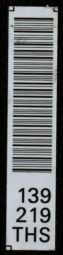

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Suzanne Linda Shunn

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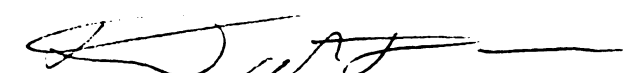

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11/23/05

Date

DNA BASED ANCESTRY ANALYSIS OF HUMAN SKELETAL REMAINS FROM FORT MICHILIMACKINAC (1743-1781)

By

Suzanne Linda Shunn

A THESIS

Submitted to
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ABSTRACT

DNA BASED ANCESTRY ANALYSIS OF HUMAN SKELETAL REMAINS FROM FORT MICHILIMACKINAC (1743-1781)

By

Suzanne Linda Shunn

Ancestry analysis was performed on 15 sets of human skeletal remains interred in Fort Michilimackinac, Michigan, between 1743 and 1781. Fort Michilimackinac was built by the French in order to help regulate the fur trade. Intermarriage between Native American women and French men was reportedly common in communities centered on the fur trade. These unions produced allies, and created kin networks between French and Native American traders. Therefore, while male remains from the fort were likely European, female remains were more apt to be Native American. Consequently, analysis of subadults' mtDNA would also be predicted to be Native American since it is maternally inherited. Analysis of geographically specific mtDNA haplogroups was used to determine the ancestry of 13 adults (8 males, 4 females, 1 undetermined) and 2 subadults from Fort Michilimackinac. A method for detecting mtDNA haplogroups for Native Americans (haplogroups A, B, C, D and X) and some Europeans (haplogroups H and J) was created using SNP analysis of the mtDNA coding region. None of the burials from Fort Michilimackinac were found to be Native American (although one has an unknown ancestry). These results provide a new tool for understanding the life and culture of Fort Michilimackinac, and a method for detecting ancestry using SNP analysis.

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INTRODUCTION

The analysis of genetic data from ancient skeletal remains holds the potential for uncovering characteristics of a past culture, people, or individual that would otherwise be unobtainable. When skeletal remains are found several questions must be answered, the first of which is whether they are human or non-human (Byers 2002). Next, it must be determined if they are contemporary (died within 50 years) or noncontemporary, in order to establish their medicolegal significance. The context of remains when found, personal belongings, body modifications (such as dental work) and preservation can help solve both these questions. For instance, bones found buried in graves in an area where interments were known to occur in the 1700's are likely to be human and noncontemporary.

The discovery of contemporary remains can initiate a legal investigation, with a goal of determining the identity of the person. The investigation may start with estimating the person's biological profile (age, sex, ancestry, and stature), looking for signs of injury or disease, estimating time since death, and determining cause and manner of death (Byers 2002). These methods are used to tentatively match the remains to a missing person. Next, positive identification of the individual may be accomplished through odontology, radiography, skull-photo superimposition, or the use of nuclear DNA analysis, all of which require antemortem information or samples from an individual for comparison. When genetic analysis is used, reference samples may also be obtained from family members in order to determine familial relationships or attempt to identify the individual if antemortem material is not available.

If bones are noncontemporary, much of the same information can be obtained, but it is used in a different context. For ancient remains the biological profile can be employed to help understand who settled in the area, and the interaction among people of different ancestries, ages, and sexes. Signs of injury may be important in reconstructing behavior, and stresses encountered during life; while body position and interment conditions may demonstrate cultural burial practices. Cause and manner of death would not be used to prompt a police investigation, but instead could be helpful in understanding the way of life during a certain time period. One avenue of research not often attempted with ancient bone is producing a positive identification; however, genetic analyses may still be beneficial in determining familial relationships, and the ancestry of the remains.

It is also possible for noncontemporary skeletal material to be involved in legal proceedings. For instance, in 1996 a nearly complete human skeleton named the "Kennewick Man," dating from 9,300 years before present (YBP), was found in Washington State. On one side, researchers claimed that the individual exhibited Caucasoid characteristics despite its age (http://www.washington.edu/burkemuseum/kman/kman_home.htm). On the opposing side, Native American groups argued that the skeleton was from a time before European settlement of America, and therefore, the Kennewick Man should be returned to Native American descendents under the Native American Graves Protection and Repatriation Act (NAGPRA) (http://www.cr.nps.gov/archeology/kennewick/c14memo.htm). NAGPRA is a Federal law enacted in 1990 which "provides a process for museums and Federal agencies to return certain Native American cultural items—human remains, funerary objects, sacred

objects, and objects of cultural patrimony—to lineal descendants, culturally affiliated Indian tribes, and Native Hawaiian organizations" (http://www.cr.nps.gov/nagpra/). In 2002 the courts ruled that the bones should be studied instead of being returned to one of the four Native American groups claiming him as an ancestor, a ruling that was upheld in 2004 (http://seattlepi.nwsource.com/local/231401_kennewick06.html). This case demonstrates a use of noncontemporary bones in court, and the importance from a legal standpoint of the capability to detect Native American ancestry.

The ability to distinguish between Native American and European remains becomes even more challenging if the remains were deposited after European settlers began living in America. For example, in the Great Lakes Region during the colonial period, the fur trade prompted daily interactions between these two groups (Sleeper-Smith 2001), potentially calling into question the ancestry of individuals buried in that area. The purpose of this thesis research was to develop a method for determining ancestry using SNP analysis (see details below) on DNA from human remains interred under the floor of the Church of St. Anne in Fort Michilimackinac, Michigan, between 1743 and 1781.

History of Fort Michilimackinac

Fort Michilimackinac (1715–1781) was located on the northern tip of Michigan's Lower Peninsula, just south of the Straits of Mackinac near present-day Mackinaw City. The creation, settlement and destruction of the fort was directly tied to the fur trade, which was established between the French and Native Americans in the Great Lakes and St. Lawrence River valley areas in the 1500's (Kent 2004). Many Great Lakes Native

American groups participated in this exchange, including the Ottawa, Huron, Tionontates, Ojibwas, Iroquois and Potawatomis. The French first arrived at the Straights of Mackinac when Jean Nocolet, a fur trade ambassador, traveled through in 1634. In 1664, Fr. Jacques Marquette established a Jesuit Mission in Sault St. Marie on the north coast of Michigan's Upper Peninsula, and then relocated the mission to Mackinac Island. The mission was next moved to the Upper Peninsula mainland (north of the straights) at St. Ignace in order to have more land for crops and a greater availability of game than was present on the island (Kent 2004). A French garrison was erected in St. Ignace called Fourt de Baude, which was commanded by Antoine de la Mothe Cadillac between 1694 and 1701, when the French capital in the west was relocated to Detroit (Havighurst 1966). The main purpose of the military at St. Ignace was to regulate the fur trade, although the stated purpose also included protecting the Jesuits. In 1696 the French government revoked all fur trade licenses, which were used to regulate trade and interactions between French traders and Native Americans, in an attempt to compel Native Americans to bring their furs to ports further east to be traded (Havighurst 1966; Peterson 1982). Because they were no longer needed to check licenses and supervise trade, French troops were removed from Fourt de Baude. Although the regulated fur trade was suspended, many illegal French traders (*coureurs de bois*), some with Native American wives, remained outside of established forts (Peterson 1982). After Fourt de Baude was deserted, the Jesuit mission at St. Ignace was also abandoned and razed in 1706 (Havighurst 1966).

Because of the Straights of Mackinac's strategic location, the French government only briefly vacated it. Following the re-opening of the licensed fur trade, a new

settlement arose on the northern tip of the Lower Peninsula across from St. Ignace (Havighurst 1966; Peterson 1982). Fort Michilimackinac, a stockade fort, was built at this location in 1715. The Church of St. Anne de Michilimackinac was erected within the fort's walls as a Jesuit church in the early 1740's, and burials were placed under the floor of the church (Figure 1; May1964; Maxwell and Binford 1961). The Mackinac Register—part of the collections of the State Historical Society of Wisconsin—contains a record of interments, marriages and baptisms performed by priests at the church (Thwaites 1910). Included in the register are accounts of burials of individuals with Native American names, or people referred to as "savage," suggesting that some Native Americans were interred in the church.

Figure 1: **Reconstructed Church of St. Anne de Michilimackinac**

Reconstruction of the Church of St. Anne located within the reconstructed Fort Michilimackinac, which is a part of the Mackinac State Historic Parks. Buildings and stockade walls were rebuilt over excavated sites starting in the 1960's (http://vm.mackinacparks.com/).

Culturally, Fort Michilimackinac was a mix between a Native American village and a French community (Peterson 1982). Instead of creating a purely French settlement

in Michigan, inhabitants of the fort adapted to the environment of the region, through their clothing, the types of food eaten, and the use of goods made by Native Americans. Also, because the culture was based on the fur trade, the different classes did not show much disparity in material status; for example, merchants and voyageurs lived in the same type of houses. Before 1763, most of the households were located within the walls of Fort Michilimackinac, where farming was not possible. Instead, corn and other supplies were bought from local Native American groups, such as the Ottawa (Peterson 1982). Due to the small French military presence, Fort Michilimackinac was also dependent on Native Americans in adjacent villages for protection (Sleeper-Smith 2001).

Although the French fort was the largest in the area, Fort Michilimackinac normally housed fewer than 35 military men (Peterson 1982). "By 1722 there were thirty French families — of soldiers and officers — in the fort and thirty traders' families outside" (Havighurst 1966). A report from September 1749 indicated that 40 people lived within the stockade at Fort Michilimackinac, including 10 French families, three of which had mixed ancestry (Peterson 1982). The population near Fort Michilimackinac varied widely since French voyager traders and visiting Native Americans lived near the fort in the summer in order to trade goods, and then left in the winter to hunt. Permanent residents of the area included troops, families of traders or military men, merchants, clerks, retired traders and their relatives, laborers, black and Native American (Panis) slaves and more. Most well-off French traders owned at least one Native American slave (Armour 1967).

In the Great Lakes region, intermarriage between European men, especially voyager traders, and Native American women has been reported to be very common,

resulting in children with mixed ancestry (Peterson 1982). In Canada and the Northwestern United States, this mixing, which had foundations in the Great Lakes area, subsequently led to the creation of a new native group called Métis. Intermarriage benefited the fur trade by creating allies, increasing a trader's safety, increasing access to furs, and turning trading into an exchange between kin (Sleeper-Smith 2001). Of the 62 marriages recorded in the Mackinac Register between 1698 and 1765, 48% of them were unions between Canadian men and Native American or Métis women, and 32% were between Canadian men and Canadian women (Peterson 1982). The rest of the recorded marriages involved Indians, Métis, a combination of both, or individuals of unknown ancestry. Consequently, many Métis children were also being born, as suggested by the large number of recorded baptisms of Métis children (about 39% of the baptisms were of people at least 1/8 Native American). Métis females typically married French men, while Métis males typically married Native American women (Sleeper-Smith 2001). Records from the Mackinac Register however, would likely not include non-baptized children, previously married couples, or marriages which were not performed in the church, including those between European men and Native American women who did not convert to the Catholic faith. Also, some men may have had a legal wife elsewhere and a Native American wife in Michigan, or have produced illegitimate children with Native American slaves at the fort (Peterson 1982).

European women were not entirely absent from Fort Michilimackinac or the Great Lakes area. For example, Cadillac brought his wife with him from St. Lawrence, and a map of Fort Michilimackinac in 1717 reported that the fort contained "a commandant, a few settlers, and even some French women" (Peterson 1982). One trader

reported that in the 1770's most of the wives of Frenchmen were European (Armour 1967). The French government made attempts to attract French families and women to the new world. For example, from 1663 to 1673 young French women called the les filles du roi (the king's daughters) were sent to Canada in order to marry settlers, increase family sizes, and balance the sex ratio in the colonies (http://www.whitepinepictures.com /seeds/i/12/sidebar.html). To entice families to the Great Lakes region, the French government offered them a farm and agricultural equipment for settling near Detroit, the only French farming settlement in Michigan; however, only 46 families accepted (Havighurst 1966; Peterson 1982). In the farming community, intermarriage between French men and Native American women was reportedly less common than in regions that were based around the fur trade, such as Fort Michilimackinac.

The French and Indian War was fought between 1754 and 1761 as a result of conflict between the English and the French. With the French loss in the war, Fort Michilimackinac was relinquished to the British in 1761, although many French inhabitants remained (Havighurst 1966; Kent 2004a; Maxwell and Binford 1961). French and British policies regarding interactions with Native Americans were very different. For example, under English occupation the number of settlers living outside of the fort's stockade increased, impinging on Native American land, and material goods were imported instead of being obtained from Native Americans (Peterson 1982). Also, the English did not participate in native customs, such as gift giving (Kent 2004a). Lastly, British settlers did not take advantage of the influence that intermarriage had on the fur trade and the creation of alliances between traders and Native Americans (Peterson 1982). Angry with the English, Ottawa war chief, Pontiac organized a joint Ottawa,

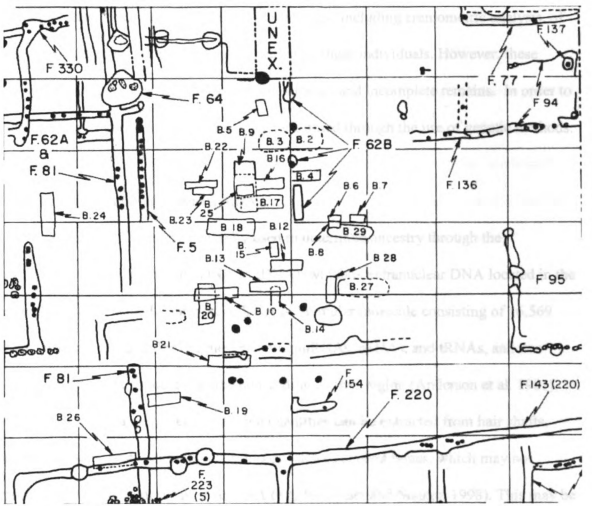
Chippewa, and Potawatomis attack against multiple English forts in 1763 (Kent 2004a). At Fort Michilimackinac the attack started while Chippewa and Sac played a lacrosse-like game outside the fort to distract the English soldiers, and resulted in the British losing control of the fort for a year, while French traders were allowed to remain.

During the revolutionary war, English commander Patrick Sinclair moved Fort Michilimackinac and the surrounding communities to a more militarily strategic location on Mackinac Island (Kent 2004a). Other factors that contributed to the fort's relocation were the wind and thin soil, which made farming difficult at the fort's mainland site (Peterson 1982). The Church of St. Anne was moved in the winter of 1781 and reconstructed on the island (May 1964); however, burials located under the church floor were left in their original sites. After moving or salvaging as many buildings as possible, the remains of the old fort were razed (Maxwell and Binford 1961).

Current Fort Michilimackinac

In 1959 the Mackinac Island State Park Commission and the Michigan State University Museum undertook an excavation of the area where Fort Michilimackinac once stood near what is now Mackinaw City (Maxwell and Binford 1961). As a result of the excavation, the Department of Anthropology at Michigan State University gained possession of human remains found under where the Church of St. Anne used to be positioned (Figure 2). Currently, reconstructed fort walls and buildings, including a church, are present at the Fort Michilimackinac site as a part of the Mackinac State Historic Parks (Figure 1; Stone 1974).

Figure 2: Map of Excavation of Fort Michilimackinac



Map from the 1959 – 1966 excavation of Fort Michilimackinac. Burials are labeled "B." Burials B-10, B-12, B-14, B-16, B-17, B-18, B-19, B-20, B-21, B-22, B-24, B-26, B-27, B-29 are pictured (B-11 and B-34 not shown). Feature F. 62B is labeled as the Church area (from Stone 1974).

Excavated remains from Fort Michilimackinac include male and female adults, as well as subadults, with unclear ancestries. Due to the reports of intermarriage between European men and Native American women in the literature, it is possible that the males were European, while the females and children were predominately Native American. At least one burial (B-34) has known ancestry from the historical record. This is a commingled burial of six individuals reported to be Native Americans who died from

smallpox (Thwaites 1910). In addition to the use of historical records, anthropologists have previously examined the skeletal morphology, including craniometric analysis, of these samples in an effort to estimate ancestry of these individuals. However, these methods can be problematic especially for subadult and incomplete remains. In order to assist, ancestry determination can also be performed through the use of genetic methods.

Characteristics of Mitochondrial DNA

Genetic analyses are generally used to determine ancestry through the examination of mitochondrial DNA (mtDNA), which is extranuclear DNA located in the mitochondria of cells. In humans, mtDNA is a circular molecule consisting of 16,569 basepairs, including multiple genes encoding mRNAs, rRNAs, and tRNAs, and two hypervariable regions located in the non-coding control region (Anderson et al. 1981). MtDNA is advantageous because usable quantities can be extracted from hair shafts, putrefied tissue, and old biological samples, such as ancient bones, which may not produce usable amounts of nuclear DNA (e.g. Rousselet and Mangin 1998). This may be due to mtDNA being present in multiple copies per cell (approximately 1000 molecules), mtDNA being more protected from degradation by exonucleases because it is circular, or mtDNA being less susceptible to environmental damage through its enclosure in the mitochondrion (Bogenhagen and Clayton 1974; D. Foran personal communication).

Another distinguishing characteristic of mtDNA is that it is haploid while nuclear DNA is diploid. Therefore, although there are multiple copies of mtDNA per cell, all of the copies are the same. In some rare individuals however, heteroplasmy can occur, in which different mitochondria in the same person contain a different DNA sequence or

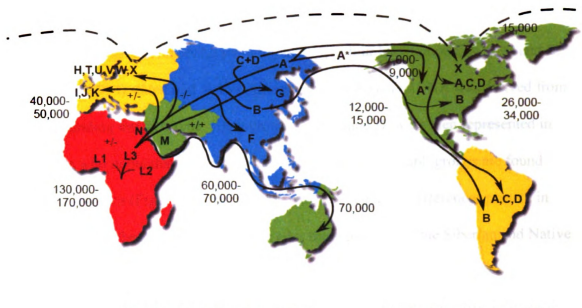
haplotype. A haplotype refers to the genetic sequence of a haploid DNA molecule in an individual (e.g. mtDNA or the Y chromosome).

MtDNA is inherited solely through the mother (Giles et al. 1980) since mitochondria in sperm are located in the sperm's tail, which does not enter the egg during fertilization. Consequently, a person shares the same mtDNA sequence with all their maternal relatives (including their mother, siblings, maternal grandmother, aunts and uncles on their mother's side, etc.). Therefore, mtDNA may be used to identify familial relationships, or determine an individual's maternal ancestry (see details below).

Ancestral mtDNA Haplogroups

A mtDNA haplogroup is a set of similar mtDNAs with the same haplotype at certain defined locations. These haplogroups can be used to track the ancestral movement of humans based on phylogenetic studies (Figure 3). Generally, Africans show the most DNA sequence divergence, indicating a longer time since common ancestry (Cann et al. 1987). Estimates of divergence times for different haplogroups are shown in Figure 3 in years before present (YBP); however, divergence times are not well established and vary widely among sources. For example, in contrast to the times in Figure 3, Torroni et al. (1996) report haplogroup H and J have a divergence time of 17,000-30,000 YBP and haplogroups A, B, C and D have an overall divergence time of 16,750 – 33,500 YBP. Also, three studies cited in Reidla et al. (2003) indicate haplogroup X has a divergence time of 17,000-30,000 YBP, 13,700 –26,600YBP or no more than 11,000 YBP.

Figure 3: **Migration of Human mtDNA**



Map showing migration of human mtDNA sequences from their origin in Africa, and their differentiation to separate geographic areas (http://www.mitomap.org/WorldMigrations.pdf). Europeans are represented by haplogroups H, I, J, K, T, U, V, W, X, and M (not shown), while Native Americans include haplogroups A, B, C, D, and X. Note that haplogroup X is present in Europeans and Native Americans, while the other Native American haplogroups are present in Asians, indicating that haplogroup X had a different dispersal pattern. Divergence times are estimated on the map.

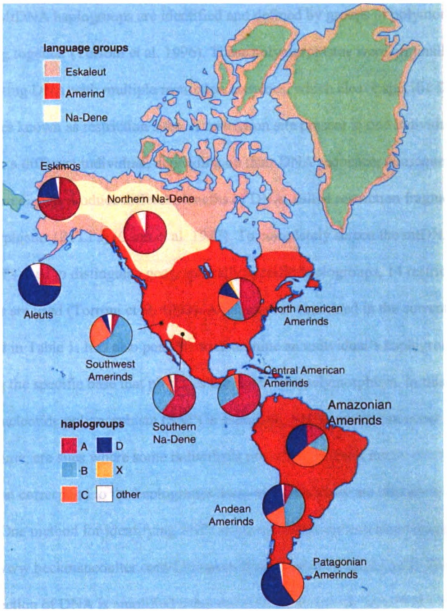
Torroni et al. (1996) described 10 haplogroups (H, I, J, K, T, U, V, W, X and M) in Europeans, with H being the most common (~41%; ~45.7% in the SWG-DAM database; Allard et al. 2002). These European haplogroups (specifically H, I, J, K, T and W) were very rarely identified in samples from other continents, which suggests they are continent specific, and indicates that they arose after Europeans were genetically separated from Africans and Asians (Torroni et al. 1996). European haplogroups are found throughout the continent due to the large amount of genetic admixture between European populations in the past and present (Dubut et al. 2004). Therefore, it is not

possible to use haplogroup frequencies to identify populations from different European regions.

Native American mtDNAs primarily belong to four haplogroups, A, B, C and D. Torroni et al. (1993) stated that Native American and Siberian mtDNA was derived from the same founding mtDNA type. Haplogroup A, B, C, and D, were then represented in the migrants who populated America from Asia, since all four haplogroups are found throughout America (Figure 4; Schurr 2000). MtDNA sequence differences present in individuals within these haplogroups arose after the separation of the Siberian and Native American populations (Torroni et al. 1993).

A fifth haplogroup, X, represents an additional founding lineage of Native Americans. This group is unique because it is also found in European and West Asian populations; however, it is not present in East Asian populations, which would correspond to the typical route of human dispersal through Asia to America (Figure 3; Brown et al. 1998; Malhi and Smith 2002). Haplogroup X is found in a frequency of about 4% in Europeans. In Native Americans, it is mostly localized to northern populations, including the Ojibwa from the Great Lakes Region, in which haplogroup X has a frequency of about 25% (Brown et al. 1998). Reidla et al. (2003) reported haplogroup X has a divergence time no later than 11,000 YBP, which is consistent with dispersal around or after the last glacial maximum.

Figure 4: **Native American Haplogroup Distribution**



Map indicating the frequency distribution of Native American haplogroups. Haplogroups A, B, C, and D are generally widely distributed throughout both continents, while haplogroup X is localized to North American Amerinds (from Schurr 2000).

Ancestry Analysis of MtDNA Haplogroups

MtDNA haplogroups are identified and defined by groups of polymorphic sites occurring together (Torroni et al. 1996). These polymorphisms were originally identified by digesting DNA with multiple restriction enzymes, which cleave specific DNA sequences known as restriction sites. A restriction site present in one individual may be absent in a different individual, depending on their DNA sequence; consequently, restriction digests produce different lengths of DNA, called restriction fragment length polymorphisms (RFLPs) (Cann et al. 1987). To completely screen the mtDNA genome for RFLPs used to distinguish geographically specific haplogroups, 14 restriction enzymes are used (Torroni et al. 1993). A list of sites examined in the current study can be found in Table 1. It is also possible to determine an individual's haplogroup by assaying the specific base that produces the restriction polymorphism. In this case, a single nucleotide polymorphism (SNP) is examined. SNPs, which can occur anywhere in the genome, are sites where some individuals possess a different nucleotide base. The SNPs that correspond to the haplogroups assayed in this study are also shown in Table 1.

One method for identifying SNPs is the use of primer extension reactions (http://www.beckmancoulter.com/Literature/BioResearch/A-1928A.pdf). First, a small target section of DNA is amplified using polymerase chain reaction (PCR). In order to allow greater amplification of low copy DNA without creating non-specific products, PCR products may be reamplified with primers internal to the original PCR primers, in a process called nested PCR. Semi-nested PCR can also be performed, in which only one internal primed is used. Non-nested, nested or semi-nested PCR products serve as the template DNA in the SNP-primer extension reaction. Next, a SNP-primer that ends one

Table 1: **Diagnostic Polymorphic Sites for Haplogroups in this Study**

Polymorphic sites identified by RFLP and SNP analyses (except haplogroup B, which is identified by a 9bp deletion), the gene the site is located within, and the percentage of the haplogroup in the continent, are shown. For the Polymorphic Sites for RFLP Analysis, "+" indicates a restriction site for that enzyme is gained at that position in the haplogroup, and "-" indicates that a restriction site is lost at that location. Haplogroup B DNA is 9bp shorter than non-B. The second SNP base listed is present in individuals of that haplogroup, while the first base is present in all others. A=adenine, C=cytosine, T=thymine and G=guanine. Haplogroup X is not included in the percentage for America. This is not an exhaustive list of haplogroups present in Europe, so the percents for Europe do not add to 100. MtDNA polymorphisms other than those listed may also be used to define these haplogroups (Compiled from www.mitomap.org and Brown et al. 1998).

Continent	Haplogroup	Polymorphic Site for RFLP Analysis	Polymorphic Site and Base for SNP Analysis	Gene	% of Continent
America	A	663+HaeIII	664 A-G	12S ribosomal RNA (MTRNR1)	44
America	B	8271-8281 9bp deletion	8271-8281	Non-coding nucleotides between MTCO2 and MTTK (MTNC7)	22
America	C	13259-/13262+ HincII/AluI	13263 A-G	NADH dehydrogenase subunit 5 (MTNC5)	18
America	D	5176- AluI	5177 G-A	NADH dehydrogenase subunit 2 (MTND2)	16
America or Europe	X	1715- DdeI	1719 G-A	16S ribosomal RNA (MTRNR2)	4 (European) ~25 (Ojibwa)
Europe	H	7025- AluI	7027 T-C	Cytochrome c oxidase subunit I (MTCO1)	39
Europe	J	13704- BstOI	13707 G-A	NADH dehydrogenase subunit 5 (MTND5)	9
Europe	K	9052- HaeII/HhaI	9052 G-A	ATP synthase F0 subunit 6 (MTATP6)	8

base before the SNP site is annealed to the template DNA. This primer is extended one base through addition of a fluorescently labeled dideoxy nucleotide complementary to the base of interest. The dideoxy nucleotide lacks a 3' hydroxyl group which prevents further extension of the DNA strand. Different colored fluorescent dyes are used to distinguish between the four possible bases (ddATP in red, ddGTP in green, ddUTP in blue and ddCTP in yellow).

The above analysis of haplogroups focuses on polymorphisms within the mtDNA coding region. However, information regarding ancestry can also be obtained by sequencing the two hypervariable regions located within the non-coding control region. For example, haplogroup J is defined in the coding region by a *Bst*0I restriction site loss at position 13704 due to the SNP guanine (G) to adenine (A) transition at nucleotide 13707; alternatively, haplogroup J can be defined in the control region by specific bases at three polymorphic sites: a T at position 16069, a C at position 16126 and a T at position 295 (Torroni et al. 1996, Allard et al. 2002). Haplogroup X is completely characterized by polymorphisms in both the coding and non-coding regions; namely the RFLP sites 1715 –DdeI, and 16517 +HaeIII, and the control region mutations 16223T and 16278T (Brown et al. 1998). Native American haplogroup X is differentiated from its European counterpart through the control region mutations 16213A and 200G (Brown et al. 1998; Malhi and Smith 2002).

Goals of this Study

The goal of this research was to determine the ancestry of remains interred in Fort Michilimackinac between 1743 and 1781. In order to accomplish this, a method of

detecting Native American and European mtDNA haplogroups was created using SNP-analysis of the mtDNA coding region. A working hypothesis is that male remains were European, while female and subadult remains were predominately Native American, due to the location of the burials within a French fort; reports of a high frequency of intermarriage between Native American women and French men; and information on the origins of the Métis people.

MATERIALS AND METHODS

Samples

Skeletal remains from the 1959 excavation of Fort Michilimackinac were obtained from Dr. Norman J. Sauer of the Department of Anthropology at Michigan State University.

Burial number and skeletal samples obtained are listed in Table 2. These included a femur and tibia from burials B-10, B-11, B-12, B-18, and B-29, proximal femur and tibia from burial B-19, both femurs from burial B-17, femur and humerus from burial B-24, and femur only from burials B-14, B-16, B-20, B-21, B-22, B-26, B-27, B-34 and B-34B. Burial B-34 was a commingled burial containing six individuals, corresponding to records of an interment of six Native Americans who died of smallpox. Femora from an adult (B-34B) and a subadult (B-34) were obtained in order to ensure that two different individuals were being tested. Overall, skeletal material from 14 adults and 3 subadults (B-19, B-22, and B-34) were acquired.

Known Native American DNA samples of haplogroups A, B, C, and D were obtained from Dr. Connie J. Mulligan of the Department of Anthropology at the University of Florida.

Table 2: **Skeletal Samples from Fort Michilimackinac**
Bones used in this study, selected from material excavated at Fort Michilimackinac, Michigan.

Burial Number	Skeletal Material	Burial Number	Skeletal Material
B-10	Right Femur	B-20	Right Femur
	Right Tibia	B-21 (Box 12)	Left Femur
B-11c	Right Femur	B-22	Right Femur (subadult)
	Right Tibia	B-24	Left Femur
B-12	Left Femur		Left Humerus
	Right Tibia	B-26	Right Femur
B-14	Left Femur	B-27	Right Femur
B-16	Left Femur	B-29	Left Femur
B-17	Right Femur		Right Tibia
	Left Femur	B-34	Left Femur (subadult)
B-18	Right Femur	B-34B	Left Femur
	Right Tibia		
B-19	Proximal Right Femur		
	(subadult)		
	Right Tibia (subadult)		

DNA Extraction

Bone powder collection was performed in a PCR hood (blower not turned on), which was cleaned with bleach and UV irradiated prior to use and between burials. The Dremel tool was wiped with bleach then UV irradiated, and all removable drill components were soaked in bleach then UV irradiated before use and between bones. Weigh paper, microcentrifuge tubes, bench paper and pipettes were also UV irradiated before use.

The top layer of bone was sanded off using a Dremel tool and separate sanding bands for each bone in order to remove surface contaminates which may inhibit PCR, and to remove possible DNA contamination resulting from the handling of the bones since excavation. Bones were drilled near the middle of the shaft using a 1/16-inch drill bit. The bone powder was collected on a piece of weigh paper and poured into a 1.5 ml centrifuge tube. Five to 15 holes were drilled into each bone taking care not to drill into

the center of the shaft. Drilling continued until 0.007 g to 0.01 g of bone powder was obtained.

The bone powder was placed in a sterile 1.5 ml centrifuge tube, and 400 µl or 500 µl of filter sterilized digestion buffer (20 mM Tris, 50 mM EDTA, pH 7.5) and 5 µl of 20 mg/ml proteinase K were added. A reagent blank was started using the same reagents. The solution was vortexed and incubated overnight (greater than 16 hours) in a 55°C incubator.

Following incubation an equal volume of phenol was added to each sample (400 µl or 500 µl). The sample was vortexed and centrifuged at full speed for 5 minutes. The top, aqueous layer was transferred to a sterile 1.5 ml centrifuge tube and the bottom phenol layer was discarded. These steps were repeated with phenol, and then with chloroform:isoamyl alcohol (20:1). The aqueous layer was purified using a Microcon YM-30 column. The column was centrifuged for 10 minutes or greater at 14,000 rcf until all of the liquid had flowed through the column, with the flow through discarded. The DNA (retentate) was washed 3 times using 200 µl of TE (10 mM Tris, 1 mM EDTA), and the centrifugation process was repeated. The DNA was retrieved using 15 – 18 µl TE depending on the initial mass of bone powder (~2 µl/mg), by pipetting the TE onto the top of the Microcon column and then transferring it into a sterile 1.5 ml centrifuge tube.

Polymerase Chain Reaction

Non-nested PCR product sizes ranged from 62 to 118 bp (Table 3, including primer sequences, and PCR product sizes). PCR primers were designed using Primer3 (http://fokker.wi.mit.edu/primer3) with the criteria of a small product size and little

complementarity (the ability of a primer to base pair with other primers) or secondary structure (the ability to form base pairs within a primer). Primers were synthesized at Michigan State University's Macromolecular Structure Facility, except for the haplogroup B forward primer, which was synthesized by Proligo Primers and Probes with a D4 blue dye attached to the 5' end. Five to 15 thymine bases (PolyT tail) were synthesized on the 5' end of the SNP-primers for haplogroups X, J, and K in order to lengthen the product, which would allow for possible multiplexing in the future.

PCR was performed for haplogroups A, C, D, X, H, J, and K using the forward and reverse primers from Table 3 in 10 µl reactions consisting of 1 unit HotMaster Taq (Eppendorf), 0.4 µM forward and reverse primer, 0.2 mM dNTPs, and 1X HotMaster Reaction Buffer with 20 µM $MgCl_2$ (Eppendorf). A positive PCR control of DRF (male DNA from saliva) or SLS (female DNA from saliva), and a negative PCR control omitting DNA were prepared for each reaction. PCR was also performed on the reagent blanks for at least one set of primers. Original reactions were performed with 5 times greater primer concentrations; however, it was observed that 1/5 of the primer concentration also produced successful amplification, so the more dilute concentration was used for subsequent reactions.

Table 3: **PCR Primers and Product Size for each Haplogroup**

Nucleotide location of the SNP site, bases in question, PCR and SNP primer sequences used, and expected PCR product sizes for each of the haplogroups tested. Sequences are written 5' to 3'. The second base listed is present in individuals of that haplogroup, while the first base is present in all others. For forward SNP-primers, the bases present in the polymorphic site were added to the premix. For the reverse SNP-primers the complementary bases were added.

Haplogroup	Polymorphic Site and Base	PCR Primer Sequence	PCR Product Size
A	664 A-G	Forward: cccataaacaaataggttt Reverse: actggaacggggatgctt SNP(Forward): ccataaacaaataggtttggtcct	Non-nested: 79bp Nested: 77bp
B	8271-8281	Forward: (dyeD4)-agggcccgtatttaccctat Reverse External: aagttagctttacagtgggctct Reverse Internal: gtcactttacggggttgattt	Non-nested: 118bp or 109bp Nested: 64bp or 55bp
C	13263 A-G	Forward: tcgtagccttctccacttca Reverse: ttggttgatgccgattgtaa SNP(Reverse): gattgtaactattatgagtcctag	Non-nested: 62bp Nested: 50bp
D	5177 G-A	Forward: cttaaactccagcaccacga Reverse: gagaggagggtggatggaat SNP(Forward): accctactactatctcgcacctgaaacaa	Non-nested: 90bp Nested: 71bp
X	1719 G-A	Forward: ccaaacccactccaccttac Reverse: gcgccaggtttcaatttcta SNP(Forward): tttttccaccttactaccagacaacctta	Non-nested: 88bp Nested: 82bp
H	7027 T-C	Forward: agacatcgtactacacgacacg Reverse: tgatggcaaatacagctcct SNP(Reverse): tattgataggacatagtggaagtg	Non-Nested: 80bp Nested: 61bp
J	13707 G-A	Forward: aacgaaaataaccccacccta Reverse: aatcctgcgaataggcttcc SNP(Reverse): ttttttttttttttgcgaataggcttccggctg	Non-Nested: 68bp Nested: 77bp
K	9052 G-A	Forward: actgcaggccacctactcat Reverse: agagggaaggttaatggttga SNP(Reverse): tttttatggttgatattgctagggtggcg	Non-Nested: 72bp Nested: 64bp

Serial dilutions of DNA (1 µl DNA, 1:10 dilution, and 1:100 dilutions) were amplified in order to determine if PCR inhibitors were present in the sample, and to determine the optimal DNA concentration for each DNA extraction. The lowest concentration of DNA that was amplifiable was used for subsequent reactions, and the corresponding reagent blank was diluted the same amount. Four µl of 10 mg/ml bovine serum albumin (BSA) was added to the PCR reactions if inhibition was detected that could not be elimination through diluting the sample. The DNA was denatured for 2 minutes at 94°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 57°C, and DNA extension for 30 seconds at 72°C, with a final DNA extension for 5 minutes at 72°C. In some instances the number of cycles in the PCR reaction was reduced to 30 or 35 in order to ensure the negative PCR control was not contaminated.

After PCR amplification of haplogroups A, C, D, X, H, J and K, the PCR products were separated on 3% agarose gels, stained with ethidium bromide, and photographed on an ultraviolet light source.

Semi-nested Polymerase Chain Reaction

Semi-nested PCR was performed using the SNP-primer and complementary forward or reverse primer for each haplogroup, if no amplification product was visible, or if only faint bands were present on the agarose gel. Ten µl PCR reactions were performed with the same reagents as the non-nested reactions described above, except that BSA was not included. The DNA was amplified as above, with the number of cycles reduced to 10

– 30 cycles. Semi-nested PCR products were then separated on 3% agarose gels, stained with ethidium bromide, and photographed on an ultraviolet light source.

PCR Product Purification

PCR products were purified using 2 µl (2 units) shrimp alkaline phosphatase (SAP) (USB or Roche), and 1 or 0.5 µl (10 units) exonuclease1 (Exo1) (USB or NEB). The reaction was incubated at 37°C for 1 hour, and then heated to 75°C for 15 minutes for enzyme inactivation. When exonuclease from NEB was used, the heat inactivation step was increased to 80°C for 20 minutes as per manufacturer's instructions. Some samples were purified using 2 µl ExoSAP-IT (USB). These reactions were incubated at 37°C for 1 hour, and then heated to 80°C for 15 minutes for enzyme inactivation.

SNP-Primer Extension

SNP-primer extension reactions were performed using a modified protocol from Beckman Coulter's CEQ SNP-Primer Extension Kit. A 10 µl (half) reaction was used compared to the manufacturers suggested volumes. A premix of 1 µl reaction buffer, 1 µl of each of the appropriate ddNTPs, 0.5 µl polymerase and 2 µl water (substituted for the other two ddNTPs), was made for each reaction (a list of appropriate ddNTPs for each haplogroup is included in Table 3). Original reactions were performed with all four ddNTPs in the premix. It was subsequently observed that reactions using only the two appropriate ddNTPs produced more clear results, so two ddNTPs were used for subsequent reactions. Premix was originally made before each use; however, when a

stock of premix was made, pipetting errors caused by the glycerol in the polymerase were reduced, and the polymerase was not consumed before the other reagents in the kit.

In a PCR tube, 5.5 µl premix, 0.5 or 1 µl DNA (depending on PCR band intensity), 1 µl of 2 µM or 0.2 µM SNP-primer, and water to a final volume of 10 µl were combined. Reagents were added in the order of water, premix, DNA, then SNP-primer, and all reagents were stored on ice during reaction preparation as per manufacturer's instructions. The DNA underwent 25 cycles of denaturation for 10 seconds at 96°C, primer annealing for 5 seconds at 50°C or 55°C, and DNA extension for 30 seconds at 72°C.

SNP-Primer Extension Product Purification

SNP-primer extension products were purified using 1 µl (1 unit) SAP. The reaction was incubated same as above.

CEQ 8000 Genetic Analysis System

SNP-primer extension reactions were separated on the CEQ 8000 Genetic Analysis System and analyzed using CEQ 8000 Software. 0.5 µl purified SNP-primer extension product, 0.5 µl Size Standard 80 (Beckman Coulter) and 39 µl Sample Loading Solution (SLS, Beckman Coulter) were combined for each well. Size Standard 80 contains 13 nt and 88 nt fragments labeled with red dye (D1). In some runs, 0.25 µl of Size Standard 80 was used to conserve materials; however the decreased peak size made analysis and correct calling of the size standard peak sizes more difficult. In some reactions, the amount of purified SNP-primer extension product was increased to 1 µl in

order to increase the size of the peaks in the electropherogram; SNP-primer extension product was decreased to 0.5 µl, or diluted up to 1:10 in order to decrease the size of the peaks in the electropherogram.

SNP-primer extension reactions were electrophoresed using the default program SNP-1 or modified programs, which I named SNP-long and SNP-15. The running parameters for the default program were: Capillary: temperature 50°C, wait for temperature Yes; Denature: temperature 90°C, duration 60 seconds; Inject: voltage 2.0 kV, duration 30 seconds; Separate: voltage 6.0 kV, duration 16.0 minutes; Pause: 0 minutes. The duration of separation was increased to 18 minutes in the modified program SNP-long, in order to ensure that the 88 nt fragment of the Size Standard 80 eluted during the run. The duration of injection was reduced to 15 seconds in the modified program SNP-15, in order to reduce the size of the peaks in the electropherogram.

The data were analyzed using the Fragment Analysis mode of the CEQ 8000 Software, using the DefaultSNPAnalysisParameters. If the correct 13 nt and 88 nt peaks from the Size Standard 80 were not identified, the slope threshold was altered or the dye mobility correction was removed. If the size standard peaks were still not identified correctly, then the electrophoresis was repeated.

Haplogroup B Protocol

PCR was performed for haplogroup B using the labeled forward and external reverse primers (Table 3) with 10 µl reactions consisting of 1 unit HotMaster Taq, 0.4 µM forward and reverse primer, 0.2 mM dNTPs, and 1X HotMaster Reaction Buffer with 20 µM $MgCl_2$. A positive PCR control of DRF or SLS DNA, and a negative PCR control

omitting DNA, were prepared for each reaction. Four µl of 10 mg/ml BSA was added to the PCR reactions if inhibition was detected in previous PCR reactions. The DNA was denatured for 2 minutes at 94°C, followed by 30 or 35 cycles of denaturation for 30 seconds at 94°C, primer annealing for 30 seconds at 57°C, and DNA extension for 30 seconds at 72°C, with a final DNA extension for 5 minutes at 72°C. The number of cycles was reduced from the 40 cycles used for the other haplogroups in order to ensure that no detectable amplification occurred in the negative PCR control. PCR products were run on a 1.5% or 2% agarose gel, stained with ethidium bromide, and photographed on an ultraviolet light source.

Semi-nested PCR was performed as above using the labeled forward primer and internal reverse primer for haplogroup B, if no amplification could be seen, or if only faint bands were present on the agarose gel.

PCR reactions were diluted 1:200 or 1:400 after bands were visible for the non-nested and semi-nested products on an agarose gel. No PCR product purification was performed. One µl diluted PCR product, 0.25 µl Size Standard 400 (Beckman Coulter) and 38.75 µl SLS were combined for each well. Size Standard 400 contains 60, 70, 80, 90, 100, 120, 140, 160, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380, 400, and 420 nt fragments labeled with red dye (D1). Samples were separated on the CEQ 8000 Genetic Analyzer using the program Frag-3 that has the running parameters: Capillary: temperature 50°C, wait for temperature Yes; Denature: temperature 90°C, duration 120 seconds; Inject: voltage 2.0kV, duration 30 seconds; Separate: voltage 6.0kV, duration 35 minutes; Pause: 0 minutes.

After electrophoresis, the data were analyzed using the Fragment Analysis mode of the CEQ 8000 Software and the DefaultFragAnalysisParameters.

Known DNA Samples

Analyses described above were first performed on known Native American DNA from haplogroups A, B, C, and D in order to ensure that these haplogroups were correctly identified before being applied to bone samples from Fort Michilimackinac. Protocols were optimized using positive control DNA (female SLS DNA, or male DRF DNA), with 1 µl of 20 µM, 2 µM, 0.2 µM, and 0.02 µM SNP-primer in the primer extension reaction in order to determine the optimal concentrations used in this study.

Haplogroup B assays were performed as described above using the labeled forward and internal reverse primer, instead of the external reverse primer, or semi-nested PCR. The product was then diluted 1:200. One µl diluted PCR product, 0.25 µl Size Standard 80 and 38.75 µl SLS were combined for each well. The samples were separated on the CEQ 8000 Genetic Analyzer using the default program SNP-1 and the data were analyzed using the Fragment Analysis mode of the CEQ 8000 Software using the DefaultSNPAnalysisParameters.

MtDNA Control Region Sequencing

A portion of the mitochondrial hypervariable region 1 (HV1) was sequenced for the bone which was identified once as haplogroup X using the SNP-primer extension method previously described, as well as the other bone sample from the same burial. Primer sequences are listed in Table 4. DNA was amplified in 20µl reactions containing 1

unit HotMaster Taq, 0.4 μM F16144 forward primer, 0.4 μM R16410 reverse primer, 0.2 mM dNTPs, and 1X Hot Master Reaction Buffer with 20 μM $MgCl_2$. Four μl of 10 mg/ml BSA was added to the PCR reactions if inhibition was detected in previous PCR reactions. The DNA was denatured for 2 minutes at 94°C, followed by 38 cycles of denaturation for 30 seconds at 94°C, primer annealing for 1 minute at 55°C, and DNA extension for 1 minute at 72°C, with a final DNA extension for 5 minutes at 72°C. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and photographed on an ultraviolet light source.

Table 4: **Control Region PCR Primers**
PCR primer sequences for amplification of a section of the control region are shown. Primer names include F for forward, R for reverse and the number of the base where the sequence begins. Sequences are written 5' to 3' (from http://www.afip.org/Departments/oafme/dna/afdil/protocols.html).

PCR Primer Name	PCR Primer Sequence
F16144	tgaccacctgtagtacataa
F16190	ccccatgcttacaagcaagt
R16322	tggctttatgtactatgtac
R16410	gaggatggtggtcaagggac

Semi-nested PCR was performed using the primer pairs F16190 and R16410 or F16144 and R16322. Twenty μl PCR reactions were performed with the same reagents as the non-nested reactions described above, except that BSA was not included. The DNA was amplified as above, with the number of cycles reduced to 20. Semi-nested PCR products were then separated on 3% agarose gels, stained with ethidium bromide, and photographed on an ultraviolet light source.

PCR reactions were purified using Montage PCR Centrifugal Filters according to manufacturer's instructions, except that DNA was recovered from the column by pipetting 15 μl of TE onto the column and removing it into a sterile 1.5 ml centrifuge tube.

Sequencing reactions were performed with 4 µl Quick Start Buffer (Beckman Coulter), 2 µM of one primer used for semi-nested PCR, 0.5 or 1 µl PCR product, and water to a final volume of 10 µl. The DNA underwent 30 cycles of denaturation for 20 seconds at 96°C, primer annealing for 20 seconds at 50°C, and DNA extension for 4 minutes at 60°C. Sequencing reactions were stopped using 1.2 µl of 3 M sodium acetate (NaAc), 0.24 µl of 500 mM EDTA, and 0.6 µl glycogen in a final volume of 3 µl per 10 µl sequencing reaction.

Thirty µl of cold 95% ethanol was added, vortexed and centrifuged at 14,000 rpm for 15 minutes and the supernatant was removed. Two hundred µl of cold 70% ethanol was added, vortexed, centrifuged at 14,000 rpm for 3 minutes, and removed using a pipette being careful not to disturb the pellet. The last step was repeated, and samples were vacuum dried for 30-40 minutes. Samples were resuspended in 40 µl of SLS.

Samples were separated on the CEQ 8000 Genetic Analyzer using the program LFR1-45 which has the running parameters: Capillary: temperature 50°C, wait for temperature Yes; Denature: temperature 90°C, duration 120 seconds; Inject: voltage 2.0kV, duration 15 seconds; Separate: voltage 4.2kV, duration 45 minutes; Pause: 0 minutes. The data were analyzed using the Sequence Analysis mode of the CEQ 8000 Software, and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to verify base designations and align the sequence to the reference sequence from Anderson et al. (1981). Sites of interest included positions 16223 and 16278, which define haplogroup X, and 16213, which defines Native American haplogroup X.

RESULTS

DNA Extractions

MtDNA was successfully extracted from each bone sampled. An extraction was considered successful if the DNA was amplified by PCR (regardless of if dilution, the addition of BSA, or a nested PCR step, were required) and if a primer extension reaction could be performed. For haplogroups where two different bones from the same individual were analyzed, results were consistent, with the exception of assays for haplogroup H, where inconsistencies were found in nine individuals within the same bone and between the two bones analyzed (see details below).

Method Optimization

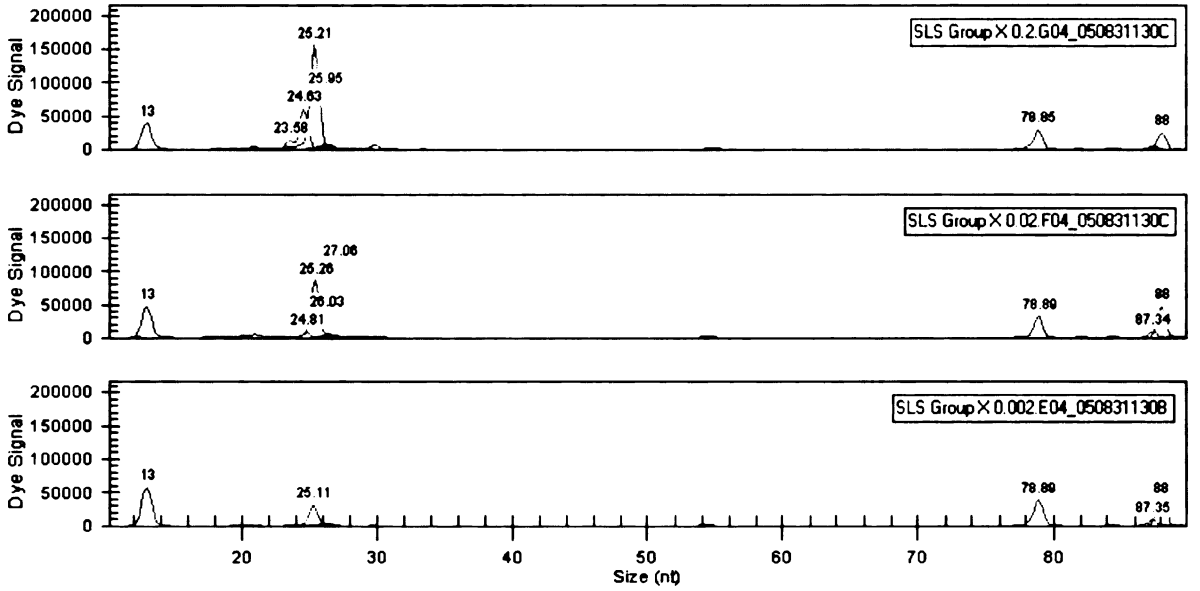
The target peak height for the SNP-primer extension product in the raw data was less than 130,000 relative fluorescence units (rfu), but greater than about 5,000 rfu. Extremely high peaks resulted in pull-up in the other dye channels (Figure 5a and b). When the injection time was lowered from 30 to 15 seconds, or the amount of SNP-primer extension product was decreased by reducing the volume of product or diluting the sample before loading, results were clean. Samples with very small peak heights, about the size of baseline noise, became more distinct when the primer concentration in the SNP-primer extension reaction was increased. Raising the amount of SNP-primer extension product loaded above 0.5 µl did not increase peak rfu enough to be beneficial. Particularly, when greater than 1 µl of SNP-primer extension product was loaded, product peaks became even smaller or were eliminated.

The final concentration of primer (2 – 0.002 μM) in the SNP-primer extension method was optimized using control DNA (Figure 5a and b). Too much primer caused numerous artifact and off-scale peaks, resulting in pull-up and split peaks in the analyzed data. As the primer concentration was lowered, the pull-up and non-target artifact peaks decreased or disappeared, although many small background peaks barely above the baseline were still called. Too little primer caused all peaks, including the peak of interest, to be much smaller. A final primer concentration of 0.02 μM was used unless the peaks on the electropherogram had small rfu values, in which case 0.2 μM was used.

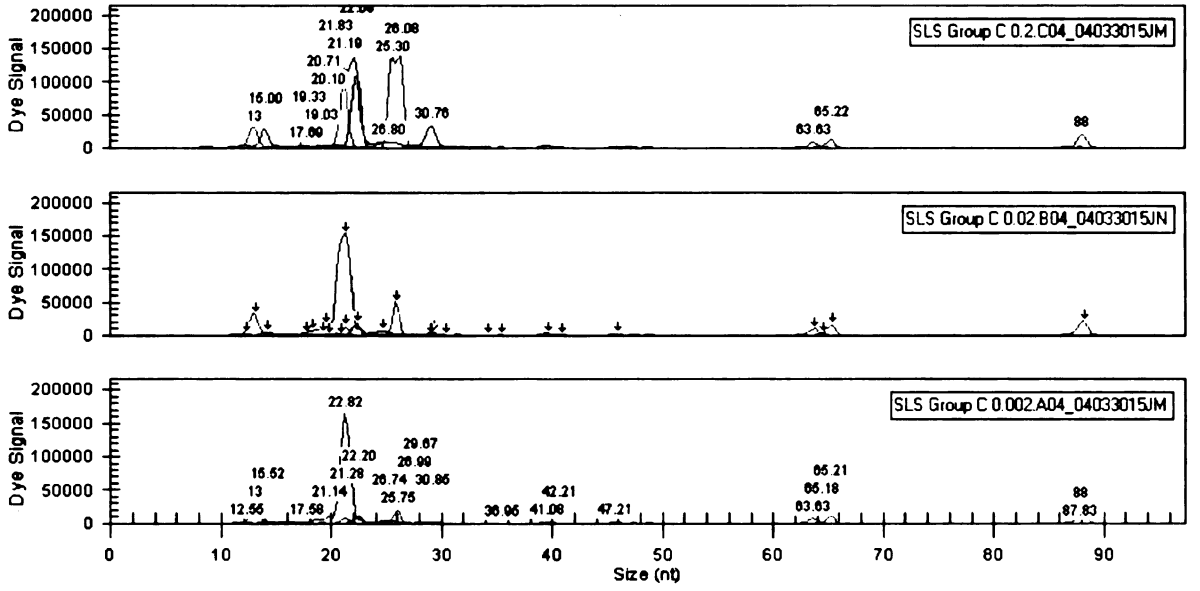
For all electropherograms (Figures 5-12, excluding Figure 9a), red size standard peaks are located at 13 nt and 88 nt. All other peaks correspond to fluorescently labeled nucleotides with ddATP in red, ddGTP in green, ddUTP in blue, and ddCTP in yellow (shown in electropherograms as black). The mobility of the dye during electrophoresis caused peaks to be labeled as slightly, but consistently, smaller in nucleotide (nt) size than the actually size of the primer plus one labeled base. Differences in nucleotide size of product within the same haplogroup are also due to differing mobility of the various dyes.

Figure 5: **Primer Concentration Optimization**

(a) Decreasing SNP Primer Concentration



(b) Decreasing SNP Primer Concentration with Offscale Peaks



Optimization of SNP-primer concentration using final concentrations of 0.2 µM, 0.02 µM and 0.002 µM: (a) non-X result when peak of interest was not offscale (<130,000rfu in unanalyzed data), and (b) non-C result when peak of interest was offscale (>130,000rfu in unanalyzed data). [Note: Y-axis scale is standardized to be the same for each electropherogram.]

At the highest primer concentration in (a) (top panel) ddGTP product peak is visible in green at ~29 nt, with red pull up. As the primer concentration was lowered, the pull-up decreased or disappeared; however, the dye signal (peak height) of the peak of interest was also reduced to a level that may not be useable. At the highest primer concentration in (b) (top panel) ddUTP product peak is visible in blue, however the peaks have split tops, red and yellow pull-up peaks were present, and large non-target artifact peaks were present (for example, green at ~25 nt and blue at ~30 nt). As the primer concentration was lowered, the pull-up and non-target artifact peaks decreased or disappeared, although many small background peaks barely above the baseline were still labeled.

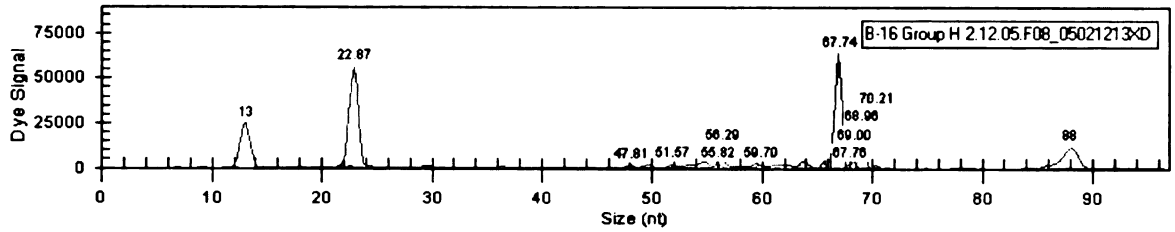
The 13 nt and 88 nt peaks from the Size Standard 80 were sometimes identified incorrectly by the CEQ 8000 software. For example, a small artifact peak, or a primer peak labeled with red dye, was occasionally identified as a size standard peak. Results with visible size standard in the unanalyzed data were reanalyzed with an adjusted slope threshold or removed dye mobility correction. If the size standard peaks were still not recognized correctly, or if it was not apparent which peaks seen in the raw data corresponded to the size standard, then electrophoresis was repeated. Altering the slope threshold in the analysis parameters also affected the labeling of peaks other than the size standard. When the slope threshold was increased, the number of small non-specific peaks identified during analysis decreased.

Method Optimization – Artifact Peaks

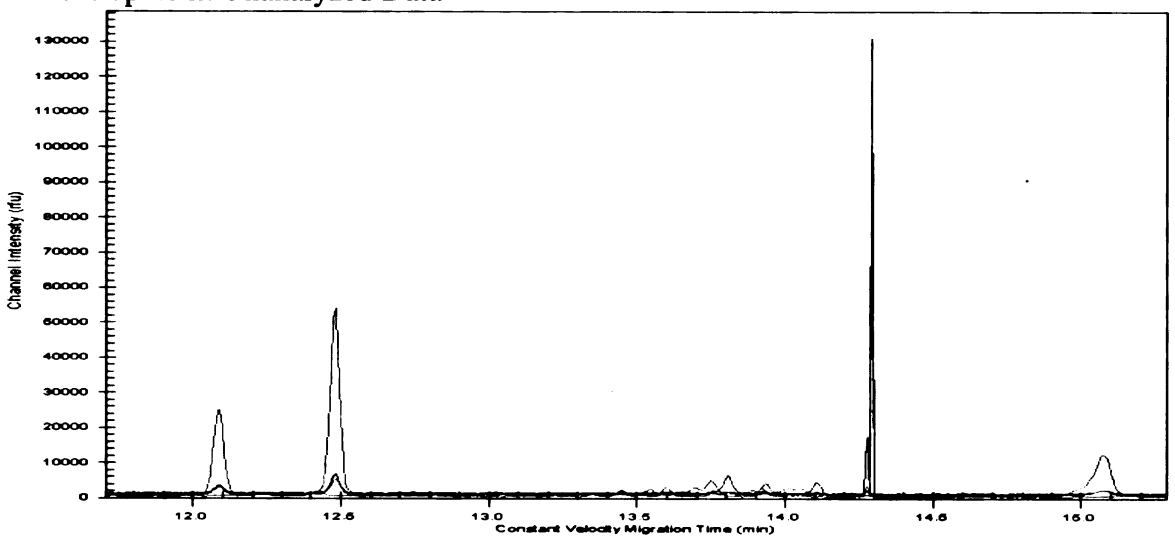
Artifact peaks were observed which were random, or only present in tests for specific haplogroups. Random artifact peaks included spikes, which were identified most easily in the unanalyzed data as large, very steep peaks (Figure 6a). These samples were re-injected. Non-target peaks were also found which were attributed to excess labeled ddNTPs, and were lessened after the premix was made using only the two ddNTPs of interest (Figure 6b). The last type of random artifact peak occurred when the SNP-primer extension product purification was unsuccessful and demonstrated that the purification was normally effective (Figure 6c).

Figure 6: **Examples of Random Artifact Peaks**

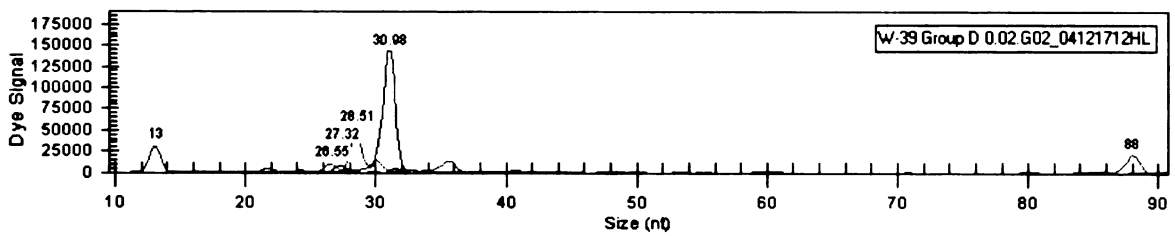
(a) Current Spike in Analyzed Data



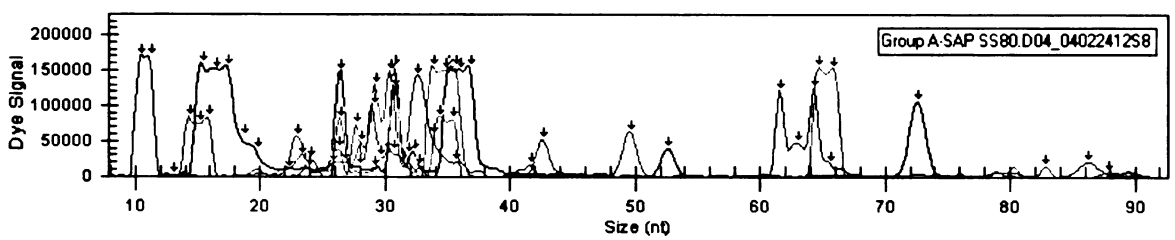
Current Spike in Unanalyzed Data



(b) Non-target Peaks with All Four ddNTPs



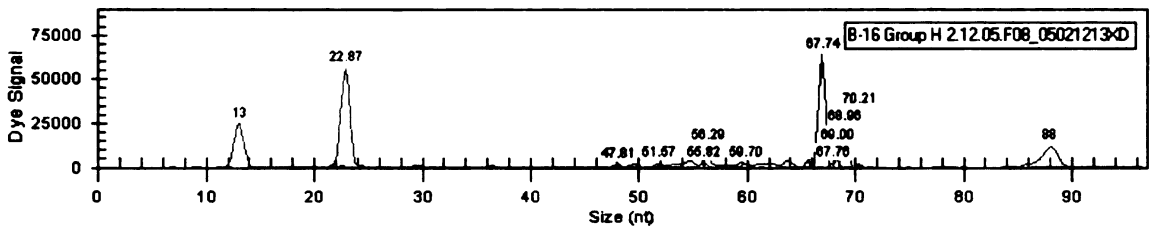
(c) Omitting SNP-primer Extension Product Purification



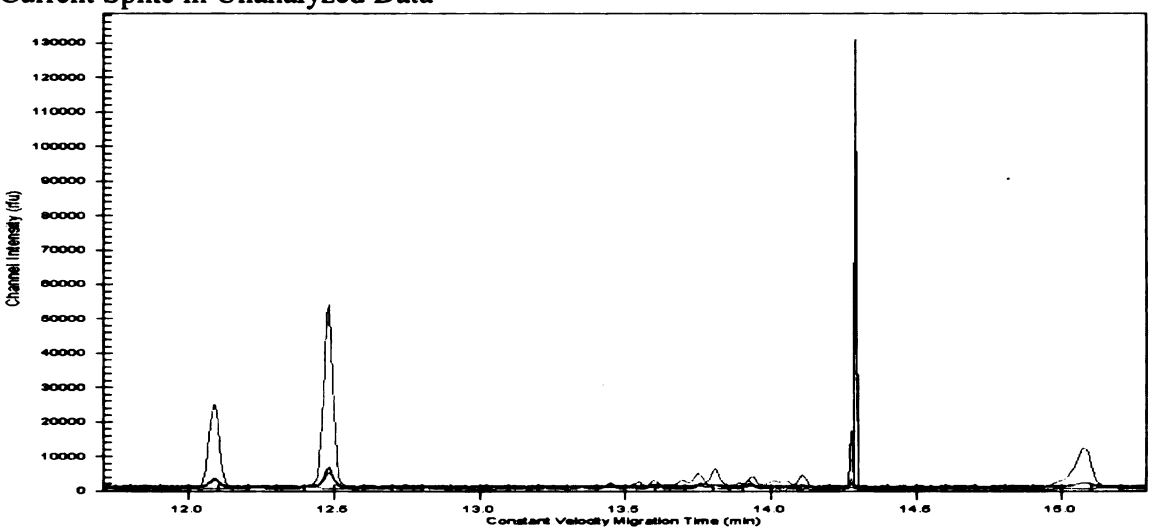
Examples of random artifact peaks: (a) blue current spike at about 67 nt in analyzed data and 14.3 minutes migration time in the unanalyzed data, (b) non-target peaks when all 4 ddNTPs were added to SNP primer extension reaction, and (c) omitting the SNP-primer extension product purification.
In (a) the red ddATP non-H product peak is seen at approximately 23 nt and the blue current spike is seen at approximately 67 nt. The ddUTP non-D product (blue) is visible in (b) at approximately 31 nt, along with ddATP (red) and ddGTP (green) non-target peaks. Multiple peaks present in (c) obscure the product peak of interest.

Figure 6: **Examples of Random Artifact Peaks**

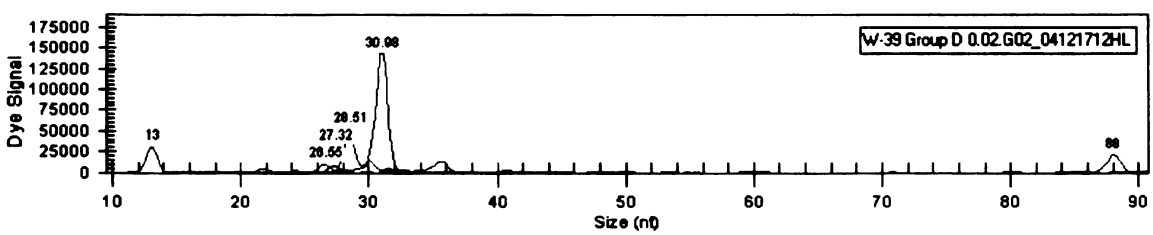
(a) Current Spike in Analyzed Data



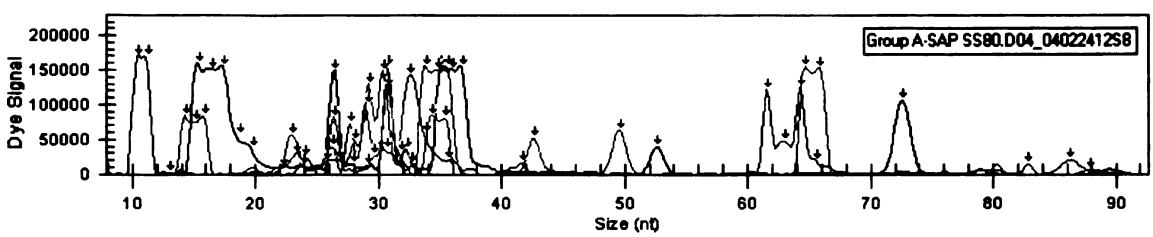
Current Spike in Unanalyzed Data



(b) Non-target Peaks with All Four ddNTPs



(c) Omitting SNP-primer Extension Product Purification



Examples of random artifact peaks: (a) blue current spike at about 67 nt in analyzed data and 14.3 minutes migration time in the unanalyzed data, (b) non-target peaks when all 4 ddNTPs were added to SNP primer extension reaction, and (c) omitting the SNP-primer extension product purification.
In (a) the red ddATP non-H product peak is seen at approximately 23 nt and the blue current spike is seen at approximately 67 nt. The ddUTP non-D product (blue) is visible in (b) at approximately 31 nt, along with ddATP (red) and ddGTP (green) non-target peaks. Multiple peaks present in (c) obscure the product peak of interest.

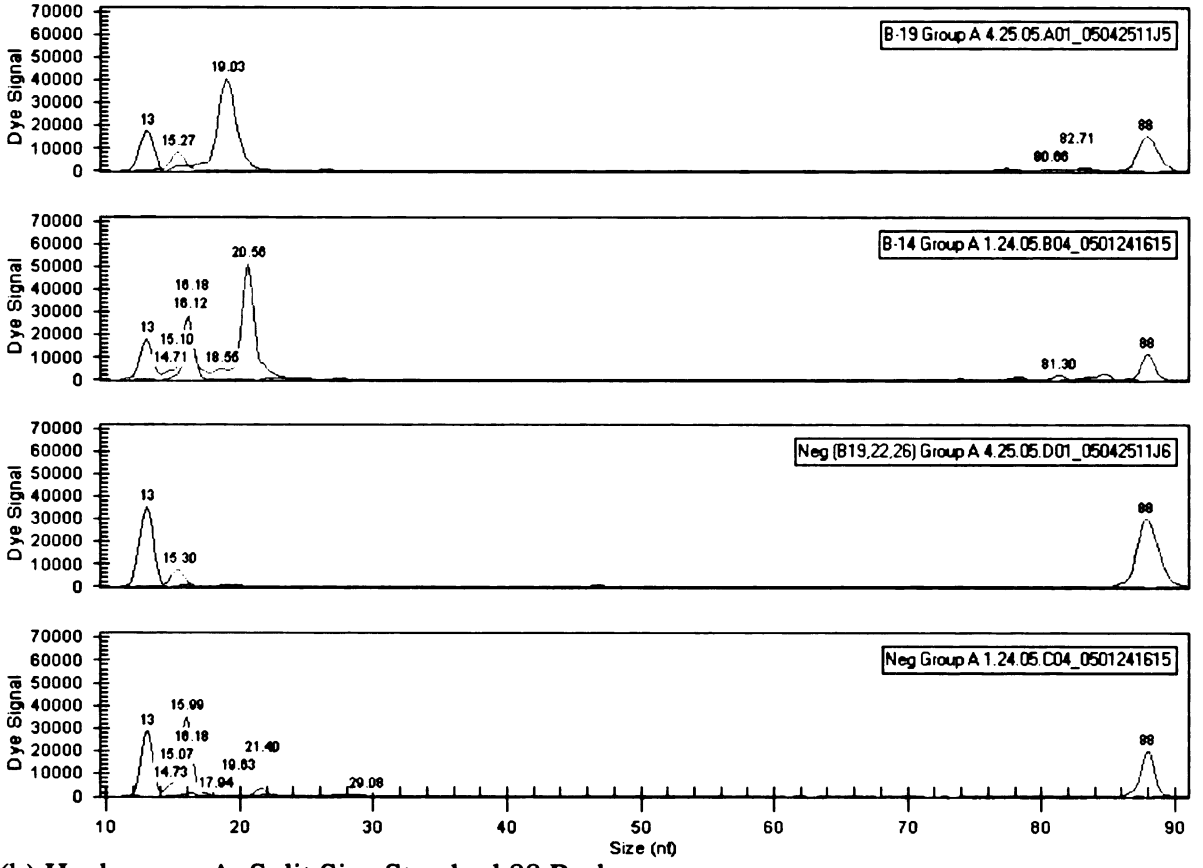
Artifact peaks were also observed which were only present in tests for haplogroup A or haplogroup D (Figure 7). Non-target peaks were seen at approximately 16 nt in tests for haplogroup A, which could not be eliminated (Figure 7a and b). These peaks were not of the expected size, did not interfere with the identification of the size standard peaks or the peak of interest, and were present when no product was in the reaction (reagent blanks and negative controls), indicating that they were a result of the reaction mix, not the sample, and were thus disregarded. Also, in some results for haplogroup A, the 88 nt size standard peak appeared to be split into three peaks, which sometimes resulted in the size standard peaks being labeled incorrectly as already described (Figure 7b). Results for haplogroup D included an artifact peak at approximately 19 nt in reagent blanks and negative controls only (Figure 7c). This peak was also disregarded since it was only seen when no sample was present, indicating it was an artifact caused by the reaction mix.

Figure 7: **Artifact Peaks Only Present in Haplogroups A or D**

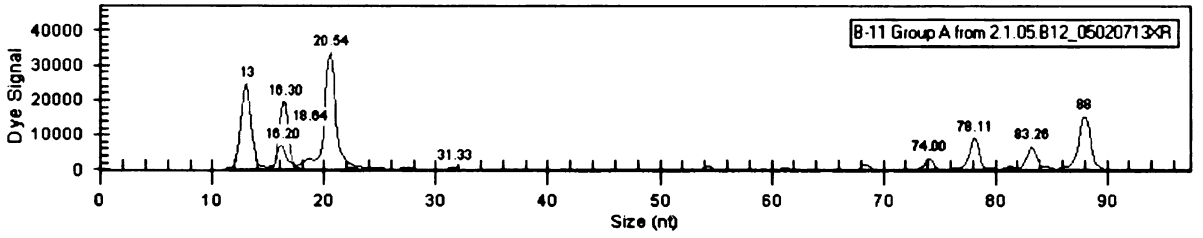
Examples of artifact peaks specific to tests for certain haplogroups: (a) haplogroup A, non-target red or green peak at about 16 nt in all reactions including negative controls (b) haplogroup A, split size standard 88 nt peaks (the 16 nt artifact peak is also present) (c) haplogroup D, non-target green peak at about 19 nt in negative control or reagent blank (bottom), but not present in samples (top) [Note: Y-axis scale in (a) and (c) is standardized to be the same for each electropherogram.]

Figure 7: **Artifact Peaks Only Present in Haplogroups A or D**

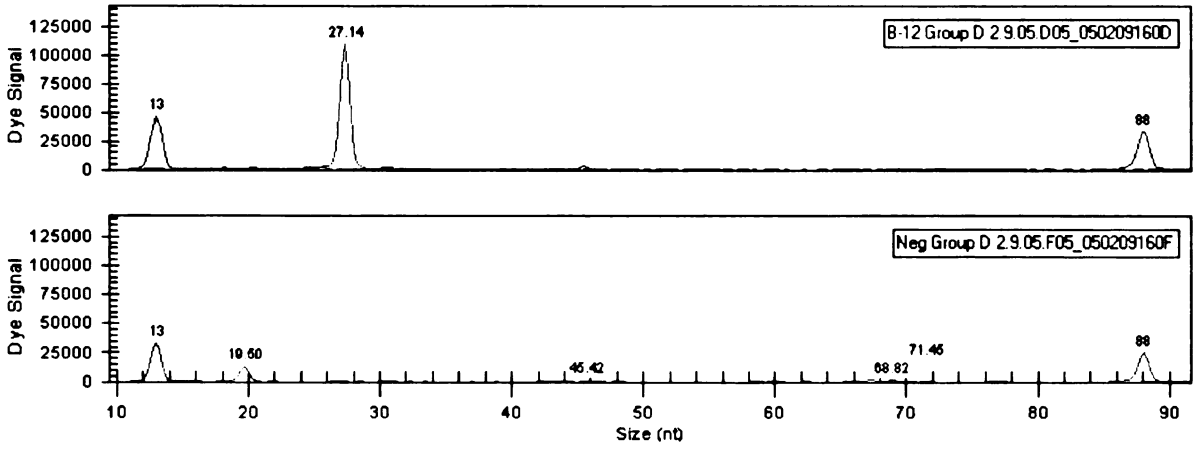
(a) Haplogroup A, Non-target Peak at About 16 nt



(b) Haplogroup A, Split Size Standard 88 Peaks



(c) Haplogroup D, Non-target Peak at About 19 nt

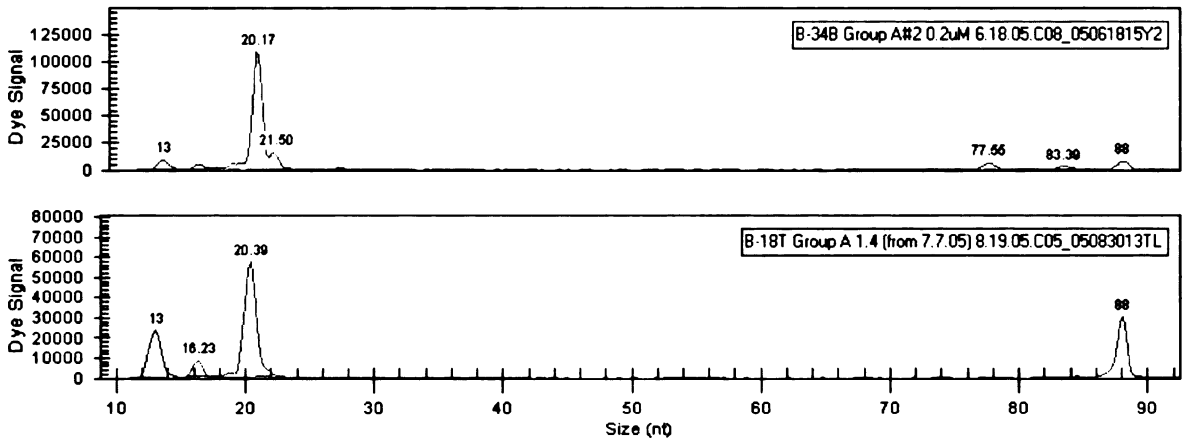


Haplogroup Determination Overview

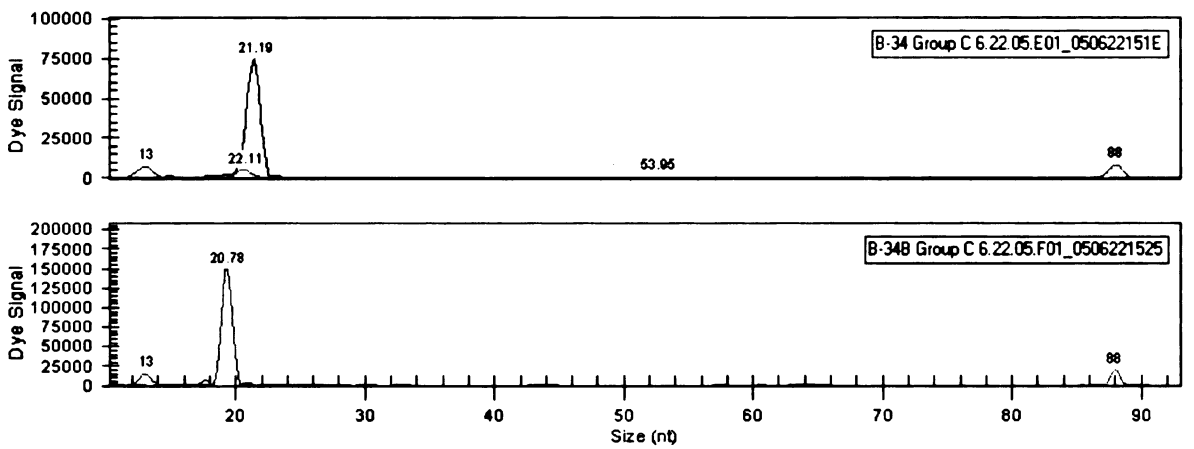
Tests for haplogroups B, C, D, X, and J generally produced unambiguous results on the first attempt. Figure 8 shows examples of both possible outcomes for each of these haplogroups, excluding haplogroup D, which was not seen in any of the burials. Figure 9 shows both outcomes for haplogroup B (defined by a 9bp deletion) using two different primer pairs. Tests for haplogroup A produced ambiguous results for one burial (B-29), in which both peaks were found in equal intensity (Figure 10). Haplogroup H gave ambiguous results in several cases, in which both peaks were seen, though peaks often differed in intensity among runs (Figure 11, Table 6). Also, within a single burial, haplogroup H, non-H, and ambiguous results were observed (Figure 12). An individual was called haplogroup H only if the result was clean twice, and no non-H results were seen. The burial was typed as non-H if the result was clean, or if some results were clean and others ambiguous, while haplogroup H results were not found. All other cases were typed as 'possibly haplogroup H'. Negative controls in tests for haplogroup K produced PCR product in all cases, including use of new primers, indicating the original primer stock was contaminated. Thus, these results were disregarded.

Figure 8: **Possible Outcomes for Each SNP-Primer Extension Reaction**

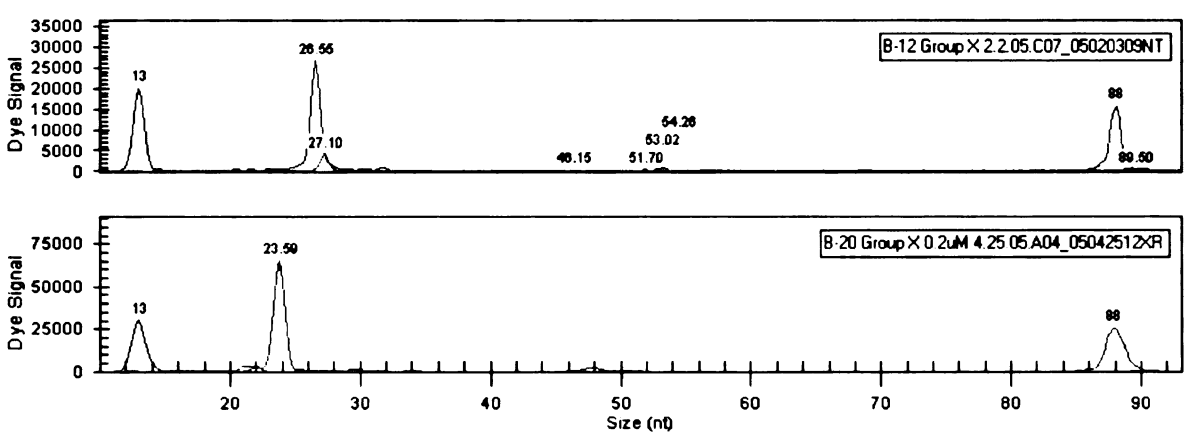
(a) Example of Haplogroup A and Non-A



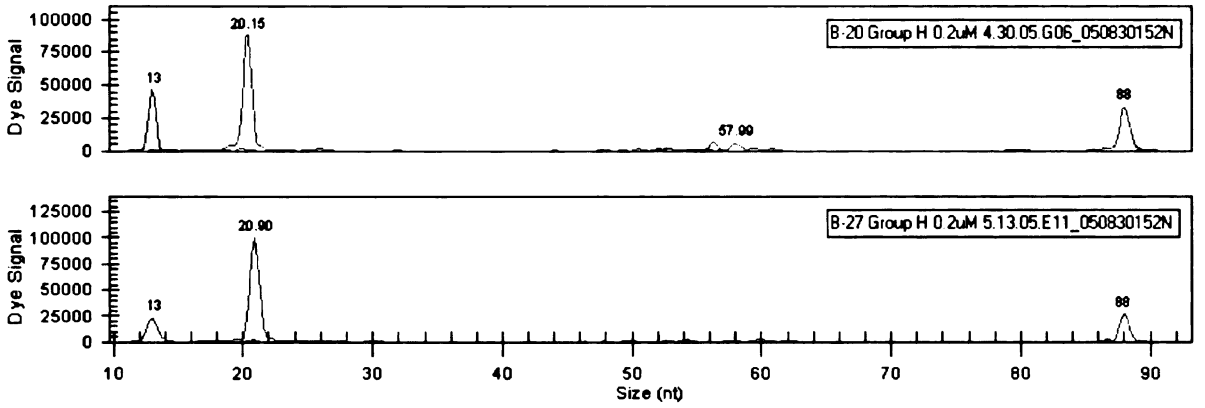
(b) Example of Haplogroup C and Non-C



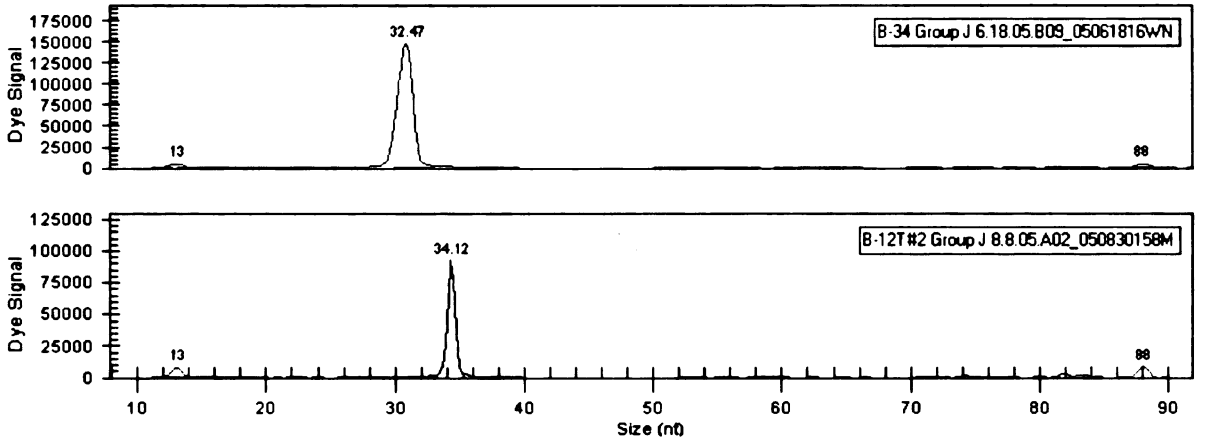
(c) Example of Haplogroup X and Non-X



(d) Example of Haplogroup H and Non-H



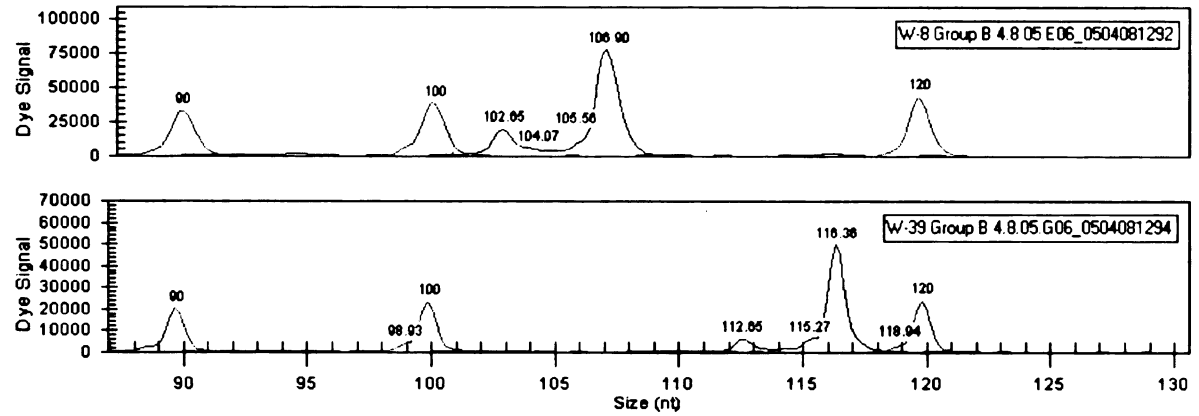
(e) Example of Haplogroup J and Non-J



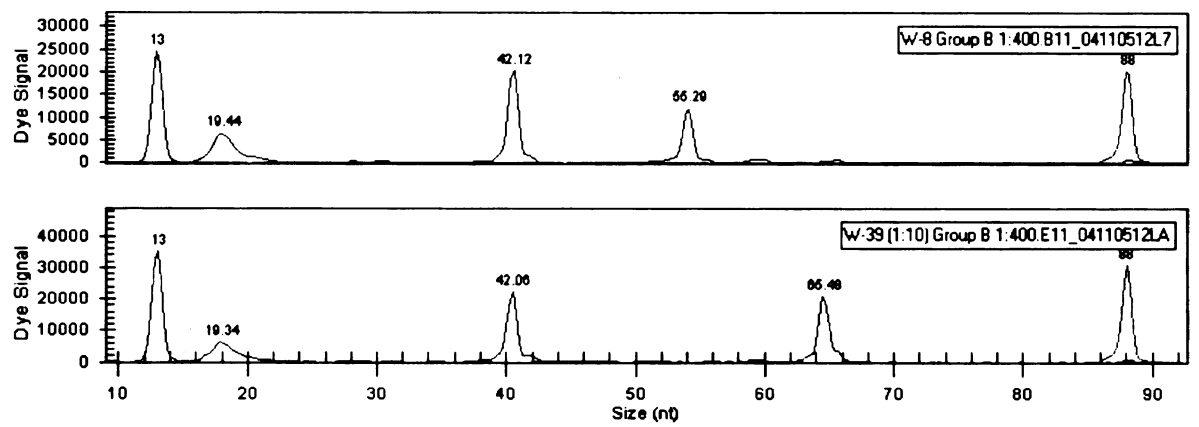
Examples of possible outcomes for each SNP-primer extension reaction from Fort Michilimackinac bone samples: (a) haplogroup A and non-A, (b) haplogroup C and non-C, (c) haplogroup X and non-X (a detailed discussion of this haplogroup X result for B-12 is in the Haplogroup Assignment Resolved by Control Region Sequencing section), (d) haplogroup H and non-H, and (e) haplogroup J and non-J.

Figure 9: **Possible Outcomes for Haplogroup B**

(a) Example of Haplogroup B and Non-B Using Labeled Forward and External Reverse Primers



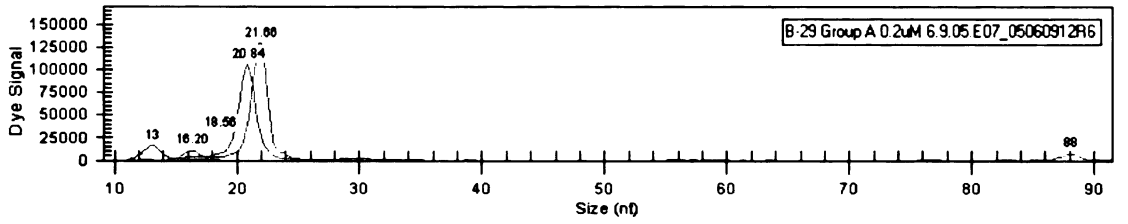
(b) Example of Haplogroup B and Non-B Using Labeled Forward and Internal Reverse Primers



Examples of both possible outcomes for haplogroup B from known Native American DNA: (a) haplogroup B and non-B using labeled forward and external reverse PCR primers, (b) haplogroup B and non-B using labeled forward and internal reverse PCR primers.

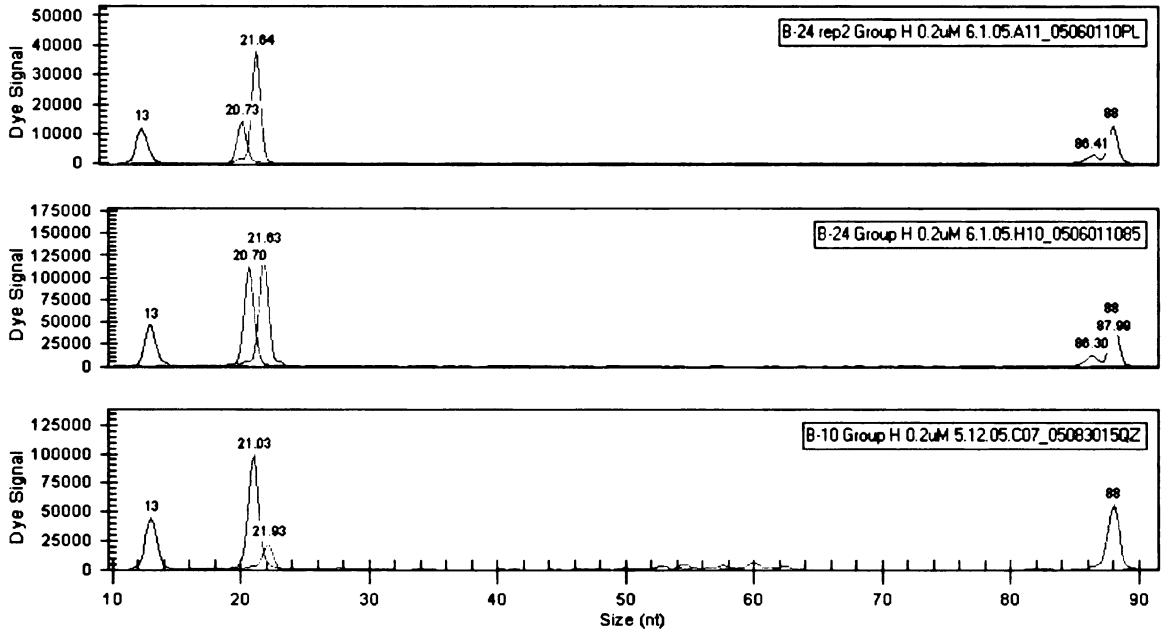
In (a) red size standard 400 peaks are visible at 90 nt, 100 nt and 120 nt. Labeled blue PCR product is visible at approximately 106 nt in haplogroup B, and 9 nt larger in non-B. The small blue peak approximately 4 nt smaller than the product peak is an artifact seen only in the known Native American DNA samples, and not in any of the bone samples. In (b) blue primer peaks are seen at about 20 nt, and blue primer dimer peaks are present at about 42 nt. Labeled blue PCR product is visible at approximately 55 nt in haplogroup B, and 9 nt larger in non-B.

Figure 10: **Ambiguous Results for Haplogroup A**



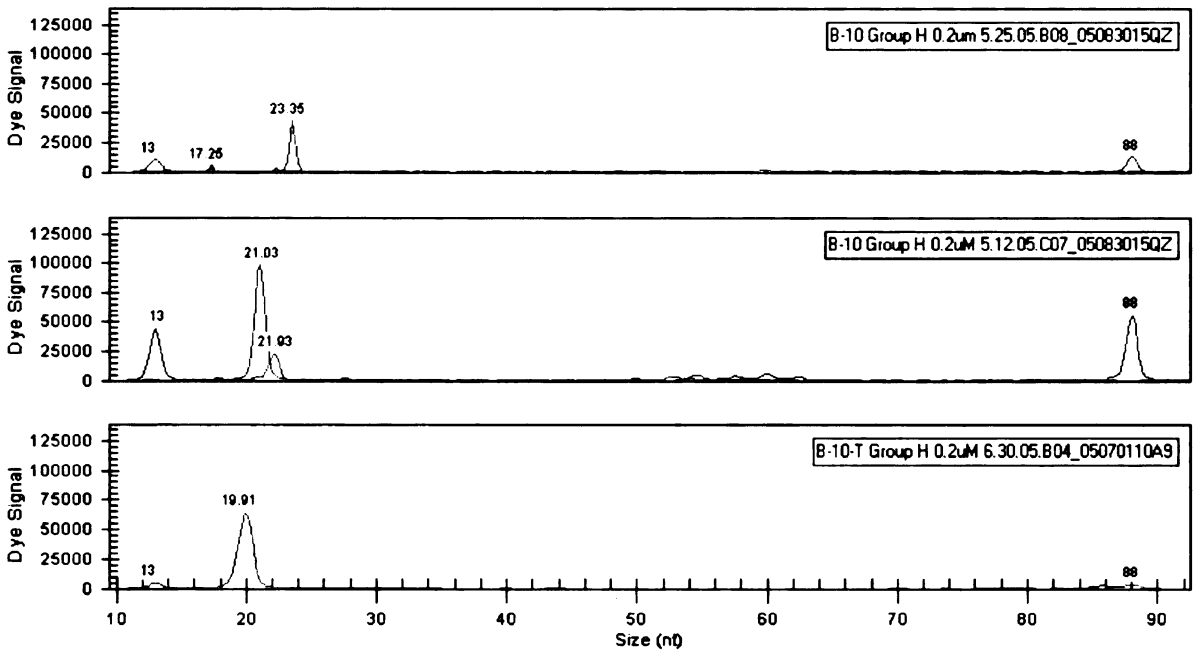
Example of an ambiguous result for haplogroup A, which was only seen for burial B-29. The ddGTP product peak in green and the ddATP product peak in red are present at approximately 21 nt. For all reactions of B-29 for haplogroup A, both product peaks were present at approximately equal peak heights; therefore, B-29 could not be included in haplogroup H or non-H.

Figure 11: **Ambiguous Results for Haplogroup H**



Examples of ambiguous results for haplogroup H showing the range of peak height imbalance, with the haplogroup H ddGTP product peak in green and non-H ddATP product peak in red. The top electropherogram, from B-24, shows a stronger haplogroup H peak, the middle electropherogram, from B-24, illustrates balanced peak heights, and the bottom, from B-10, shows a stronger non-H peak.

Figure 12: **Ambiguous Results for Haplogroup H Within a Single Burial**



Examples of ambiguous results for haplogroup H from the same individual (B-10), with the haplogroup H ddGTP product peak in green, and non-H ddATP product peak in red. The top electropherogram shows a clean haplogroup H result for the right femur from burial B-10, the middle electropherogram shows an ambiguous result for the same femur, and the bottom electropherogram shows a clean non-H result for a tibia from the same individual. [Note: Y-axis scale is standardized to be the same for each electropherogram.]

Ancestry and Haplogroup Assignments

The known Native American DNA samples for haplogroups A, B, C and D typed as the haplogroup expected, and as none of the other haplogroups tested in this study. The adult and subadult from the commingled burial, B-34, typed as haplogroup A, and haplogroups C and J (see details below) respectively, demonstrating the ability to detect Native American haplogroups from bone samples from the fort.

Fourteen out of the fifteen individual burials were not Native American. Five of these typed as European, four showed ambiguity in their haplogroup H results, and the

rest did not type as any of the haplogroups tested. Haplogroups, ancestry assignments, sexes estimated by skeletal morphology, and age groups are presented in Table 5.

Table 5: **Haplogroup Assignment**
Age group and sex as estimated by skeletal morphology, haplogroup assignment, and ancestry for each burial. Haplogroups A and C are specific to Native Americans, while haplogroups H and J are found in Europeans.

Burial Number	Age Group	Sex	Haplogroup	Ancestry
B-10	Adult	Male	Possibly H[b]	Not Native American
B-11c	Adult	Female	Possibly H[b]	Not Native American
B-12	Adult	Possibly Female	Unknown[a]	Not Native American
B-14	Adult	Likely Male	Unknown[a]	Not Native American
B-16	Adult	Male	J	European
B-17	Adult	Male	H and J	European
B-18	Adult	Possibly Female	Possibly H[b]	Not Native American
B-19	Subadult	Unknown	Unknown[a]	Not Native American
B-20	Adult	Male	H	European
B-21 (Box 12)	Adult	Male	J	European
B-22	Subadult	Unknown	Unknown[a]	Not Native American
B-24	Adult	Likely Female	Possibly H[b]	Not Native American
B-26	Adult	Male	H	European
B-27	Adult	Unknown	Unknown[a]	Not Native American
B-29	Adult	Likely Male	A/non-A[c]	Undetermined
B-34	Subadult	Unknown	C and J	Undetermined
B-34B	Adult	Unknown	A	Native American

[a]Typed as none of the haplogroups tested.

[b]Typed as none of the haplogroups tested, except ambiguous for haplogroup H.

[c]Typed as none of the haplogroups tested, except showed both A and non-A bases at approximately equal quantities in the same reaction.

The European individuals included burials B-16 and B-21 as haplogroup J, burials B-26 and B-20 as haplogroup H (results from two replicates), and burial B-17 as haplogroup H and J. Burials B-10, B-11c, B-18, and B-24 could not be assigned as haplogroup H or non-H; however, they did not belong to any of the other haplogroups tested and were assigned the ancestry 'Not Native American'. Five burials, B-12, B-14, B-19, B-22 and B-27, did not type as any of the haplogroups tested and were therefore,

also assigned the ancestry 'Not Native American'. These individuals may be European with a haplogroup other than H or J, or they could possibly be African or Asian. More details on all burials with ambiguous results or multiple haplogroup assignments are presented below.

The final single burial (B-29), produced haplogroup A and non-A results at approximately equal concentrations in all tests (three replicates total) from femur and tibia, indicating the individual may be heteroplasmic (see details in Discussion; Figure 10). Burial B-29 did not type as any of the other haplogroups tested, could not be excluded from being Native American, and therefore, had an undetermined ancestry.

Ambiguous Haplogroup Assignments

Tests for haplogroup H generated ambiguous results for nine individuals, four of which could not be assigned as haplogroup H or non-H (Table 6 and 5 respectively; Figures 11 and 12). These four burials, B-10, B-11, B-18 and B-24, did not type as any of the other haplogroups tested and were designated 'Not Native American,' ambiguous for haplogroup H.

Table 6: **Ambiguous Results for Haplogroup H**
Haplogroup results for each burial that exhibited some ambiguity. F_L=left femur, F_R=right femur, T=tibia, and H=humerus.

Burial Number	Haplogroup H	Ambiguous	Non-H
B-10	1 F_R	1 F_R	1 T
B-11	1 F_R	5 F_R, 1 T	1 F_R, 1 T
B-12		2 F_L	1 F_L, 1 T
B-17	2 F_R	2F_L	
B-18	1 F_R, 1 T	1 F_R	3 F_R
B-19		3 F_R	1 T
B-20	2 F_R	1 F_R	
B-24	1 H	4 F_L	
B-29		1 F_L, 1 T	1 F_L

In two instances burials produced results indicating that they belonged to two separate haplogroups. Burial B-17 typed as haplogroup H, although some results were ambiguous (Table 6), and haplogroup J. Given that both haplogroups were European, B-17 was designated as European. Burial B-34 gave results for Native American haplogroup C, and European haplogroup J; therefore, B-34 could not be excluded from being Native American or European, and has an undetermined ancestry.

Haplogroup Assignment Resolved by Control Region Sequencing

Burial B-12 was typed as haplogroup X in one DNA extraction from femur (Figure 8). However, in subsequent testing of the femur, as well as tibia, the burial was classified as non-X. When a section of HV1 was sequenced, both bone samples included a T and a C at base 16311 (indicating heteroplasmy), and otherwise did not differ definitively from the sequence published by Anderson et al. (1981). The sequence did not contain the 16223T or 16278T, which are used to characterize haplogroup X (Brown et al. 1998). B-12 also did not contain the 16213A present in haplogroup X of Native Americans. Therefore, control region sequencing confirmed that B-12 was non-X. This burial also typed as none of the other haplogroups tested, although some results for haplogroup H were ambiguous (Table 6). Therefore, B-12 was designated as 'Not Native American'.

Haplogroup Determination Sorted by Sex of Individual

The sexes of the individuals used in this study were estimated by members of the Department of Anthropology at Michigan State University (Table 5; R. Tubbs personal

communication). The discrete burials included four adult females and eight adult males, as well as two subadults and one adult whose sex was not estimated. The sexes of B-34 and B-34B were unspecified since these individuals were from a commingled burial (R. Tubbs personal communication).

The four female individuals were not Native American (three with ambiguous haplogroup H). Both of the subadults, which were buried independently, were not Native American and did not belong to any of the haplogroups tested. Five of the male individuals were European (haplogroup H, J or typing as both), two were not Native American (one with an ambiguous haplogroup H), and one had an undetermined ancestry. The non-commingled adult with unknown sex was not Native American.

DISCUSSION

The goal of this research was to determine the ancestry of remains interred in Fort Michilimackinac between 1743 and 1781. In order to accomplish this, a method of detecting Native American and European mtDNA haplogroups was created using SNP-analysis. The bone samples used in this study were over 200 years old and of historical significance; therefore, much care was taken to account for DNA degradation and the sample type. For instance, in order to minimize damage to the skeletal material, as few holes were drilled into the bone as possible, and those that were, were very small.

MtDNA was chosen for analysis because it can often be extracted in useable amounts from noncontemporary skeletal material, and because it allows determination of ancestry. Ancient DNA analysis, however, contains a number of potential pitfalls, including limited DNA quantity, degraded DNA, and the presence of substances that may inhibit the chemical reactions used in analysis. Extracted DNAs were resuspended in a minimal amount of TE buffer to keep the DNA as concentrated as possible, although this limited the number of reactions that could be conducted on each sample. Also, in spite of attempts to keep DNA concentrated, in many instances PCR results were negative. Therefore, semi-nested PCR was often necessary to increase the amount of DNA amplification to detectable levels. In order to utilize as much template DNA from each extraction as possible, including degraded fragments, PCR primers were designed to produce small amplicons.

The limited DNA quantities also made diluting samples to reduce inhibition problematic. The source(s) of this inhibition was not determined, although it might have included substances from the bone itself, or components of soil, both of which are known

to inhibit PCR. For example, calcium carbonate in bone may bind to the polymerase and prevent it from functioning, while humic acids from soil are also inhibitory. Due to inhibition, it was often necessary to add BSA to PCR reactions. In the end, all bones produced successful extractions either alone or when a combination of nested PCR, sample dilution, and/or addition of BSA were performed.

Method Optimization and Artifact Peaks

The SNP assay was developed using known Native American DNA samples belonging to haplogroups A, B, C and D, which were all identified correctly. Each haplogroup assay required optimization. Optimal SNP-primer concentrations were found to be 0.02 μM or 0.2μM, which varied among assays. Some SNP-primers seemed to be less 'robust' than others; therefore haplogroups A, X and H assays generally called for the higher primer concentration. Variables such as target peak height, incorrect labeling of size standards and random artifact peaks, were dealt with on sample-to-sample bases by performing the analysis again with a modified procedure as described in the results section.

Artifact peaks that were regularly present in tests of a specific haplogroup were disregarded. The artifact peak in analyses of haplogroup A, found at about 16 nt, and the artifact peak in tests for haplogroup D, at about 19 nt, probably resulted from components of the reaction mix, as they were present in reactions containing no sample (reagent blanks and negative controls). Time did not allow further analysis of these artifacts, but tests such as running reactions without primer or other components of the reaction mix could be conducted to help determine their cause. The split 88 nt size standard peaks

occurring in tests for haplogroup A were also disregarded if the largest peak (in nucleotide size and peak height) was correctly labeled as the size standard. Otherwise, the sample was rerun. It is possible that these extraneous peaks were a result of the SNP reaction and not the size standard. This could be tested by performing the primer extension reaction without the ddATP (labeled red), or by omitting the size standard. If the extra peaks are still present when ddATP is excluded, then the peaks resulted from the size standard; if the peaks are present when the size standard is omitted, then they resulted from the reaction itself. In all, while some artifacts were seen, those that appeared consistently could be disregarded, and those that appeared intermittently could be reanalyzed, resulting in successful analysis of all skeletal remains.

Ancestry Assignment

All five haplogroups present in Native Americans (haplogroups A, B, C, D, and X) were analyzed in this research, making it possible to classify remains as Native American or not Native American. At least 13 other haplogroups (and far more subgroups) from around the world have been described in the literature; therefore, it was not practical to do an exhaustive examination of the remains to definitively identify the haplogroup of each. Instead, only the most common European haplogroups, H and J, were tested. Haplogroup H encompasses ~ 40% of European mtDNA, while haplogroup J encompasses ~ 9% (www.mitomap.org). Haplogroup K (~8%) was also designed to be tested, but the PCR primers for this assay appeared to be contaminated (negative controls consistently gave results), thus this assay was abandoned. European haplogroups were examined as it seemed probable that non-Native American individuals were European

due to the context of the burials within Fort Michilimackinac. However, black slaves were known to reside at the fort, and the burial register includes interments of some slaves (Peterson 1982; Thwaites 1910). Consequently, in some instances ancestry determination was limited to 'Not Native American', presumably because either the individual was not European, or was of an untested European haplogroup.

Fifteen femora, representing 15 individuals buried at Fort Michilimackinac, were originally received for analysis. Additional bone samples (femur, tibia, or humerus) were added for eight burials that were producing ambiguous results. Among the 15 individuals, 14 tested as not Native American, while one (B-29) could not be excluded as Native American (see details below). Of the non-Native Americans, five tested as European (two individuals were haplogroup H, two individuals were haplogroup J, and one individual typed as both haplogroups H and J). For the remaining nine individuals a specific haplogroup could not be discerned, including four burials which produced ambiguous haplogroup H and non-H results (see details below).

After obtaining results from the original burials and discovering that none tested as Native American, the question was raised if the assay used could successfully detect Native Americans from Fort Michilimackinac remains. In order to investigate this, femora from two individuals (B-34 and B-34B), originating from a 6 person mass grave at Fort Michilimackinac, were obtained. This commingled burial was assumed to contain Native American remains, given that the only record of a burial of six individuals at the fort was of Native Americans who died of smallpox. The adult sample from the commingled burial (B-34B), typed as Native American haplogroup A. The subadult individual (B-34) typed as Native American haplogroup C, as well as European

haplogroup J (Table 5) in multiple tests. Although, a European haplogroup was also identified (see below), both individuals from the mass burial produced results for a Native American haplogroup, demonstrating that the method successfully detected these Native American SNP sites in the bone samples.

While the reproducible dual result for burial 34-B was surprising, instances of one individual producing two haplogroup findings have been reported in the literature. Torroni et al. (1993) described a mtDNA sample with the 663 +HaeIII characteristic of haplogroup A and the 9bp deletion characteristic of haplogroup B. They concluded that the 9bp deletion was a derived mutation that occurred in the haplogroup A mtDNA background. Additionally, Torroni et al. (1996) reported that the polymorphism at position 7025 diagnostic of haplogroup H has been sporadically observed in non-Europeans; therefore, a false positive may occur in mtDNA typing when a sample actually originated from a different haplogroup. In this study, B-34 could not be excluded from being Native American or European, and as such was assigned an undetermined ancestry. Analysis of additional SNP sites in the coding or control regions, which further characterize haplogroups C or J, could be useful in assigning a haplogroup and ancestry to this burial. (Burial B-17 also produced positive results for two haplogroups, H and J; however, as these are both European haplogroups, they did not interfere with assigning the individual European ancestry).

Ambiguous Haplogroup Results

Nine burials generated ambiguous results in at least one assay for haplogroup H (Table 6). Among samples that tested as not Native American, four could not be assigned

to haplogroup H or non-H due to ambiguities (Table 5). These showed varying amounts of peak height imbalance with each PCR attempt or DNA preparation (Figures 11 and 12). In some instances, two peaks were plainly present at the SNP site, while retesting the same DNA, or testing a new DNA isolation from the same bone or another bone in the burial, resulted in one or the other peak. Variable findings in DNA analyses, with fluctuating results, is characteristic of "stochastic effects", or inconsistent findings resulting from unequal sampling of small amounts of starting DNA; one DNA molecule (or small number of molecules) is randomly sampled one time, the other may be sampled the next time, or both may be present at different ratios.

The ambiguous haplogroup H results necessitate two different DNA molecules being present in the bone. MtDNAs are generally found as identical molecules within an individual; however, post-mortem mutation of DNA may alter this. Gilbert et al. (2003) proposed a C/T mutation process in which the base cytosine (C), which normally pairs with guanine (G) during DNA replication, undergoes deamination (loss of an amine group) thus mutating into uracil (U; a base that replaces thymine (T) in RNA). During PCR replication these uracils will pair with adenine (A), not G. As this new molecule is replicated, the A pairs back with T, resulting in a C to T transition on the original strand, and a G to A transition on the complementary strand. This process will most likely not occur in each DNA molecule, and if large amounts of DNA are present, the results from non-mutated DNA may make a small amount of mutated DNA undetectable. However, as the bone ages, the level of DNA degradation and cytosine deamination may increase, resulting in both low quantities of DNA, and relatively high levels (ratios) of mutated material to non-mutated. Two different molecules and low DNA levels can result in

stochastic effects, producing the ambiguous results and peak height variability seen in tests for haplogroup H. Although such postmortem DNA damage could possibly affect any of the SNPs tested, this type of ambiguous result was only common for haplogroup H. This indicates that the specific nucleotide that defines haplogroup H, or perhaps the region of mtDNA containing that site, may be particularly susceptible to post-mortem mutation.

To test for postmortem DNA mutations caused by cytosine deamination, DNA samples can be treated with uracil-N-glycosylase (UNG), which removes uracil bases, and effectively destroys all DNA strands containing them (Hofreiter 2001). For ambiguous H haplogroup typing, UNG treatment should result in clean, haplogroup H results, since cytosine deamination could create non-H results from haplogroup H DNA, but not vice versa. Two replicates of burials B-18 and B-24 were treated with UNG prior to PCR amplification, and the SNP subsequently tested. In both cases, ambiguous results were still produced (results not shown). For the samples examined here, it appears that postmortem DNA damage may be occurring, but by a mechanism other than cytosine deamination. Further analysis of postmortem damage would be required to understand the mechanism that resulted in the frequent haplogroup H ambiguity. Fortunately, this ambiguity did not influence the ability to makes calls for Native or non-Native Americans.

A unique ambiguous result was observed in burial B-29, occurring in assays for haplogroup A. These results differed from the haplogroup H ambiguities, in that both A and non-A peaks were in a constant ratio among tests, as well as in different bones from the burial (Figure 10). Because the results were highly consistent, the polymorphism

likely existed antemortem in the form of heteroplasmy, and occurs in all DNA from that individual. Heteroplasmy, while rare, is widely described in the literature (Holland and Parsons 1999). Owing to the consistent presences of A and non-A peaks in B-29 (and a lack of any other defining haplogroup results), its specific ancestry was not assigned.

One ever-present reason for ambiguous results, where a sample generates two product peaks, is contamination with DNA from the molecular biologist or anthropologists who worked with the bone samples. In the cases presented here, this is improbable because the outer surface of the bone was removed to eliminate potential contamination due to past handling, precautions were taken to avoid contamination by the researcher, and contamination would be required to occur multiple separate times for each ambiguous burial and not at all for unambiguous burials. Further, the contamination would need to be highly specific to haplogroup H (or A in one instance). Overall, the reproducibility of the ambiguous findings indicates that these results are real, and stem from the skeletal material itself.

MtDNA vs. Craniometric Ancestry Assignment

Previous research into the ancestry of burials from Fort Michilimackinac was performed by Dr. Russell Nelson of the University of Michigan (R. Nelson personal communication). That research used craniometric analysis (measurement of human skulls) to create a database of 2,200 individuals from around the globe, including mainland Asians, American Indians from several regions, central and western Europeans, sub-Saharan Africans and others. No subadults were included in these analyses as they are considered not useful in craniometric studies (R. Nelson personal communication).

Six burials from Fort Michilimackinac with compete skulls were analyzed by Dr. Nelson in 1996. When the cranial measurements for these individuals were compared to the database, three (B-24, B-29, and B-34) sorted with American Indians, two (B-17, and B-21) sorted with Europeans, and one (B-18) indicated a Native American/European mixture.

The ancestry assessments using craniometrics were mainly in concordance with the results of this study using mtDNA analysis, with the exception of burial B-24. Burials B-17 and B-21 were found to be European using both methods. Burial B-17 had some indications of Indian ancestry using craniometrics; however, it grouped mainly with Europeans (R. Nelson personal communication). As mentioned, burial B-34 was a commingled burial of 6 individuals; therefore, it is not possible to determine if the adult femur from this study (B-34B) belonged to the same individual as the adult skull investigated using craniometrics. Both studies suggested the remains representing B-34 were Native American, which is consistent with the historical record that indicated the mass interment of six Native Americans. Burial B-29 sorted with Native Americans using craniometrics and had an undetermined ancestry using mtDNA, due to heteroplasmy at the SNP site for Haplogroup A. Since burial B-29 could not be included in or excluded from Native American haplogroup A, it is possible that the individual belongs to that Native American mitochondrial haplogroup, and thus had Native American ancestry in concordance with the craniometric results.

Craniometric analysis of burial B-18 sorted with both American Indians and Europeans, suggesting a mixture. Because mtDNA analysis only examines maternally inherited DNA, an individual with mixed ancestry would type as the ancestry of their

mother using this approach. Therefore, if burial B-18 had a European mother and a Native American father, the European mtDNA haplogroup and mixed ancestry craniometric results would be in concordance. However, at Fort Michilimackinac many more male Europeans (trappers, soldiers etc.) were present than female Europeans, making this specific combination unlikely. Even if the mixture occurred further back in this individual's family (if they were not first generation Métis), the European contribution would have to be female at some point in order to incorporate the European mtDNA lineage. Overall, craniometric and mtDNA ancestry estimations for burial B-18 do not contradict each other, but do create a seemingly unlikely scenario with regard to Fort Michilimackinac.

The final individual examined using both mtDNA and craniometrics (B-24) produced potentially contradictory results. Craniometric analysis indicated the individual was of Native American ancestry, although it also sorted fairly highly with mainland Asians. The current study found that mtDNA from burial B-24 did not belong to any of the Native American or European haplogroups tested, with ambiguous results for European haplogroup H, thereby indicating the individual was not Native American. However, since not all Asian mtDNA haplogroups were examined (for example haplogroup G, see Figure 3), these results could be reconciled if the individual had mixed Native American and Asian ancestry with an untested Asian mtDNA haplogroup being inherited maternally. Again, although this mixture is possible and would reconcile the craniometric and genetic results, the combination would appear unlikely given the population present at Fort Michilimackinac.

Implications for the History of Fort Michilimackinac

Fort Michilimackinac was a stockade fort on the northern tip of Michigan's Lower Peninsula built by the French in 1715. Inside the fort, the Church of St. Anne was erected in 1743 and burials were placed under the church floor (Maxwell and Binford 1961). Although the fort was a military structure, it mainly served as a cultural/commercial center used in the fur trade. Inhabitants of the fort included a small number of military personnel, a large number of individuals involved in the fur trade, merchants, black and Native American slaves, and others. Some families of troops or traders were also present at the fort. These families are assumed to have included Native American women and Métis children, since intermarriage has been reported to be an important component of communities involved in the fur trade (Peterson 1982).

In this research, 15 individuals excavated from Fort Michilimackinac were analyzed, none of which were found to be Native American (one has an unknown ancestry). The lack of Native American men is consistent with Fort Michilimackinac primarily housing French troops, fur traders, and craftsmen. Native American men, while often present near the fort to exchange fur and other goods, would likely be buried by their own people and not interred by members of the Catholic Church in the fort. The absence of Native American women among the four female remains analyzed implies that Native American females were less common in the church burials than historical accounts of its social organization, and records of baptisms, marriages and interments, would indicate.

Unfortunately, interpreting the incomplete or non-detailed records of life at Fort Michilimackinac is difficult. For example, marriage listings from the Mackinac Register

would not include French women who were married before arriving at the fort (and some officers were known to bring wives and families). Consequently, these women might be underrepresented in the records, causing Native American females to appear more prevalent. The details of interments in the Mackinac Register are also often unclear. Interments at the church, ranging from 1743 to 1773, appear to indicate a maximum of 12 adult females (four European, three Native American and five slaves) (Thwaites 1910). Due to incomplete records however, some of these may be subadults (age not given), some slaves or Native Americans may be male (sex not given), and some individuals with French names could be slaves or converted Native Americans. At least two slaves were identified as Panis, indicating that they were Native American; however, the others may be Native American or black.

The reason no Native Americans were found in the burials from the church, beyond the ambiguous records, is unclear. It is possible that Native Americans who did not convert to Catholicism, potentially including traders wives or slaves, may have been returned to their families or tribes to be buried, and therefore, would not be identified by this research. The church records indicate that many individuals, including Native Americans, were baptized or involved in other church customs prior to burial. For example, one entry reads, "On the same day and in the same place I interred a little girl savage whom I had privately baptized yesterday," while another states a twenty year old male was interred, "after receiving all the sacraments and having been assisted with the prayers of the church" (Thwaites 1910). Also, there may have been a preference for burying French women as opposed to Native American converts beneath the church. Interment records from the Mackinac Register often do not give specific locations of

burials, and instead only record information such as "interred in the same place" (Thwaites 1910); consequently, church officials may have interred Native Americans in a different location than the remains from this study. Native American burials were not entirely absent from the Fort, as demonstrated by the commingled burial (B-34) that was known to be Native American from historical records and confirmed for two individuals by this study. However, since these six individuals were buried in a mass grave as a result of a smallpox outbreak, their presence may be the result of unusual circumstances.

Given that the adult remains in this study were not Native American, it is probable that the two subadults buried beneath the church (B-19 and B-22) would also be not Native American. However, this result is less expected when the large numbers of recorded Métis births (as inferred from baptismal records) are considered. The discrepancy could again be due to preferential burial of European children within the church. Also, most illegitimate children with Native American slaves as mothers and French men as fathers were treated as Native American slaves themselves (Armour 1967); therefore, although slaves and their children were present at the fort, they were not necessarily converted Catholics, or considered worthy of church burial and may have been buried at other locations.

Conclusions

The genetic analysis presented here provides a new method, in addition to the historical record, for examining and understanding the life and culture at Fort Michilimackinac. The close interaction between the French Europeans who built and ran the fort and the Native Americans who traded, married, and produced children with its

inhabitants, indicates a strong mixing of cultures. However, this mixing was not confirmed by the 15 burials from the fort investigated in this study, 14 of which were found to be not Native American, with the final burial having an undetermined ancestry. This genetic evidence of ancestry-based segregation accompanying burial practices at Fort Michilimackinac may lead to uncertainty of whether this integration was actually carried out. By using genetic data to investigate burial customs, further questions arise about the potential conflicts between Catholic religious beliefs and the inclusion of Native Americans into the cultural practices of Fort Michilimackinac inhabitants.

Ancestry determination based on genetic analysis is not limited to samples from Fort Michilimackinac. The method presented here to distinguish between Native Americans and non-Native Americans may be used on non-contemporary remains as in this study, as well as on modern material. The same general method may be expanded to differentiate individuals from other regions and to analyze all known European haplogroups. This research provides an alternative approach to mtDNA haplogroup determination, including the design of novel primers for PCR and SNP-primer extension reactions, with the potential of future incorporation into multiplex reactions. The study design may be especially beneficial in the forensic science community because it utilizes SNP technology with current detection methods instead of older RFLP techniques traditionally used in mtDNA haplogroup analysis.

When human skeletal remains are found, whether contemporary or non-contemporary, a biological profile including ancestry can provide a tremendous amount of information about the specific individual, as well as the society they belonged to. Ancestry determination from genetic analysis may be instrumental in creating a

biological profile, particularly in instances where skeletal remains are incomplete, making anthropological estimation of ancestry problematic. Subadult remains also complicate the estimation of ancestry via skeletal morphology; however, a genetic method may alleviate this obstacle. Genetic ancestry determination, as was used in this study, can augment anthropological examinations, or greatly increase the historical, scientific and/or legal value of otherwise potentially uninformative biological samples.

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