obtained through a saline mouthwash (L.A. Allison, personal communication, 2011). The cells were collected by centrifugation and resuspended in a solution of 10% Chelex (1 g BioRad Chelex 100, 10 mL 50 mM Tris, pH 11). Chelex binds to metal ions responsible for the inhibition of PCR. During a boiling water incubation, cells are lysed, releasing the
DNA. Cell debris, Chelex beads and metal ions are pelleted by centrifugation; the supernatant, which contains the DNA, is removed and stored in a 1.5 mL eppendorf tube at 4°C.

d. PCR

The entire HV I, from positions 15,975 to 16,420, of each sample was amplified using PCR. PCR preparation was conducted in an isolated AirClean 600 PCR Workstation (Atlantic Technical Systems) within the ancient DNA facility. Performing PCR setup in a separate environment within the ancient DNA facility reduced the risk of contamination with any DNA, modern or ancient, exposed during extraction. In addition to the two negative controls (alcohol and silica) from the extraction, each PCR sample included two additional controls: a no template control to test for contamination and activity of PCR reagents and a positive control to test for contamination by laboratory personnel.

The total volume in each PCR tube was 25 μL. Each tube contained 1 μL each of two HPLC purified primers: L15995 5’-TCCACCATTAGCACCCAAAG-3’ (F) and H16420 5’-GCACTCTTTGTGCAGGATATT-3’ (R) (Integrated DNA Technologies, diluted to 100μM stock in nuclease free water). Between 0.5 and 3.0 μL of sample DNA template was used depending on the reaction. Platinum PCR SuperMix (Invitrogen) was added to each tube so that the reaction volume of the tube with the largest amount of sample DNA template totaled 25 μL. Nuclease free water was added to all other tubes to bring total volume to 25 μL.
PCR was carried out in the Applied Biosystems 2720 Thermal Cycler. Samples were denatured for 3 minutes at 94°C. Amplification cycles consisted of a 15 second denaturation step at 94°C, a 45 second annealing step at 55°C and a 30 second extension step at 72°C, for a total of 60 cycles. After a final extension step at 72°C for 3 minutes, samples were stored at 4°C.

e. Goose Bone Extraction

MtDNA was extracted from ancient goose bones (courtesy of Brian Kemp, Washington State University) using combined alcohol and silica extraction, as well as silica extraction only. Goose mtDNA was amplified using PCR, which was set up in the ancient DNA facility as described above. In addition to the negative controls from the alcohol and silica extractions, each goose sample included four additional controls: two positive controls — one with laboratory personnel DNA, one with goose DNA and human primers — to test for contamination by laboratory personnel, and two no template controls to test for contamination and activity of PCR reagents for both goose and human primers.

PCR was carried out in the Applied Biosystems 720 Thermal Cycler as described above. Goose mtDNA was amplified using 1 μL each of HPLC purified primers Goose BSP1 5’-ATACACTACACCGCAGACAC-3’ (F) and Goose R 5’-GCCGTAGTAGAGG CCTCGT-3’ (R) (Integrated DNA Technologies, diluted to 100μM stock in nuclease free water). Either 1 μL or 3 μL of goose DNA template was used. 20 μL of Platinum PCR
SuperMix (Invitrogen) was added to each tube, and nuclease free water was added to PCR tubes with only 1 μL of DNA template to bring total volume to 25 μL.

f. DNA Gel Electrophoresis

Gel electrophoresis was used to visualize amplified ancient DNA and modern DNA. 10 μL of PCR product was added to 2 μL of Crystal 5x DNA Loading Buffer Blue (BIOLINE) on Parafilm “M” (Pechiney Plastic Packaging). 10 μL of this mixture was loaded in a 1.2% agarose gel in 1X TBE (89 mM Tris base, 2.7 mM EDTA, 89 mM boric acid), along with 7 μL of 100 bp ladder (0.1 μg/μL). Small gels were made with 0.6 g agarose (Fisher Scientific), 50 mL 1X TBE and 3 μL ethidium bromide. Large gels were made with 1.2 g agarose, 100 mL 1X TBE and 6 μL ethidium bromide. Gels were run for 20 minutes at 120 V on the BioRad Power Pac 1000. Images of all gels were captured using the BioRad Gel Doc XR imaging system with Quantity One analysis software (v4.6.1).

g. REPLI-g Mitochondrial DNA Kit

Select samples were amplified using the REPLI-g Mitochondrial DNA Kit (QIAGEN), which specifically amplifies mtDNA. REPLI-g Reaction Buffer was added to PCR tubes in the PCR Workstation in the ancient DNA facility. Samples were added to each PCR tube and then carried to a separate location in the ISC for amplification. After a 5-minute incubation at 75°C in the thermal cycler, REPLI-g polymerase was added to
each tube and PCR resumed for 8 hours at 33°C, followed by a final extension step at 65°C. Samples were stored at 4°C. PCR amplification of HVI was conducted with the same HPLC primers and Platinum PCR SuperMix, but two reaction tubes were prepared for each sample, the first with 1μL of sample, the second with 3μL. DNA gel electrophoresis was performed as previously described.

h. DNA Sequencing

Prior to sequencing, 5 μL of PCR product from samples that showed amplified DNA during gel electrophoresis were purified for sequencing using ExoSAP-IT PCR Product Clean-Up (Affymetrix). Samples were sequenced in-house with the Applied Biosystems 3500 Genetic Analyzer. All sequences were analyzed using Applied Biosystems Sequencing Analysis software (v5.4).

i. Haplotyping

All sequences were compared to the rCRS using CLC Main Workbench (v6.8), which aligns samples sequences with reference sequences and flags positions with conflicting nucleotides. The rCRS was downloaded from the National Center for Biotechnology Information GenBank (sequence number NC_012920). Three separate sources were used to identify the haplogroup of ancient and modern mtDNA samples: the Haplogroup Prediction Tool, part of National Geographic’s Genographic Project, the “Updated comprehensive phylogenetic tree of global human mitochondrial DNA
variation” established by van Oven and Kayser at phyloTree.org, and the “MtDNA Haplogroup Specific Control Region Mutation Motifs” published by the Yonsei DNA Profiling Group.

Single nucleotide polymorphisms were entered into the Haplogroup Prediction Tool, which predicts the most likely principle haplogroup for a set of mutations. The branch under which this predicted haplogroup would be found was selected on phyloTree.org. The “Find” command was used to search for specific sample mutations among the haplogroups listed in that particular branch of the tree, and samples were matched with the haplogroup containing the same set of mutations (van Oven and Kayser, 2009). These results were cross-referenced against the haplogroups and associated mutations listed in the control region mutation motifs. Sample mutations were located among the motifs again by using the “Find” command.

III. Results

a. Ancient DNA Extraction

Of the 6 samples available, only 3 yielded DNA: sample 30bu61, sample 30bu62 and sample 30bu141. Despite multiple extraction attempts, only one extraction was successful for each of these three samples, and none of the extractions were successful for samples 30bu54, 30bu72 and 30bu147 (Table 2, 3). DNA extracted from sample 30bu141 was amplified and sequenced twice, but the DNA extracted from sample 30bu61 and sample 30bu62 was amplified and sequenced only once.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Date of Extraction</th>
<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>30bu54</td>
<td>skull</td>
<td>8.8.11</td>
<td>562 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu61</td>
<td>femur</td>
<td>8.8.11</td>
<td>370 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu62</td>
<td>molar</td>
<td>8.8.11</td>
<td>80 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu72</td>
<td>femur</td>
<td>8.8.11</td>
<td>422 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu141</td>
<td>molar</td>
<td>8.8.11</td>
<td>142 mg</td>
<td>92 days</td>
<td>positive</td>
</tr>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>8.8.11</td>
<td>907 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Date of Extraction</th>
<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
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<td>47.5 mg</td>
<td>7 days</td>
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<td>N/A</td>
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<td>N/A</td>
<td>negative</td>
</tr>
<tr>
<td>30bu72</td>
<td>femur</td>
<td>9.10.12</td>
<td>92 mg</td>
<td>82 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu141*</td>
<td>molar</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>10.22.12</td>
<td>532 mg</td>
<td>111 days</td>
<td>negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Date of Extraction</th>
<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
</tr>
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<tbody>
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<td>565 mg</td>
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<td>negative</td>
</tr>
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<td>30bu61</td>
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<td>377 mg</td>
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<td>30bu147</td>
<td>skull</td>
<td>11.28.12</td>
<td>535 mg</td>
<td>145 days</td>
<td>negative</td>
</tr>
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**Table 2—Alcohol and Silica Extractions**

Conditions and results of extractions of each sample using both alcohol and silica extraction methods. Date of extraction refers to the date on which the first round of pK was added to the sample. Incubation refers to the length of time each sample was left dissolving in EDTA.

*In the case of molars, dissolving enough bone mass to extract DNA usually involves dissolving the entire specimen. In order to preserve molar remains, fewer extractions were performed on these samples than others.*
<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Date of Extraction</th>
<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
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<tbody>
<tr>
<td>30bu54</td>
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<td>50 mg</td>
<td>92 days</td>
<td>negative</td>
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<td>30bu61</td>
<td>femur</td>
<td>8.8.11</td>
<td>50 mg</td>
<td>92 days</td>
<td>positive</td>
</tr>
<tr>
<td>30bu62</td>
<td>molar</td>
<td>8.8.11</td>
<td>41 mg</td>
<td>92 days</td>
<td>negative</td>
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<tr>
<td>30bu72</td>
<td>femur</td>
<td>8.8.11</td>
<td>65 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
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<td>30bu141</td>
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<td>8.8.11</td>
<td>52 mg</td>
<td>92 days</td>
<td>negative</td>
</tr>
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<td>30bu147</td>
<td>molar</td>
<td>8.8.11</td>
<td>45 mg</td>
<td>92 days</td>
<td>negative</td>
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<table>
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<th>Sample</th>
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<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
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<td>N/A</td>
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<td>N/A</td>
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<td>92 mg</td>
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<td>negative</td>
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<td>30bu141*</td>
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<td>30bu147</td>
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<td>80 mg</td>
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<table>
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<th>Sample</th>
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<th>Date of Extraction</th>
<th>Mass of Sample</th>
<th>Length of Incubation</th>
<th>Results</th>
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<tbody>
<tr>
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<td>skull</td>
<td>10.22.12</td>
<td>59 mg</td>
<td>111 days</td>
<td>negative</td>
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<tr>
<td>30bu61</td>
<td>femur</td>
<td>10.22.12</td>
<td>92 mg</td>
<td>111 days</td>
<td>negative</td>
</tr>
<tr>
<td>30bu62*</td>
<td>molar</td>
<td>N/A</td>
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<td>30bu72</td>
<td>femur</td>
<td>10.22.12</td>
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<td>111 days</td>
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<td>11.28.12</td>
<td>83 mg</td>
<td>145 days</td>
<td>negative</td>
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</table>

**Table 3—Silica Extractions**

Conditions and results of extractions of each sample using only the silica extraction method. Date of extraction refers to the date on which the first round of pK was added to the sample. Incubation refers to the length of time each sample was left dissolving in EDTA.

*In the case of molars, dissolving enough bone mass to extract DNA usually involves dissolving the entire specimen. In order to preserve molar remains, fewer extractions were performed on these samples than others.
Sample 30bu61 was extracted using silica extraction. When sequenced, it showed 3 mutations from the rCRS in HV I: 16172C, 16219G and 16278T (Figure 5). Sample 30bu62 was extracted using alcohol and silica extraction and showed, upon sequencing, 6 transition mutations from the rCRS: 16185T, 16189C, 16223T, 16260T, 16298C and 16317T (Figure 6). When first sequenced, sample 30bu141 showed 5 discrepancies from the rCRS; the second sequencing trial showed only 4 mutations, which matched 4 of the 5 mutations from the first sequencing attempt (Figure 7, 8). The extraneous mutation of the first sequence likely is due to a sequencing error, the result of working with such poor, degraded samples, or possibly heteroplasmy within the individual. A consensus of these two sequences, therefore, indicates four transition mutations from the rCRS: 16223T, 16298C, 16325C and 16327T.

b. Positive Controls

In order to identify whether contamination occurred during extraction, DNA from laboratory personnel was used as a positive control. When sequenced, DNA from this author showed four transition mutations from the rCRS: 16126C, 16294T, 16296T and 16304C. Two other personnel who also had access to the ancient DNA facility and worked directly with the bone samples showed 6 mutations and no mutations from the rCRS, respectively.
Figure 5—Sample 30bu61 vs. rCRS
Base comparison of sample 30bu61 sequence with HV I of the rCRS. Sample 30bu61 exhibited three transition mutations from the rCRS: 16172C, 16219G and 16278T.
Figure 6—Sample 30bu62 vs. rCRS
Base comparison of sample 30bu62 sequence with HV I of the rCRS. Sample 30bu62 exhibited six transition mutations from the rCRS: 16185T, 16189C, 16223T, 16260T, 16298C and 16317T.
Figure 7—Sample 30bu141 vs. rCRS, Amplification #1

Base comparison of first sequencing results of sample 30bu141 with HV I of the rCRS. This sequencing trial showed five transition mutations from the rCRS: 16223T, 16298T, 16325C and 16327T.
Figure 8—Sample 30bu141 vs. rCRS, Amplification #2
Base comparison of second sequencing results of sample 30bu141 with HVI of the rCRS. This sequencing trial showed four of the five same transition mutations from the rCRS as the first sequencing results of sample 30bu141: 16223T, 16298T, 16325C and 16327T.
c. Ancient DNA Control

To confirm the ancient DNA facility was free of modern DNA to the extent that extraction of ancient samples could be performed without contamination, and also to establish it was possible to extract ancient DNA with these methods and this author, DNA was extracted from ancient goose bones. Goose mtDNA was extracted successfully using combined alcohol and silica extraction. After sequencing, a BLAST search showed a 98% match with the *Anger gannicus* mtDNA genome (Figure 9).

d. Optimizing Extraction Methods

i. Pre-extraction Modifications

After producing negative results from several extraction attempts, steps were taken to optimize conditions for DNA extraction, beginning with bone preparation and dissolution.

Operating under the assumption that the mass of bone fragment dissolved plays a role in the ability to extract DNA — samples with too little mass may not have enough DNA to extract, while samples of larger mass may have too many PCR inhibitors — two sets of samples were prepared for each bone sample. Because the amount of bone dissolved directly correlates to the amount of DNA available for extraction, each of these sets of samples — one of smaller mass, one of larger mass — was incubated in EDTA for over three months in order to dissolve as much bone fragment as possible. Unfortunately,
Figure 9—Goose 236B vs. Anser canagica
Base comparison of amplified ancient goose DNA with goose mitochondrial genome. BLAST search results indicate a 98% match (124/126 bases matching).
extractions of all samples, regardless of mass, extended incubation or method, yielded no DNA.

**ii. Repeat Silica Extraction**

When working with ancient samples, it can be difficult to determine whether the lack of visible bands in DNA gel electrophoresis is the result of a true lack of DNA in the sample or negative amplification results due to the presence of PCR inhibitors in the sample. A new protocol published by Kemp et al. (2006) describes a method for both determining the presence of PCR inhibitors and further removing them from samples: If no bands are visible on the agarose gel, samples are extracted again via silica extraction using Wizard Minicolumns. When repeat extracted samples are prepared for PCR, two tubes are prepared per sample, one of which is spiked with 1 μL of previously amplified and sequenced extracted ancient DNA. PCR reactions are set up and run exactly the same otherwise.

In these reactions, the ancient DNA spike acts as an indicator of the presence of PCR inhibitors. If no DNA amplifies in either of the two reactions for each sample, PCR inhibitors likely are present, thus preventing amplification of the ancient DNA spike that has been shown to amplify previously. If DNA amplifies in the spiked sample but not the pure sample, PCR inhibitors most likely are not present, and therefore it is unlikely there is any DNA in the sample to amplify. These results, of course, can be confirmed by sequencing the spike sample PCR product to ensure the mutations of the spike sample match the mutations of the ancient DNA spike. Samples must be spiked with another
ancient sample because PCR inhibitors associated with ancient DNA do not prevent amplification of modern DNA samples.

Repeat silica extractions in this study indicated both the presence of PCR inhibitors and the absence of DNA in ancient samples. Because the bone samples most likely did not belong to the same individual despite their discovery within the same excavation site, and because sample 30bu141 had been amplified and sequenced previously (by L. Epp), sample 30bu141 was used as the ancient DNA spike. Samples 30bu54, 30bu61 and 30bu62, extracted using silica extraction as well as alcohol and silica extractions, were subject to repeat silica extractions.

After the first repeat silica extraction only spiked sample 30bu54 was amplified; all other lanes showed no bands (Figure 10.1). These data indicated there was no DNA extracted from sample 54 using silica extraction alone and that PCR inhibitors were preventing amplification in all other samples. After a second round of repeat silica extraction, sample 30bu54, as extracted using alcohol and silica extractions and spiked with 30bu141, was the only sample to produce bands. All other lanes were empty (Figure 10.2). From these data it was concluded no DNA was extracted from 30bu54 using the combined alcohol and silica extractions, and inhibitors prevented PCR amplification in all other samples.

After a third round of repeat silica extraction, for a total of four silica extractions per sample, two lanes produced bands: 30bu61, extracted using alcohol and silica extractions and spiked with sample 30bu141, and sample 30bu62, extracted using alcohol and silica extractions but without any ancient DNA spike (Figure 10.3). These data indicated 30bu61 extracted with both methods had yielded no DNA, and all other
Figure 10.1—DNA Gel Electrophoresis of First Repeat Silica Extraction
Amplification in lane 4, sample 30bu54 extracted using silica extraction and spiked with ancient DNA, indicates no DNA was extracted from 30bu54 using silica extraction. Lane 15 shows amplified positive control (KH) DNA.

Figure 10.2—DNA Gel Electrophoresis of Second Repeat Silica Extraction
Amplification in lane 2, sample 30bu54 extracted using both alcohol and silica extraction methods and spiked with ancient DNA, demonstrates no DNA was extracted from 30bu54 using the combination of alcohol and silica extraction methods. Lane 14 shows amplified DNA from the ancient DNA spike, and lane 15, amplified positive control (LM1) DNA.

Figure 10.3—DNA Gel Electrophoresis of Third Repeat Silica Extraction
Amplification in lane 4, sample 30bu61 extracted using both alcohol and silica extraction methods and spiked with ancient DNA, indicates no DNA was extracted from 30bu61 using the combination of alcohol and silica extraction methods. Amplification in lane 7, sample 30bu62 extracted using both methods in the absence of the ancient DNA spike, indicates DNA was extracted from 30bu62 using both alcohol and silica extractions. Lane 13 shows amplification of positive control (KH) DNA.
samples still contained PCR inhibitors. However, no further repeat extractions were performed despite the lack of conclusive results for all samples regarding the presence of PCR inhibitors or the absence of DNA. After four extractions or more, while there likely are no PCR inhibitors, there also likely is no DNA. Each extraction inevitably removes DNA as well as PCR inhibitors. Upon sequencing the PCR products, both samples of 30bu54 and 30bu61 showed the same mutations as 30bu141, confirming the amplified DNA in those samples was that of 30bu141.

iii. REPLI-g Amplification

In case the lack of bands during DNA gel electrophoresis was due to low concentration of extracted mtDNA, select samples were amplified using the REPLI-g Mitochondrial DNA Kit (QIAGEN), which specifically amplifies mtDNA. None of the samples treated with the REPLI-g kit showed amplified HV I mtDNA except previously extracted and sequenced 30bu141 DNA (Table 4). These results suggested the REPLI-g Mitochondrial DNA Kit did work in the presence of ancient mtDNA and allowed for repeat sequencing of sample 30bu141.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Method of Extraction</th>
<th>Amount of DNA Template</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>alcohol and silica</td>
<td>1 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>silica only</td>
<td>1 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu61</td>
<td>femur</td>
<td>alcohol and silica</td>
<td>1 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu141</td>
<td>molar</td>
<td>alcohol and silica</td>
<td>1 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>alcohol and silica</td>
<td>3 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu147</td>
<td>skull</td>
<td>silica only</td>
<td>3 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu61</td>
<td>femur</td>
<td>alcohol and silica</td>
<td>3 μL</td>
<td>negative</td>
</tr>
<tr>
<td>30bu141</td>
<td>molar</td>
<td>alcohol and silica</td>
<td>3 μL</td>
<td>positive</td>
</tr>
</tbody>
</table>

Table 4—Results of REPLI-g PCR Amplification Kit
REPLI-g Mitochondrial DNA Kit was used with samples recently extracted at the time of the kit’s arrival (30bu147) and samples previously extracted, amplified and sequenced using alcohol and silica extraction methods. After REPLI-g amplification, PCR amplification of HV I was conducted using 3 μL of DNA template as well as the standard 1 μL of template in an attempt to optimize the DNA concentration produced by REPLI-g amplification. Only sample 30bu141 yielded any amplified DNA, which was later sequenced.
IV. Discussion

a. Determining Haplogroups and Origins

Mutations observed in modern mtDNA samples corresponded exactly with European haplogroups that match the known maternal ancestry of laboratory personnel. This author was haplotyped as haplogroup T2b; other personnel were haplotyped as haplogroup H (the rCRS itself) and haplogroup J1b1 (Table 5).

Haplotyping the mutations from the rCRS observed in ancient DNA samples proved more difficult, especially for those samples from which neither multiple extractions nor multiple amplifications were achieved. While single sequence analyses can produce reliable results, double and triple analyses are better: without reproducible sequencing trials, it is difficult to differentiate between true mutations from the rCRS and mutations that are the result of sequencing errors due to the degraded quality of the DNA template, as was the case for sample 30bu62 (Bar et al., 2000).

The six mutations observed in sample 30bu62 do not correspond with any one haplogroup; in fact, one of the mutations, 16317T, is not seen in any of the haplogroup motifs published by the Yonsei DNA Publishing Groups. While this may be a mutation specific to this person, or perhaps his or her family, it also suggests the possibility of sequencing errors within this sequence, already suggested by the lack of reconciliation between the mutations observed in sample 30bu62 with any haplogroup. When compared to worldwide mtDNA populations individually, four of the six mutations (16185T,
<table>
<thead>
<tr>
<th>Sample</th>
<th>Mutations from rCRS</th>
<th>Haplogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH (author)</td>
<td>16126C</td>
<td>T2b</td>
</tr>
<tr>
<td></td>
<td>16294T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16296T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16304C</td>
<td></td>
</tr>
<tr>
<td>LM1</td>
<td>16069T</td>
<td>J1b1</td>
</tr>
<tr>
<td></td>
<td>16126C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16145A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16172C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16222T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16261T</td>
<td></td>
</tr>
<tr>
<td>LM2</td>
<td>none</td>
<td>H</td>
</tr>
</tbody>
</table>

Table 5—Mutations and Haplogroups of Laboratory Personnel
MtDNA from all laboratory personnel was sequenced and haplotyped to act as a positive contamination control. Two lab members (LM1 and LM2) and this author were haplotyped as haplogroups J1b1, H and T2b, respectively, three European haplogroups.
16189C, 16223T and 16317T) presented the highest match probability with Kenyan populations (Table 6). The transition mutation at position 16260 showed the highest match probability with Chinese populations (0.0501, closely followed 0.0490 in Kenyan populations), and the transition mutation at 16298 showed the highest match probability with Hispanic populations. These match probability data suggest the likelihood of sequencing errors, as it is nearly impossible for the remains to be of Asian origin.

When comparing sample mtDNA sequences to reference sequences, just one nucleotide difference is enough evidence to suggest that sample does not belong to a particular maternal lineage or haplogroup (Bar et al., 2000). The sequence obtained from sample 30bu62, therefore, ultimately is inconclusive; however, ancient DNA sequences are accepted more readily when they fit into the appropriate context (Edwards et al., 2004). With this in mind, the mutations in sample 30bu62, while they do not match any haplogroup, suggest the remains are of African origin and the inability to haplotype them unequivocally is the result of damage to the DNA template, as well as sequencing errors caused by the degraded quality of the sample.

Despite the inconclusive results of sample 30bu62, the single sequence of sample 30bu61 produced seemingly reliable results. The three mutations of sample 30bu61 correspond with haplogroup U6a. Principle haplogroup U is a northern and western European haplogroup; sub-haplogroup U6, however, is indigenous to North Africa (Salas et al., 2005). While the rest of the U haplogroups evolved out of Africa to Europe, U6 itself stayed within North Africa, exhibiting only minor diffusion to the Iberian Peninsula. Individually, when compared to world mtDNA populations, each of the mutations exhibited the highest worldwide match probability in African populations.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Type</th>
<th>Mutations from rCRS</th>
<th>Highest Probability of Match in Worldwide Populations</th>
<th>Haplogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td>30bu61</td>
<td>femur</td>
<td>16172C</td>
<td>0.3627 Kenyan</td>
<td>U6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16219G</td>
<td>0.0574 N. African</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16278T</td>
<td>0.6160 U.S. African</td>
<td></td>
</tr>
<tr>
<td>30bu62</td>
<td>molar</td>
<td>16185T</td>
<td>0.0392 Kenyan</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16189C</td>
<td>0.4902 Kenyan</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16223T</td>
<td>0.9608 Kenyan</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16260T</td>
<td>0.0501 Chinese</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16298C</td>
<td>0.2137 Hispanic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16317T</td>
<td>0.0196 Kenyan</td>
<td></td>
</tr>
<tr>
<td>30bu141</td>
<td>molar</td>
<td>16223T</td>
<td>0.9608 Kenyan</td>
<td>C1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16298C</td>
<td>0.2317 Hispanic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16325C</td>
<td>0.2941 U.S. Hispanic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16327T</td>
<td>0.2392 U.S. Hispanic</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6—Mutations and Haplogroups of Sequenced Barbados Samples**

3 of the 6 bone samples yielded mtDNA sequences, of which the mutations from the rCRS are presented here, alongside the population in which there is the highest probability of finding that particular mutation. Haplogroup predictions for samples 30bu61 and 30bu141 are listed. No haplogroup matched the set of mutations present in sample 30bu62, and as such, no haplogroup is listed for that sample.
These data, therefore, suggest sample 30bu61 belongs to the remains of a slave taken to Barbados from North Africa.

Successfully haplotyping sample 30bu141 also was possible, largely due to the multiple sequences produced for this sample. The four mutations of sample 30bu141 correspond exactly with haplogroup C1, a Native American haplogroup found in populations in both North and South America (Figure 11). When compared to worldwide frequencies, three of the four mutations exhibited the highest match probability with Hispanic and Venzuelan haplogroups; the fourth mutation presented highest match probability with Kenyan populations. All together, these data suggest sample 30bu141 belongs to the remains of one of the enslaved 40 Amerindians, or the descendants of one of the enslaved Amerindians, given to Henry Powell by the Dutch governor of Guyana.

b. Preservation and Degradation of Ancient Samples

Sporadic amplification, in which amplification occurs in one extraction from one sample, but not in all extractions from all samples or even all extractions from that particular sample, as seen in this study, largely can be attributed to excessive damage accrued when remains are poorly preserved (Edwards et al., 2004). Excessive damage to remains renders preserved DNA unsuitable to act as a DNA template. In ancient samples, two types of DNA damage are common: hydrolytic damage and oxidative damage (Hoss et al., 1996). Hydrolytic damage includes deamination, depurination and depyrimidination; oxidative damage includes modification of purine and pyrimidine
Figure 11—Migration of mtDNA Haplogroups
All haplogroups evolved out of Africa, depicted here by the migration patterns of human mtDNA.
bases through the interaction of oxygen free radicals with DNA (Beckman and Ames, 1997).

Depurination and depyrimidination involve the release of purine and pyrimidine bases, respectively, upon cleavage of the glycosidic bond between the nucleic bases and the deoxyribonucleoside sugar (Lindahl, 1993). For DNA without active repair mechanisms, DNA is weakened at points of uncorrected base loss, and the DNA strand is cleaved at these sites within a few days. Because repair mechanisms are no longer functional in deceased tissues, depurination and depyrimidination are two sources of ancient DNA degradation.

Pyrimidines, particularly thymine, are more sensitive to oxidative damage than purines, and the oxidative damage to pyrimidines accounts for much of the base modification observed in ancient samples, which can present themselves as false mutations in the mitochondrial genome (Paabo, 1989). Oxidative damage also is responsible for the production of PCR inhibitors: Cytosine and thymine are oxidized to hydantoins, which act as PCR inhibitors by blocking chain amplification by DNA polymerase. Without functional DNA polymerase, PCR amplification cannot occur (Hoss et al., 1996).

Unfortunately for this study, samples with lowest amounts of damage and highest amounts of amplifiable DNA across species are those remains preserved in regions of low temperature (Hoss et al., 1996; Edwards et al., 2004). Samples observed with lowest levels of base damage are those from the Antarctic and sub-Antarctic; a decrease in temperature of just 20°C can induce a 10 to 25-fold reduction in the rate of decay of nucleotide bases. Samples from Egypt, Europe, South Africa and the warm regions of the
Americas statistically produce fewer samples with reproducible amplifications. The degree to which remains are submitted to hotter, semi-arid weather in these regions also plays a role in degradation of samples: The rate of desiccation immediately after death affects the rapidity of the post-mortem endogenous hydrolytic processes that degrade DNA (Hoss et al., 1996; Paabo, 1989). Air-dried samples remain partially hydrated and therefore are susceptible to higher rates of hydrolysis during decay (Lindahl, 1993). The samples in this study, buried in shallow soil that offered little protection from the elements, likely were subjected highly to the weather on Barbados; in Fontabelle and St. Michael specifically, this meant being subjected to high levels of humidity and heat year round (Handler, 1989; Schomburgk, 1848). Temperature, therefore, more than any other factor, may account for lack of DNA extracted and amplified from the Fontabelle, St. Michael, Barbados remains.

c. Future Directions

While it appears nothing more can be done with this particular selection of remains, it would be worth attempting DNA extraction from another set of remains from Barbados, especially given the sporadic nature of amplification observed with poorly preserved remains. If remains of higher quality yielding multiple positive extractions were obtained, sequencing HV II in addition to HV I might be considered; sequencing more of the non-coding region of mtDNA identifies more mutations from the rCRS, which allows for more specific haplotyping results. Perhaps most significantly for the future, the establishment of a facility specifically for the extraction of ancient DNA
allows for the widespread pursuit of molecular archaeology projects at the College of William and Mary.

V. Acknowledgments

Working on this project and working with ancient human remains was an unbearably sad, indescribably surreal and immensely rewarding experience. This project and these people mean so much to me, and as no woman is an island unto herself, even when working with remains from an island in the Caribbean, I would like to take the time to express my gratitude to everyone who made my work possible.

I would like to thank the Barbados Museum & Historical Society, particularly Kevin Farmer, for their collaboration and for entrusting us with a piece of their nation’s history. Thank you to Christopher Crain for making this collaboration possible, and thank you to Dr. Allison for allowing me to work on such an important project and giving me such a unique opportunity. I could never thank you enough, but let me start by thanking you for your guidance, your generosity, your encouragement, and for believing in and accepting the girl who walked into your office and told you she loved writing as much as she loved science.

I owe an enormous thank you to Lidia Epp who taught me everything I know, from pipetting to sequencing. Thank you for teaching me and brainstorming with me, but also for chatting with me and getting to know me in the in-between moments. I am indebted hugely to Dr. Brian Kemp for his protocols and his tutelage, both in person and via email, and also for the elusive guanidine chlorite resin. I would like to thank Dr. Kurt
Williamson, Dr. Shanta Hinton and Professor Chelsey Johnson for their patience and for serving on my committee, and also Tom Meier for helping build the facility that made working with ancient DNA plausible.

From the Allison lab, I would like to thank Vinny Roggero for answering all of my questions, no matter how simple or how many times I asked them, and for not laughing too hard when I spit on my shoes. I also would like to thank all of the Allison lab members for their feedback and support, especially when my confidence was wearing thin. In particular I would like to thank: Caroline Feigert, for introducing me to new sequencing software and accepting my tears and frustrations over 400-year-old remains in stride; Heidi Schoomaker, my partner in crime, for scrubbing away mold and keeping me company for hours on end, for drawing unicorns and connecting the dots; and Hallie Nelson, without whom I would not have survived the past year. Thank you for being my companion in all things: snacks, movies, traveling, posters, presentations, and late-night lab sessions. I would have been lost without you.

Last but not least, thank you to all of my friends and family, for your hugs, your love and your faith in me.

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VI. References


