MITOCHONDRIAL DNA ANALYSIS OF THE OHIO HOPEWELL OF THE HOPEWELL MOUND GROUP

DISSERTATION

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By

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ABSTRACT

The Hopewell Earthworks (33RO27) near Chillicothe Ohio have inspired awe and curiosity since their discovery. The mounds have been the focus of numerous research projects which have posed the classic questions of archeology. Mortuary analysis and biological profiling have been the main tools used by researchers to examine the Hopewell skeletal material. With the invention of Polymerase Chain Reaction (PCR), it has become possible to apply molecular biotechniques to archaeological questions. This dissertation focuses on the results of research which examines the following questions:

1. What are the phylogenetic relationships and affiliations between the Ohio Hopewell Mound Group mtDNA lineages to other ancient and contemporary North Native American mtDNA lineages ?

2. What information does mtDNA add to what is known biologically about the prehistoric groups of the Ohio River Valley ?

3. Is there evidence to support matrilineal descent among the Ohio Hopewell of the Hopewell Mound Group ?

4. Is there segregation of individuals interred within the Ohio Hopewell Mound Group based upon mtDNA lineages; specifically within Mounds 2 and 25 ?

Ancient mtDNA was extracted from the teeth of thirty-four individuals excavated by H.C. Shetrone and interred in Mounds 2,4,7,25 and 26, as well as those unable to be assigned to a mound. Both Phenol-Chloroform (Paabo 1993) and Yang Protocol C (1998) extraction methods were utilized to test for restriction site polymorphisms (RFLP) to distinguish the five maternal mtDNA haplogroups (A,B,C,D, and X) and sequence Hypervariable region I (HVI) from 16047 to 16429.

Results have identified four of the possible five mtDNA haplotypes known to be involved in the initial peopling of the New World. HV I data from 50 modern and ancient Native American sample populations generated Neighbor Joinging trees allowing the placement of the Ohio Hopewell Mound Group sample in the context of existing modern mtDNA variation in North America and grouping them with individuals who share similar haplotype mutations. In a search of GENE BANK, the Ohio Hopewell Mound Group sample shares unique mutations with mtDNA lineages in China, Korea, Japan and Mongolia. Genetic analysis was also completed utilizing Arlequin to calculate, π, theta and Tajima's D indicating that the Ohio Hopewell have polymorphic mtDNA lineages.

Dedicated to my family, especially my mother, Faye Mills

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Without these individuals, this research would not have been possible.

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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

The mounds and earthworks of southern Ohio have long inspired awe and wonderment from the public and scientific community. For over one hundred years, scientists have been fascinated not only with the mounds and earthworks but with the ancient people who were responsible for their construction. The earliest scientific inquiry focused on determining who built the mounds and earth works and then concentrated on the lifeways of the population deemed "Ohio Hopewell". Within the field of Anthropology, the majority of research has been from the sub-field of archaeology in an attempt to recover as much of the material remains of the Ohio Hopewell as possible. Physical anthropology has made contributions in the areas of human skeletal analysis and diet reconstruction. Cultural anthropology has focused on reconstructing the level of social organization necessary to provide the infrastructure that would support the erection of the mounds and earth works. Linguistics provided hypotheses to explain population movement and possible origins of the Ohio Hopewell from analyses of modern Native American languages and their distribution. This study, via biological anthropology, simply adds another chapter to what is already known about the Ohio Hopewell and takes that knowledge to the molecular level.

The study draws from all four of the above listed sub-fields of anthropology. Beginning with the mapping and excavation of the study's primary focus, the Hopewell Mound Group site, Chapter One reviews the archaeology which has contributed a considerable amount of information to the understanding of the Hopewell. Chapter Two, the Ohio Hopewell, goes into detail about what is known to date about the lifeways of the Hopewell in Ohio. Beginning with a brief history of the search for the "Mound Builders" in Ohio, the chapter then progresses to what is known about the subsistence of the Ohio Hopewell. The Hopewell were once thought to be maize agriculturists and this chapter discusses the rejection of this idea and the change of focus to the Eastern Agricultural Complex (native cultigens) and diet reconstruction. From subsistence, the chapter proceeds to Ohio Hopewell settlement patterns and two competing hypotheses in this area, the Dispersed Sedentary Community and a continuation of earlier Archaic settlement patterns. Archaeological and biological continuity in the Ohio River Valley begins with the difficulty early archaeologists experienced with the correct chronology for the Ohio River Valley. It goes on to further discuss some of the human skeletal and material remains data that played a role in the separation of populations in Ohio. The chapter then summaries the archaeology of Ross County, Ohio, the location of the Hopewell Mound Group site, and progresses to a summary of the site itself, its chronology, layout and excavation history. Finally, the chapter ends with a description of mortuary analysis and its utilization on Mound 25 of the Hopewell Mound Group site.

Biological Anthropology is a field which has come into its own in the last twenty years. With the invention of the Polymerase Chain Reaction (PCR) technique that allows

researchers to retrieve the genetic identity of an individual from the nucleus of only one cell, the possibility of a vast and new array of research was made available that could shed light on questions that earlier would not have been deemed probable to test. In Chapter Three, the structure of Mitochondrial DNA and the mapping of the human mitochondrial genome is explained. The chapter then progresses to the properties which make Mitochondrial DNA suitable for lineage studies. The history of two methods, Restriction Fragment Length Polymorphisms and Direct Cycle Sequencing, are then discussed as well as how those two methods were applied to Native American populations. Other ancient Native Americans studies are then outlined as well as their possible connection to the Ohio Hopewell. Hypotheses, both archaeological and linguistic, that focus on ancestor/ descendant relationships with contemporary Native Americans are also outlined and discussed with potential data to shed light on Ohio Hopewell origins. Finally, hypotheses generated from osteological and dental data, offer ideas to test with Restriction Fragment Length Polymorphisms and Direct Sequencing data from the Hopewell Mound Group site.

Chapter Four, Materials and Methods, goes through the steps that are necessary to extract, recover, amplify and test mitochondrial DNA. The chapter begins with the sample origins for this study as well as sample selection parameters. After sample selection, extraction of mitochondrial DNA is outlined as well as the Phen-Chloroform and Yang protocol C methods. Procedures to clean sample extracts and increase possible sample recovery are discussed as well as the technology behind the liberating method of Polymerase Chain Reaction. Sample screening and Polymerase Chain Reaction

purification techniques are put into the context of direct cycle sequencing and Restriction Fragment Length Polymorphism testing. Finally, protocols to deal with sample contamination and a new procedure to separate out contamination are introduced along with the steps after the separation.

Chapter Five, Results, begins with the overall amplification success rate for this study, which will then be broken down by individual mound. After the amplification success rate, the variation with the Ohio Hopewell Mound Group will be explored via RFLP and HV I sequence data. The RFLP data will focus on the distribution of Native American haplotypes within Hopewell Mound Group mounds 2 and 25 while further breaking down the data by sex. HVI sequence data will concentrate on unique haplotypes and those mutations which make them unique within the Ohio Hopewell at Hopewell Mound Group. Through a search of the database GenBank, modern and ancient populations were also located that shared the Ohio Hopewell unique mutations. The next section will concentrate on answering four questions which explore the genetic variation present in the Ohio Hopewell and are discussed in Chapter Three:

(1) What are the phylogenetic relationships and affiliations between the Ohio Hopewell Mound Group mtDNA lineages to other ancient and contemporary North Native American mtDNA lineages ?

(2) What information does mtDNA add to what is known biologically about the prehistoric groups of the Ohio River Valley (Glacial Kame, Adena, Hopewell and Fort Ancient)?

(3) Is there evidence to support matrilineal descent among the Ohio Hopewell of the Hopewell Mound Group ?

(4) Is there segregation of individuals interred within the Ohio Hopewell Mound Group based upon mtDNA lineages; specifically within Mounds 2 and 25 ?

For each question, a brief outline of the method utilized to answer the question will be given along with the source of the method. Finally, genetic indices such as π, theta and Tajima's D will be presented for the Ohio Hopewell of the Hopewell Mound Group.

Chapter Six, Conclusions, reiterates the focus of this research. A multidisciplinary approach combining hypotheses from archaeology, linguistics, and physical anthropology is the model for the research. The chapter provides a reiteration of the proceeding chapters along with the present research's contributions to the body of knowledge known about the Ohio Hopewell. Also included are additional ideas for future work which could contribute and build upon the foundation of this research.

CHAPTER 2

OHIO HOPEWELL

In the late 18th century, as the European immigrants made their way into today what is known as Ohio, they could not help but notice the large number of earthworks. The shear number and magnitude of some of the individual mounds would have been breathtaking. Various groups, who were designated the "Mound Builders", were given credit for the buildings of the earthworks. The Phoenicians, the Toltecs, the Mormons and maybe one of the Lost Tribes of Israel were but a few of the groups designated as those responsible for the construction of the earthworks. Anyone, it seemed but the ancestors of the Native Americans who were occupying Ohio at the time could be the "Mound Builders". This theme of "anyone but the Native Americans" went on for many years as more and more European immigrants poured into Ohio and as more mounds were discovered and described by the public. It was not until 1894, with the publication of *Report on the Mound Explorations of the Bureau of Ethnology, 1890-1891* by Thomas, that the idea that ancestors of present day Native Americans were in fact the constructors of the earthworks. Having established that Native Americans were the architects of the mounds, intensive surveys and excavations of the different mounds began in earnest. This chapter deals with what has been defined as " Ohio Hopewell". The chapter begins with the life ways of the Ohio Hopewell, starting with subsistence practices and

integrating settlement patterns. The chapter then goes on to discuss specifics of what is known about the Ohio Hopewell focusing on the Hopewell Mound Group, from its archaeological excavation history, to the time span and layout of the site, and its place within the Hopewell Interaction sphere. Finally, the chapter discusses social organization and a detailed mortuary analysis of Mound 25 of the Hopewell Mound Group, analyzed by Greber and Ruhl(1989).

Subsistence

As stated earlier, some antiquarians and the public were fixated upon the notion that non-Native Americans were the architects of the numerous mounds and earthworks in Ohio and the surrounding areas. After the 1890's, it was accepted that ancestors of the present day Native Americans were responsible for the construction of the mounds. However, as more and more excavations occurred a new fixation arose, the search for maize. It was assumed that maize would have provided the necessary energy source and surplus for those building the massive earthworks and mounds (Thomas 1894). So, with each subsequent archaeological excavation, the search for maize continued within Ohio. Unfortunately, maize has only been recovered at one Ohio site, Edwin Harness. However, after maize was still consistently not recovered in any great quantities during archaeological excavation, archaeologists consulted with physical anthropologists in an effort to put the question of maize to the test. Two avenues of study were explored, stable carbon isotope ratios and analysis of dental wear and pathologies in the form of caries. Maize utilizes a C4 or Hatch-Slack photosynthetic pathway, which results in the increase of C13 within the bone collagen of individuals who have ingested it (Bender et

al. 1981). The stable carbon isotope ratio, C13 to C12, indicates whether or not an individual has been ingesting a C4 plant (maize in the Ohio Valley). If maize has been consumed consistently, the amount of C13 in bone collagen will be high, if however, the level is low, little C4 has been consumed in an individuals diet. Smith (1992) and Sciulli (1997) both indicate that the levels of C13 to C12 in bone collagen are too low to suggest that maize contributed significantly to the diet of Eastern Native Americans until after B.P. 1000. Sciulli (1997) further provides evidence of a decrease in dental wear and an increase in caries prevalence from the Late Archaic to the Late Prehistoric. Utilizing 40 Ohio Valley samples, consisting of a total of 3613 individuals, Sciulli (1997) suggests that over a 3000 year time span from Late Archaic to Late Prehistoric, the rate of tooth wear decreased while the prevalence of caries remained fairly constant until about 1000 B.P., the beginning of the Late Prehistoric period when maize agriculture is also documented by carbon isotopes. Sciulli (1997) offers two innovations that might explain the decrease of tooth wear and increase of caries. At the beginning of the Woodland period, the introduction of ceramics lead to the softening of food during cooking and a decrease in the amount of grit which adhered to the food thus decreasing wear on the crowns. Finally, during the Late Prehistoric, an increased reliance on maize increased the simple carbohydrates in the diet which would have accounted for the increase in caries (Sciulli 1997). Overall, maize does not seem to be a major component in the diet of any Ohio Valley population, including the Ohio Hopewell, prior to 1000 B.P..

Thus, if the Ohio Hopewell were not the large scale maize agriculturists envisioned by early archaeologists, what were they subsisting upon? In 1989, Smith put forth the

hypothesis that a group of native eastern Noth American plants were domesticated. He designated this group as the Eastern Agricultural Complex (EAC). Smith (1992) indicates the following plants make up the Eastern Agricultural Complex (EAC): gourds (*Cucurbita*); squash (*C. pepo.*); sumpweed or marshelder (*Iva annua*); sunflower (*Heliantus annuus*); maygrass(*Phalaris caroliniana*); erect knotweed (*Polygonium erectum*); and goosefoot (*Chenopodium spp.*). The above plants prefer a disturbed, riverine environment and Smith (1992) suggests that by about 5000 to 3000 B.P. humans were intervening in the life cycle of the above plants, deliberating planting and storing seed stocks. Plant domestication began by taking advantage of already "naturally" occurring flood plain stands of plants, and then a slow progression of human intervention eventually leading to the active, intentional encouragement of the plant by humans (Smith 1992).

Smith (1992) points to four important technological advances in archaeology for increasing essential information on agriculture in eastern North America. He states that water floatation technology helped increase recovery of small carbonized seeds and other plant parts while the scanning electronic microscope (SEM) improved the ability to document morphological changes (Smith 1992). Accelerator Mass Spectometer (AMS) provided an accurate dating technique and stable carbon isotopes allowed for the direct analysis of collagen within bone thus enabling specific diet information to be recovered from skeletal analysis (Smith 1992).

In 1992, Wymer completed a detailed analysis of the paleoethnobotanical record in the Mid-Ohio Valley. Three sites from the Middle Woodland were surveyed, Newark Campus, Murphy and Dow Cook. Wymer (1992) examined the combined wood charcoal, nutshell, nut meat densities and the total number of identified plant taxa present at the above sites and seven other sites ranging from Early Woodland to Late Late Woodland. Eastern Agricultural Complex (EAC) plants were found at all three sites, with starchy members of the EAC contributing the majority (around 60% to 80%) of the seed assemblages and oily EAC plants contributing 1% to 2% (Wymer 1992). Wymer (1992) concludes that a trend of increasing dominance of a few species within the crop system, specifically goosefoot and sunflower, continues into the Fort Ancient tradition agricultural systems. In 1996, Wymer offered a summary of a decade of paleoethnobotanical research within the Ohio River Valley. She concluded that "Hopewellian populations practiced a sophisticated system of agriculture based upon a wide variety of crops and most likely utilizing some form of swidden or garden plot rotation" (Wymer 1996:47). She also suggests that EAC species made up a large portion of the Hopewell diet, with gathered fruits, berries, nuts and hunting supplementing the EAC species (Wymer 1997).

However, others have questions concerning whether or not the Ohio Hopewell were agriculturalists, horticultarists or simply hunter/gathers. Utilizing microwear analysis of chipped stone artifacts from the Murphy site, Yerkes (1990) suggests that the Hopewell tool kit lacked tools that would have allowed for specialization in agriculture/horticulure. Instead, the analysis of Hopewell bladlets demonstrated that they were unspecialized or

general and used for many purposes (Yerkes 1990). Yerkes (2000) further casts doubts as to whether the Ohio Hopewell were in fact practicing agriculture/horticulture and intervening in the life cycle of the EAC plants. He points to evidence that sumpweed, sunflower, chenpodium, knotweed and maygrass were most likely domesticated 900 or more years, well before the Hopewell culture (Yerkes 2000). He goes on to state that based upon the definition of farming by Harris (1997), the Hopewell could not be considered farmers. According to Harris (1997) "farming is defined as a system of agricultural crop production that employs systematic soil preparation and tillage". Yerkes (2000) further hypothesizes that the Ohio Hopewell were incorporating EAC plants as a supplement to their ancestral diet of wild nuts, native plants, fish and game.

Based upon site excavations, lack of paleobotanical evidence, stable isotope ratios and dental wear and pathology, the Ohio Hopewell were not maize agriculturists. Smith (1992) suggests that maize might have played a ceremonial role within Ohio Hopewell culure. Maize did not make a substantial contribution to the diet until after 1150 B.P.. Whether, as Wymer suggests, the Ohio Hopewell were swidden agriculturists relying heavily on EAC, or as Yerkes proposes, the EAC plants were just a small part of a seasonal hunter/gatherer subsistence pattern, EAC plants were a part of the Ohio Hopewell diet. The extent of that involvement is still in debate.

Settlement

At one time it was thought that in order to sustain large scale maize agriculture, one must also have large scale nucleated villages housing individuals who would be working in the nearby fields and generating surplus food supplies. Archaeologists looked for these large scale nucleated villages in Ohio, during their excavations of the earthworks, mounds and areas surrounding both. However, the Ohio Hopewell did not have large scale nucleated villages.

In 1964, Prufer, in an attempt to organize Hopewell settlement data into coherent patterns, put forth the idea of Vacant Ceremonial Centers drawn from Mesoamerica parallels. He suggested that Ohio Hopewell subsisted largely on agricultural products and habitation sites were located in agriculturally suitable bottom land along rivers in small settlements or farmsteads, not large nucleated villages (Prufer 1964). All of the small farmsteads were related to the greater ceremonial centers adjacent to their small communities. Dancey (1992) in a comparison of 2 sites, one from the Middle Woodland (Murphy) and the other from Late Woodland (Water Plant) noted a difference in settlement patterns and locations between the sites. For the Middle Woodland Period, he noted that communities were made up of dispersed household units occupying unprotected settlements on the valley floor (Dancey 1992). However, by the Late Woodland, communities were living together in defendable multi-household villages on bluffs. Thus came the end of the small Hopewell hamlet by about 1500 B.P. and the decline of the Hopewell in Ohio.

Dancey and Pacheco (1997) further fleshed out Prufer's idea into the Dispersed Sedentary Community model suggesting small dispersed sedentary hamlets surrounding the vacant earthwork-mound centers. They proposed that the hamlets were occupied year round by one or several households connected with a surrounding territory. The ceremonial centers would be periodically visited by some or all in the group and those individuals were most likely bound together by kinship and tradition through several generations (Pacheco 1996). The ceremonial centers served as a means to reduce the risk of subsistence failure and share resources through reciprocal relations with other groups visiting the center.

Others (Yerkes 1990,2000,2002; Lepper and Yerkes 1997; and Cowan 2002) however, have expressed difficulties with the idea that the Ohio Hopewell were sedentary. Yerkes (2002) catalogues archaeological evidence that would have to be recovered in order for sedentism to be suggested. First, he points out the lack of recovery of substantial dwellings and thick midden deposits. Both would be necessary to indicate year long, multi-generational occupations by the Ohio Hopewell (Yerkes 2002). Yerkes also suggests that the archaeological recovery of seasonal flora and fauna covering the entire year would indicate that the hamlets were being utilized winter, spring, summer and fall. Finally, Yerkes (1990) indicates the general nature of the Ohio Hopewell tool kit, with no specialization to process specific plants or animals.

Yerkes proposes another model that would explain the settlement of the Ohio Hopewell. Yerkes (2002) states that the Ohio Hopewell continued the settlement and subsistence pattern of earlier Woodland traditions. The Ohio Hopewell were not sedentary farmers but were in fact still utilizing hunter/gatherer subsistence in small, most likely family groups (Yerkes 2002). During the summer months when food sources were at their highest, the Ohio Hopewell would gather at the ceremonial areas to work on the mounds, exchange mates and generally socialize before breaking up again into small family groups. These small family groups or extended family groups would have been better able to withstand the lack of food resources in the harsh winter environment. Yerkes (2002) postulates that the Ohio Hopewell were a mobile, egalitarian, decentralized society which continued to utilize the settlement and shelter of their ancestors.

Archaeological and Biological Continuity in the Ohio River Valley

Early on, as more and more sites were being excavated, an outpouring of theories were being generated to account for differences with each new site excavation. Clearly, there were differences in cultural materials and skeletal remains that were being excavated. Mills (1902) was the first to make distinctions between the Glacial Kame, Adena, Hopewell and Fort Ancient and designate them "cultures". Early archaeologists then went about establishing a temporal order for the four cultures. Mills looked at the variation present at the sites and thought that Fort Ancient and Hopewell were contemporaneous and that the Fort Ancient population had conquered the Hopewell and destroyed their artifacts (Griffin 1997). Shetrone (1926) also followed the idea that Fort Ancient and Hopewell were at least in part contemporaneous, but he did not know which

had appeared first or which was last to disappear from Ohio (Griffin 1997). Griffin (1943) thought that the Hopewell arose from not one source but from many donors in the eastern United States. However, the Ohio Hopewell were thought to have originated from an Adena Base. A major part of the difficulty in arriving at the correct temporal organization of the Adena, Fort Ancient and Hopewell, is that it is unimaginable that a society with such massive amounts of exotic goods, such as the Hopewell, would then move into a period where the quantity and quality of the exotic declined, as with Fort Ancient. It wasn't until 1922, when Willoughby, based on the Turner site, suggested that the Fort Ancient population followed the Hopewell. The chronology, based upon carbon 14 dates for the Ohio Valley, is as follows: Late Archaic (ca. 5000-2800 B.P.); Adena (ca. 2800 to 1900 B.P.); Hopewell (ca. 1900 to 1400 B.P.) and Fort Ancient (ca. 1000 to300 B.P.).

Physical anthropologists and later archeologists continued to fine-tune the characteristics that define Late Archaic, Glacial Kame/Red Ocher, Adena, Hopewell and Fort Ancient cultures of Ohio. The Glacial Kame/Red Ocher traditions represent two regional methods for treatment of the dead. During the Late Archaic, the Red Ocher burial tradition drew its name from burials found in a flexed position in a pit within a natural knoll or ridge, usually within sand or gravel, which were capped by powdered red ocher. The mineral red ocher ranges in colors from a mustard yellow to a bright red. Also associated with the burials were a distinct point type, the turkey tail point, getting its designation from its unusual shape. The burials were also accompanied by other grave goods, consisting of chert caches, galena crystals, copper and ground stone artifacts, along with marine shell

necklaces (Converse 1980). The Glacial Kame tradition is thought to predate the Red Ocher tradition, and did not utilize turkey tail point in their burial process but included gorgets with their other burial artifacts of marine shells, and beads. Rothschild (1979) likens the Adena to a small scale egalitarian society where status is distributed according to age, sex or personal achievement. Brose (1985) characterizes the Adena as generalized hunters/gatherers/fishermen who occupied major riverine areas west to east in the Ohio Valley from 3050 B.P. to 1250 B.P.. Brose (1985) suggests that the Adena evolved away from their egalitarian social organization as ceremonial trade and activities began to play a greater role. He also suggests that a greater status distinction within the society began that involved ritual exchange, and that acquired status through economic and social processes developed by the close of the Early Woodland (Brose 1985). Greber (1991), utilizing archaeological site data from the central Scioto, suggests three characteristics that separate the Adena from the Hopewell in Ohio. She notes a change in the use of vertical space from single groups within the Adena to multi-groups with the Hopewell (Greber 1991). As part of their ceremonial/ritual activities, the Hopewell have a greater incorporation of more exotic goods such as copper, mica and marine objects (Greber 1991). Finally, the Hopewell simply increased the complexity and size of the mounds and earthworks, they were constructing, thus increasing their archaeological visibility and the likelihood of recoverable archaeological remains (Greber 1991). The Ohio Hopewell are characterized, among other features, by the following material remains: mounds, ceramic vessels, obsidian, copper, mica, platform and pan pipes, and cut animal jaws and teeth (Seeman 1995). Overall in terms of their material remains, the Hopewell seem to have taken the original Adena mortuary program to its extreme. The exact opposite is true

when looking at the transition from Hopewell to Fort Ancient. In southern Ohio, where the Hopewell Mound Group is located, the Late Woodland Newtown tradition follows the Hopewell in time but does not continue the Hopewell mortuary program. Construction of large geometric earthworks and importation of exotic raw materials ceases at that time. The burials are simple inhumations without the large quantities of material remains, which so defined the Ohio Hopewell. Again, the overall theme in mortuary behavior has been changed along with the decline of exotic goods accompanying those burials. Housing and pottery styles change and stone grinding implements are recovered in large nucleated villages focused on maize agriculture (Seeman 1993). New hunting technology, in the form of bows and arrows, allow for increased hunting effeciency at longer distances.

Early physical anthropologists were also interested in exploring the biological variation of the Ohio River Valley populations. Webb and Snow (1945), based mainly upon cranial morphology, suggested that the Ohio Hopewell were characterized by three cranial shapes. About 80% of their sample were dolicocephalic or long-headed,while 10% to 15% of the sample was brachycephalic or round-headed, leaving the remaining sample as unidentifiable (Webb and Snow 1945). Snow (Webb and Snow 1945) defined the dolicocephalic type as Hopewell type 1 while the brachycephalic were considered "Adena-like" in their shape and designated Hopewell type 2. Based upon the cranial types, Snow postulated that the Hopewell type 1 cranial shapes were descendants of indigenous populations of the Ohio River Valley, while the people with the Adena type cranial shapes (Hopewell type 2) had migrated into the area (Webb and Snow 1945). He

further suggests the possibility that the Hopewellian type 2 cranial shapes were a result of admixture between indigenous Hopewell and the invading Adena (Webb and Snow 1945). Dragoo (1964) first proposed two migrations for the ancestors of the populations within the Ohio River Valley. The first by the "Lenid People" of the Late Archaic Burial Complex, including the Glacial kame culture, located near the Great Lakes, who intermixed with the Adena-Red Ocher people of central Indiana-north central Illinois. The second migration was similar to the first in that the "Lenid People" intermixed with the Adena of the Ohio River Valley to produce the Ohio Hopewell. All of these ideas suggest distinct biological gaps between the Glacial Kame, Adena and Hopewell as outside populations migrate into Ohio. Reichs (1975,1984), utilizing metric and discrete cranial characteristics, examined the hypothesis that the Ohio Hopewell populations and culture originated from Hopewell populations in Illinois. Her results demonstrated that the Illinois and Ohio Hopewell populations were distinct from each other biologically and had no significant genetic exchange during the Middle Woodland. Sciulli and Mahaney (1986) extended Reichs' work within the Ohio River Valley by looking at distinct local populations within Ohio from the Glacial Kame Late Archaic populations (ca. 3000-2500 B.P.), Adena Early Woodland (ca. 3000-2000 B.P.) and Hopewell Middle Woodland (ca. 2000-1700 B.P.). Sciulli and Mahaney (1986), based upon analysis of cranial measures and discrete cranial trait frequencies, concluded that the Hopewell Mound Group site sample demonstrated the same configuration of cranial size and shape variation and discrete trait expression as the Archaic Glacial Kame population. There results suggest, at

least, biological continuity for the Ohio Hopewell, at the Hopewell Mound Group site and lack of support for large scale migrations from Illinois to Ohio or vice versa by the Hopewell groups.

Ross County ,Ohio

Prior to a specific discussion concerning the Hopewell Mound Group site, a brief discussion of the importance and significance of the area in which the site is found, Ross County, Ohio, is appropriate. According to the State of Ohio's preservation office, Ross County has approximately 950 Ohio Archaeological Inventory forms with Middle Woodland components. These Ohio Archaeological Inventory forms have been generated from isolated finds, lithic scatters, habitiation sites, individual burials, mounds and earthworks. Along the Scioto-Paint Creek confluence, within a 30 kilometer radius around Hopewell Mound Group (HMG), there are at least 9 major mounds and earthworks. Simply put, Ross County is the epicenter for Ohio Hopewell. Ross County archaeological sites were central to defining major aspects of regional prehistory beginning in the 1840's with the pioneering investigations of Squier and Davis. Subsequent professional work of such pioneering archaeologists as Thomas, Putnam, Moorehead and Mills defined and extended major aspects of regional prehistory (Seeman 1995). The known mounds and earthworks in Ross County can be counted in the hundreds, and exceed densities for any comparably sized area in eastern North America (Seeman 1995).

The Hopewell Mound Group (33RO27)

The Hopewell Mound Group (HMG), designated 33RO27, has been one of the most comprehensively surveyed, excavated, and studied Hopewell sites in Ohio. Caleb Atwater(1820) was the first to describe and map the earthworks. Squier and Davis (1848), supplied the first detailed survey of the site. At the time of their survey, the site was owned by W.C. Clark and was known as the "Clark's Works". In order to collect materials for the 1892 World Columbian Exposition in Chicago, Warren K. Moorehead in 1891, directed the first intensive archaeological excavation of the site. At the time of Moorehead's excavation the site had passed in ownership to Mordecai Cloud Hopewell, therefore, it was then known as the "Hopewell site" and materials excavated from it designated "Hopewell". Moorehead mapped and excavated fifteen mounds on the site. In 1922, the Ohio Historical Society sent Henry Shetrone to completely map and excavate the site, continuing Moorehead's excavation.

The Hopewell Mound Group (HMG) is located within the central Scioto River valley, along the North Fork of Paint Creek in Ross County, Ohio (Figure 1). The site itself is immense, incorporating 40 mounds, and a large rectangular and a D-shaped earthwork. The mounds range in size from about 30 centimeters in height (Mounds 32 to 36) to the massive Mound 25 which is 9 meters in height and 150 meters in length. A large rectangular earthwork, more than 99 acres (40 hectares) in area unfolds along the contours of the second and third terraces of the North Fork of Paint Creek and a small

unnamed stream. Known as the Great Enclosure, it conjoins with another enclosure located along the eastern side. A D-shaped earthwork is within the Great Enclosure and surrounds the largest mound, Mound 25 (Figure 2).

Archaeologists have attempted to assign a time depth to the Hopewell Mound Group and other Hopewell sites in Ohio. Prufer (1964) produced a chronological breakdown of Ohio Hopewell based on trait comparison and supportive radiocarbon data. The chronological breakdown of the sites was as follows:

Early Hopewell: Mound City

Middle Hopewell: Hopewell,Harness,Seip

Late Hopewell: Ater, Russell Brown

Greber and Ruhl (1989) state that chronologically, the central mound at Hopewell Mound 25 is older than Seip. Maslowski et al. (1995) provides a range of C14 dates from Mounds 17 and 25 of the Hopewell Mound Group. For Mound 17, the C14 dates range from 2285 +/- 335 B.P. to 1620 +/- 140 B.P., while at Mound 25 the C14 dates range from 2044 +250-94 B.P. to 1951 +200-1 B.P.. Greber (2003) has collected 335 dates from middens, pits, rock shelters, small and large mounds, embankment walls and samples from within and near embankment walls. From those samples, she has a rounded mean frequency C14 distribution date of 1740 +/- 290 B.P. for the Ohio Scioto Valley. Greber (2003) was able to secure AMS dates from materials in Moorehead's 1891 to 1892 excavation. Two dates from charred wood found with Moorehead double Burial 260/261 gave a weighted average of 1730 +/- 61 B.P. and three altar dates gave a

weighted average of 1803 +/- 72 B.P.. As with other Hopewell sites, dating of the Hopewell Mound Group site is difficult due to the complicated and complex nature of the site, and early archaeological excavation techniques.

Hopewell Interaction Sphere

Ohio Hopewell communities are well known for bringing in large quantities of only the best quality exotic goods. Many of the exotic items were made from non-local raw materials such as hematite, obsidian, hornstone, copper and mica. These raw materials were fashioned into cutouts of animals, celts, headdresses, breastplates, ear spools, blades, and bladelets (Struever and Houart 1972). As a means of explaining the significance of exotic materials hundreds of miles from their origin, Struever and Houart (1972) proposed that the Hopewell Interaction Sphere as coined by Caldwell (1964) was not an ideological network that held the Hopewell together but an economic distribution mode. Struever and Houart (1972) thought that exotic raw materials and finished items would move along riverine networks from regional or interlocal transaction centers to local transaction centers in outlying areas. Through the distribution of these exotic goods, which were displayed in mortuary contexts, subsequent political leaders were able to respond to societal needs while also increasing their own status. Seeman (1979) analyzed a key component of the Struever and Houart model, the proposed evenly spaced distribution centers in Ohio and found that the data did not support the model. In fact, the Ohio Hopewell distribution sites appeared to cluster and appeared to be more one sided trading, with groups in the Scioto River Valley area occupying a pivotal position in the trade network. Seeman (1979) also concluded the system was not a dynamic or

integrated exchange system. Overall, Seeman (1979) was able to show that the Struever and Houart model was not supported by the available evidence and that the distribution sites ,which were so key to their analysis , did not occur along a riverine network.

Mortuary Analysis

In an effort to uncover social organgization, Saxe (1970) and Binford (1972) proposed the analysis of mortuary practices as a primary means of investigating past social systems. They argued that variations in types of society and social complexity could be analyzed via mortuary analysis. For example, Binford (1972) argued that:

"We would expect that other things being equal, the heterogeneity in mortuary practice which is characteristic of a single sociocultural unit would vary directly with the complexity of the status hierarchy, as well as with the complexity of the overall organization of the society with regard to membership units and other forms of sodalities" (p. 221-2).

and

"It is proposed that there are two general components of the social situation to be evaluated . . . First is what we may call, with Goodenough (1965, p. 7) the “social persona” of the deceased. This is a composite of the social identities maintained in life and recognized as appropriate for consideration at death. Second is the composition and size of the social unit recognizing status responsibilities to the deceased and the number of persons having duty-status relationships vis-a-vis the deceased "(p. 225-6).

According to Binford (1972), two main features are detectable by archaeologists with regard to prehistoric social organization from an analysis of mortuary practices: (1) the type of organization present, whether or not the presence of distinct and/or competing corporate groups is indicated; (2) the complexity of past social systems or, in other words, how much structural differentiation there appears to be. Some characteristics that may be utilized in order to recognize social persona or role are age, sex, social affiliation, position, condition and location of the dead. Rothschild (1979) goes on to further detail assumptions underlying the Saxe-Binford program of mortuary analysis. First, in the Saxe-Binford model, it is possible to determine aspects of social organization from methodological analysis of mortuary practices (Rothschild 1979). Secondly, the model assumes that distinctions visible in mortuary practices reflect status distinctions visible during life (Rothschild 1979). Thirdly, if patterns do exist in mortuary practices, it is assumed that they relate to structural divisions in the society and that analysis of behavior of the whole society reveals patterns; those patterns demonstrate significant factors in social structure (Rothschild 1979). Finally, the model assumes that details of the burial program, be it artifact inclusion in graves or differential treatment of the body, indicates ways in which status divisions were drawn within the society (Rothschild 1979).

The identification of pattern and its correlation with social complexity is the general strategy of mortuary analysis. Specific questions are asked of the mortuary data. Those questions might include the following: what is the artifact distribution and do certain artifacts regularly occur with others; is this occurrence specific to individual graves, or

with a particular age or sex; what is the directional orientation of the burials and are all burials orientated in the same direction or are some facing another direction; is the arrangement of burials in a cemetery random or regularly patterned; does the spatial organization of burials differ within and between cemeteries; what are the demographic parameters of deceased populations. In an attempt to calculate measures of effort expenditure attention has centered on treatment of the deceased (Tainter 1975). Buikstra (1981) focused on physical indicators of social differences within skeletal populations. Overall, mortuary analysis draws from the theoretical perspective of structural-functionalism and role theory. Radcliffe-Brown (1952) viewed social structures in terms of three basic questions: What are the structures within the society and how may they be categorized? How do the structures function and maintain themselves? How are the structures established ? Radcliffe-Brown (1952) views the basic unit of structure as the "family" and other kin ties or structural relationships can be probed utilizing that basic unit. According to role theory, an individual's social persona is formed as a response to different situations. The roles can change depending upon whether they were achieved or ascribed, what context they are occurring in and their temporal enactment (Shanks and Tilley 1987). One individual can have various roles or persona throughout their life span and at the same time.

Mortuary analysis has been criticized on many fronts. Kerber (1986) states that Saxe mistakenly assumes that all non-random variability in mortuary ritual to be representation of the dead ego's role in social structure. According to Kerber (1986) in Saxe's model, mourners are motivated by obligations owed the deceased, and are moved to symbolize

the deceased's status in traditional ways through mortuary offerings. Kerber (1986) also criticizes the implications of the model including the notion that a finite number of social identities exists to be symbolized and that the symbols employed have significance. He also questions that the obligation to the deceased is a sufficient cause for the ritual work to be done (Kerber 1986). Shanks and Tilley (1987) state that the entire theoretical perspective is based on structural-functionalism and role theory and criticize mortuary analysis for its reliance on correlation as explanation. Carr (1995) states that emphasis has been placed on the reconstruction of social organization and the determination of the status of individuals interred together while little attempt has been made to test hypotheses based on ideological tenets.

Brown (1995) raises criticisms of the reliance on social factors for understanding mortuary practices and usefulness of cross-cultural comparison. However, Brown (1995) does suggest that although Binford and Saxe based their theoretical position on global arguments, they do offer the ability to articulate specific arguments. Thus, according to Brown (1995:10), "mortuary analysis becomes a middle-range-theory -- a body of theory whose generalities are useful for advancing part of a larger argument in favor of some explanation about specific issues". Specifically, contemporary and historical mortuary practices of Native Americans have been observed and studied in order to look for correlations between those practices and ones found in the archaeological record. Finally, Brown (1995:10) states "that Saxe's information theory approach to the organization of patterned differences in the physical remains of the dead complimented Binford's focus on the cross-cultural strength of any proposition linking systematic

differentiation in the range of the disposal-of-the-dead practices with social complexity". Overall, the Saxe-Binford method allows the archaeological record to provide some insight into the cultural processes of the Ohio Hopewell.

Greber (1979) and Greber and Ruhl (1989) apply Saxe-Binford mortuary analysis of mortuary items to two Ohio Hopewell sites: Seip Mound 1 and the Hopewell Mound 25. Greber (1979) compiled data sets from both sites incorporating nonperishable associated artifacts, type of inhumation, spatial location, age, sex, and detailed grave attributes (Greber 1979). Ordinal scales and ranking procedures for the comparisons of individuals and groups within a site were utilized because they enable the preservation of the most detail for the majority of the basic variables available in the sets of burial data (Greber 1979). Another advantage of using ordinal scales is the possibility of combining information from sets of attributes into a rank sum (Greber 1979). Greber (1979) then utilized nonparametic comparison procedures once sets of rankings have been established for each individual, in order to facilitate the comparison of groups. Greber (1979) first utilized the above methods in her dissertation, where she was able to formulate a model of social organization based on mortuary analysis of associated artifacts, physical characteristics of the burial, tomb construction, and location of the burials. Greber (1979) suggests that at Seip Mound 1 individuals of high rank may represent kin-based units within the mound, which based on her analysis has three main hierarchical social divisions. She also proposes an internal complexity probably paralleling their respective ranks (Greber 1979). Greber's analysis further states that social distinctions associated with membership within the divisions were stronger within the mound population than

distinctions which the society may have associated with sex. Based on her mortuary analysis, she also concludes that their status was most likely ascribed, not inherited (Greber 1979).

Hopewell Mound 25

Greber and Ruhl (1989) further extend Greber's dissertation work to the Hopewell Mound Group, specifically Mound 25. Greber and Ruhl (1989) summarized the burials excavated by Moorehead (1922) and Shetrone (1926). The goal of the analysis was to use ranksum analysis of the burial to determine the complexity of the social structure at Hopewell Mound 25. Greber and Ruhl (1989) begin their analysis by first looking at the placement of the burials, stating that it is a common Ohio Hopewell practice to place burials on the floor of a previously erected structure. They go on to describe the burial floor plan of Mound 25 beginning with four extended burials lying on beds of gravel which were found separately 1.2 to 2 meters above the mound floor in the western side of the central mound (Greber and Ruhl 1989). Greber and Ruhl (1989) illuminate the distribution of the burials in Mound 25 into 7 subgroups based upon their spacial location (Figure 3). Group A1 is redeposited cremation placed on a platform in the south side of the mound next to Group A2, also in the south, which is a double extended burial interred in a shallow grave (Greber and Ruhl 1989:51). Group B is southeast and contains 6 cremated burials found together under a small mound (Greber and Ruhl 1989:51). Group F, on the western floor, is made up of 2 extended and 2 redeposited cremated

burials which were within a wooden structure (Greber and Ruhl 1989:51-2). Groups C, D, E, the 3 major groups, contain primary extended burials (all individuals within Mound 25) and redeposited cremations (Greber and Ruhl 1989:52).

Mortuary Analysis of Hopewell Mound 25

At Hopewell Mound 25, Greber and Ruhl unveiled little or no differences in social ranking among the major groupings (B, C, D, and E) based upon statistical analysis of grave goods (Greber and Ruhl 1979:56). However, they do state that there are differences within the groups which indicate internal social groupings but all groups are well supplied with grave goods (Greber and Ruhl 1989:293). Greber and Ruhl interpret the social status of the majority of those individuals as being tied by biological or social kinship to an individual most likely interred near them (1989:60).

According to Greber and Ruhl, "the Seip Mound 1 represents social relationships which appear to be ranked internally and divided into 3 major groups" (1989:292). However, "each group did not deposit the same quantities of grave goods which would suggest an allocation of precious grave goods which could be linked to the individuad's social status" (Greber and Ruhl 1989:292). Greber and Ruhl suggest that "the Seip Mound 1 social structure appears to be much more rigid while the social structure at Hopewell Mound 25 seems more fluid" (1989:292). Chronologically, the central mound at Hopewell Mound 25 is older than Seip Mound 1, which may indicate that the social structure of the Ohio Hopewell of the Central Scioto changed significantly over time.

Overall archaeology has been able to provide clues to the lifeways of the Ohio Hopewell, from their place in history, to their relationship to other Native American groups, to information on subsistence, settlement and social structure. After many decades of archaeological research and carbon 14 dates from many sites, the Hopewell take their place in the chronology of Ohio, following the Glacial Kame and Adena traditions and preceding the Fort Ancient tradition. Based upon shared material remains, it appears that the Ohio Hopewell have a close relationship with the Adena, taking their mortuary program and expanding it greatly. The relationship between the Ohio Hopewell and Glacial Kame groups is not as clear, as there is still some debate about the relationship between Glacial Kame groups and the Adena. The same holds true for the affiliation between the Ohio Hopewell and Fort Ancient. It is difficult to comprehend that the descendants of the Ohio Hopewell stopped practicing the elaborate mortuary program as did the Fort Ancient groups. This very decline of earthworks and decrease in imported mortuary items demarcates an end to the Ohio Hopewell tradition. With the addition of a new hunting technology, bows and arrows and an increased reliance upon maize agriculture, Fort Ancient traditions make the transitions to large nucleated villages that early archaeologists searched for with the Hopewell. Mortuary analysis contributes a view of the Ohio Hopewell social organization which seems to be achieved rather than ascribed. The Ohio Hopewell do not have the full scale chiefdoms found in the later Mississippian traditions, but there are indications of differential access to imported materials incorporated into the mortuary program. Overall, the Ohio Hopewell appear to be compiled of small groups most likely extended families, who practiced early horticulture and lived in small dispersed communities.

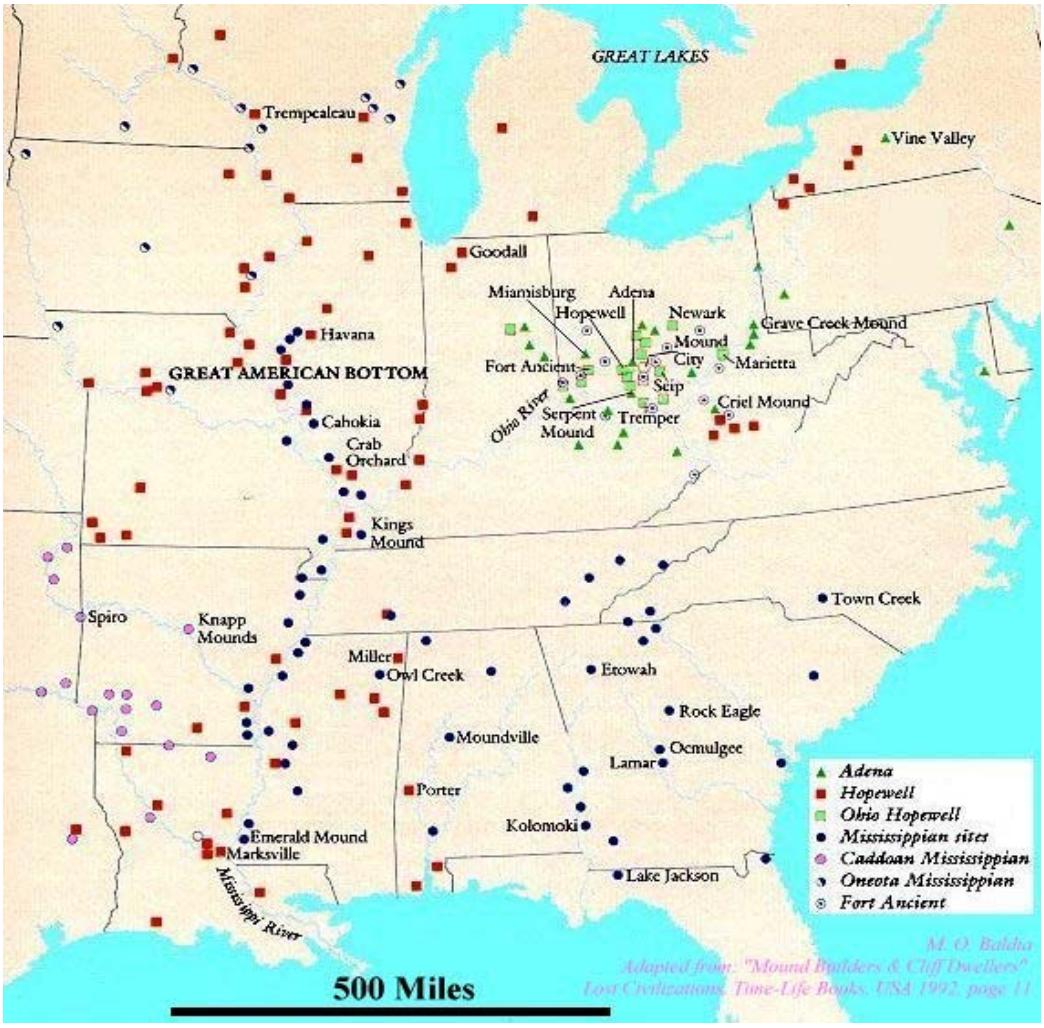


Figure 1: Map of eastern United States showing the Hopwell Mound Group site and its relation to Adena and other Hopewell sites (www.comp-archaeology.org)

Figure 2: Hopewell Mound Group Site Layout (the 3 mounds in center of the D enclosure were consolidated into 1,Mound 25)
(www.comp-archaeology.org)

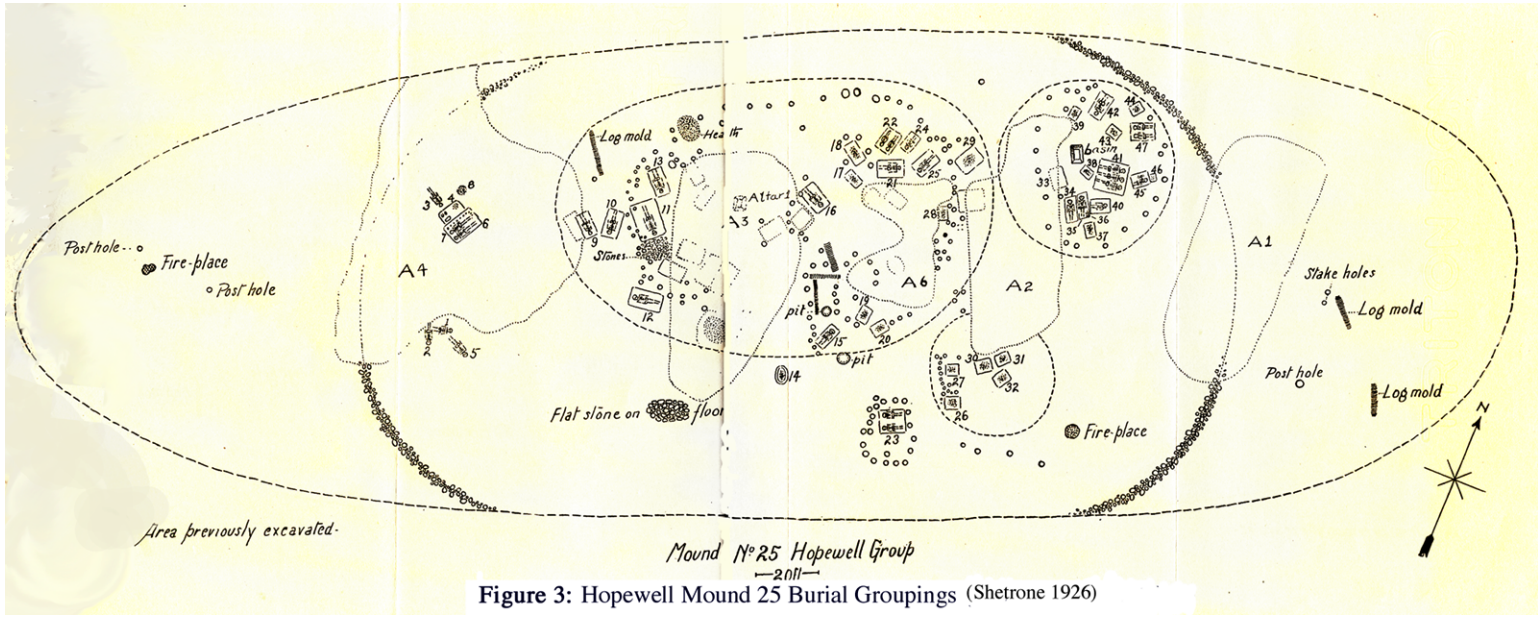


Figure 3: Hopewell Mound 25 Burial Groupings (Shetrone 1926)

CHAPTER 3

MITOCHONDRIAL DNA

In the 1980's, a plethora of new methods and techniques were developed within molecular genetics to examine population diversity. The human mitochondrial genome was completely diagrammed and sequenced and the diversity within that genome was unlocking new information at a vast rate. Other methods were developed to assist in the amount of sample that could be attained from an individual making up that population. This chapter will discuss the explosion of new information and techniques and how they were implemented in order to study Native American contemporary and ancient populations. This chapter begins with the Cambridge Reference sequence by Anderson, and then discusses the properties that make mitochodrial DNA suitable for genetic studies. The history of Restriction Fragment Length Polymorphisms as they apply to ancient and contemporary Native American populations, as well as studies utilizing Hypervarible Region I will also be analyzed within this chapter. A molecular genetic review of ancient Native American populations will provide an overview of the work that has been done in this area. Finally, a statement of the molecular genetic questions that this dissertation will ask concerning the Ohio Hopewell will end the chapter.

Cambridge Reference Sequence

In 1981, Anderson et al., were the first to successfully describe the organization of the human mitochondrial genome as a single type of circular double stranded DNA and to completely sequence all of the nucleotides. The sequence is referred to as the Cambridge Reference sequence and all other subsequent sequences are compared to it. Overall, the human mitochondrial DNA (mtDNA) genome is 16569 base pairs in length and is composed of 44% guanine (G) +cytosine (C). The two DNA strands consist of different base composition with the heavy (H) strand entailing a high concentration of guanines and the light (L) strand composed mostly of cytosines. Due to the additional synthesis of a segment of mtDNA, 7S DNA or D-loop, the double stranded mtDNA molecule has a small section defined by a triple DNA strand structure. A high coding sequence density results from the absence of introns and the close apposition of all genes, including a section where genes overlapping; ATPase 8 partially overlaps ATPase 6. Anderson et al. (1981) describe polypeptide encoding genes such as NADH dehydrogenase subunits 1 to 6 (ND1-ND6), cytochrome C oxidase subunits (CO1-CO3) and cytochrome B (CYB). MtDNA genome organization also included genes for 12S, 16S and 22 tRNA's as well 8 coding genes (Figure 4). The non-coding region, which is the major control region is 1.1 kilobase long and extends from base pair position 16024 to 00576. It contains the displacement (D) loop comprised of 2 hypervariable regions, HV-I and HV-II (Cantatore and Attardi 1980; Anderson et al. 1981). The majority of the variability of mtDNA exists

within the two hypervariable segments (HVI and HVII) of the D-loop control region which accumulates mutational changes 5 to 10 times faster than its counterpart, nuclear DNA (Brown et al. 1979; Ferris et al. 1981).

Why MtDNA

The human body contains two different types of human genome, nuclear DNA and mitochondrial DNA (mtDNA). MtDNA was chosen for this study because of its unique characteristics. First, the mitochondrion provides the power source of the cell in the form of ATP. Thus it is found in every cell within the body containing approximately 1500 copies of mtDNA, with an average of 2.6 copies per mitochondrion (Hagelberg 1993). Because there are more copies of mtDNA than nuclear DNA, it is more likely that mtDNA to be recovered rather than nuclear DNA in ancient samples (Rogan and Salvo 1998). During zygote formation the sperm cell contributes nuclear but only very rarely mtDNA therefore males only very rarely provide any mtDNA to the next generation. MtDNA is usually solely maternally inherited and during mitotic cell division two exact copies of the original maternal mtDNA form two daughter cells. MtDNA does not undergo recombination and is transmitted intact from mother to offspring. The only changes that may occur to mtDNA are due to point mutations, insertions and deletions, with insertions and deletions being rare. Soodyall et al. (1998) detected high fidelity with no mutations in over 100 transmissions from mother to offspring. However, when a new mutation arises and spreads in the mtDNA genome, such as in heteroplasmy, it will lead to two significantly frequent mtDNA genotypes. Jenuth et al. (1996) show that human mtDNA heteroplasmy has been found in association with a heterogenous group of disorders referred to as mitochondrial encephalomyopathics. Analysis of human

Figure 2. Human mtDNA genome, showing the approximate location of the five mtDNA markers that delineate New World haplogroups. Gene abbreviations: ATPase – components of ATPase complex; COI – COII – cytochrome c oxidase subunits; cyt b – cytochrome b; D-Loop – displacement loop; N1 – N6 – NADH-dehydrogenase subunits; rRNA – ribosomal RNA.

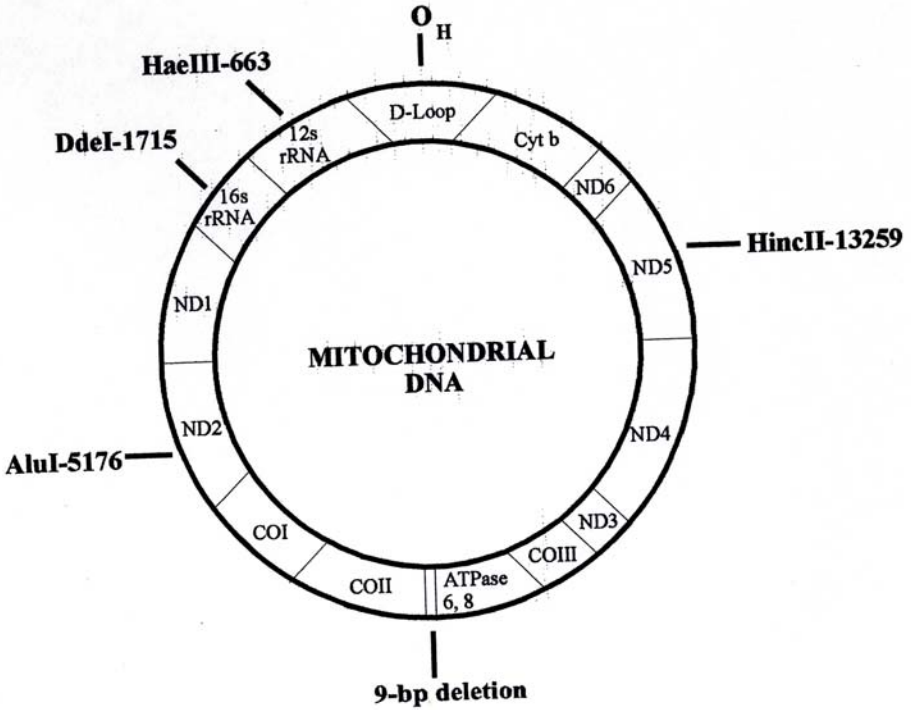


Figure 4: Diagram of Mitochondrial DNA (Napier 2000)

pedigrees with segregating pathogenic point mutations in mtDNA coding sequences demonstrates that complete segregation of mild mutations (homoplasy), and large shifts in proportion of mutant mtDNA associated with severe phenotypes (always heteroplasmic), can occur in as little as 3-4 generations (Jenuth et al. 1996). The proportion of mutant mtDNAs in a heteroplasmic individual must generally exceed 85-90% before a biochemical or clinical phenotype is expressed. Below this threshold mutant mtDNAs should be invisible to selection and would likely segregate randomly. In 1994, heteroplasmy came to the forefront in the possible forensic identification of the Romanov family. What was supposed to be a certain identification turned into a problem when Czar Nicholas's mtDNA didn't quite match his relatives. It was widely held that most humans carry only one type of mtDNA passed down from their mothers but the czar had two types of mtDNA. The same site sometimes contained a cytosine and sometimes a thymine while his relatives had only the thymine (Giles et al. 1994). The controversy was put to rest in 1996 when the analysis was published in *Nature Genetics*, from the exhumation of Nicholas's brother ,who also had two different sequences of mtDNA from their mother (Giles et al. 1996). Heteroplasmy is found to occur in at least 10% and probably 20% of humans. If this is the case, as heteroplasmy is caused by mutations, this high rate of incidence suggests that mtDNA might mutate as much as 20-fold faster (Gill et al. 1994). Finally, mtDNA shares several features with most prokaryotic genomes: small size, absence of introns, a high percentage of coding DNA, lack of repeated DNA sequences and small prokaryotic-like rRNA genes (Anderson et al. 1981).

Mitochondria are thought to have originated as a result of endocytosis by anaerobic eukaryotic precursor cells of an aerobic eubacterium (Anderson et al. 1981). The evolution of the altered mitochondrial genetic code is thought to be a response to the gradual loss of coding potential by gene transfer from the original endosymbiontic to the nuclear genome. Thus, the mitochondrial genome most likely evolved as a result of reduced selection pressure as a response to a diminished coding capacity. As a result , the rate of evolution is approximately 5 to 10 times faster than that of nuclear DNA making it ideal to study questions of recent migration, kinship, and phylogenetic relationships (Brown et al. 1979; Ferris et al. 1981).

History of RFLP and mtDNA Sequence

In an effort to examine the variability within DNA, Botstein et al. (1980) developed a recombinant DNA technique utilizing single-copy DNA probes hybridized to restriction digests. The mapping technique would allow for the detection of DNA polymorphisms and define individual loci for these polymorphisms. Restriction enzymes are bacterially derived proteins that recognize and cut at short specific nucleotide sequences. A restriction fragment length polymorphism (RFLP) result from mutations, both nuclear and mitochondrial. Genetic variation between individuals is due to the existence of alternative alleles associated with restriction fragments that differ in size from each other. When DNA is digested with a restriction enzyme, fragment sizes may differ depending on the presence or absence of the particular recognition sequence for that particular enzyme. RFLP's can be visualized via gel electrophoresis by separating fragments based

upon size, with larger fragments separating first and smaller fragments traveling farther down the gel. The simplest RFLP's are caused by single base-pair substitutions. However, insertions, duplications, deletions or translocations can also produce RFLP's.

Utilizing the methods of Botstein et al. (1980), Johnson et al. (1983) established that human mtDNA RFLP's demonstrated groupings according to geographical regions and that possible relationships between lineages might be recognized based upon these groupings. Wallace et al. (1985) were the first to apply mtDNA RFLP techniques to three Native American populations in order to trace possible Asian sources for the mtDNA lineages. As researchers were beginning to determine the amount of mtDNA variation present in contemporary Native American populations, at the same time, one of the single most important molecular biology techniques was being developed, polymerase chain reaction (PCR) (Mullis et al. 1986). PCR allows sensitivity, in that single DNA molecules may be detected and analyzed for sequence content. (Please see Material and Methods section for detailed description of the PCR technique)

With this new technique, PCR, an explosion of research began to document mtDNA variation present in contemporary Native American populations. Researchers began by defining Native American haplotypes as unique combinations of RFLP's or mutations in a sequence found in the D-loop (Merriwether 2002). Schurr et al. (1990) demonstrated that three contemporary Native American populations from North, South and Central America all clustered into one of four distinct groups. In 1992, Wallace and Torroni defined the four distinct Native American haplotypes as three RFLP's and one 9-base

pair deletion. They designated the haplotypes as A,B,C, and D and defined them as follows:

1. Haplotype A by the presence of a *Hae III* cut at nucleotide position (np) 663.

2. Haplotype B by the presence of a 9-B.P. deletion of DNA in Region V of the mtDNA genome located in the non-coding regions between genes that code for cytochrome oxidase II and the tRNA for lysine (Merriwether 2000).

3. Haplotype C by the presence of a *Hinc II* cut at nucleotide position (np) 13259.

4. Haplotype D by the presence of a *Alu I* cut at nucleotide position (np) 5176.

Extensive mtDNA research continued on contemporary Native American populations as well as other populations of Asian descent (Torroni et al. 1993A, 1993B, 1994A,1994B; Shields et al. 1993; Horai et al. 1993; Szathmary 1993; Merriwether et al. 1993,1995,1996; Monsalve et al.1994; Easton et al. 1996; Forster et al. 1996; Lorenz and Smith 1996; Bonatto and Salzano 1997 and Brown et al. 1998). Researchers were not only interested in the variation to be found in contemporary Native American populations but in the source of that variation from Asia. Three other haplotypes have also been identified, X6, X7 and Brown's X in North America. Easton et al. (1993) defines X6 and X7 as haplotypes that lack the diagnostic markers of haplotypes A, B, C or D. X6 is differentiated by not having a Hae III site at nucleotide position 16517 while X7 cuts at that site. Torroni et al. (1993) was the first to identify X6 in a Makiritare individual and identified the haplotype as "AM83". AM83 differed slightly from X6 in that it has a loss at the Rsa I site located at nucleotide position 16049. Brown et al. (1998) defined

Brown's X as having a loss at the Alu I site located at nucleotide position 10397 and at the Dde I site located at nucleotide position 10394 while also lacking the Hae III site located at nucleotide position 663 of haplotype A. Brown's X is also non-deleted making it different from haplotype B.

Along with utilizing RFLP data, researchers were also exploring the available variability of the displacement loop (D-loop) or hypervariable region I (HV I) sequence data. Researchers found that specific nucleotide mutations corresponded with defined haplotypes. Haplotype A corresponded with 4 transistions within HVI (Ward et al. 1991; Torroni et al. 1993A; Horai et al. 1993; Ginther et al. 1993; Balliet et al. 1994; Forster et al. 1996) (Table 1). Haplotype B corresponded with 3 transversions within HVI (Ward et al. 1991; Torroni et al. 1993A; Horai et al. 1993; Ginther et al. 1993; Balliet et al. 1994; Forster et al. 1996; Easton et al. 1996) (Table 1). Haplotype C corresponded with 4 transistions within HVI (Torroni et al. 1993A; Horai et al. 1993; Balliet et al. 1994; Forster et al. 1996; Easton et al. 1996) (Table 1). Haplotype D mutations do not cluster as tightly as the other haplotypes. However, haplotype D does roughly correspond with 4 transistions within HVI (Forster et al. 1996; Easton et al. 1996) (Table 1). Haplotype X6 has 2 transitions within HVI, a Cytosine to Thymine at nucleotide position 16234 and a Thymine to Cytosine at nucleotide position 16092 that are unique (Easton et al. 1996).

Haplotype X7 also has 2 transitions within HVI, a Cytosine to Thymine at nucleotide positions 16256 and 16353 that are unique (Easton et al. 1996). Brown's X displays 1 transition of a Cytosine to Thymine at nucleotide position 16278 (Forster et al. 1996) (Table 1).

Nucleotide Position within HVI	16111	16223	16290	16319	16362	16183	16189	16217	16223	16298	16325	16327	16179	16223	16256	16294	16325	16362	16187	16223	16278	16325
Reference	C	C	C	G	T	A	T	T	C	T	T	C	C	C	C	C	T	T	C	C	C	T
Substitution	T	T	T	A	C	C	C	C	T	C	C	T	A	T	T	T	C	C	T	T	T	C
Easton et al 1996						B	B	B	C	C	C	C	D	D	D	D	D	D				X
Forster et al 1996	A	A	A	A	A				C	C	C	C							X	X	X	

Table 1: MtDNA HVI sequence substitutions that correspond to specific Native American haplotypes. Reference refers to Cambridge Reference Sequence (Anderson et al. 1981) and the substitution can refer to either a transition or a transversion mutation within HVI.

As more and more contemporary Native American and Asian populations were sequenced and RFLP data was produced, correspondence between geographical location and haplotypes began to display patterns. Torroni et al. (1993) compared these sequences with Asian D-loop sequences and found that these group-specific mutations were shared between populations of the two geographic regions, while other mutations seen in the D-loop are not. They suggested that the group-specific mutations probably arose in Asia while the other mutations occurred after Native Americans separated from Asian

populations (Torroni et al. 1993). Merriwether et al. (1995) suggests that the frequencies of Lineage C and D vary widely in North, Central and South America. However, Lineage A does show a decreasing frequency from North to South while Lineage B has an increasing frequency from North to South. The increase in frequency of Lineage B with the decline in latitude is striking on both sides of the Bering Strait, highlighting the near absence of lineage B around the Bering Strait. Two separate migrations out of Asia/Siberia could explain lack of lineage B populations that decided not to stay in Beringia and just pass through. Current Eskimos, Aleuts and Siberians could be from recent migrations or from groups that migrated back into the area from New World. The fact that lineage B occurs against a shared mtDNA D-loop sequence background in both Asia/Oceania and the New World indicates that lineage B is most likely due to descent from a common ancestral deleted haplotype (Merriwether et al. 1995). Shields et al. (1993) study showed a zero percentage of Lineage B for all circumarctic populations on both sides, except in Old Harbor Eskimos and Altai of Siberia.

Ancient Native American mtDNA

At the same time researchers were revealing the mtDNA variation within contemporary Native American populations, researchers were also expanding their search and focusing in on ancient DNA. Ancient DNA is DNA that has begun the decaying process at time of death, be it in recent forensic cases or in remains thousands of years old. The sources of the ancient DNA vary; the DNA can be found in naturally preserved soft tissue, mummies, hair, bone or teeth. Pääbo (1985) was the first to successfully achieve the molecular cloning of a 2,400 year old section of the human nuclear family *Alu*. He was

able to perform DNA hybridization and nucleotide sequencing on the soft tissue of an Egyptian mummy. However, Pääbo (1985) cloned the ancient nuclear DNA, which had an effect on the yield and integrity. As Polymerase Chain Reaction (PCR) had had a liberating effect on the study of contemporary DNA, Mullis's (1986) method emancipated ancient DNA. The specificity and sensitivity of PCR allows for the selective amplification of a small number of intact and unmodified DNA molecules. Unlike cloning, PCR has no capability for repair of the damaged DNA molecules.

After PCR, various researchers continued utilizing soft tissue from both natural occurring mummies and human constructed mummies as a source of ancient DNA. Doran et al. (1986) extracted mtDNA from the brain tissue of an 8,000 year old Florida peat bog mummy, discovering RFLP haplotypes X6 and X7. Pääbo (1988) extracted mtDNA from a 7,000 year old brain and Lawlor et al. (1991) extracted HLA genes from 7,500 year old soft tissue. Individuals who had been mummified by other humans were also an excellent source of soft tissue DNA (Pääbo et al. 1988; Horai et al. 1991; and Rogan and Salvo 1993). However, archaeological excavations rarely yield soft tissue remains; bone and teeth are more likely to preserve and survive. Hagelberg et al. (1989) was the first to successfully extract DNA from ancient bone, opening up a plentiful source of testable material for ancient DNA. In the western United States, researchers have utilized ancient mtDNA to answer questions of ancestor/descendant relationships and ancient migrations of Native Americans (Parr et al. 1996; Kaestle 1998; and Carlye et al. 2000).

Ohio Hopewell Relevant Populations

Combining mtDNA studies of both ancient and contemporary Native American populations, with mtDNA analysis of individuals interred within the mounds of the Hopewell Mound Group this research seeks to address the following questions:

1. What are the phylogenetic relationships and affiliations between the Ohio Hopewell Mound Group mtDNA lineages to other ancient and contemporary North Native American mtDNA lineages ?

2. What information does mtDNA add to what is known biologically about the prehistoric groups of the Ohio River Valley ?

3. Is there evidence to support matrilineal descent among the Ohio Hopewell of the Hopewell Mound Group ?

4. Is there segregation of individuals interred within the Ohio Hopewell Mound Group based upon mtDNA lineages; specifically within Mounds 2 and 25 ?

The first two questions will be outlined in this chapter, while the last two questions will be discussed in Chapter Five. Question 1 deals with phylogenetic relationships between the Ohio Hopewell Mound Group lineages and other ancient and contemporary Native American lineages. First, it is important to look at what is defined as a "population or sample " in terms of ancient DNA studies. It is not a complete or random sampling of every individual and in some cases, it is a subset of a subset of individuals. In ancient DNA studies, the first subset of the sample are those individuals who were interred vs those who were not interred for whatever reason. This can leave out the very young who might not be culturally recognized as members of the group at the time of their death or

anyone who have passed away when it is not possible to transport them to the mounds for interment. Also during archaeological recovery, especially those from earlier periods when information gathering techniques were not as developed, a sampling bias could have developed because material objects were of primary interest to archaeologists of that time. However, in the case of the Ohio Hopewell of the Hopewell Mound Group, there does not seem to be an internment bias within the sample. Based upon a biological profile of skeletal material recovered by Moorehead and Shetrone, equal numbers of males and females make up the sample as well as a normal distribution of age groups (Johnston 2002). Therefore, based upon this analysis there does not seem to be an unequal internment of males vs females or old vs young within those interred in the mounds of the Hopewell Mound Group. The final sample subset is based upon what samples will amplify, which is based upon conditions of interment, age of samples, molecular techniques and sometimes simply luck. It is possible to start out with a beginning sample of 200 and only get 40 to amplify. All things considered, the samples that are utilized in this study and others are not true "samples".

Ancient Native American samples which were utilized to explore phylogenetic relationships among the Ohio Hopewell Mound Group lineages include two populations from the western United States, Pyramid Lake and Stillwater March, and one population from Illinois, the Oneota. Kaestle (1998) RFLP typed 18 individuals from the Pyramid Lake region of Nevada, dating from 860 (+/- 75) to 9200 (+/- 60) B.P. and 21 individuals from the Stillwater Marsh region of Nevada, dating from 290 (+/- 80) to 3290 (+/- 90) B.P. (Table 2). Both populations overlap the time range of the Ohio Hopewell Mound

Group population and are geographically located near areas where source materials for Ohio Hopewell burial items such as grizzly bear claws and teeth, chalcedony and obsidian have been located. Geographically, the closest and largest sample to date of ancient mtDNA, is Stone's 1996 Ph.D dissertation on the 700 B.P. Norris Farm Oneota cemetery site in Illinois. Stone (1996) extracted mtDNA from 152 Oneota, RFLP typed 108 Oneota, and HVI sequenced 52 Oneota (Table 2).

Contemporary Native American populations that can provide both information concerning phylogenetic relationships and the origin and affiliation of the Ohio Hopewell include the Mohawks, who are classified in the Amerind language family, within the sublevel Iroquoian. Parker (1916) was the first to suggest that the Iroquois had originated around the mouth of the Ohio River Valley during the Mississippian development and had crossed the Detroit River pushing northeast to the farthest eastern vicinity occupied presently by the Mohawk. It was later found that Parker ignored sites that didn't accommodate his hypothesis. Ritchie (1969) hypothesized that the Point Peninsula culture from New York could be the founding population for both the Mohawks and the Ohio Hopewell. However, Snow (1995) suggested that the Northern Iroquoians (including the Mohawk) originated from the Clemson Island Culture in central Pennsylvania around 1000 B.P., which geographically is close to Ohio. Snow (1995) also suggests that the Iroquians migrated into the northeast from the southeast, specifically from areas occupied by the Cherokee. Merriwether and Ferrell (1996) provide mtDNA haplotype frequencies for the Mohawk.

Sample				RFLP Haplotype Frequencies								
Population	**Region**	**Language**	**Time Period**	**A**	**B**	**C**	**D**	**X6**	**X7**	**X**	**N**	**Ref**
Creek	Southeast	Muskogean	Modern	.36	.15	.21	.28	0	0	0	39	1
Chocktaw	Southeast	Muskogean	Modern	.73	.19	.04	.04	0	0	0	27	1
Mohawk	Northeast	Iroquioan	Modern	.57	.17	.24	.02	0	0	0	123	2
Nuu-Chah –Nulth	Northwest coast	Wakashan	Modern	.40	.07	.13	.27	0	0	.13	15	3
Bella Coola	Northwest coast	Salishan	Modern	.50	.08	.17	.25	0	0	0	36	3
Oklahoma Cherokee	Southeast	Iroquioan	Modern	.21	.21	.53	.05	0	0	0	19	4
Stillwell Cherokee	Southeast	Iroquioan	Modern	.11	.46	.43	0	0	0	0	37	4
Pyramid Lake	Southwest	Unknown	Ancient	.11	.33	.06	.5	0	0	0	18	5
Stillwater Marsh	Southwest	Unknown	Ancient	.05	.38	0	.57	0	0	0	21	5
Braden DeMoss	Plateau	Unknown	Ancient	0	.43	0	.57	0	0	0	7	6
Congdon	Plateau	Unknown	Ancient	0	.50	.37	.13	0	0	0	8	6
Norris Farms	Midwest	Unknown	Ancient	.32	.12	.42	.08	0	0	.06	108	7
Hind Site	Midwest	Unknown	Ancient	.13	.63	.19	.06	0	0	0	16	6
FortAncient	Northeast	Unknown	Ancient	0	0	1.0	0	0	0	0	8	8
Robbins Mound	Northeast	Unknown	Ancient	.17	0	.17	.49	0	0	.17	6	9
Wright Mound	Northeast	Unknown	Ancient	0	0	.5	.5	0	0	0	8	9
Ohio Hopewell	Northeast	Unknown	Ancient	.41	.09	.29	.21	0	0	0	34	10

References:

(1) Bolnick and Smith (in press)
(2) Merriwether and Ferrel (1996)
(3) Ward et al. (1991)
(4) Lorenz and Smith (1996); Malhi et al. (2001)
(5) Kaestle (1998)
(6) Schultz et al. (2001)
(7) Stone (1996)
(8) Merriwether et al. (1995)
(9) Bolnick (2002)
(10) Mills (this study)

Table 2: MtDNA RFLP Haplotype Frequencies of Modern and Ancient Native American Populations

Some Cherokee oral traditions state that the ancestors of the Cherokee were the builders of the Ohio Hopewell mounds (Mooney 1900). Even if the Cherokee are not the ancestors of the Ohio Hopewell, there may have been trading interaction between the Ohio Hopewell and another group thought to be the ancestors of the Cherokee, the Connestee (Walthall 1990). The Connestee (2150 B.P. to 2550 B.P.) located in western

North Carolina may have provided the Ohio Hopewell with raw materials such as mica, quartz crystals, steatite and chlorite schists for their elaborate and exotic mortuary rituals. It is possible that as they were trading material items they were also trading genetic material. It might also be that the trading may be a result of reciprocal obligation and formal gift-giving between lineages or clans that controlled specific geographical areas. So that lineages or clans controlled an area and its resources, and at the time of death of that clan leader, raw materials for mortuary gifts or mortuary gifts were placed with the individual at the time of special internment within the mound. Lorenz and Smith (1996) and Malhi et al. (2001) collected and provided the mtDNA haplotype frequencies for the Oklahoma Red Cross Cherokee and Stillwell Cherokee samples. Both groups are similar to the Mohawks in that they belong to the Amerind language family and within the sublevel language of Iroquoian. Also included from the southeast are RFLP data from Bolnick and Smith (in press) from the Creek and Chocktaw. Both groups belong to the Muskogean language family and in early historic times, the Creek inhabited much of Alabama and Georgia while the Chocktaw were in southern Mississippi.

Contemporary and ancient Native American samples which might address the first two questions concerning phylogenetic relationships, origins and affiliations and genetic information about the prehistoric Ohio River Valley include populations from the northwest United States. Based upon linguistic evidence, Siebert (1967) proposed that the region between Lake Huron and Georgian Bay, with a northern boundary of Lake Nipissing and a southern boundary of Lake Ontario, as the homeland of the Proto-Algonquian people. Snow (1976) utilizing archaeological evidence indicates that the

Proto-Algonquian homeland should be larger, extending to the west by Niagara Falls. Berman (1982) however, suggests a western homeland for the Proto-Algonquian based upon further linguistic data. He suggests that based upon similarities between the Proto-Algonquian and Proto-Salish vowel systems, one could place the Proto-Algonquian homeland near that of the Proto-Salish, which is located in the Northwest culture area (Berman 1982). Based upon a combination of archaeological and linguistic evidence, Denny (1991) has added support to Berman's idea of a western homeland for the Proto-Algonquian. Denny (1991) however suggests that the Red Ocher/Glacial Kame Twin Burial Complex populations might be descended from populations of the Western Idaho Archaic Burial Complex. According to Denny (1991), glottochronological evidence suggests that the appearance of the Red Ocher/Glacial Kame Twin Burial Complex in the Great Lakes occurs at about the same time as Proto-Algonquian begins to undergo modifications into Algonquian. Denny (1991) also suggests that about 4,000 B.P., the people of the Western Idaho Archaic Burial Complex migrated to the Great Lakes and were Proto-Algonquian language speakers. Goddard (1994) utilizing linguistic data focusing on words connected to subsistence adds further support to Denny's idea of a western origin. Goddard (1994) states that Algonquian should be viewed as a cline, from west to east, with the greatest time depth in the west and the earliest in the east reinforcing the hypothesis of a western homeland for Proto-Algonquian.

Three ancient samples from the proposed Algonquian homeland are the Braden, De Moss and Congdon sites (Table 2). The Braden and De Moss sites are located along the Snake River in the Plateau and date to approximately 6000 B.P.. They are both considered part

of the Western Idaho Archaic Burial Complex. Farther northwest, near the Columbia River in the Plateau, the Congdon site dates to approximately 3000 B.P. and overlaps the time range of the Glacial Kame population in the Great Lakes region. Schultz et al. (2001) have provided preliminary RFLP data on a small number of samples from the Braden , De Moss and Congdon sites, combining the data from the Braden and De Moss sites (Table 2). Two contemporary Native American populations from the Pacific northwest rime are the Bella Coola and Nuu-Chah-Nulth. The Bella Coola are classified in the Salishan language family and inhabit theNorthwest coast while the Nuu-Chah-Nulth are Wakashan speakers, which is the neighboring language group and are located in the Columbia Plateau near the ancient Braden, De Moss and Congdon sites. Ward et al. (1991) provides mtDNA RFLP and sequence data from both populations (Table 2).

To explore the second question of what information mtDNA analysis can add to what is known biologically about the prehistoric Ohio River Valley, it is important to start with hypotheses relating to biological affinities. Webb and Snow (1945), based mainly upon cranial morphology, suggested that the Ohio Hopewell were characterized by three cranial shapes. About 80% of their sample were dolicocephalic or long-headed,while 10% to 15% of the sample was brachycephalic or round-headed, leaving the remaining sample as unidentifiable. Snow defined the dolicocephalic type as Hopewell type 1 while the brachycephalic were considered “Adena-like” in their shape and designated Hopewell type 2. Based upon the three cranial types, Snow postulated that the Hopewell type 1 cranial shapes were descendants of indigenous populations of the Ohio River Valley, while the Adena type cranial shapes had migrated into the area. He further suggests the

possibility that the Hopewellian type 2 cranial shapes were a result of admixture between indigenous Hopewell and the invading Adena. However, Prufer (1964) did not observe a clear change from brachycephalic to dolicocephalic and suggested instead a migration of the Illinois Hopewell dolicocephalic types into the Ohio River Valley. Dragoo (1964) first proposed two migrations. The first by the "Lenid People" of the Late Archaic Burial Complex located near the Great Lakes, who intermixed with the Adena-Red Ocher people of central Indiana-north central Illinois. The second migration was similar to the first in that the "Lenid People" intermixed with the Adena of the Ohio River Valley to produce the Ohio Hopewell. All of these ideas suggest distinct biological gaps between the Glacial Kame, Adena and Hopewell as outside populations migrate into Ohio. Reichs (1975,1984), utilizing metric and discrete cranial characteristics, examined the hypothesis that the Ohio Hopewell populations and culture originated in Illinois utilizing populations of both Illinois and Ohio Hopewell. Her results demonstrated that the Illinois and Ohio Hopewell populations were distinct from each other biologically. Sciulli and Mahaney (1986) extended Reich's work within the Ohio River Valley by looking at distinct local populations within Ohio from the Glacial Kame Late Archaic populations (ca. 3000-2500 B.P.), Adena Early Woodland (ca. 3000-2000 B.P.) and Hopewell Middle Woodland (ca. 2000-1700 B.P.). Sciulli and Mahaney (1986), based upon analysis of cranial measures and discrete cranial trait frequencies, concluded that the Hopewell Mound Group site samples demonstrated the same configuration of cranial size and shape variation and discrete trait expression as the Glacial Kame. The Adena samples were only included in

non-metric testing due to the cultural deformation of the skulls. Suggesting, at least, biological continuity for the Ohio Hopewell Mound Group site and lack of support for large scale migrations from Illinois to Ohio by the Ohio Hopewell.

Ancient Native American mtDNA samples have been selected to further explore Sciulli and Mahaney's hypothesis of biological continuity within the Ohio River Valley. Unfortunately no molecular genetic analysis for Ohio River Valley populations within Ohio is available. Thus, I have chosen samples that may reflect the Ohio River Valley traditions. Schultz et al. (2001) provide mtDNA RFLP frequencies from the Hind site of southern Ontario (Table 2). The site is a Glacial Kame site dating to 3000 B.P.. Bolnick (2002) contributes preliminary mtDNA RFLP frequencies from two Adena mound sites, Robbins Mound and Wright Mound (Table 2). Both mounds are located in Kentucky and date to the Early Woodland period. This study provides mtDNA RFLP frequencies and sequence data for the Ohio Hopewell Mound Group (Table 2). Finally, Merriwether et al. (1995) contributes mtDNA RFLP data from the Mann site, a Fort Ancient site of West Virginia. The Fort Ancient tradition follows the Late Woodland period within the Ohio River Valley. Overall, the above contemporary and ancient Native American samples provide a solid basis from which to test the first three questions.

CHAPTER 4

MATERIALS AND METHODS

Sample origin

The samples for this study are from the archaeological excavation of the Hopewell Mound Group of Ross County, Ohio (33RO27). A portion of the site was first mapped and excavated by Ephraim Squire and Edwin Davis in 1845 as part of the field work for their book, *Ancient Monuments of the Mississippi Valley* (Squire and Davis 1848). As part of the Chicago Columbian Exposition, under Fredrick W. Putnam, Warren K. Moorehead continued mapping and excavating the site from 1891 to 1892. H.C. Shetrone continued excavation at the site from 1922 to 1925. My samples are from the Shetrone excavation and have been curated at the Ohio Historical Society since their extraction from the mounds.

Sample Selection Parameters

Why teeth

In order to perform non-destructive testing and allowing for the possibility of reconstruction, teeth rather than bone were chosen for this study. Several studies (Alvarez et al. 1996; Ginther et al. 1992; Mornstad et al. 1999; Pfeifferetal 1999; Potsch et al. 1992; Schwartz et al. 1991; and Smith et al. 1993) have shown that teeth are an

excellent source of nuclear DNA and mitochondrial DNA (mtDNA). After root formation is complete, the tooth structure itself acts as a barrier to the outside environment, which is of special importance in an archaeological setting. The enamel of the tooth provides protection within a burial environment, rich in outside microbes and chemical processes occurring during decomposition. One study in particular, Mornstad et al. (1999), provides solid evidence of mtDNA or fragments of mtDNA being intermingled between calcium phosphate crystals thus existing in the dentine.

Criteria

The criteria set up by the Ohio Historical Society (OHS) for tooth selection stated that no individual could be chosen from the Hopewell Collection who had fewer than four teeth.

Mound No. in Hopewell Mound Group	Total number of individuals per mound	Number of individuals with less than 4 teeth	Final number of possible individuals	Number of Individuals chosen
2	6	1	5	4
4	4	0	4	1
7	2	0	2	1
25	41	3	38	31
26	1	0	1	1
27	1	0	1	0
Unknown	29	10	19	11
Total	84	14	70	49

Table 3: Number of teeth chosen from each mound at the Hopewell Mound Group

Of the possible 84 individuals recovered from the Hopewell Mound Group, 17% were ruled out for having fewer than 4 teeth, leaving 83% of the sample remaining from which to choose. From those 70 possible individuals, 49 were chosen for the study and 2 teeth from each individual were selected for extraction. However, in the case of three samples 150043, 150066 and 150083, a clerical error occurred and three teeth were collected from 15066, while only 1 tooth was collected from both 150043 and 150083**.** Teeth selected for the study were free of cracks, caries and had completed roots (apex complete). However, if the only tooth available had a crack, carie or unfused root, the tooth was chosen and precautionary steps were taken during extraction. Of the teeth chosen for the study, 1st, 2nd and 3rd molars were given preference due to their larger pulp chambers. Anterior and posterior premolars and canines were selected if a molar was unavailable or if the molars were extremely worn or had caries(Table 4).

Type of tooth	**Total Number chosen**
First Molar	18
Second Molar	22
Third Molar	47
Total Number of Molars	**87**
Anterior Premolar	2
Posterior Premolar	3
Total Number of Premolars	**5**
Total Number of Canines	**6**
Total Number of teeth	**98**

Table 4: Number of each type of tooth selected for extraction

In order to preserve the morphology of the tooth for possible future study, photographs of the occlusal and buccal surface of each tooth were taken prior to extraction. Also a reversable hydrocollid mold was made of each individual tooth. From that mold, a polymethylmethacrylate replica was constructed to aid in future morphological studies. Finally, during the extraction, each tooth was split into 2 equal halves to allow for reconstruction and to preserve the integrity of the tooth.

Extraction

All equipment used in the mitochondrial DNA extraction procedure was UV irradiated for 15 minutes prior to utilization in a Stratlinker UV 1800 at 312 nanometers. This included the following:

15 milliliter (ml) Sarstedt conical tubes, toothbrush, plastic non-reusable pipettes, double distilled water, Sears Craftsman drill, casings and drill bits, 1.5 ml Fisherbrand eppendorf tubes, Dasco Pro chisel, Stanley hammer, plastic non-reusable weight trays, and 0.5 M EDTA (ph 8.0). All dedicated ancient extraction room counter surfaces were washed down with 10% bleach and the floor was also mopped with 10% bleach. The room was then UV irradiated for 30 minutes prior to each extraction.

Each extraction consisted of 4 teeth and 1 negative control. MtDNA was extracted utilizing the EDTA Tooth Extraction Procedure by Merriwether (1993). Each tooth was first cleaned of loose dirt with a toothbrush soaked in 3% Hydrogen Peroxide (HOH). The toothbrush was cleaned in between teeth with 10% bleach and double distilled water to prevent cross contamination of later teeth. Each tooth was soaked in 3% HOH for 5

minutes. Those teeth with broken or unfused roots were not soaked in the HOH. The remaining dirt was scrubbed off each tooth with a toothbrush soaked in HOH. The 15ml Sarstedt conical tube containing the tooth and the tooth were rinsed with UV irradiated double distilled water. Each tooth was soaked in 10% bleach for 10 minutes to remove outside contamination from the surface of the tooth. The tooth was washed off with UV irradiated double distilled water to remove the bleach. Following the rinse, each tooth was UV irradiated in a Stratalinker UV 1800 for 5 minutes on each side and allowed to completely dry. After drying, the tooth was placed on a plastic non-reusable weight tray and split into two equal halves by a Dasco Pro Chisel.

Half of the tooth was set aside on an UV irradiated plastic non-reusable weight tray and placed back inside the Stratlinker UV 1800 to protect it from aerosol tooth powder. The first half of the tooth's root and pulp chamber was drilled (Sears Craftsman) into a fine powder weighting between 0.1 and 0.3 grams. The UV irradiated plastic non-reusable weight tray was soaked with 1.0 ml of 0.5M EDTA (ph 8.0) which mixed with the powdered tooth pulp and made static electricity from the drilling nonexistent. The aqueous solution was transferred via pipette to a 1.5ml Fisherbrand eppendorf tube leaving an air pocket in the top of the tube to allow for movement of the solution. The tube was sealed with parafilm, vortexed to thoroughly mix and placed on a spinner within an incubator set at 56 º Celsius to progress on to the Phenol-Chloroform method. Along with the samples, a negative tube of just 0.5M EDTA (ph 8.0) was also placed with each extraction. The samples were allowed to digest overnight and the EDTA checked in the morning for color. Each tube was centrifuged in a Savant SFA13K at 12,000 x g for 1

minute and checked for brown color. If a tube displayed brown color, old EDTA was removed and saved in a labeled, UV irradiated 15ml Sarstedt conical tube. Fresh UV irradiated 0.5M EDTA (ph 8.0) was added to the 1.5ml Fisherbrand eppendorf tube, sealed with parafilm, vortexed and placed back into the 56 ºC spinning incubator for 12 hours. If after the 12 hours the 0.5M EDTA (ph 8.0) was still brown the process was repeated until a clear 0.5M EDTA (ph 8.0) color was obtained indicating that the sample was clean. Because of the sealed environmental system of the tooth, all samples were clear after the overnight incubation.

Yang Protocol C (Yang et al. 1998)

The second half of the tooth was removed from the Stratlinker UV 1800 after the counter tops had been washed with 10% bleach and new bench paper was put down to avoid cross contamination. The Sears Craftsman drill was wiped with 10% bleach and allowed to dry between each tooth. The second half of the tooth's root and pulp chamber was drilled (Sears Craftsman) into a fine powder weighting between 0.1 and 0.3 grams. 8.0 ml of the Yang Modified Protocol C Extraction Buffer (Yang et al. 1998) was added to the powdered tooth pulp in the UV irradiated plastic non-reusable weight tray and transferred into a 15ml Sarstedt UV irradiated conical tube. The Yang Modified Protocol C Extraction Buffer consisted of 0.5M EDTA (ph 8.0), 0.5% sodium dodecyl sulfate (SDS), and 100 ug/ml proteinase K. The 15ml Sarstedt conical tube lid was sealed in parafilm, votexed, and rotated in a incubator at 55 º C overnight. A negative control of just Yang Modified Protocol C Extraction Buffer was included with the extractions. After 24 hours, tubes were removed from the incubator and spun in a IEC Centra GP8R

centrifuge at 2000 x g for 5 minutes. The aqueous supernatant was filtered through a 2ml Amicon YM-3 Centricon Centrifugal Filter Device at 7,500 x g for 30 minutes per 2ml until 8ml of supernatant was completely filtered. After final 2ml filtration, a new UV irradiated cap was placed on the tube and centrifuged for 5 minutes at 7,500 x g . Caps were separated from Centricon tubes and a QIA quick purification protocol was performed on the filtered supernatant. Five volumes of QIA quick Buffer PB was added to 1 volume of filtered supernatant and mixed well. Using the QIA quick spin columns, 750ul of the above aqueous solution was centrifuged through the filtered columns at 12,800 x g for 1 minute. The QIA quick spin columns were reloaded until supernatant was gone. 750ul of QIA quick PE Buffer was centrifuged for 1 minute at 12,8000 x g through the QIA quick columns in order to wash the DNA. QIA quick column was transferred to final labeled UV irradiated 1.5ml eppendorf tube and eluted with 100ul of TE Buffer (10mM Tris-HCL, 1mM EDTA (ph 7.5)). Samples were stored at –20 ºC.

Phenol-Chloroform (Pääbo 1990)

The 1st half of the tooth which was demineralized overnight at 56 ºC in the EDTA Buffer was centrifuged at 12,000 x g for 2 minutes. The tooth powder was washed with UV irradiated double distilled water, spun down at 12,000 x g for 2 minutes and the supernatant removed with a UV irradiated pipette. The supernatant was washed 3 times with UV irradiated double distilled water and preserved in an UV irradiated 15ml Sarstedt conical tube and stored at –20 º C. After the 3rd wash, a Proteinase K Extraction Buffer (Merriwether 1993) was added to each sample and the negative control. The

Proteinase K Extraction Buffer consisted of 10mM Tris-HCL (ph 8.0), 2mM EDTA, 10mM NaCl, 10 mg/ml DTT, 0.2% SDS, 500 ug/ml Proteinase K and UV irradiated double distilled water. 1ml of Pro-K Buffer was added to each sample, including the negative control. Each tube was then vortexed thoroughly, parafilmed, and incubated on a continuous spinner at 37 º C.

After the overnight digestion, samples were centrifuged for 10 minutes at 12,000 x g. Three 15ml Sardtedt conical tubes for each sample and negative, transfer pipettes, beakers for liquids, and tube holder were UV irradiated for 15 minutes. The aqueous solution from each of the tubes and the negative were transferred to UV irradiated 15ml Sardtedt conical tubes and an equal volume of phenol was added to the sample and negative control. The tubes were inverted to mix, and centrifuged for 25 minutes at 5000 x g in order to separate the two solutions. The supernatant was removed with UV irradiated transfer pipette to a second UV irradiated 15ml Sardtedt conical tube and an equal volume of phenol/chloroform (1:1 weight/volume) was added to each tube. The tubes were inverted to mix and centrifuged for 25 minutes at 5000 x g in order to separate the two solutions. The final supernatant was removed with UV irradiated transfer pipette to a third UV irradiated 15ml Sardtedt conical tube and an equal volume of 24:1 chloroform :isoamylalcohol was added to each sample and the negative. The tubes were inverted to mix, and centrifuged for 25 minutes at 5000 x g in order to separate the two solutions. It is very important during this next step of removing the supernatant that no chloroform is picked up by the transfer pipette. The chloroform will dissolve the filter of the Amicon Centricon-30 Microconcentrator. Thus the supernatant was removed to a

1.5ml Fischerbrand eppendorf tube and centrifuged for 10 minutes at 1200 x g in order to separate the two solutions further. A UV irradiated and bleached 200ul pipette was used to remove the final supernatant, careful not to draw up any chloroform. The final supernatant was transferred to a Amicon Centricon-30 Microconcentrator. All supernatant was filtered through and washed with UV irradiated double distilled water. 100ul of TE Buffer (10mM Tris-HCL, 1mM EDTA (ph 7.5)) was added to the reservoir of the tube and centrifuged for 3 minutes at 12,000 x g into a new UV irradiated 1.5ml eppendorf tube and stored at –20 º C.

Sample Dilution

Archaeological samples, such as the Hopewell, frequently exhibit inhibition which may be due to the presence of humic acid in the soil in which they were interred (Pääbo 1990; Richards et al. 1993). After both extraction procedures, the modified Yang Protocol C (Yang et al. 1998) and the Phenol-Chloroform (Pääbo 1990), each extract was diluted with UV irradiated double distilled water in order to increase sample quantity and decrease possibility of inhibition. Each extract was diluted 1 to 10 (1/10), 1 to 50 (1/50), and 1 to 100 (1/100).

Proteinase-K Wash

A PCR Buffer was made from 50mM KCL, 15mM Tris-HCL (ph 7.5), and 2.5mM MgCl(2). A K Buffer was made from 0.5% Tween 20 and 20 mg/ml Proteinase K. The two Buffers were combined in a 1ml solution with 0.005 ml of Tween 20 and 0.005ml of Proteinase K added to 0.990 ml of the PCR Buffer (Kaestle 1998; Stone 1996). 10ul of

the extracts and their dilutions were added to 40ul of Proteinase K (Pro-K) wash buffer and incubated for 1 hour at 55 º C and heated to 95 º C in the Perkins 9600 Thermocycler for 10 minutes in order to heat inactivate the Proteinase K. At the beginning and end of each Pro-K plate, a negative control of just Pro K buffer, was set up to check for contamination. If either negative control tested positive for DNA, the Pro-K plate was redone with new extract. The Pro-K plate became the master plate of extracts and dilutions from which all subsequent testing of samples was utilized for Restriction Fragment Length Polymorphisms (RFLP) and Hypervariable Region I (HVI).

Polymerase Chain Reaction (PCR)

All extracts, dilutions and negative controls were screened for specific mitochodrial DNA (mtDNA) Native American mutations utilizing Polymerase Chain Reaction (PCR) (Mullis et al. 1986). By mimicking in vivo cellular mechanisms, PCR allows specific in vitro amplifications of exact DNA regions (Mullis et al. 1986). By utilizing short specific manufactured single stranded DNA sequences, oligonucleotides, to bind to unique areas flanking the area of interest, specific regions can be primed by the polymerase enzyme. The two oligonucleotides overlap so that complimentary DNA strands can then act as a template for additional replications. The original template is amplified geometrically through many cycles to allow for even the most severally degraded or damaged sample to be amplified by PCR. Theoretically, even a single DNA molecule can be amplified utilizing the PCR process (Higuchi 1989; Innis et al. 1990).

Screening

Utilizing PCR, a specific region of mtDNA HVI from 16192 to 16375 was amplified in order to screen for specific Native American mutations that correspond with specific Native American RFLP haplotypes. There are five Native American Haplotype A, B, C, D and X that were tested for in HVI. Haplotype A has two transitions, one at np 16290 Cytosine to Thymine , and the second at np 16319 Guanine to Adenine . Haplotype B has a transition at 16217 Adenine to Guanine. Haplotype C has two transitions, one at np 16298 Thymine to Cytosine, and the second at np 16327 Cytosine to Thymine. Haplotype D has a transition at 16325 Thymine to Cytosine in this region. Haplotype X has a transition at np 16278 Cytosine to Thymine. Therefore, by utilizing HVI sequence area 16192 to 16375, all five Native American Haplotype were represented and could be screened for in 20ul PCR's.

A 20ul PCR reaction consisted of 3.0ul Proteinase K extract, 2.0ul 10X PCR Buffer. 0.4ul 10mM dntp mix, 0.2ul each of the 2 10mM primers (16192Forward and 16375 Reverse) (Table 5) , 0.6ul 50mM MgCl(2), 0.1ul of Platinum Taq DNA Polymerase (Gibco) and 13.5ul UV irradiated double distilled water. The cocktail was made without the extract or the Platinum Taq and weighted before being UV irradiated in the Stratalinker UV 1800 for 20 minutes to destroy any possible contamination in the cocktail. After which the evaporated double distilled water was replaced with fresh UV irradiated double distilled water and Platinum Taq added to the cocktail which was vortexed to mix thoroughly. 17ul of the cocktail was added to each well, along with 3ul of either the extract, dilution or negative extract control. Two new negative controls were

added to the PCR plate, one at the beginning and one at the end to check for contamination of the cocktail and the PCR. Also a positive control of a known sample which had worked in the past was added in order to make sure the PCR had worked and as a reference.

Because Platinum Taq is heat activated a cold start, touchdown PCR method was done. For primers 16192F and 16375R, each cycle of amplification consisted of denaturation for 2 minutes at 94 º C, annealing for 0.15 minutes at 55ºC, and elongation for 0.15 minutes at 74ºC for 45 cycles on a Perkin Elmer 9600 Thermocycler. 10ul of the PCR sample was mixed with 2ul of Blue loading dye and electrophoresed on a 2% agarose gel containing 10X Buffer and 0.5 ug/ml ethidium bromide to determine if amplified DNA was present. On the agarose gel, 1 lane was loaded with 5 ul of size standard. The gel was visualized under a UV transilluminator and photographed to determine amplification of DNA.

If all of the PCR negative controls were negative and the positive control amplified indicating the PCR was successful, a 50ul PCR was done for all of those extracts that amplified on the gel. The 50ul PCR reaction consisted of 5.0ul Proteinase K extract, 5.0ul 10X PCR Buffer, 2.0ul 10mM dntp mix, 1.25ul each of the 2 10mM primers (16192Forward and 16375 Reverse) (Table5), 1.5ul 50mM MgCl(2), 0.5ul of Platinum Taq DNA Polymerase (Gibco) and 33.5ul UV irradiated double distilled water. The cocktail was made without the extract or the Platinum Taq and weighted before being UV

Primer	Sequence (5' to 3')	Temperature in Celsius	Haplotype or HV I
591For	ACC TCC TCA AAG CAA TAC ACT G	56	Hap A
698Rev	GCA TGT GTA ATC TTA CTA AGA G	56	Hap A
8244For	AAA TAG GCG CCG TAT TTA CCC	63	Hap B
8244For	AAA TAG GCG CCG TAT TTA CCC	63	Hap B
13236For	AAT CGT AGC CTT CTC CAC TTC A	58	Hap C
13335Rev	GGC GTG GGT ACA GAT GTG CAG G	58	Hap C
5146For	CGA CCC TAC TAC TAT CTC GC	57	Hap D
5259Rev	ATT TGG GCA AAA AGC CGG TTA	57	Hap D
10284For	CCA TGA GCC CTA CAA ACA ACT	61	Hap X
10484Rev	GTA AAT GAG GGG CAT TTG GTA AAT	61	Hap X
16047For	GGG TAC CAC CCA AGT ATT GAC T	58	HVI
16106For	GCC AGC CAC CAT GAA TAT TGT	58	HVI
16192For	CCA TGC TTA CAA GCA AGT	55	HVI
16204For	GCA AGT ACA GCA ATC AAC CC	58	HVI
16204 (16223MUT) For	GCA AGT ACA GCA ATC AAC CT	58	HVI
16214Rev	GCT GTA CTT GCT TGT AAG CAT	58	HVI
16251Rev	GGA GTT GCA GTT GAT GTG TGA T	58	HVI
16301For	CAG TAC ATA GTA CAT AAA GCC AT	58	HVI
16375Rev	GTC ATC CAT GGG GAC GAG AA	58	HVI
16429Rev	GCG GGA TAT TGA TTT CAC GG	58	HVI

Table 5: RFLP and HVI Sequence Primers and their optimal temperature

irradiated in the Stratalinker UV 1800 for 20 minutes to destroy any possible contamination. After which the evaporated double distilled water was replaced with fresh UV irradiated double distilled water and the Platinum Taq added to the cocktail which was vortexed to mix thoroughly. 45ul of the cocktail was added to each well, along with 5ul of either the extract, dilution or negative extract control. Two new negative controls were added to the PCR plate, one at the beginning and one at the end to check for contamination of the cocktail and the PCR. Also a positive control of a known sample which had worked in the past was added in order to make sure the PCR had worked and as a reference.

Because Platinum Taq is heat activated a cold start, touchdown PCR method was utilized. For primers 16192F and 16375R, each cycle of amplification consisted of denaturation for 2 minutes at 94 º C, annealing for 0.15 minutes at 55ºC, and elongation for 0.15 minutes at 74ºC for 45 cycles on a Perkin Elmer 9600 Thermocycler. 10ul of the PCR sample was mixed with 2ul of Blue loading dye and electrophoresed on a 2% agarose gel containing 10X Buffer and 0.5 ug/ml ethidium bromide to determine if amplified DNA was present. On the agarose gel, 1 lane was loaded with 5 ul of size standard. The gel was visualized under a UV transilluminator and photographed to determine amplification of DNA.

PCR Product Purification

All extracts, dilutions, and negative extract controls that amplified were purified using the Qiagen QIAquick PCR Purification Kit protocol to remove the primers. In the PCR tube

well, 200ul of Buffer PB was added to the remaining 40ul of PCR product and mixed thoroughly. The samples were centrifuged through a 2ml Qiagen spin column for 1 minute at 13,000 x g and the flow through discarded. 750ul of Buffer PE (ethanol) was added to wash the samples and centrifuged for 1 minute at 13,000 x g. Columns were opened and allowed to set open for 10 minutes to allow all of the ethanol to completely evaporate and dry. QIA quick column was placed in a 1.5ml eppendorf tube and 50ul of Elution Buffer was added to the center of the QIA quick column membrane. The columns stood for 1 minute and were centrifuged for 1 minute at 13,000 x g . Purified samples were stored in a –20ºC freezer.

Dye-deoxy terminator cycle sequencing

After removing the primers, Dye-deoxy terminator cycle sequencing was performed on the purified samples. In order to sequence the samples, 2 cycle sequencing reactions, 1 forward and 1 reverse, were performed for each sample utilizing the FS Dye Terminator Kit (ABI). A Dye-deoxy cocktail was made for each primer, forward and reverse. The cocktail consisted of 1.5ul ABI Kit Big Dye and 0.075ul primer which were vortexed to mix thoroughly and centrifuged for 1 minute at 13,000 x g. 1.7ul Dye-deoxy sequencing cocktail was added to 2.25ul purified DNA placed at the bottom of the tube well. The sample plate was shaken for 1 minute and centrifuged for 1 minute at 13,000 x g to concentrate sample and cocktail at the bottom of the well.

The procedure requires a hot start Thermocycler method which starts the Perkins Elmer 9600 Thermocycler at 96ºC and it stays at 96ºC for 10 seconds, 50ºC for 5 seconds and

60ºC for 2 minutes for 30 cycles of amplification. The reaction is light sensitive, so the plate was covered with aluminum foil when removed from the Thermocycler. Plate was stored in a –20ºC freezer within a plastic freezer bag to inhibit evaporation.

In order to remove the unincorporated dye terminators, the cycle sequencing product was purified 30 minutes after pouring a ABI Long Ranger Single Gel. 20ul of 75% Isopropanol alcohol was added to each tube well and mixed by vigorously vortexing for 45 seconds. The plate was covered with aluminum foil and left at room temperature (25ºC) for 20 minutes. In a well-balanced centrifuge, plate was centrifuged for 45 minutes at 2000x g. Carefully remove plate without jostling tube wells. Invert the plate onto a paper towel and centrifuge for 1 minute at 400 x g. Tubes were completely dry when 0.75ul loading dye was added to each tube (6:1 de-ionized fomamide: EDTA/Dextran Blue) and tightly capped to prevent evaporation. The plate was tapped several times to ensure dye settled to the bottom of the tube and centrifuged for 45 seconds 400 x g . Plate was covered with aluminum foil and shaken on vortexer at medium speed for 15 minutes. 0.70ul from each well was loaded onto the ABI Applied Biosystems 377 Automated Sequencer.

Sequences were aligned and corrected using ABI Software Sequence Analysis 3.3 and Sequence Navigator Program 1.0.1 to compare to the Cambridge Reference Sequence (Anderson et al. 1981). If an extract exhibited the above transitions demarcating any of the five Native American haplotypes, it was completely sequenced for mtDNA HVI. Primers from 16047 to 16429 for a total of 382 bases were utilized to sequence specific regions of HVI. The primers are listed in Table 5. Due to the damaged and fragmentary

nature of ancient DNA, primers 16047F to 16251R did not perform well, most likely due to a base length of 204 bases. Primers 16047F to 16214R, with only 167 bases, were utilized on those samples which failed with the longer primer set for that region of HVI. The remaining sequences were also aligned and corrected using ABI Software Sequence Analysis 3.3 and Sequence Navigator Program 1.0.1 to compare to the Cambridge Reference Sequence (Anderson et al. 1981)

RFLP Testing

Those extracts and dilutions that were completely sequenced for mtDNA HVI, were also tested for Restriction Fragment Length Polymorphisms (RFLP). A 40ul PCR reaction was done on each extract and dilution consisting of 5.0ul Proteinase-K extract, 4.0ul 10X PCR Buffer, 0.8ul of 10mM dntp mix, 0.4ul each of the 2 10mM primers, 1.2ul 50mM MgCl(2), 0.2ul Platinum Taq DNA Polymerase (Gibco) and 28.0ul UV irradiated double distilled water. The cocktail was made without the extract or the Platinum Taq and weighted before being UV irradiated in the Stratalinker UV 1800 for 20 minutes to destroy any possible contamination. After which the evaporated double distilled water was replaced with fresh UV irradiated double distilled water and the Platinum Taq added to the cocktail which was vortexed to mix thoroughly. 35ul of the cocktail was added to each well, along with 5ul of either the extract, dilution or negative extract control. Two new negative controls were added to the PCR plate, one at the beginning and one at the end to check for contamination of the cocktail and the PCR. Also a positive control of a known sample which had worked in the past was added in order to make sure the PCR had worked and as a reference.

Because Platinum Taq is heat activated a cold start, touchdown PCR method was utilized. The PCR conditions involved each cycle of amplification consisting of denaturation for 2 minutes at 94 º C, annealing for 0.15 minutes at temperature for that primer set (see Table 6), and elongation for 0.15 minutes at 74ºC for 45 cycles on a Perkin Elmer 9600 Thermocycler. 10ul of the PCR sample was mixed with 2ul of Blue loading dye and electrophoresed on a 2% agarose gel containing 10X Buffer and 0.5 ug/ml ethidium bromide to determine if amplified DNA was present. On the agarose gel, 1 lane was loaded with 5 ul of size standard. The gel was visualized under a UV transilluminator and photographed to determine amplification of DNA. The following primers delineate the 5 Native American Haplotypes (Table 7). Each Native American Haplotype corresponds with a specific digestion site location on the mtDNA genome. Those extracts and dilutions which amplified were digested with the following enzymes (Table 8).

Primers	Number of bases	Temperature
16047F-16251R	204	58ºC
16047F-16214R	167	58ºC
16106F-16251R	145	58ºC
16192F-16375R	183	55ºC
16301F-16429R	128	58ºC

Table 6: Table of HVI primers with the size and annealing temperatures

Haplotype	Primers	Temperature	Size of Primers
Haplotype A	591F-698R	56ºC	107
Haplotype B	8244F-8313R	63ºC	69
Haplotype C	13236F-13335R	58ºC	99
Haplotype D	5146F-5259R	57ºC	113
Haplotype X	10284F-10489R	61ºC	205

Table 7: Table of RFLP primers with the size and annealing temperatures for Native American Haplotypes

Haplotype	Restriction Digest Enzyme	Gain or Loss of Site
Haplotype A	Hae III nt 663	Gain at nt 663
Haplotype B	9 base pair deletion	Loss
Haplotype C	Hinc nt 13259	Loss at nt 13259
Haplotype D	Alu I nt 5176	Loss at nt 5176
Haplotype X	Dde I nt 10394 and Alu I 10397	Loss at nt 10394 and nt 10397

Table 8: Table of RFLP sites for Native American Haplotypes

Cocktails for digestion consisted of 4.0ul manufacturer's enzyme buffer, and 1ul (10,000u/mg) restriction enzyme added to 30ul of DNA sample. Digestion was incubated overnight (minimum of 16 hours) in a 37ºC water bath. Also included with DNA products was a negative control of a known sample which did not cut for the enzyme and a positive control of a known sample which did cut for the specific enzyme. The negative and positive controls were utilized to first test if the enzyme was working properly and secondly as a reference for those samples which were cut or not cut by the enzyme on the gel. 10ul of the Enzyme sample was mixed with 2ul of Blue loading dye and electrophoresed on a 3% Nu-Sieve-1% agarose gel containing 10X Buffer and 0.5 ug/ml ethidium bromide until seperation was complete The gel was visualized under a UV transilluminator and photographed to determine amplification of DNA. Samples were scored based upon cut or no cut of the DNA product and compared to the negative and positive control on the gel (Figure 5). If partial digestion occurred the remaining digestion product was placed back into the 37ºC water bath for another 8 hours. After that period of time the digestion was checked again with the above procedure until digestion was determined to be complete.

Haplotype	9B.P. deletion	Hae III np 663	Alu I Np 5176	Hinc II np 13,259	Dde I np 10394 /Alu I np10397
A	-	+	+	+	-
B	+	-	+	+	-
C	-	-	+	-	+
D	-	-	-	+	+
X	-	-	+	+	-

Table 9: RFLP restriction sites (gain/loss) for Native American Populations

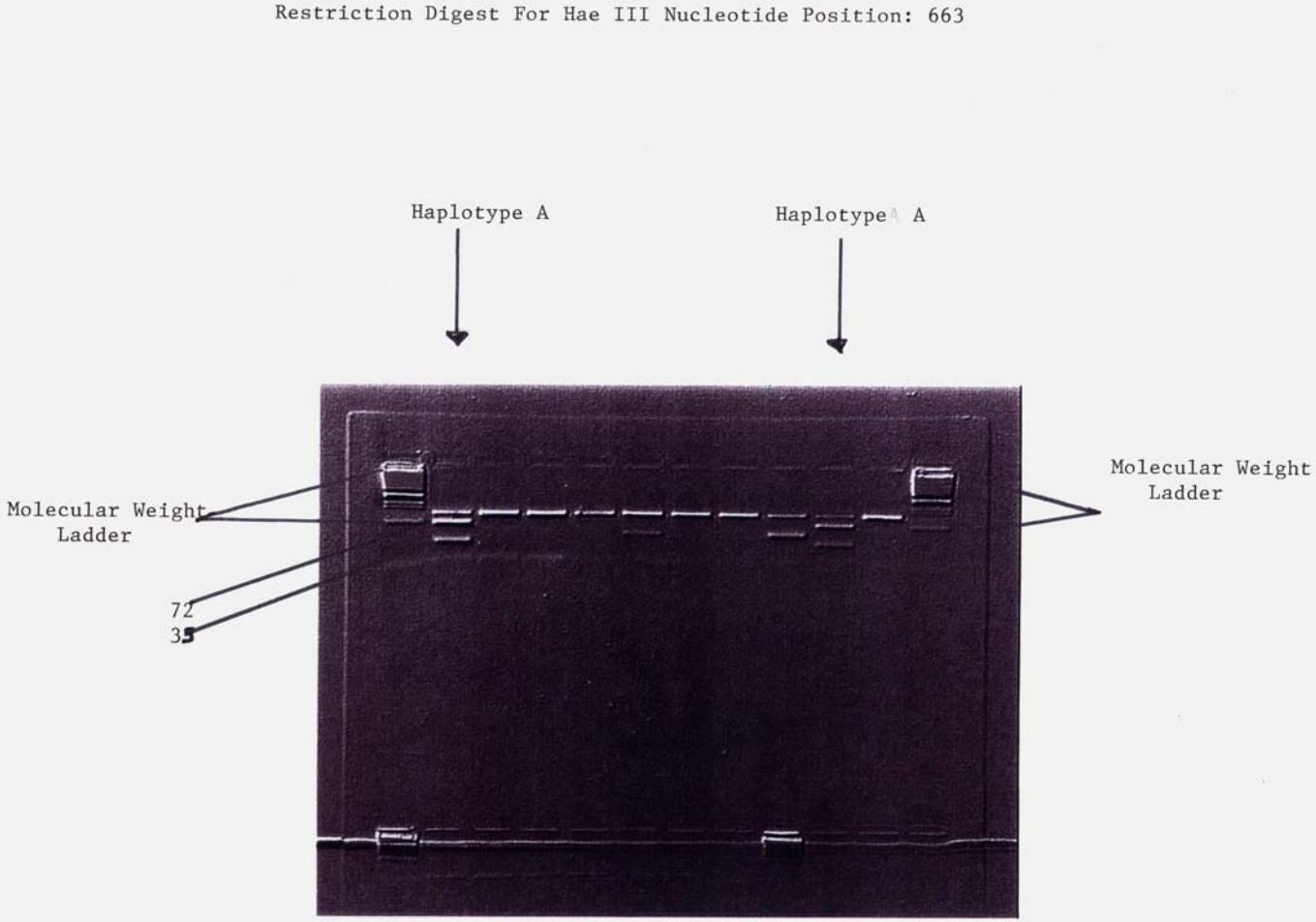


Figure 5: Photo of Haplotype A gel

Contamination

According to Handt et al. (1996) there are two types of contamination which affect ancient DNA studies. The first type can be caused by the permeation of exogenous DNA in extraction and amplification reagents and will systematically affect all of the amplifications. The second type can be caused by previous handling of the samples or by modern DNA or PCR product introduced during the extraction or PCR procedure. The second type will occur sporadically in PCR negatives and can be controlled for by redoing the PCR. In order to control for the two types of contamination, the following protocols were implemented:

1. The room for ancient DNA extraction and the room for ancient DNA PCR procedures were physically separated.
2. Before any work was done in either room, the rooms were washed down with 10% bleach, including the wiping of all counter surfaces and mopping the room.
3. Before entering either room, researcher was covered with a Kappler Pro/Shield I body suit, including head and foot cover, face mask, and double latex gloves.
4. Before any procedure, rooms were UV irradiated for 30 minutes.
5. All equipment including centrifuges, pipettes, and drills were wiped down with 10% bleach.
6. Aerosol barrier pipettes were utilized for every procedure and wiped with 10% bleach.
7. During PCR procedures, PCR cocktail without Platinum Taq was UV irradiated for 20 minutes.
8. Negative controls were placed at the front and end of each PCR run in order to check if contamination was in cocktail or in extract procedure.
9. Any PCR in which cocktail negative control was positive was discarded and rerun until the cocktails negative control was negative.

10. During PCR procedure, samples were added first to PCR wells and sealed with a cap. Caps were then washed with 10% Bleach to prevent cross contamination. The cocktail was then added with researchers gloved fingers bleached in between sealing cap tops.

11. Two rows or 16 wells were done with 1 cocktail. Six rows or 64 wells were done with each PCR run, each having its own separate cocktail and negative controls.

12. The researcher and all other laboratory personnel were completely sequenced for the mtDNA HVI region 16047 to 16429.

13. The researcher, who is of English maternal descent, is an exact match for the Cambridge Reference Sequence in HVI.

14. Those negative extract controls which were positive were sequenced for HVI in order to determine the source of the contamination.

15. All sample and dilutions sequences were compared and in agreement.

Despite all of the above precautions to combat contamination, systematic contamination occurred in 34 of the possible 52 extraction negative controls. The following 18 extract controls were negative: Extract 2 Yang, Extract 3 Phenol, Extract 4 Yang and Phenol, Extract 5 Yang, Extract 9 Yang and Phenol, Extract 10 Yang and Phenol, Extract 12 Yang, Extract 15 Yang, Extract 16 Yang, Extract 17 Phenol, Extract 18 Yang , Extract 19 Yang and Phenol , Extract 25Yang, and Extract 26 Yang. The negative controls were sequenced and the Cambridge Reference Sequence occurred indicating the researcher as the source of the contamination. Of a possible sample size of 392, made up of extract samples and their dilutions, 330 samples amplified, leaving 62 (16 %) with no amplification at all (Appendix A). Of the 330 amplified samples, 57 (17 %) displayed

Native American mutations during sequencing. The remaining 273 (83 %) samples were sequenced and the Cambridge Reference Sequence appeared, again indicating the researcher as the source of the contamination.

After sequencing the amplified samples, something interesting could also be seen during the visual inspection of the electropharagrams. Double peaks, a second peak of about 50% the height of the first peak, occurred in several locations such as 16217, 16223, 16298, 16325, and 16327 indicating Native American mtDNA mutations (Figure 6).

In order to try to separate the two samples, primers were designed whose 3 prime base ended on a mutation at nt 16223 and another primer for the contaminant (Table 10). Mutations were chosen closest to the end of the strand so that phase could be assigned to separate the sequences. Contaminated Haplotype A and C sequences had a mutation at nt 16223. Haplotype B sequences had a mutation at nt 16217 and Hplotype D had a mutation at nt 16325. The primer chosen was 16204 forward for the contaminant and 16204 forward 16223 forward mutation with the mutated end for the Native American sequence. 16204 forward (16223 mutation) has a mutation at the end of the primer at 16223 of a T instead of the wild type, which ended with a C. The 5' to 3' sequence for the two primers is listed in Table 10.

Primers	5' to 3' sequence
16204 Forward	GCA AGT ACA GCA ATC AAC C**C**
16204 Forward (16223 Mutated)	GCA AGT ACA GCA ATC AAC C**T**

Table 10: HVI Primers and annealing temperatures used for HVI sites of contamination

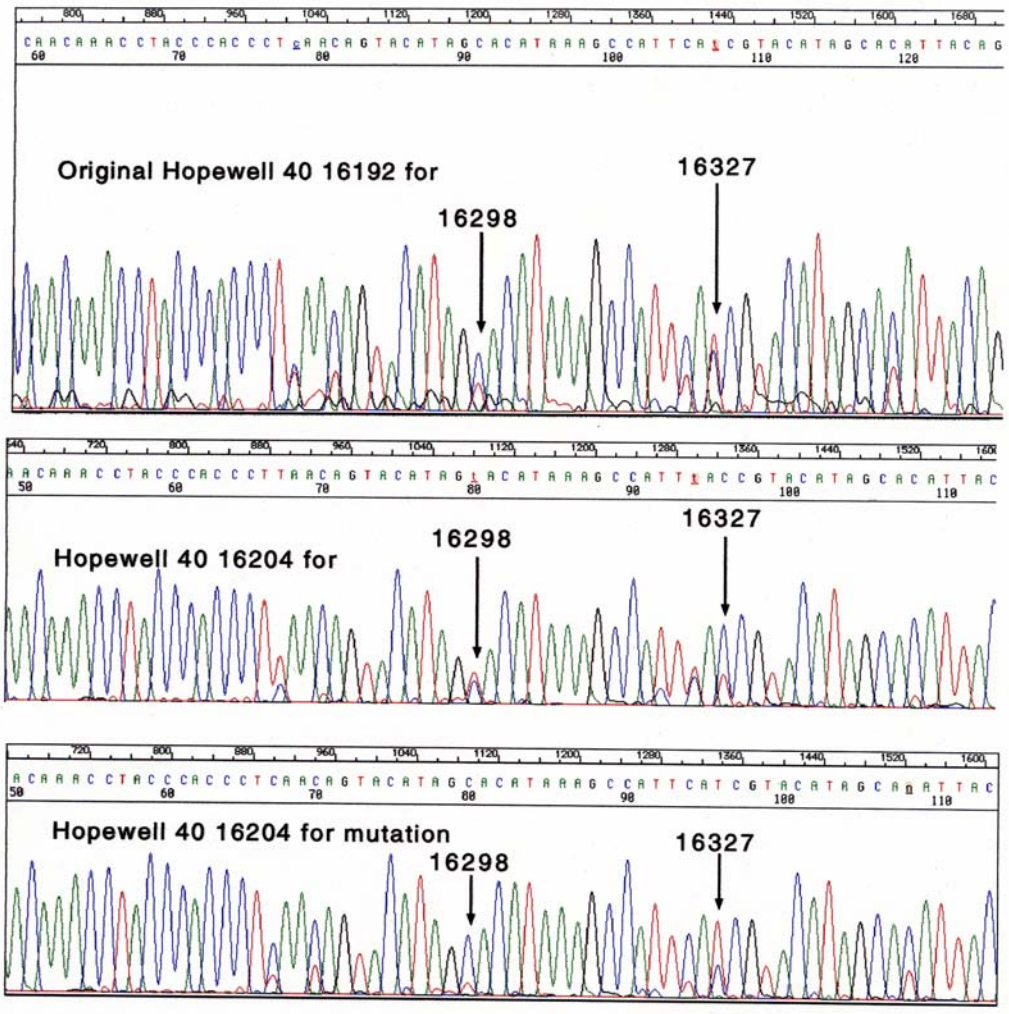


Figure 6: Molecular Primer Method for Seperation of Contaminants from original HW150040 Sequence (16192f-16204f)

A 50ul PCR cocktail was done with HVI primers, 16192 Forward and 16375 Reverse. The 50ul PCR reaction consisted of 5.0ul Proteinase K extract, 5.0ul 10X PCR Buffer, 2.0ul 10mM dntp mix, 1.25ul each of the 2 10mM primers (16192Forward and 16375 Reverse) (Table 5) , 1.5ul 50mM MgCl(2), 0.5ul of Platinum Taq DNA Polymerase (Gibco) and 33.5ul UV irradiated double distilled water. The cocktail was made without the extract or the Platinum Taq and weighted before being UV irradiated in the Stratalinker UV 1800 for 20 minutes to destroy any possible contamination. After which the evaporated double distilled water was replaced with fresh UV irradiated double distilled water and the Platinum Taq added to the cocktail which was vortexed to mix thoroughly. 45ul of the cocktail was added to each well, along with 5ul of either the extract or the dilution. Two new negative controls were added to the PCR plate, one at the beginning and one at the end to check for contamination of the cocktail and the PCR. Also a positive control of a known sample which had worked in the past was added in order to make sure the PCR had worked and as a reference.

Because Platinum Taq is heat activated, a cold start, touchdown PCR method was utilized. For primers 16192F and 16375R, each cycle of amplification consisted of denaturation for 2 minutes at 94 º C, annealing for 0.15 minutes at 55ºC, and elongation for 0.15 minutes at 74ºC for 45 cycles on a Perkin Elmer 9600 Thermocycler. 10ul of the PCR sample was mixed with 2ul of Blue loading dye and electrophoresed on a 2% agarose gel containing 10X Buffer and 0.5 ug/ml ethidium bromide to determine if

amplified DNA was present. On the agarose gel, 1 lane was loaded with 5 ul of size standard. The gel was visualized under a UV transilluminator and photographed to determine amplification of DNA.

All extracts, and dilutions that amplified were purified using the Qiagen QIAquick PCR Purification Kit protocol to remove the primers. In the PCR tube well, 200ul of Buffer PB was added to the remaining 40ul of PCR product and mixed thoroughly. The samples were centrifuged through a 2ml Qiagen spin column for 1 minute at 13,000 X G and the flow through discarded. 750ul of Buffer PE (ethanol) was added to wash the samples and centrifuged for 1 minute at 13,000 X G. Columns were opened and allowed to set open for 10 minutes to allow all of the ethanol to completely evaporate and dry. Qiagen column was placed in a 1.5ml Fischerbrand eppendorf tube and 50ul of Elution Buffer was added to the center of the QIA quick column membrane. The columns stood for 1 minute and were centrifuged for 1 minute at 13,000 X G. Purified samples were stored in a –20ºC freezer.

Dye-deoxy terminator cycle sequencing

After removing the primers, Dye-deoxy terminator cycle sequencing was performed on the purified samples. In order to sequence the samples, 2 cycle sequencing reactions, 1 forward and 1 reverse, was performed for each sample. A Dye-deoxy cocktail was made for each primer, forward and reverse. Instead of primers 16192 forward and 16375 reverse, 16204 forward and 16204 forward (16223 mutation) were utilized for the Dye-deoxy terminator cycle sequencing. The cocktail consisted of 1.5ul ABI Kit Big Dye and

0.075ul 16204 Forward and 16204 Forward (16223 mutated) primers which were vortexed to mix thoroughly and centrifuged for 1 minute at 13,000 x g. 1.7ul of Dye-deoxy sequencing cocktail was added to 2.25ul of purified DNA placed at the bottom of the tube well. The sample plate was shaken for 1 minute and centrifuged for 1 minute at 13,000 x g to concentrate sample and cocktail at the bottom of the well.

The procedure requires a hot start Thermocycler method which starts the Thermocycler at 96ºC and it stays at 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 2 minutes for 30 cycles of amplification. The reaction is light sensitive, so the plate was covered with aluminum foil when removed from the Thermocycler. The plate was stored in a –20ºC freezer within a plastic freezer bag to inhibit evaporation.

In order to remove the unincorporated dye terminators, the cycle sequencing product was purified 30 minutes after pouring a ABI Long Ranger Single Gel. 20ul of 75% Isopropanol alcohol was added to each tube well and mixed by vigorously vortexing for 45 seconds. The plate was covered with aluminum foil and left at room temperature (25ºC) for 20 minutes. In a well-balanced centrifuge, centrifuge plate for 45 minutes at 2000 x g. Carefully remove plate without jostling tube wells. Invert the plate onto a paper towel and centrifuge for 1 minute at 400 x g. Tubes were completely dry when 0.75ul loading dye was added to each tube (6:1 de-ionized fomamide: EDTA/Dextran Blue) and tightly capped to prevent evaporation. The plate was tapped several times to ensure dye settled to the bottom of the tube and centrifuged for 45 seconds. Plate was

covered with aluminum foil and shaken on vortexer at medium speed for 15 minutes. 0.70ul from each well was loaded onto the ABI Applied Biosystems 377XL Automated Sequencer.

During the visual reinspection of the electropharagrams, Figure 6, separation of the Native American sample from the European contaminant had occurred. The contaminant was now 50% the height of the Native American sample. Sequences were aligned and corrected on the ABI Sequence Navigator program using the Cambridge Reference Sequence (Anderson et al. 1981). This procedure offers the ability to separate identified contaminants from research samples. However, the following information must be known before procedure can be utilized.

1. The source of contamination must be known along with the mutations that make up the contamination.
2. The contamination separation procedure works best when the population of maternal origin of both the contaminant and study population have different mutations.
3. In order to be cost efficient, the contamination separation procedure works best with small number of samples.

However, it does offer hope that all is not lost to contamination.

CHAPTER 5

RESULTS

The overall amplification success rate for this study was 34 of 49 individuals or 69%. Table 11 displays the distribution of the success rate per each mound. All mounds, and samples including no mound affiliation, had a success rate over 50% with the no mound affiliation having the lowest of 55%. 100% success was achieved with mounds 4,7,and 26 however, only 1 individual was chosen from each of the mounds. The largest sample came from Mound 25 which had a success rate of 71%. Overall, 69% is comparable to other ancient DNA studies (Parr 1996; Stone 1996; Kaestle 1998 and Napier 2000).

In order to detect the level of mtDNA variation present within the Ohio Hopewell of the Hopewell Mound Group, RFLP testing for the five Native American haplotypes was conducted on the samples. Table 12 shows the overall distribution of the five Native American haplotypes. Haplotype X was not found while 41% of the individuals were haplotype A. Haplotype C had the second highest RFLP distribution, with haplotype D next and only 9% of the sample had haplotype B. The Ohio Hopewell of the Hopewell Mound Group are similar in RFLP frequencies to other Amerindian populations

Mounds of Hopewell Mound Group	Total number of individuals per mound	Number of individuals with less than 4 teeth	Final number of possible individuals	Number of Individuals chosen	Number of Individuals Amplified	Percent of Ind. Success Amplified
2	6	1	5	4	3	75
4	4	0	4	1	1	100
7	2	0	2	1	1	100
25	41	3	38	31	22	71
26	1	0	1	1	1	100
27	1	0	1	0	0	0
No Mound	29	10	19	11	6	55
Total	84	14	70	49	34	69

Table 11: Amplification Success Rate for Ohio Hopewell Mound Group per Mound

(Schurr et al. 1990;Lorenz and Smith (1996); Merriwether (1993;2002) and Wallace and Torroni 1992). The Ohio Hopewell of the Hopewell Mound Group do not fit the trend of decreasing haplotype A and increasing haplotype B from north to south. In fact, it is the exact opposite of this trend.

HAPLOTYPE	A	B	C	D	X	TOTAL
Number of Individuals	**14**	**3**	**10**	**7**	**0**	**34**
Percentage of Individuals	**41**	**9**	**29**	**21**	**0**	**10**

Table 12: Native American RFLP Haplotype Distribution for the Ohio Hopewell of the Hopewell Mound Group

The RFLP frequencies were further broken down by sex. Sex estimation (male,female, and indeterminate) was taken from the skeletal analysis of Johnston (2002). Haplotype A distribution by sex is displayed in Table 13 and shows an approximately equal division between males and females. Haplotype B frequencies are provided in Table 14 and it shows more interesting results with no males at all having that haplotype and only 1 female. What is most interesting about the lone female (Mound 25, Burial 41M) is that she is the middle burial of a triple burial located in the northeast section of Mound 25. Haplotype C frequencies are provided in Table 15 and are similar to haplotype A in that the distribution is approximately equal between males and females. Finally, haplotype D frequencies follow a similar trend of haplotypes A and C.

SEX	MALE	FEMALE	INDETERMINATE	TOTAL
Number of Individuals	5	6	3	14
Percentage of Individuals	36	43	21	100

Table 13: Native American RFLP Haplotype Distribution by Sex Haplotype A

SEX	MALE	FEMALE	INDETERMINATE	TOTAL
Number of Individuals	0	1	2	3
Percentage of Individuals	0	33	67	100

Table 14: Native American RFLP Haplotype Distribution by Sex Haplotype B

SEX	MALE	FEMALE	INDETERMINATE	TOTAL
Number of Individuals	4	2	4	10
Percentage of Individuals	40	20	40	100

Table 15: Native American RFLP Haplotype Distribution by Sex Haplotype C

SEX	MALE	FEMALE	INDETERMINATE	TOTAL
Number of Individuals	2	3	2	7
Percentage of Individuals	26	44	28	100

Table 16: Native American RFLP Haplotype Distribution by Sex Haplotype D

When examining the distribution of Native American haplotypes within the individual mounds, several mounds (4,7, and 26) only have 1 individual and will not be surveyed. However, Mounds 2 and 25 have the majority of the RFLP typed individuals and Table 17 and 18 display their Native American haplotype frequencies. With only 3 samples, Mound 2 (Table 17) has only haplotypes A and C, with no B, D or X. Mound 25 (Table 18), however, has the greatest percentage of haplotype A with 50% and haplotype B with 9%. It also has a higher frequency of haplotype D of 27% and 14% haplotype C.

HAPLOTYPE	A	B	C	D	X	TOTAL
Number of Individuals	1	0	2	0	0	3
Percentage of Individuals	33	0	67	0	0	100

Figure 17: Mound 2 Native American RFLP Haplotype Distribution

HAPLOTYPE	A	B	C	D	X	TOTAL
Number of Individuals	11	2	3	6	0	22
Percentage of Individuals	50	9	14	27	0	100

Figure 18: Mound 25 Native American RFLP Haplotype Distribution

Table 19 contains a combination of RFLP and HVI cycle sequencing data in the analysis of the HVI region. HVI was completely sequenced from nucleotide position 16049 to 16429. The first five columns in Table 19 designate the five Native American haplotypes and the remaining columns exhibits those nucleotide positions which differ from the Cambridge Reference Sequence. All other nucleotide positions not shown agree with the Cambridge Reference Sequence. After collapsing the data for sequences that were similar into one sequence, the Ohio Hopewell have 21 haplotypes with 24 polymorphic sites within 341 loci.

	00663+	9B.P.del+	10394+	13262+	05176-	16111	16126	16166	16179	16189	16217	16223	16235	16247	16259	16265	16270	16275	16290	16298	16304	16309	16311	16319	16325	16327	16335	16357	16362
Camb. Ref.	C	C	C	C	C	C	T	A	C	T	T	C	A	A	C	A	C	A	C	T	T	A	T	G	T	C	A	T	T
HW150036	A											T							T					A					
HW150061	A											T							T					A					
HW150069	A											T							T					A					
HW150124	A											T							T					A					
HW150209	A											T							T					A					
HW150057	A											T					T	G	T				C	A					
HW150112	A											T	G						T			G		A					
HW150122	A					T						T			T				T					A					C
HW150123	A							G											T					A					C
HW150143	A					T						T							T					A					C
HW150165	A						C					T							T					A					C
HW150210	A																		T					A					
HW150213	A											T							T				C	A					
HW150219	A											T							T				C	A					
HW150043		A								C	C											G							
HW150050		A								C	C											G							
HW150053		A								C	C																		
HW150028			A	A								T								C					C	T			
HW150037			A	A								T								C					C	T			
HW150058			A	A								T								C					C	T			
HW150109			A	A								T								C					C	T			
HW150164			A	A								T								C					C	T			
HW150212			A	A								T								C					C	T			
HW150215			A	A								T								C					C	T			
HW150040			A	A								T								C			C		C	T			
HW150055			A	A								T								C			C		C	T			
HW150137			A	A								T								C	C				C	T	G		
HW150062			A		A							T		G		G									C				
HW150066			A		A							T													C				C
HW150068			A		A																				C				C
HW150115			A		A																				C			C	
HW150116			A		A							T													C				
HW150121			A		A																		C		C				C
HW150166			A		A				T			T													C				

Table 19: Ohio Hopewell of the Hopewell Mound Group mtDNA RFLP and HVI Sequence Data

A search of GenBank, a genetic database with over 4 million sequences, was conducted to look for other populations, both modern and ancient, that share both the RFLP haplotype designation and unusual mutation location with the Ohio Hopewell. Haplotype A has 6 sequences with unique transitions. Beginning with a transition from T to C at nucleotide position 16126, several modern Asian populations from China, Taiwan, Japan and Korea, as well as Mongolian populations share this mutation with the Ohio Hopewell. Also several modern Native American populations, such as Iowa, Objibwa, Pawnee, and Yakima, have the mutation. One ancient Native American population, Pyramid Lake, also has the transition at 16126. Continuing with Haplotype A, another transition of a A to G at nucleotide position 16235 is found in several modern Asian populations from China, Taiwan, Japan and Korea. However, the mutation is not found in any other modern or ancient populations in North America. It is found in South America in Bolivia with the Aymara. Another transition within haplotype A is a C to T at nucleotide position 16259 which is found within Central America in Puerto Rico and Brazil. A transition of a C to T at nucleotide position 16270 is found only in North America in the modern population of Nuu-chah-nulth. One transition of T to C at nucleotide position 16357 is found only in an ancient Native American population of Pyramid Lake. Finally, a transition of a T to C at nucleotide position 16311 is found in modern populations from China, Russia and Korea while also being found in South America in Bolivia and Guatemala. In North America, Native American modern populations of Canadian Inuit, Alaskian Eskimo and Nug Finals also share the transition.

Haplotype B, with only 3 samples, has a surprising amount of genetic variation. A transition of a A to G at nucleotide position 16309 is found in the modern Asian population of China, as well as the Native American population of Navajo in North America. In South America, the transition is found in several modern Native American populations in Bolivia, such as the Aymara, Mataoc, Pilago, and Yanomama. At the Hopewell Mound Group, the mutation was established in a female (Mound 25, Burial 41M) ,who was part of a triple burial in Mound 25. What is most interesting about this mutation in haplotype B is that Napier (2000) found the mutation at Cahkoia in 5 of 8 individuals interred within 72Sub2 charnel house. Napier goes on to utilize the mutation as evidence of elite status for the five individuals (Napier 2000).

Haplotype C and D only have 1 unique mutation each within the Ohio Hopewell. Haplotype C has a transition of a C to T at nucleotide position 16311. The mutation is found in modern populations in India, China, Siberian Russia, and Mongolia while also found in Native American populations in South America in the Aymara, Cashinawa, and Huilliche. The mutation is in Native American populations in North America in both modern populations of Apache, Chippewa, Navajo, Pima and Seri while also in the ancient Native American population of Norris Farms. Haplotype D has only 1 transition of a C to T at nucleotide position 16179 which is shared with only modern populations found in the Macushi and Brazil of South America.

The next section will examine the four questions put forth in Chapter 2 :

1. What are the phylogenetic relationships and affiliations between the Ohio Hopewell Mound Group mtDNA lineages to other ancient and contemporary North Native American mtDNA lineages ?

2. What information does mtDNA add to what is known biologically about the prehistoric groups of the Ohio River Valley ?

3. Is there evidence to support matrilineal descent among the Ohio Hopewell of the Hopewell Mound Group ?

4. Is there segregation of individuals interred within the Ohio Hopewell Mound Group based upon mtDNA lineages; specifically within Mounds 2 and 25 ?

For each question, the method utilized to answer the question will be outlined as well as the theoretical basis behind the method. Also included will be the components involved in the method, such as which populations were utilized, location of the population and size of the sample, abbreviation of sample in table ,maps and genetic trees and references for each sample.

In order to answer question 1, phylogenetic trees were generated based upon the Kimura 2-parameter model (Kimura 1980). Jukes and Cantor(1969) developed the Jukes-Cantor 1-parameter model which states that the probability of any nucleotide changing to any other nucleotide was equal. However, the 1-parameter model was thought to be simplistic because all changes do not occur at the same rate. The Ohio Hopewell are a perfect example of this difference. Table 19 displays the mutations that occur in the Ohio Hopewell, and when examined only 1 transversion occurs while the rest of the mutations

are transistions. Kimura (1980) 2-parameter model recognizes this phenomenion and sets the 2 rates differently, the rate of transistions is set at alpha, which for mtDNA was input at 15 and the rate of transversions is set at beta, which for mtDNA was input as 1.

Fifty Native American populations from North and Central America were chosen to explore the phylogenetic and affiliate relationships of the Ohio Hopewell. Eight hundred and thirty-one aligned HVI sequences were exported to Mac Clade 3.08a (Maddison and Maddison 2000) to be filtered. All identical sequences were removed so only one copy of a sequence represented the duplicates and cropped and the right and left sides of longer sequences were reduced so that all sequences were the same length as the shortest sequence which was from 16084 to 16408. Kimura-2 parameter genetic distance matrices (Kimura 1980) were generated using PHYLIP 3.6's DNAdist program (Felsenstein 2002). A transistion : transverion rate of 15:1 was specified for mtDNA. A Neighbor-Joining tree (Saitou and Nei 1987) was built utilizing the distance matrices in PHYLIP 3.6's Neighbor. Neighbor-Joining (Saitou and Nei 1987) was chosen because it assumes a minimum number of changes to explain data. A "treefile" was generated and viewed in Tree View 1.6.6 (Page 1996).

Table 20 displays the name of each sample, its language classification, reference source, Neighbor-Joining tree abbreviation and the number of individuals within each sample. Figure 7 shows the location of 33 of the above 50 samples. Anderson (1980) refers to the first human mtDNA sample completely sequenced, which was of English ancestory.

Sample	Language	Reference	Abbrev. of Samp.	Samp. Size
1.Alaskan Athabaskin	Athabaskin	Shields et al. 1993	AlaAth	21
2.Alaskan Central Yupik	Yupik	Simonson et al. 1997	CYupik	25
3. Apache	Athabaskin	Budowle B 2002	Apache	187
4. Bella Coola	Bella Coola	Ward et al 1993	BelCol	44
5. Chaluka Eskimo	Eskimo-Aleut	No Data	Chal	5
6. Chippewa	Algonquian	Malhi et al 2001	ChippSW	8
7. Choctaw	Muskogean	Lorenz	Choctaw	1
8. Chumach	Chumachan	Lorenz	Chumach	3
9. Dogrib	Athabaskin	Torroni et al 1993	Dogri	2
10. Haida	Haida	Ward et al. 1993	Haida	43
11. Ohio Hopewell	Unknown	this study	HW150	34
12. Inupiaq Eskimo	Eskimo-Aleut	Simonson et al. 1998	Inupiaq	15
13. Iowa	Siouan	Malhi et al 2001	Iowa	3
14. Kickapoo	Algonquian	Malhi et al 2001	Kickapoo	5
15. Micmac	Algonquian	Malhi et al 2001	Micmac	4
16. Nahua	Uto-Aztezian	Malhi et al 2002	Nahua	5
17. Navajo	Navajo	Budowle B 2002	Navajo	169
18. Norris Farms	Unknown	Stone A 1998	NF	23
19. Nug Finals	No Data	No Data	Nug	2
20. Nuu-chah-nulth	Wakashan	Ward et al. 1991	NuuCN	63
21. Ojibwa	Algonquian	Lorenz	Ojibwa	2
22. Old Harbor Eskimo	Eskimo-Aleut	No Data	OldHarb	20
23. Oklahoma Cherokee	Iroquoian	Malhi et al 2001	ORCCh	15

Table 20: 50 Populations utilized in analysis (Continued)

Table 20: Continued

Sample	Language	Reference	Abbrev. of Samp.	Samp. Size
24. Pawnee	Caddoan	Malhi et al 2001	Pawnee	3
25. Pyramid Lake	Unknown	Kaestle	PL-	19
26. Sac&Fox	Algonquian	Malhi et al 2001	Sac&Fox	1
27. Shawnee	Algonquian	Malhi et al 2001	Shawnee	2
28. Sisston/ Wahpeton Sioux	Siouan	Malhi et al 2001	SWSio	16
29. Stillwell Cherokee	Iroquoian	Malhi et al 2001	StilwChe	15
30. St. Lawrence	Algonquian	No Data	Sleg	18
31. Tlingit	Tlingit	Simonson et al. 1998	Tlingit	8
32. Yakima	Sahaptian	Lorenz	Yakima	3
33. Baja Yuma	Yuman	Lorenz	Yuman	4
34. California Uto-Aztecian	Uto-Aztezian	Lorenz	UtoAztec	1
35. Cochimi	Cochimi-Yumsn	Malhi et al 2002	Cochimi	1
36. Jemez	Kiowa-Tanoan	Malhi et al 2002	Jemez	8
37. Kiliwa	Yuman	Malhi et al 2002	Kiliwa	1
38. Kumeyaa	Yuman	Malhi et al 2002	Kumeyaa	1
39. Paipais	Yuman	Malhi et al 2002	Paipais	2
40. Pima	Uto-Aztezian	Malhi et al 2002	Pima	9
41. Quapaw	Siouan	Malhi et al 2001	Quapaw	3
42. Seri	Seri	Malhi et al 2002	Seri	8

(Continued)

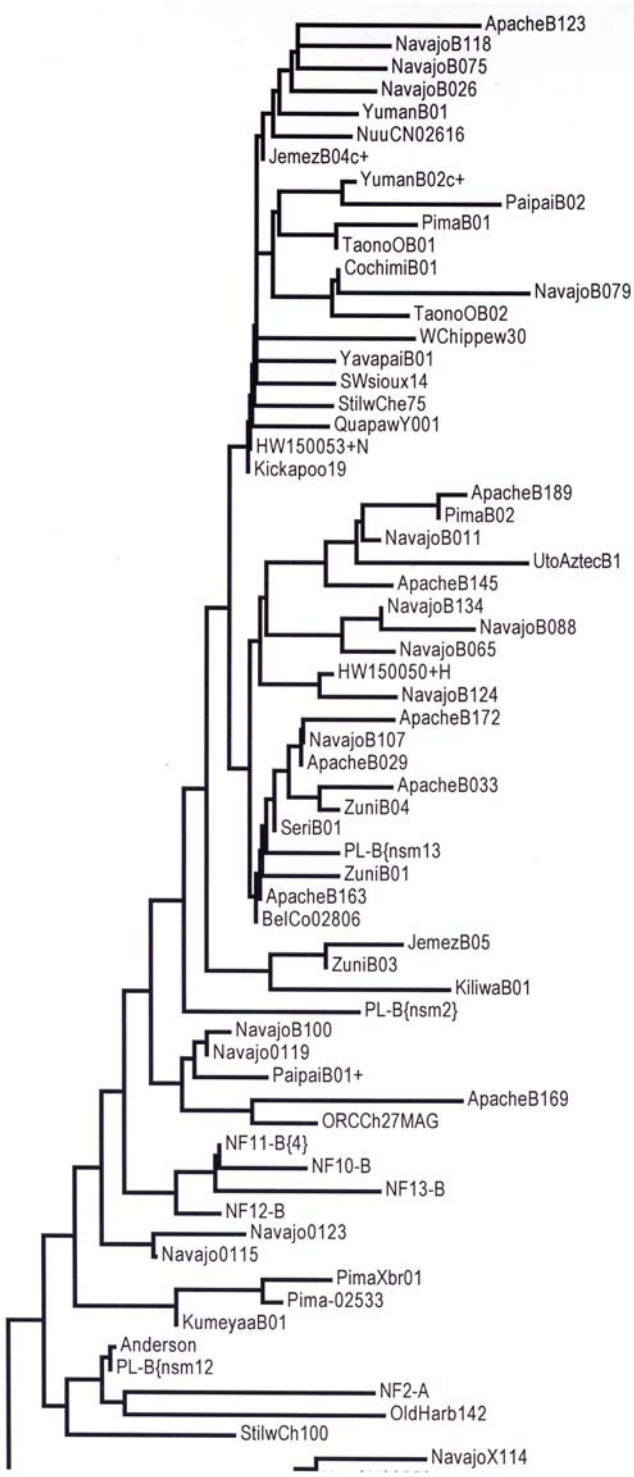
Table 20: (Continued)

Sample	Language	Reference	Abbrev. of Samp.	Samp. Size
43. Taono O'odham	Taonoan	Malhi et al 2002	TaonoO	3
44.Wisconsin Chippewa	Algonquian	Malhi et al 2001	WChipp	19
45. Yavapais	Yuman	Malhi et al 2002	Yavapais	1
46. Zuni	Zuni	Malhi et al 2002	Zuni	5
47. Cheyenne/Arapo	Algonquian	Malhi et al 2001	CheyArap	4
48. Maya	Penutian	Torroni et al 1993	Maya	31
49. St. Paul	Eskim-Aleut	No Data	St. Paul	2
50. Turtle Mt. Chipp	Algonquian	Malhi et al 2001	TMChipp	2

Legend for Map of 33 Native American Samples

1. Alaskan Athabaskin
2. Apache
3. Bella Coola
4. Chaluka Eskimo
5. Chippewa
6. Choctaw
7. Dogrib
8. Haida
9. Ohio Hopewell
10. Inupiaq Eskimo
11 Micmac
12. Navajo
13. Norris Farms
14. Nuu-chah-nulth
15. Ojibwa
16. Old Harbor Eskimo
17. Oklahoma Cherokee
18. Pawnee
19. Pyramid Lake
20. Sisston/Wahpeton Sioux
21. Stillwell Cherokee
22. St. Lawrence
23. Creek
24. California Uto-Aztecian
25. Cochimi
26. Jemez
27. Pima
28. Wisconsin Chippewa
29. Zuni
30. Cheyenne/Arapo
31. Maya
32. St. Paul
33. Turtle Mountain Chippewa

Figure 7: Map of 33 of 50 Samples



B

Figure 8: 50 Samples for the Kimura 2-parameter Neighbor Joning Tree continued

Figure 8: Continued

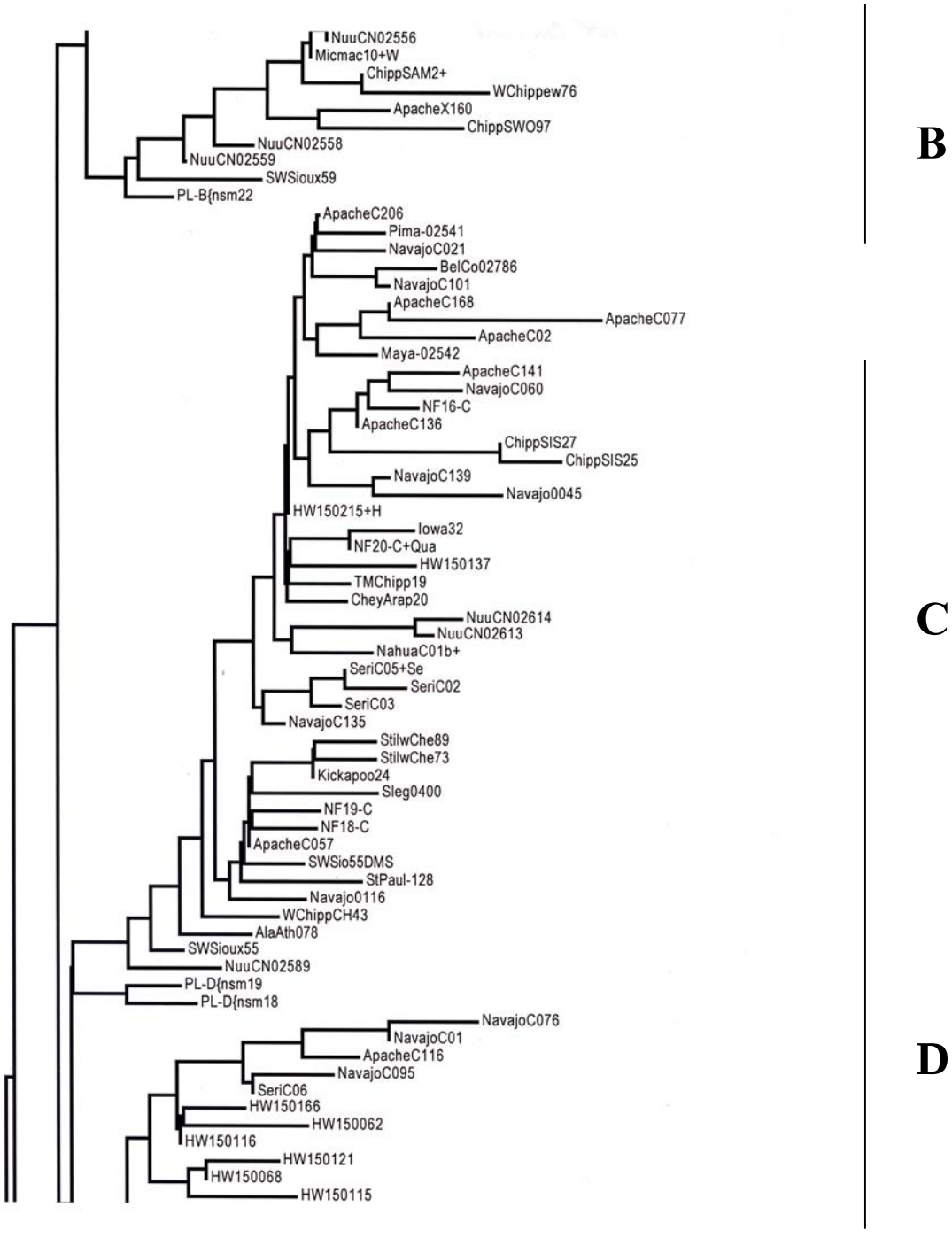


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Figure 8: Continued

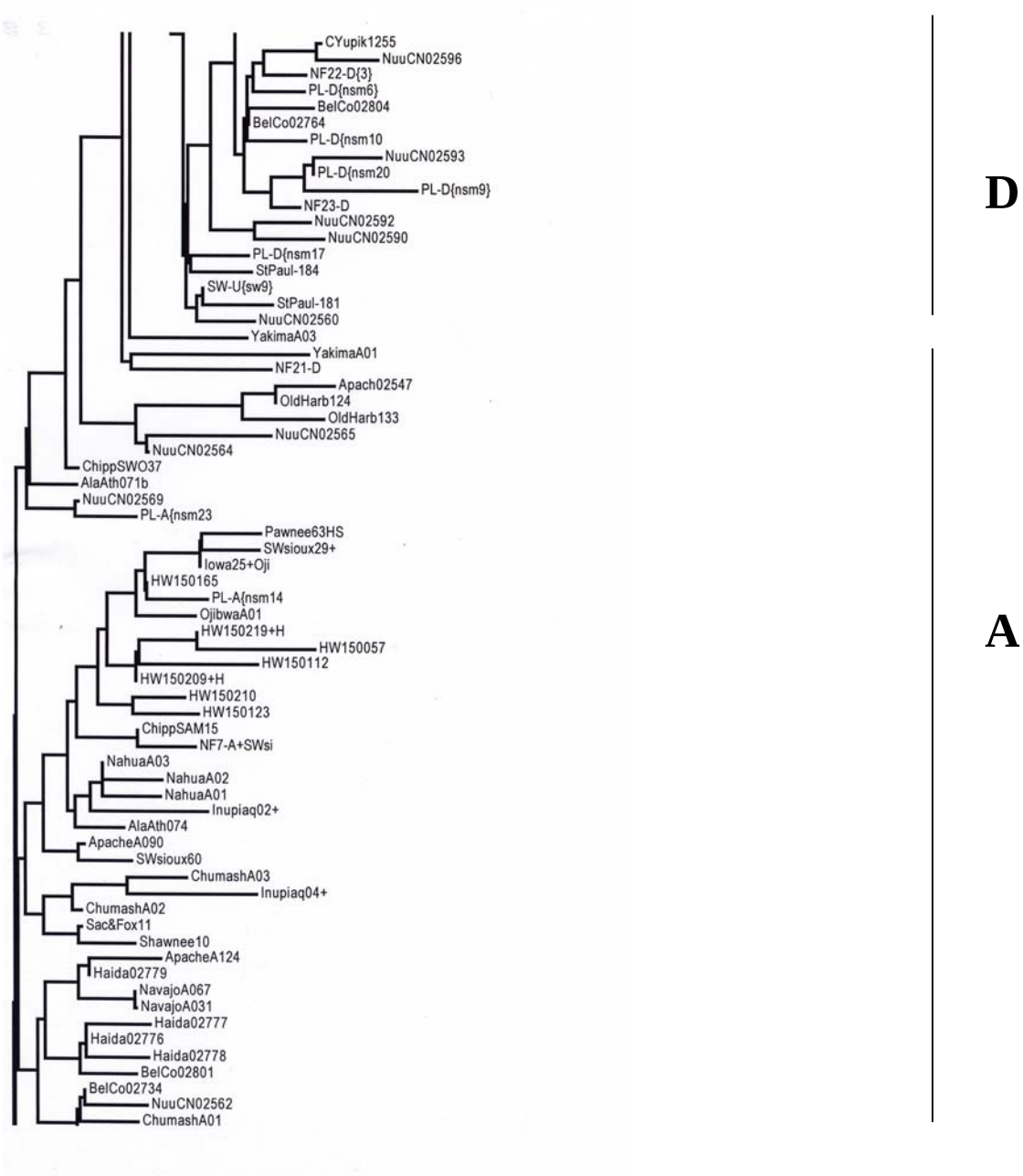

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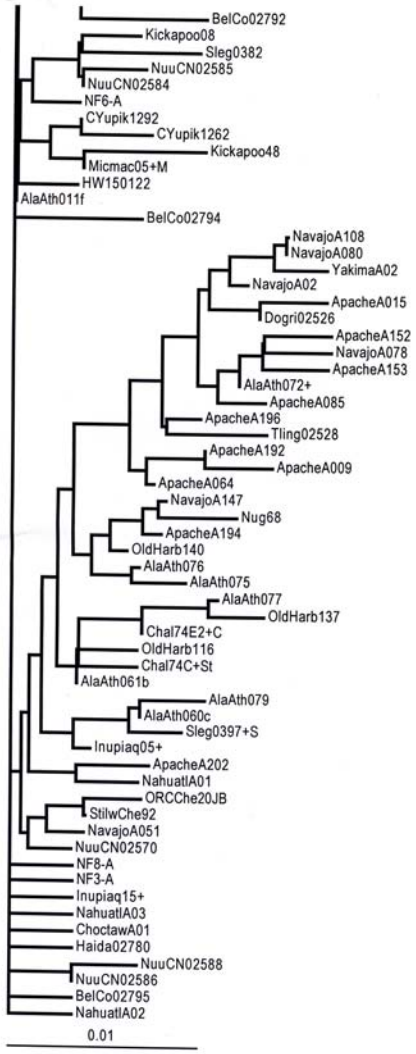
Figure 8: Continued



A

The Kimura 2-parameter Neighbor Joining tree exhibits a clustering of haplotype B, followed by haplotype C, then haplotype D and finally haplotype A. Notice that the Ohio Hopewell segregate into the four clusters demarcated by the basic polymorphisms which define each individual haplotype and are comprised of those individuals who exhibit those mutations. The results of the Kimura 2-parameter Neighbor Joining tree demonstrate that four of the five New World haplotypes are present at the Hopewell Mound Group. This is an indication of a mtDNA relationship between the Ohio Hopewell and other Native American groups both in North and Central America.

Question 1 also deals with the affiliations of the Ohio Hopewell to modern and contemporary Native American populations. Figures 9,10,11 and 12 allow for the closer examination of affiliation by taking the large and complex Kimura 2-parameter Neighbor Joining tree and breaking it down into more manageable units of each individual haplotype. Haplotype X was excluded from this analysis as the present sample Ohio Hopewell do not have the haplotype. The same analysis and method utilized to create the inclusive Kimura 2-parameter Neighbor Joining tree above was also used with each hapltoype tree. Figure 9 displays haplotype A Kimura 2-parameter Neighbor Joining tree. Notice that the Ohio Hopewell branch with ancient samples from Norris Farms and Pyramid Lake while also grouping with modern samples from Iowa, Pawnee, Southwest Sioux, Yakinma, Ojibawa and Micmac. Notice that those samples present an Eastern Woodland cluster for haplotype A. Figure 10 displays haplotype B Kimura 2-parameter Neighbor Joining tree. The Ohio Hopewell branch with no ancient samples however, they cluster with modern samples from Baja Yuma, Kickapoo and Nuu-chuh-nulth

indicating a possible recent clustering. Haplotype B is thought to be the youngest of the four major haplotypes, indicating a more recent migration because of shallow divergence time and possible coastal distribution (Torroni et al. 1992; 1993a; 1993b; 1994a; and 1994b). Figure 11 displays haplotype C Kimura 2-parameter Neighbor Joining tree. The Ohio Hopewell branch groups with no ancient samples, however they do group with modern samples from Bella Coola, Nuu-chuh-nulth, Apache, Navajo and Pima. Figure 12 displays haplotype D Kimura 2-parameter Neighbor Joining tree. It is unusual in that the Ohio Hopewell don't group with any other samples and form their own node with Anderson et al. (1980). The Anderson cluster, which refers to the Cambridge Reference sequence, represents a sequence which shares many common mutations across geographical areas.

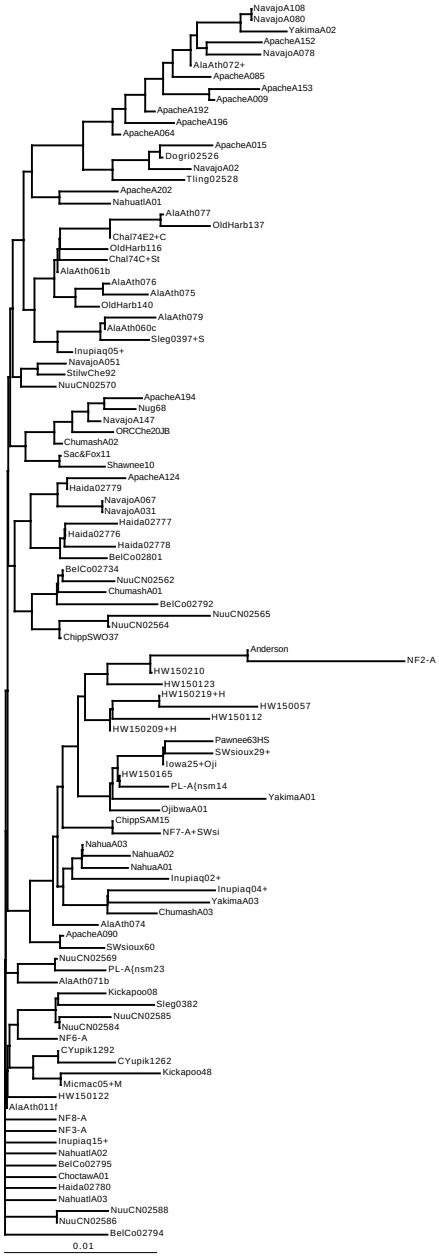


Figure 9: Kimura 2-Parameter Neighbor Joining tree for Haplotype A

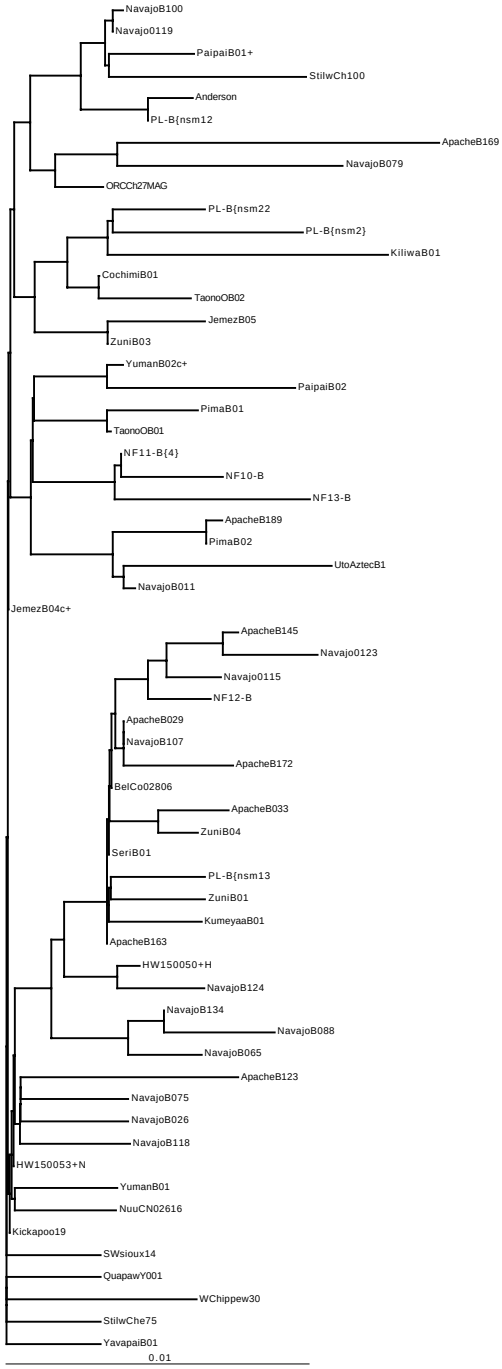


Figure 10: Kimura 2-Parameter Neighbor Joining tree for Haplotype B

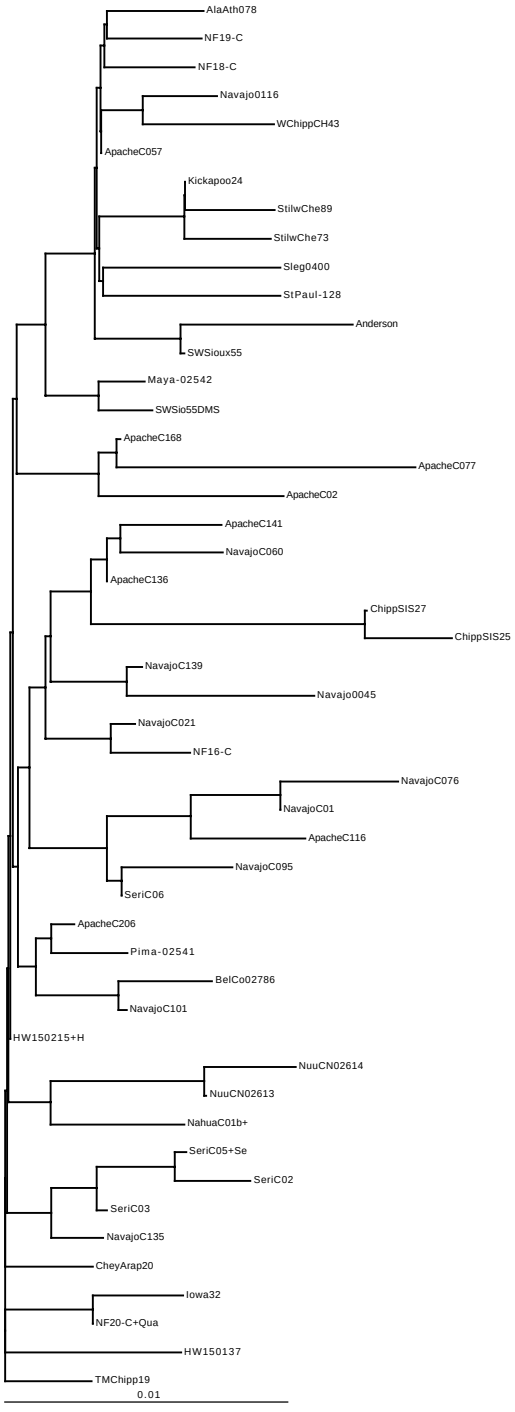


Figure 11: Kimura 2-parameter Neighbor Joining tree for Haplotype C

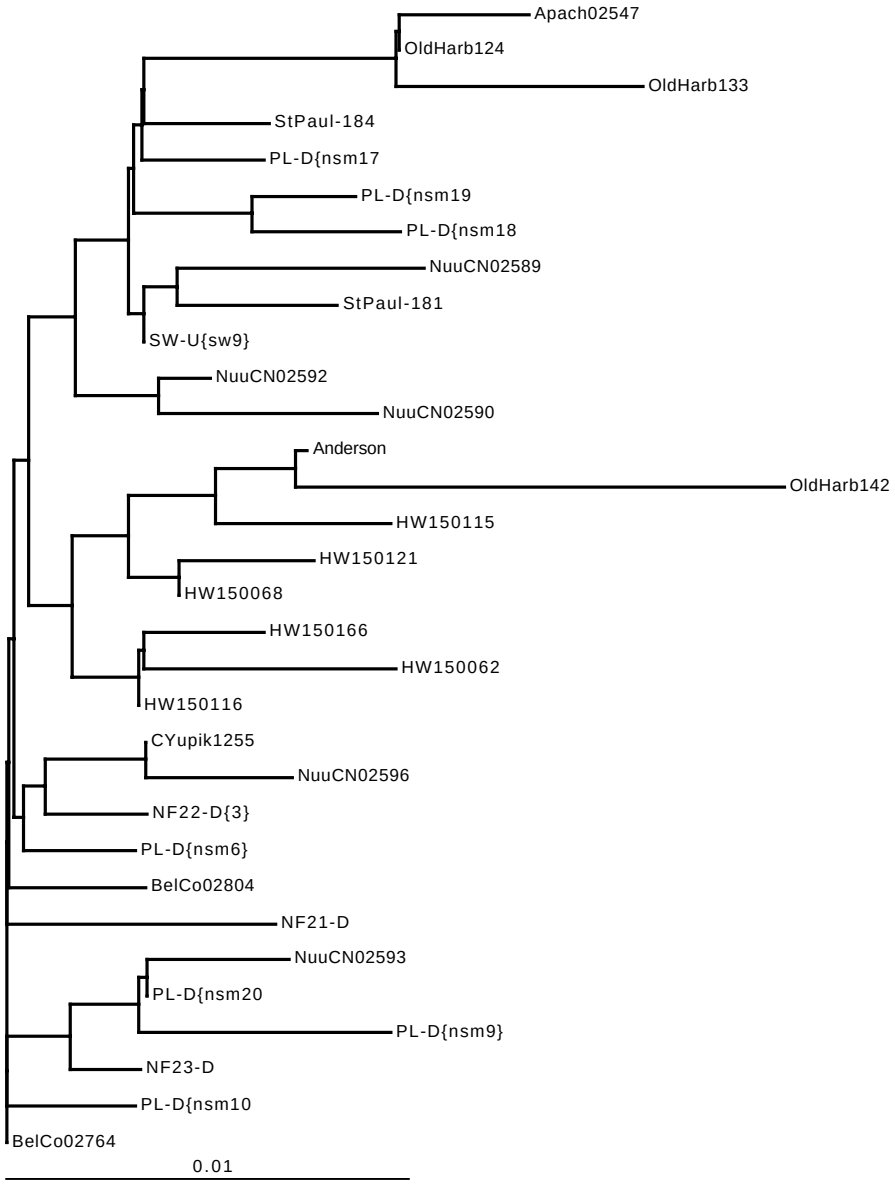


Figure 12: Kimura 2-parameter Neighbor Joining tree for Haplotype D

Data utilized to answer question 3, “What information does mtDNA add to what is known biologically about the prehistoric groups of the Ohio River Valley (Glacial Kame, Adena, Hopewell and Fort Ancient)?” originates from RFLP data located in Table 2. Nei’s (1972) genetic distance equation was calculated for each of the 16 sample populations from both ancient and contemporary Native American populations in order to shed light on possible Ohio Hopewell origins and affiliations. Nei (1972) relies upon calculated genetic distances from a set of gene frequencies from different populations. Nei’s (1972) genetic distance has several assumptions. First, all differences between populations arose from genetic drift. Nei’s (1972) indices were constructed under the infinite allele model of mutation in which each mutation leads to a completely new allele and the rate is the rate of neutral mutation. All loci also have the same rate of neutral mutation and effective population size of each population remains constant. Finally, the genetic variability in each population initially is at equilibrium between mutation and genetic driFort

Genetic distance matrices (Nei’s 1972) were generated using PHYLIP 3.6’s Gendist program (Felsenstein 2002). A Neighbor-Joining tree (Saitou and Nei 1987) was constructed utilizing the distance matrix in PHYLIP 3.6’s Neighbor. Neighbor-Joining (Saitou and Nei 1987) was chosen because it assumes a minimum number of changes to explain data. A “treefile” was generated and viewed in Tree View 1.6.6 (Page 1996).

Figure 13 displays a map of the location of the 16 sample populations utilized in the analysis. Figure 14 exhibits the Neighbor-Joining tree for the RFLP data.

1. Creek
2. Choctaw
3. Nuu-chah-nulth
4. Bella Coola
5. Oklahoma Cherokee
6. Stillwell Cherokee
7. Pyramid Lake
8. Stillwater March
9. Braden De Moss
10. Congdon
11. Norris Farms
12. Hind Site
13. FortAncient
14. Robbins Mound
15. Wright Mound
16. Hopewell

Figure 13: Map of 16 Native American sample populations

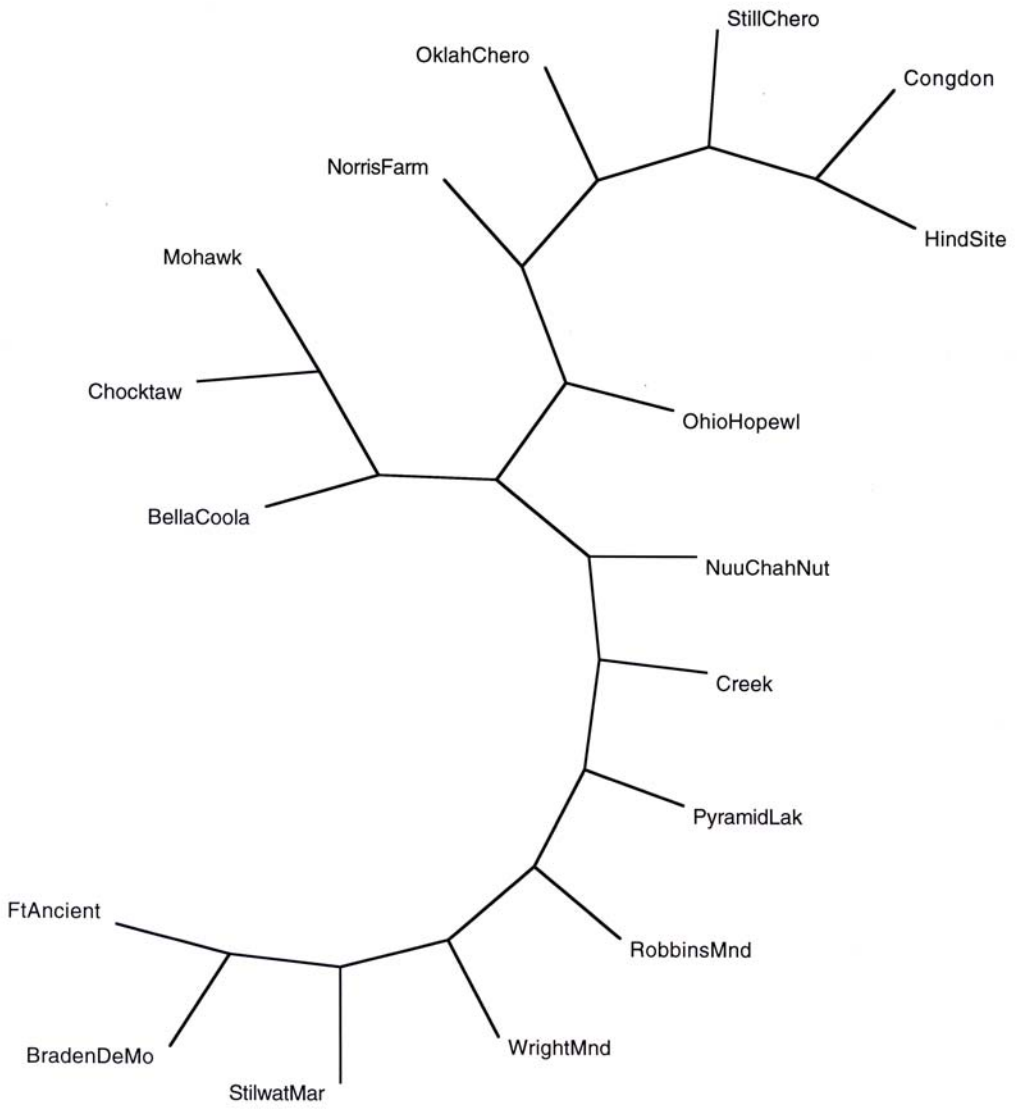


Figure 14: Neighbor-Joining tree for the RFLP data from 16 ancient and modern populations

In Chapter 3, several hypotheses are outlined for the possible origin and affiliation of the Ohio Hopewell. One idea put forth from the oral tradition of the Cherokee suggests they are the ancestors of the Ohio Hopewell. When viewing figure 14, both groups of Cherokee cluster with the Archaic samples of Congdon and Hind sites which do not cluster close to the Ohio Hopewell. When looking at several of the modern sample populations, the Nuu-chah-nulth, Bella Coola, Creek and Chocktaw, the Ohio Hopewell cluster close to those samples. However, a 2000 year difference in time makes the clustering artificial because the modern samples have been undergoing evolutionary processes. Many evolutionary processes such as natural selection, migration, mutation and most likely genetic drift have effected the modern populations. Also, after European contact a severe bottleneck took place in North America. What would have been more informative would have been the grouping of all three of the Glacial Kame sites not just the Congdon and Hind sites, while the Braden DeMoss clustered with the Fort Ancient sample. The closest ancient population is that of Norris Farms, which is the closest both geographically as well as time wise, with only 700 years separating the two samples. The Ohio Hopewell cluster fairly close to the two Adena sites, Robbins and Wright mounds, but are not near the Fort Ancient site. One possible reason why this did not occur could be due to small sample size in all three cases. What is interesting is that the Adena and Fort Ancient sites cluster close to the two sites from the western United States, Pyramid Lake and Stillwater Marsh. Overall, larger samples would give a clearer picture of ancestor descendant relationships as well as samples from other areas.

RFLP haplotype frequency distributions for each ancient site were compared to the Ohio Hopewell. Due to small sample, Robbins Mound sample size 6, the Fischer exact statistical test, similar to Chi-squared, was computed for each of the ancient RFLP frequency data sets. The null hypothesis tested was H_o: "the genotype distribution is identical across populations" (Raymond and Rousset 1995). If the p-value generated is less than 0.05, it suggests that the two populations have significantly different haplotype frequency distributions. Analysis was completed utilizing the genetic statistical program GENEPOP version 3.1b (Raymond and Rousset 1995). RFLP haplotype frequency data from the Ohio Hopewell was compared to all of the ancient samples from table 2, except Stillwater Marsh. Table 21 displays the probability value and standard error for each Fischer exact test of significance. From those 8 sample populations, 3 of the 8, were not significantly different, Robbins and Wright mounds and Norris Farms. Both Robbins and Wright mounds are slightly similar while Norris Farms is significantly similar. What is interesting is that Robbins and Wright mounds are from Adena populations which are thought to be ancestral to the Ohio Hopewell. Also figure 14 suggests the Ohio Hopewell have some relationship with Norris Farms, which is further explored with the Fischer exact statistical test. The Glacial Kame sample populations as well as the Fort Ancient have significantly different Fischer exact statistical test results.

Sample	Probability	Standard error
Braden DeMoss	0.00164	.00027
Congdon	0.01288	0.00105
FortAncient	0.00519	0.00070
Hind	0.00092	0.00026
Norris Farms	0.12192	0.00457
Pyramid Lake	0.00206	0.00034
Robbins Mound	0.12322	0.00311
Wright Mound	0.06164	0.00457

Table 21: Fischer exact statistical test results for ancient native American populations

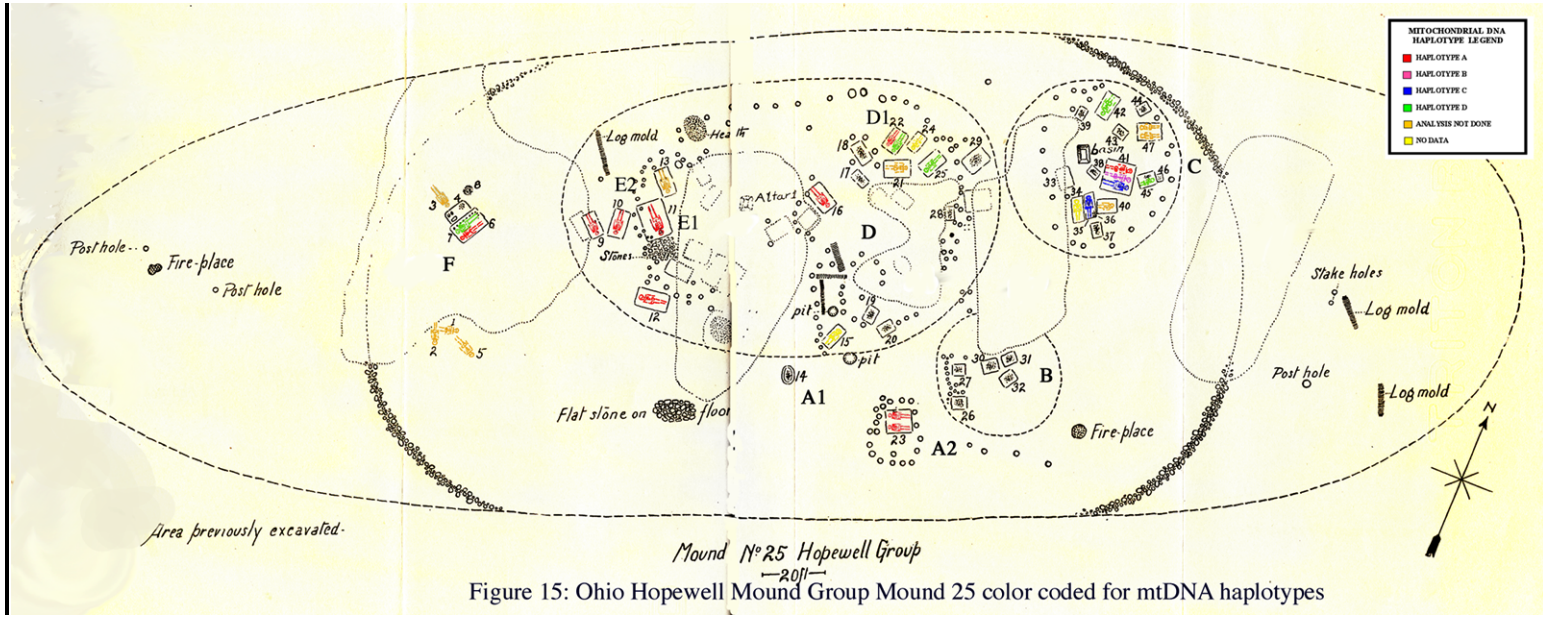


Figure 15: Ohio Hopewell Mound Group Mound 25 color coded for mtDNA haplotypes

Questions 3 and 4 will be evaluated with maps of Mound 25 of the Hopewell Mound Group. Individual burials that have been color coded for each haplotype in figure 15, which is the original Shetrone 1926 map but with Greber and Ruhl's 1989 area groupings overlayed. Questions 4 and 5 deal with kin relationships. Question 3 asks, “Is there evidence to support matrilineal descent among the Ohio Hopewell of the Hopewell Mound Group ?” and question 4 focuses in on “Is there segregation of individuals interred within the Ohio Hopewell Mound Group, specifically comparing Mounds 2 and 25 ?”. Beginning with question 4, it is important first to bring up the fact that cultural kinship is not always rooted in biological fact. It is a social construct in which any criteria could be construed as the basis for the kinship. For example, within Crow society the term for father and uncle are interchangeable. Kinship is culturally defined, not biologically determined, so that without written records or an oral tradition there is no way to know the kinship terminology or relationships for any culture. Figure 15 displays Mound 25, which has 22 successfully amplified individuals and each has been color coded for a haplotype. In order to examine the possibility of matrilineal descent, focus on the multiple burials in designated areas of Mound 25. Multiple burials exist in area A2, with burials 23N and 23S, area C , with triple burials of 41S, 41M, and 41N, and area F, with burials 6 and 7. In 3 out of the 4 multiple burials the haplotypes are different except for burials 23N and 23S. If matrilineal descent were to be based biologically, one would expect those multiple burials to be of the same haplotype, which is not the case. Greber (personal communication) suggests that burials 23N and 23S might belong to a different group than the other burials based upon unusual grave goods and possible later construction.

Question 4 focuses in on "Is there segregation of individuals interred within the Ohio Hopewell Mound Group, specifically comparing Mounds 2 and 25 ?". Tables 22 through 28 provide a break down of haplotype distribution per area.

Shetrone Burial Identification No.	Location within Mound 25	Mitochondrial Haplotype
11	E1	A
22W	D1	D
35	C	C
41S	C	A
25	D1	D
6	F	A
42	C	D
7	F	D
41M	C	B
23S	A2	A
12	E3	A
45	C	D
16	E1	A
41N	C	C
22S	D1	A
10	E2	A
23N	A2	A

Table 22: Distribution of haplotypes per area in Mound 25

Area : A2 of Mound 25	Mitochondrial Haplotype
23S	A
23N	A

Table 23: Distribution of haplotypes in area A2 of Mound 25

Area : C of Mound 25	Mitochondrial Haplotype
35	C
41S	A
42	D
41M	B
45	D
41N	C

Table 24: Distribution of haplotypes in area C of Mound 25

Area : D1 of Mound 25	Mitochondrial Haplotype
22W	D
25	D
22S	A

Table 25: Distribution of haplotypes in area D1 of Mound 25

Area : E1 of Mound 25	Mitochondrial Haplotype
11	A
16	A

Table 26: Distribution of haplotypes in area E1 of Mound 25

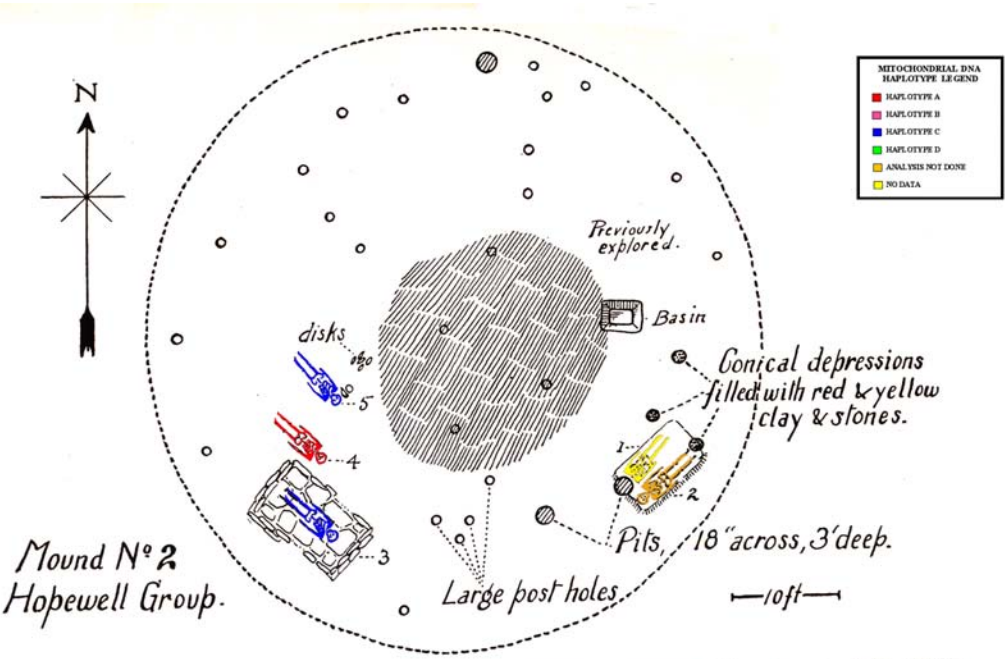
Area : E2 of Mound 25	Mitochondrial Haplotype
10	A

Table 27: Distribution of haplotypes in area E2 of Mound 25

Area : F of Mound 25	Mitochondrial Haplotype
6	A
7	D

Table 28: Distribution of haplotypes in area F of Mound 25

Unfortunately, due to small sample size no statistical analysis was available to test for segregation. However, based upon a visual inspection of figure 15 and tables 22 through 27, mtDNA haplotypes segregation does not seem to be dependent upon burial location within of Mound 25. For example, burials interred in areas A2 and E1 are the same haplotype. However, burial interred in areas C, D1, and F have a range of haplotypes suggesting the possibility of different groups coming in to bury their dead in specific areas. When viewing figure 16, color coding of Mound 2, no segregation of burials is apparent in that mound. Greber(personal communication) suggests that burial 3 of mound 2 might be related to individuals at the Turner site, due to similar burial style.



Floor Plan of Mound Number 2. Shaded Area Corresponds to location of Great Deposit of Flint Disks.

Figure 16: Color coded mtDNA haplotypes of Mound 2 (Shetrone 1926)

The last section of this chapter will examine the level of genetic diversity within the Ohio Hopwell and 12 other Native American populations, utilizing three genetic indices. The geographical location of all 13 populations is listed in figure 17 and table 20 gives the reference for each sample. 419 aligned HVI sequences were exported to Mac Clade 3.08a (Maddison and Maddison 2000) to be filtered, all identical sequences were removed so only one copy of a sequence represented the duplicates and cropped, the right and left sides of longer sequences were reduced so that all sequences were the same length as the shortest sequence which was from 16084 to 16408. All indices were calculated in Arlequin software package (Schneider et al. 1997). Each index will begin with its theoretical framework and assumptions. The first genetic index discussed will be nucleotide polymorphisms or theta. Theta is the proportion of nucleotide sites that are expected to be polymorphic (Watterson 1975). An approximation of variance can be derived under the assumption that the nucleotides at the site are functionally equivalent (Hartl and Clark 1979). The majority of mutations are assumed to be either silent or non-coding, thus not changing the amino acid, and producing the observed nucleotide polymorphism (Hartl and Clark 1979). Theta also resides under a model of neutral theory (Kimura 1980), and assumes all alleles are invisible to natural selection. This assumption creates a problem for small sample sizes due to the possibility of the sample being mutationally monomorphic such as the Fort Ancient sample of 8 which are all haplotype C. Table 30 displays the theta value, π value and Tajima D statistic for the Ohio Hopewell and 12 other sample populations. The theta values in Table 29, show an

increase as population size increases. Nucleotide diversity, π, is the proportion of nucleotide differences between all possible pairs of sequences in a sample (Hartl and Clark 1979). If all sequences are the same, the population is monomorphic.

1. Alaskan Athabascan
2. Bella Coola
3. Ohio Hopewell
4. Norris Farms
5. Nuu-chah-nulth
6. Pyramid Lake
7. St. Paul
8. Haida
9. Canadian Inuit
10. Native North Americans
11. Maya
12. Dogrib
13. Stillwater March

Figure 17: Location of 13 populations utilized in Arlequin

Sample	theta	π	D
1	.011	.014	-.69
2	.002	.017	-1.4
3	0.00	.009	0.0
4	0.00	.006	-1.6
5	.050	.019	-.55
6	.005	.017	-.12
7	.491	.017	.29
8	.035	.004	-1.2
9	0.00	.008	-1.2
10	.004	.015	-.54
11	.008	.012	-.93
12	.062	.014	-1.0
13	.001	.007	0.0

Populations

1. Ohio Hopewell
2. Northern Native Americans
3. Dogrib
4. Alaskan Athabascan
5. Maya
6. Nuu-chah-nulth
7. Bella Coola
8. Canadian Inuit
9. Haida
10. Norris Farms
11. St. Paul
12. Pyramid Lake
13. Still Water Marsh

Table 29: Distribution of Nucleotide diversity, Theta,π and Tajima's D

Notice that theta and π are in agreement for the Ohio Hopewell, which is the same case for the majority of the samples, except for the Maya, Bella Coola, Canadian Inuit, and Pyramid Lake. It might be the effect of small sample size however, the Bella Coola and Canadian Inuit have a fairly large sample. Tajima's D is an estimate of how much selection pressure has been exerted on a particular gene over the course of evolution (Tajima 1989). Tajima's D follows the infinite allele model which assumes a number of nucleotide sites is large enough that each new mutation occurs at a site that has not mutated in the past (Kimura and Ohta1969). If result is negative, there is a deficiency of

the less common type gene and if result is positive, heterozygotes were favored in the past. The Ohio Hopewell have a negative result which could be due to an excess of a common type, in this case haplotype A or and deficiency of the least common type, haplotype B.

CHAPTER 6

CONCLUSION

The results of this study add to an already growing body of research dedicated to mtDNA analysis. In recent years, research utilizing Y- chromosome and STR analysis have also begun to add to what is known about the genetic variation present in Native Americans both ancient and modern. Combine that information with mtDNA, and a complete picture of migration, gene flow and kinship begins to take shape in North America. The Ohio Hopewell through an analysis of their mutations have revealed ancestral relationships with individuals in China, Korea, Japan, Taiwan, Mongolia, Russia and South America. The Ohio Hopewell also display mutations that are uniquely their own, such as haplotype A 16166, haplotype B 16247and 16265. The unique mutations of the Ohio Hopwell of Hopewell Mound Group may have been lost through attrition or have yet to be discovered in other Native American populations. The Ohio Hopewell also share a unique mutation with the Cahokians of 72Sub2 Mound , utilized to denote a rare haplotype aligned with individuals of high status at Cahokia.

Phylogenetic analysis through Neighbor Joining trees revealed that the Ohio Hopewell group with those individuals who share the basic A through D Native American

haplotypes. Upon closer examination, the Ohio Hopewell clustered with both ancient and modern groups of Native Americans. RFLP Neighbor Joining trees revealed that the Ohio Hopewell do not group with samples from Fort Ancient populations of the Ohio River Valley, but with samples from Glacial Kame, Adena or Norris Farms, possibly indicating some relationship between the groups. This in part could be due to small sample size and a low number of sites that have been amplified. More work within all of the Ohio River Valley cultures is needed to give a clearer picture to archaeologists, linguists and biological anthropologists alike. Also examined were hypotheses detailing the migration of the Glacial Kame/Red Ocher groups into the northeast. The Ohio Hopewell did not supply evidence to support these hypotheses. This research did add to the growing body of work on the Hopewell. Matrilineal descent at least at the Hopewell Mound Group is not supported by this research. Also, based upon a visual analysis of the mtDNA haplotypes at the Hopewell Mound Group, as well as lack of homogeneity in multiple burials, segregation does not seem to be occurring at the site. More analysis is needed for the remaining burials in order to flesh out the rest of the site and compare the patterns. Finally, genetic indices were produced which present the Ohio Hopewell as a diverse and heterogenetic group, possessing four out of the five possible Native American haplotypes and presenting as an expanding population. This information also adds to the debate of the number of original waves of Native Americans to migrate into the North America. The Ohio Hopewell would suggest only one wave containing all the haplotypes that are present today in modern Native American populations.

Finally, this research provides a model for multidisciplinary research, drawing from all four fields of anthropology. Each field complementing the work of the other fields to produce a complete picture. However, due to the small sample size and restriction to mtDNA, future work could add to what is already known about the Ohio Hopewell. Contributions were also made in the struggle to deal with the bane of ancient DNA work, contamination. The procedure outlined in this research could help preserve information thought lost to contamination.

APPENDIX A

NUMBER OF POLYMERASE CHAIN REACTION TESTS PER SAMPLE

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150028 YP	1	Y	Y	5	5	4	4	9	9
150028d1-10YP	1	N	Y	0	5	1	4	1	9
150028d1-50YP	1	N	N	0	0	1	1	1	1
150028d1-100YP	1	N	N	0	0	1	1	1	1
150028(2)YPYP	9	Y	N	5	0	4	1	9	1
150028d1-10 (2) YP	9	Y	N	5	0	4	1	9	1
150d0281-50(2) YP	9	N	N	0	0	1	1	1	1
150028d1-100(2) Y P	9	N	N	0	0	1	1	1	1
150036 YP	1	Y	Y	5	5	4	4	9	9
150360d1-10 YP	1	N	N	0	0	1	1	1	1
150036d1-50 YP	1	N	N	0	0	1	1	1	1
150036d1-100 YP	1	N	N	0	0	1	1	1	1
150036(2)YP	9	N	N	0	0	1	1	1	1
150036d1-10(2) YP	9	N	N	0	0	1	1	1	1
150036d1-50(2) YP	9	N	N	0	0	1	1	1	1
150036d1-100 (2) YP	9	N	N	0	0	1	1	1	1
150037YP	1	Y	Y	5	5	4	4	9	9
150037d1-10 YP	1	N	N	0	0	1	1	1	1
150037d1-50 YP	1	N	N	0	0	1	1	1	1
150037d1-100 YP	1	N	N	0	0	1	1	1	1
150037 (2)YP	9	N	N	0	0	1	1	1	1
150037d1-10 (2) YP	9	N	N	0	0	1	1	1	1
150037d1-50(2) YP	9	N	N	0	0	1	1	1	1
150037d1-100 (2)YP	9	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150040 YP	2	Y	Y	5	5	4	4	9	9
150040d1-10 YP	2	Y	N	5	0	4	1	9	1
150040d1-50 YP	2	N	Y	5	0	4	1	9	1
150040d1-100 YP	2	Y	Y	5	5	4	4	9	9
150040 (2)YP	9	N	N	0	0	1	1	1	1
150040d1-10(2) YP	9	N	N	0	0	1	1	1	1
150040d1-50(2) YP	9	N	N	0	0	1	1	1	1
150040d1-100(2) YP	9	N	N	0	0	1	1	1	1
150042 YP	2	N	N	0	0	1	1	1	1
150042d1-10 YP	2	N	N	0	0	1	1	1	1
150042d1-50 YP	2	N	N	0	0	1	1	1	1
150042d1-100 YP	2	N	N	0	0	1	1	1	1
150042 (2)YP	10	N	N	0	0	1	1	1	1
150042d1-10(2) YP	10	N	N	0	0	1	1	1	1
150042d1-50(2) YP	10	N	N	0	0	1	1	1	1
150042d1-100(2) YP	10	N	N	0	0	1	1	1	1
150043 YP	2	Y	Y	5	5	4	4	9	9
150043d1-10 YP	2	Y	N	5	0	4	1	9	1
150043d1-50 YP	2	N	N	0	0	1	1	1	1
150043d1-100 YP	2	N	N	0	0	1	1	1	1
150043 (2)YP	10	N	N	0	0	1	1	1	1
150043d1-10(2) YP	10	N	N	0	0	1	1	1	1
150043d1-50(2) YP	10	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
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7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150043d1-100(2) YP	10	N	N	0	0	1	1	1	1
150050 YP	3	Y	Y	5	5	4	4	9	9
150050d1-10 YP	3	Y	Y	5	5	4	4	9	9
150050d1-50 YP	3	N	Y	0	5	1	4	1	9
150050d1-100 YP	3	N	Y	0	5	1	4	1	9
150050 (2)YP	10	N	Y	0	5	1	4	1	9
150050d1-10(2) YP	10	Y	Y	5	5	4	4	9	9
150050d1-50(2) YP	10	N	Y	0	5	1	4	1	9
150050d1-100 (2) YP	10	N	Y	0	5	1	4	1	9
150051 YP	3	N	N	0	0	1	1	1	1
150051d1-10 YP	3	N	N	0	0	1	1	1	1
150051d1-50 YP	3	N	N	0	0	1	1	1	1
150051d1-100 YP	3	N	N	0	0	1	1	1	1
150051 (2)YP	10	N	N	0	0	1	1	1	1
150051d1-10(2) YP	10	N	N	0	0	1	1	1	1
150051d1-50(2) YP	10	N	N	0	0	1	1	1	1
150051d1-100(2) YP	10	N	N	0	0	1	1	1	1
150059 YP	3	N	N	0	0	1	1	1	1
150059d1-10 YP	3	N	N	0	0	1	1	1	1
150059d1-50 YP	3	N	N	0	0	1	1	1	1
150059d1-100 YP	3	N	N	0	0	1	1	1	1
150059 (2)YP	10	N	N	0	0	1	1	1	1
150059d1-10(2) YP	10	N	N	0	0	1	1	1	1
150059d1-50(2) YP	10	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150059d1-100 (2) YP	10	N	N	0	0	1	1	1	1
150066 YP - 1 tooth	4	N	N	0	0	1	1	1	1
150066d1-10 YP	4	N	N	0	0	1	1	1	1
150066d1-50 YP	4	N	N	0	0	1	1	1	1
150066d1-100 YP	4	N	N	0	0	1	1	1	1
150068 YP	4	Y	N	5	0	4	1	9	1
150068d1-10 YP	4	Y	N	5	0	4	1	9	1
150068d1-50 YP	4	N	N	0	0	1	1	1	1
150068d1-100 YP	4	N	N	0	0	1	1	1	1
150068 (2)YP	11	Y	N	5	0	4	1	9	1
150068d1-10(2) YP	11	Y	N	5	0	4	1	9	1
150068d1-50(2) YP	11	Y	N	5	0	4	1	9	1
150068d1-100 (2) YP	11	N	N	5	0	4	1	9	1
150069 YP	4	Y	N	5	0	4	1	9	1
150069d1-10 YP	4	N	N	0	0	1	1	1	1
150069d1-50 YP	4	N	N	0	0	1	1	1	1
150069d1-100 YP	4	N	N	0	0	1	1	1	1
150069 (2)YP	11	Y	N	5	0	4	1	9	1
150069d1-10(2) YP	11	N	N	0	0	1	1	1	1
150069d1-50(2) YP	11	N	N	0	0	1	1	1	1
150069d1-100(2 YP	11	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150078 YP	5	N	N	0	0	1	1	1	1
150078d1-10 YP	5	N	N	0	0	1	1	1	1
150078d1-50 YP	5	N	N	0	0	1	1	1	1
150078d1-100 YP	5	N	N	0	0	1	1	1	1
150078 (2)YP	11	N	N	0	0	1	1	1	1
150078d1-10(2) YP	11	N	N	0	0	1	1	1	1
150078d1-50(2) YP	11	N	N	0	0	1	1	1	1
150078d1-100(2) YP	11	N	N	0	0	1	1	1	1
150081 YP	5	N	N	0	0	1	1	1	1
150081d1-10 YP	5	N	N	0	0	1	1	1	1
150081d1-50 YP	5	N	N	0	0	1	1	1	1
150081d1-100 YP	5	N	N	0	0	1	1	1	1
150081 (2)YP	11	N	N	0	0	1	1	1	1
150081d1-10(2) YP	11	N	N	0	0	1	1	1	1
150081d1-50(2) YP	11	N	N	0	0	1	1	1	1
150081d1-100(2) YP	11	N	N	0	0	1	1	1	1
150083 YP - 1 tooth	5	N	N	0	0	1	1	1	1
150083d1-10 YP	5	N	N	0	0	1	1	1	1
150083d1-50 YP	5	N	N	0	0	1	1	1	1
150083d1-100 YP	5	N	N	0	0	1	1	1	1

Legend

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2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
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7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150102 YP	6	N	N	0	0	1	1	1	1
150102d1-10 YP	6	N	N	0	0	1	1	1	1
150102d1-50 YP	6	N	N	0	0	1	1	1	1
150102d1-100 YP	6	N	N	0	0	1	1	1	1
150102 (2)YP	12	N	N	0	0	1	1	1	1
150102d1-10(2) YP	12	N	N	0	0	1	1	1	1
150102d1-50(2) YP	12	N	N	0	0	1	1	1	1
150102d1-100(2) YP	12	N	N	0	0	1	1	1	1
150109 YP	6	N	N	0	0	1	1	1	1
150109d1-10 YP	6	N	N	0	0	1	1	1	1
150109d1-50 YP	6	N	N	0	0	1	1	1	1
150109d1-100 YP	6	N	N	0	0	1	1	1	1
150109 (2)YP	12	Y	N	5	0	4	1	9	1
150109d1-10(2) YP	12	N	N	0	0	1	1	1	1
150109d1-50(2) YP	12	N	N	0	0	1	1	1	1
150109d1-100(2) YP	12	N	N	0	0	1	1	1	1

Legend

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8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150112 YP	6	N	N	0	0	1	1	1	1
150112d1-10 YP	6	N	N	0	0	1	1	1	1
150112d1-50 YP	6	N	N	0	0	1	1	1	1
150112d1-100 YP	6	N	N	0	0	1	1	1	1
150112 (2)YP	12	Y	N	5	0	4	1	9	1
150112d1-10(2) YP	12	N	N	0	0	1	1	1	1
150112d1-50(2) YP	12	N	N	0	0	1	1	1	1
150112d1-100(2) YP	12	N	N	0	0	1	1	1	1
150117 YP	7	N	N	0	0	1	1	1	1
150117d1-10 YP	7	N	N	0	0	1	1	1	1
150117d1-50 YP	7	N	N	0	0	1	1	1	1
151170d1-100 YP	7	N	N	0	0	1	1	1	1
150117 (2)YP	12	N	N	0	0	1	1	1	1
150117d1-10(2) YP	12	N	N	0	0	1	1	1	1
150117d1-50(2) YP	12	N	N	0	0	1	1	1	1
150117d1-100(2) YP	12	N	N	0	0	1	1	1	1
150118 YP	7	N	N	0	0	1	1	1	1
150118d1-10 YP	7	N	N	0	0	1	1	1	1
150118d1-50 YP	7	N	N	0	0	1	1	1	1
150118d1-100 YP	7	N	N	0	0	1	1	1	1
150118 (2)YP	13	N	N	0	0	1	1	1	1
150118d1-10(2) YP	13	N	N	0	0	1	1	1	1
150118d1-50(2) YP	13	N	N	0	0	1	1	1	1
150118d1-100(2) YP	13	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150121 YP	7	Y	Y	5	5	4	5	9	9
150121d1-10 YP	7	N	Y	0	5	1	4	1	9
150121d1-50 YP	7	N	Y	0	5	1	4	1	9
150121d1-100 YP	7	N	N	0	0	1	1	1	1
150121 (2)YP	13	Y	Y	5	5	4	5	9	9
150121d1-10(2) YP	13	Y	Y	5	5	4	5	9	9
150121d1-50(2 YP	13	N	N	0	0	1	1	1	1
150121d1-100(2) YP	13	N	N	0	0	1	1	1	1
150122 YP	7	Y	Y	5	5	4	5	9	9
150122d1- 10 YP	7	Y	Y	5	5	4	5	9	9
150122d1-50 YP	7	N	N	0	0	1	1	1	1
150122d1-100 YP	7	N	N	0	0	1	1	1	1
150122 (2)YP	13	Y	N	5	0	4	1	9	1
150122d1-10(2) YP	13	Y	N	5	0	4	1	9	1
150122d1-50(2) YP	13	N	Y	0	5	1	4	1	9
150122d1-100(2) YP	13	N	N	0	0	1	1	1	1
150129 YP	8	N	N	0	0	1	1	1	1
150129d1- 10 YP	8	N	N	0	0	1	1	1	1
150129d1-50 YP	8	N	N	0	0	1	1	1	1
150129d1-100 YP	8	N	N	0	0	1	1	1	1
150129 (2)YP	13	N	N	0	0	1	1	1	1
150129d1-10(2) YP	13	N	N	0	0	1	1	1	1
150129d1-50(2) YP	13	N	N	0	0	1	1	1	1
150129d1-100(2) YP	13	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150131 YP	8	N	N	0	0	1	1	1	1
150131d1- 10 YP	8	N	N	0	0	1	1	1	1
150131d1-50 YP	8	N	N	0	0	1	1	1	1
150131d1-100 YP	8	N	N	0	0	1	1	1	1
150131 (2)YP	14	N	N	0	0	1	1	1	1
150131d1-10(2) YP	14	N	N	0	0	1	1	1	1
150131d1-50(2) YP	14	N	N	0	0	1	1	1	1
150131d1-100(2) YP	14	N	N	0	0	1	1	1	1
150137 YP	8	Y	Y	5	5	4	5	9	9
150137d1P- 10 YP	8	N	N	0	0	1	1	1	1
150137d1-50 YP	8	N	N	0	0	1	1	1	1
150137d1-100 YP	8	N	N	0	0	1	1	1	1
150137 (2)YP	14	N	N	0	0	1	1	1	1
150d1371-10(2) YP	14	Y	N	5	0	4	1	9	1
150137d1-50(2) YP	14	Y	N	5	0	4	1	9	1
150137d1-100(2) YP	14	N	Y	0	5	1	4	1	9
150139 YP	8	Y	Y	5	5	4	4	9	9
150139d1- 10 YP	8	Y	Y	5	5	4	4	9	9
150139d1-50 YP	8	N	N	0	0	1	1	1	1
150139d1-100 YP	8	N	N	0	0	1	1	1	1
150139 (2)YP	14	N	Y	0	5	1	4	1	9
150139d1-10(2) YP	14	Y	Y	5	5	4	4	9	9
150139d1-50(2) YP	14	Y	N	5	0	4	1	9	1
150139d1-100(2) YP	14	N	Y	0	5	1	4	1	9

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150053 YP	15	N	N	0	0	1	1	1	1
150053d1- 10 YP	15	N	Y	0	5	1	4	1	9
150053d1-50 YP	15	N	N	0	0	1	1	1	1
150053d1-100 YP	15	N	N	0	0	1	1	1	1
150053 (2)YP	21	N	N	0	0	1	1	1	1
150053d1-10(2) YP	21	Y	Y	5	5	4	4	9	9
150053d1-50(2) YP	21	N	N	0	0	1	1	1	1
150053d1-100(2)YP	21	N	N	0	0	1	1	1	1
150055 YP	15	Y	N	5	0	4	1	9	1
150055d1- 10 YP	15	N	N	0	0	1	1	1	1
150055d1-50 YP	15	N	N	0	0	1	1	1	1
150055d1-100 YP	15	N	N	0	0	1	1	1	1
150055 (2)YP	21	Y	Y	5	5	4	4	9	9
150055d1-10(2) YP	21	N	Y	0	5	1	4	1	9
150055d1-50(2) YP	21	N	Y	0	5	1	4	1	9
150055d1-100(2) YP	21	N	Y	0	5	1	4	1	9
150056 YP	15	Y	Y	5	5	4	4	9	9
150056d1- 10YP	15	N	N	0	0	1	1	1	1
150056d1-50 YP	15	N	N	0	0	1	1	1	1
150056d1-100 YP	15	Y	Y	5	5	4	4	9	9
150056 (2)YP	21	Y	Y	5	5	4	4	9	9
150056d1-10(2) YP	21	N	N	0	0	1	1	1	1
150056d1-50(2) YP	21	N	N	0	0	1	1	1	1
150056d1-100(2) YP	21	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150057 YP	15	N	N	0	0	1	1	1	1
150057d1- 10 YP	15	Y	N	5	0	4	1	9	1
150057d1-50 YP	15	Y	N	5	0	4	1	9	1
150057d1-100 YP	15	N	N	0	0	1	1	1	1
150057 (2)YP	21	N	N	0	0	1	1	1	1
150057d1-10(2) YP	21	N	N	0	0	1	1	1	1
150057d1-50(2) YP	21	N	N	0	0	1	1	1	1
150057d1-100(2) YP	21	N	Y	0	5	1	4	1	9
150058 YP	16	N	Y	0	5	1	4	1	9
150058d1- 10 YP	16	N	Y	0	5	1	4	1	9
150058d1-50 YP	16	Y	Y	5	5	4	4	9	9
150058d1-100 YP	16	N	Y	0	5	1	4	1	9
150058 (2)YP	22	N	Y	0	5	1	4	1	9
150058d1-10(2) YP	22	N	Y	0	5	1	4	1	9
150058d1-50(2) YP	22	N	Y	0	5	1	4	1	9
150058d1-100(2) YP	22	N	N	0	0	1	1	1	1
150061 YP	16	N	Y	0	5	1	4	1	9
150061d1-10 YP	16	N	N	0	0	1	1	1	1
150061d1-50 YP	16	N	N	0	0	1	1	1	1
150061d1-100 YP	16	N	N	0	0	1	1	1	1
150061 (2)YP	22	N	N	0	0	1	1	1	1
150061d1-10(2) YP	22	Y	N	5	0	4	1	9	1
150061d1-50(2) YP	22	N	N	0	0	1	1	1	1
150061d1-100(2) YP	22	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150062 YP	16	N	N	0	0	1	1	1	1
150062d1- 10 YP	16	N	N	0	0	1	1	1	1
150062d1-50 YP	16	N	N	0	0	1	1	1	1
150062d1-100 YP	16	N	N	0	0	1	1	1	1
150062 (2)YP	22	N	N	0	0	1	1	1	1
150062d1-10(2) YP	22	N	N	0	0	1	1	1	1
150062d1-50(2)YP	22	N	N	0	0	1	1	1	1
150062d1-100(2) YP	22	N	N	0	0	1	1	1	1
150066 YP - 2 teeth	16	Y	N	5	0	4	1	9	1
150066d1-10 YP	16	N	N	0	0	1	1	1	1
150066d1-50 YP	16	N	N	0	0	1	1	1	1
150066d1-100 YP	16	N	N	0	0	1	1	1	1
150066 (2)YP	22	N	Y	0	5	1	4	1	9
150066d1-10(2) YP	22	N	Y	0	5	1	4	1	9
150066d1-50(2) YP	22	N	Y	0	5	1	4	1	9
150066d1-100(2) YP	22	N	N	0	0	1	1	1	1
150107 YP	17	N	N	0	0	1	1	1	1
150107d1- 10 YP	17	N	N	0	0	1	1	1	1
150107d1-50 YP	17	N	N	0	0	1	1	1	1
150107d1-100 YP	17	N	N	0	0	1	1	1	1
150107 (2)YP	23	N	N	0	0	1	1	1	1
150107d1-10(2) YP	23	N	N	0	0	1	1	1	1
150107d1-50(2) YP	23	N	N	0	0	1	1	1	1
150107d1-100(2)YP	23	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150108 YP	17	N	N	0	0	1	1	1	1
150108d1- 10 YP	17	N	N	0	0	1	1	1	1
150108d1-50 YP	17	N	N	0	0	1	1	1	1
150108d1-100 YP	17	N	N	0	0	1	1	1	1
150108 (2)YP	23	N	N	0	0	1	1	1	1
150108d1-10(2) YP	23	N	N	0	0	1	1	1	1
150108d1-50(2) YP	23	N	N	0	0	1	1	1	1
150108d1-100(2) YP	23	N	N	0	0	1	1	1	1
150115 YP	17	Y	Y	5	5	4	4	9	9
150115d1- 10 YP	17	Y	N	5	0	4	1	9	1
150115d1-50 YP	17	Y	N	5	0	4	1	9	1
150115d1-100 YP	17	N	N	0	0	1	1	1	1
150115 (2)YP	23	N	Y	0	5	1	4	1	9
150115d1-10(2)YP	23	Y	Y	5	5	4	4	9	9
150115d1-50(2) YP	23	N	N	0	0	1	1	1	1
150115d1-100(2) YP	23	N	N	0	0	1	1	1	1
150116 YP	17	N	N	0	0	1	1	1	1
150116d1- 10 YP	17	Y	N	5	0	1	4	1	9
150116d1-50 YP	17	N	Y						
150116d1-100 YP	17	N	N	0	0	1	1	1	1
150116 (2)YP	23	N	Y	0	5	4	1	9	1
150116d1-10(2) YP	23	N	Y	0	5	1	4	1	9
150116d1-50(2) YP	23	Y	N	5	0	1	4	1	9
150116d1-100(2) YP	23	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150123 YP	18	N	Y	0	5	1	4	1	9
150123d1- 10 YP	18	Y	N	5	0	1	4	1	9
150123d1-50 YP	18	N	Y	0	5	1	4	1	9
150123d1-100 YP	18	Y	N	5	0	1	4	1	9
150123 (2)YP	24	Y	N	5	0	1	4	1	9
150123d1-10(2) YP	24	Y	N	5	0	1	4	1	9
150123d1-50(2) YP	24	Y	N	5	0	1	4	1	9
150123d1-100(2) YP	24	Y	N	5	0	1	4	1	9
150124 YP	18	N	N	0	0	1	1	1	1
150124d1- 10 YP	18	Y	N	5	0	1	4	1	9
150124d1-50 YP	18	N	N	0	0	1	1	1	1
150124d1-100 YP	18	N	Y	0	5	1	4	1	9
150124 (2)YP	24	N	N	0	0	1	1	1	1
150124d1-10(2) YP	24	Y	N	5	0	1	4	1	9
150124d1-50(2) YP	24	N	N	0	0	1	1	1	1
150124d1-100(2)YP	24	N	N	0	0	1	1	1	1
150128 YP	18	N	Y	0	5	1	4	1	9
150128d1- 10 YP	18	N	Y						
150128d1-50 YP	18	Y	N	5	0	1	4	1	9
150128d1-100 YP	18	N	N	0	0	1	1	1	1
150128 (2)YP	24	Y	Y	5	5	4	4	9	9
150128d1-10(2) YP	24	Y	Y	5	5	4	4	9	9
150128d1-50(2) YP	24	N	N	0	0	1	1	1	1
150128d1-100(2) YP	24	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150143 YP	18	N	N	0	0	1	1	1	1
150143d1- 10 YP	18	Y	N	5	0	1	4	1	9
150143d1-50 YP	18	Y	Y	5	5	4	4	9	9
150143d1-100 YP	18	Y	Y	5	5	4	4	9	9
150143 (2)YP	24	Y	N	5	0	1	4	1	9
150143d1-10(2) YP	24	N	N	0	0	1	1	1	1
150143d1-50(2) YP	24	Y	N	5	0	1	4	1	9
150143d1-100(2) YP	24	N	Y	0	5	1	4	1	9
150164 YP	19	Y	Y	5	5	4	4	9	9
150164d1- 10 YP	19	Y	Y	5	5	4	4	9	9
150164d1-50 YP	19	N	N	0	0	1	1	1	1
150164d1-100 YP	19	N	Y	0	5	1	4	1	9
150164 (2)YP	25	N	Y	0	5	1	4	1	9
150164d1-10(2) YP	25	N	Y	0	5	1	4	1	9
150164d1-50(2) YP	25	N	N	0	0	1	1	1	1
150164d1-100(2) YP	25	N	N	0	0	1	1	1	1
150165 YP	19	N	N	0	0	1	1	1	1
150165d1- 10 YP	19	N	Y	0	5	1	4	1	9
150165d1-50 YP	19	N	N	0	0	1	1	1	1
150165d1-100 YP	19	N	N	0	0	1	1	1	1
150165 (2)YP	25	N	N	0	0	1	1	1	1
150165d1-10(2) YP	25	N	N	0	0	1	1	1	1
150165d1-50(2) YP	25	N	N	0	0	1	1	1	1
150165d1-100(2) YP	25	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150166 YP	19	N	N	0	0	1	1	1	1
150166d1-10 YP	19	Y	N						
150166d1-50 YP	19	N	N	0	0	1	1	1	1
150166d1-100 YP	19	N	Y	0	5	1	4	1	9
150166 (2)YP	25	N	N	0	0	1	1	1	1
150166d1-10(2) YP	25	N	Y	0	5	1	4	1	9
150166d1-50(2) YP	25	N	N	0	0	1	1	1	1
150166d1-100(2) YP	25	N	Y	0	5	1	4	1	9
150209 YP	19	Y	Y	5	5	4	4	9	9
150209d1-10 YP	19	Y	Y	5	5	4	4	9	9
150209d1-50 YP	19	N	Y	0	5	1	4	1	9
150209d1-100 YP	19	Y	N	5	0	1	4	1	9
150209 (2)YP	25	N	Y	0	5	1	4	1	9
150209d1-10(2) YP	25	N	Y	0	5	1	4	1	9
150209d1-50(2) YP	25	N	Y	0	5	1	4	1	9
150209d1-100(2) YP	25	N	Y	0	5	1	4	1	9
150210 YP	20	Y	Y	5	5	4	4	9	9
150210d1-10 YP	20	Y	N	5	0	4	1	9	1
150210d1-50 YP	20	Y	Y	5	5	4	4	9	9
150210d1-100 YP	20	Y	N	5	0	1	4	1	9
150210 (2)YP	25	N	Y	0	5	1	4	1	9
150210d1-10(2) YP	25	N	Y	0	5	1	4	1	9
150210d1-50(2) YP	25	N	Y	0	5	1	4	1	9
150210d1-100(2) YP	25	N	N	0	0	1	1	1	1

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	1	2	3	4	5	6	7	8	9
150212 YP	20	Y	Y	5	5	4	4	9	9
150212d1-10 YP	20	N	Y	0	5	1	4	1	9
150212d1-50 YP	20	N	Y	0	5	1	4	1	9
150212d1-100 YP	20	N	Y	0	5	1	4	1	9
150212 (2)YP	26	Y	Y	5	5	4	4	9	9
150212d1-10(2) YP	26	N	Y	0	5	1	4	1	9
150212d1-50(2) YP	26	Y	N	5	0	1	4	1	9
150212d1-100(2) YP	26	N	N	0	0	1	1	1	1
150213 YP	20	Y	N	5	0	1	4	1	9
150213d1-10 YP	20	Y	N	5	0	1	4	1	9
150213d1-50 YP	20	N	N						
150213d1-100 YP	20	N	Y	0	5	1	4	1	9
150213 (2)YP	26	N	Y	0	5	1	4	1	9
150213d1-10(2) YP	26	N	Y	0	5	1	4	1	9
150213d1-50(2) YP	26	Y	Y	5	5	4	4	9	9
150213d1-100(2) YP	26	N	Y	0	5	1	4	1	9
150215 YP	20	N	Y	0	5	1	4	1	9
150215d1-10 YP	20	Y	Y	5	5	4	4	9	9
150215d1-50 YP	20	N	N	0	0	1	1	1	1
150215d1-100 YP	20	N	N	0	0	1	1	1	1
150215 (2)YP	26	Y	N	5	0	1	4	1	9
150215d1-10(2)YP	26	N	Y	0	5	1	4	1	9
150215d1-50(2) YP	26	N	Y	0	5	1	4	1	9
150215d1-100(2) YP	26	Y	Y	5	5	4	4	9	9

Legend

1 Extraction Number
2 Amplified (Y=Yes or N=No) Yang Extraction Protocol
3 Amplified (Y=Yes or N=No) Phenol-Chloroform Extraction Protocol
4 Number of RFLP Polymerase Chain Reactions with Yang Extraction Protocol
5 Number of RFLP Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
6 Number of HVI Polymerase Chain Reactions with Yang Extraction Protocol
7 Number of HVI Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol
8 Total Number of Polymerase Chain Reactions with Yang Extraction Protocol
9 Total Number of Polymerase Chain Reactions with Phenol-Chloroform Extraction Protocol

Sample Ident.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
150219 YP	20	N	N	0	0	1	1	1	1
150219d1-10 YP	20	N	N	0	0	1	1	1	1
150219d1-50 YP	20	N	N	0	0	1	1	1	1
150219d1-100 YP	20	N	N	0	0	1	1	1	1
150219 (2)YP	26	N	Y	0	5	1	4	1	9
150219d1-10(2) YP	26	Y	Y	5	5	4	4	9	9
150219d1-50(2) YP	26	Y	N	5	0	1	4	1	9
150219d1-100(2) YP	*26*	Y	Y	5	5	4	4	9	9

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