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MATERNAL INHERITANCE IN COLONIAL LAMBAYEQUE, PERU

by

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Under the Direction of Bethany Turner-Livermore, PhD

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ABSTRACT

The Spanish invasion and colonization of Peru devastated indigenous Andean peoples. Violent takeovers, massacres, the spread of deadly European diseases, and imposing European morals and religion are some of the events that greatly affected indigenous Andeans adversely. The Spanish colonizers would systematically siphon as much of the local resources as possible, natural as well as human. Their greed forced together indigenous Peruvians and enslaved Africans into a brutal economic system run by European colonizers. This system involved harsh labor conditions and Catholic indoctrination to "westernize" the Native peoples. This colonial system mixed people from three continents—willingly or not—who bore individuals with conflicted identities, *mestizo* (mixed) and *criollo* (creole) who would experience the world within complicated social positions. This study Estimates maternal haplogroup frequencies within two colonial Catholic cemeteries within the town of Eten in the Lambayeque Valley of Peru. Maternal lineage continuity was found within and without Eten.

INDEX WORDS: Ancient DNA, Lambayeque Valley, Eten, Colonial

MATERNAL INHERITANCE IN COLONIAL LAMBAYEQUE, PERU

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DEDICATION

I dedicate this paper to all my family and friends who were integral to maintaining my mental health during my graduate studies. With a special thank you to Liz and her two cats, Tangerine and Gravy for all the head butts, excited trots, and mews.

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LIST OF ABBREVIATIONS

DNA	deoxyribonucleic acid
mtDNA	mitochondrial DNA
gDNA	Genomic DNA
EDTA	ethylenediaminetetraacetic acid
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
Numts	nuclear copies of mitochondrial DNA
NGS	next-generation sequencing
HVR1	Hyper Variable Region 1
rCRCS	revised Cambridge Reference Sequence of human mitochondrial DNA
IP	Initial Period (1600 – 800BCE)
EH	Early Horizon (900BCE -0CE)
EIP	Early Initial Period (0 – 650CE)
MH	Middle Horizon (600 – 1000CE)
LIP	Late Intermediate Period (900 – 350CE)
LH	Late Horizon (1300 -1500CE)
SCP	Spanish Colonial Period (1500-1800CE)
M	Modern (1800 - Present)

INTRODUCTION

The Spanish colonial history of Peru is a biased body of work based on ecclesiastical and high-status colonial administrative sources that fail to tell the story of the local communities that were colonized. For this reason, some archaeological projects seek to uncover Native community perspectives and lived experiences of Spanish Colonization, with a focus on Bioarchaeological evidence of community structure, European or African admixture, diets, and skeletal stress, through the lens of social theory.

In this section I will discuss the historical context surrounding this study followed by the purpose of my study.

1.1 An Overview of the History and Geography of the Pre-Hispanic Central Andes

The Central Andes has been defined as the range of land which made up the geography of the Inca Empire at its zenith. The region stretches from the Pacific Coast to the Eastern Cordillera, starting from modern-day southern Ecuador through Peru and ending within Northern Chile while including the Mid-South-West portion of Bolivia and upper South-Western Argentina (Quilter, 2014).

Archaeologists divide the time before the Inca Empire took hold of the Central Andes into three horizons and four intermediate periods based on dated assemblages of material cultures from archaeological sites within the region. A horizon is a period in which the widespread distribution of shared material styles is taken to be indicative of cultural and political uniformity. Conversely, an intermediate period is characterized by an absence of shared material styles that is taken as evidence of sociopolitical fragmentation. Although the generalizations and

assumptions imbued within this chronological framework, it serves a useful purpose in this study.

The different archaeological periods began with the Preceramic period (~ 10,000 BCE), followed by the Initial period (~1600 – 800BCE), Early Horizon (~900BCE -0CE), Early Intermediate Period (~0 – 650CE), Middle Horizon (~600 – 1000CE), Late intermediate Period (~900 – 350CE), and the Late Horizon (~1400-1532CE), respectively (Quilter, 2014).

The Initial Period in Peru is characterized by the adoption of agropastoral lifestyles as a result of improved climate and rainfall around 1600BCE in the Titicaca Basin, south Central Andes, and 1800BCE in northern Peru. These changes, according to Moseley (2001), provided a heightened need for religious ritual to continue societal prosperity and saw the spread of civic-ceremonial centers. The Early Horizon follows the Initial Period and is characterized by prolonged drought starting around 900BCE. This drought contributed to the abandonment of civic-ceremonial centers along the coast and the foundation of the civic-ceremonial center of Chavín de Huantar around 800BCE within the northern highlands (approximately 300km inland) of Peru. The Early Horizon was followed by the Early Intermediate Period (0-650CE), also known as the 'Regional Development Period' and the 'Master craftsmen Period' (Moseley, 2001). This period is characterized by the rise of several complex ceramic cultures, including the Moche in the north (expanding southward and uniting southern territories by (500CE), Lima on the central coast, and Nasca in the southern coast. The Middle Horizon (600-100CE) is characterized by the influential, expansionist highland polities of Tiwanaku on the Altiplano and Wari in the central highlands of Peru. On the north coast, heavy damage to Moche territories resulted from intense flooding in the north and drought in the south, resultant of a particularly severe El Niño event circa 560-590CE, contributing to the collapse of the Moche (Moseley,

2001). The following Late Intermediate Period follows the fall of the Wari and Tiwanaku and their dispersal into smaller kingdoms. The North contained the Lambayeque archaeological culture, also known as the Sicán, dating from AD 900 -1350, which is believed to have its origins in the Moche empire. The Lambayeque were then conquered by the Chimu empire, who reigned until they were in turn conquered by the expanding Inca towards the end of the Inca Empire around In the late 15th century CE. The central coast contained the Chancay archaeological culture, and the South Coast is known for the Chincha and the Ica cultures, also assimilated into the Inca Empire (Quilter, 2014).

The beginning of the Inca Empire's expansion has been dated as early as 1400 though most scholars would place it at 1438, reaching the North Coast around 1470. The Empire would then last roughly 100 years until the arrival of the Spanish in 1532, coinciding with widespread internal strife and civil wars (Amino, 2015; Quilter, 2014). Wealth and power in the Inca Empire were measured by the number of subjects one had and not in the ownership of land or non-human resources (Ramírez, 1996). The Empire followed a class system with the Sapa Inca at its head in the shape of a demigod and agent of the Sun God Inti, that would accept tributes from all of his subjects, starting with his royal social unit or *ayllu* termed a *panaca* to the lowly laborer class named *yanaconas* and the pariahs that refused to join the Empire known as the *mitimaes* were displaced from their lands and forced to work in a different polity. In return, the Sapa Inca was to provide for and protect his subjects forever (Amino, 2015; Quilter, 2014). In order to maintain this class system over the great expanse of the Inca Empire, subject peoples were forced to build and maintain a temple to Inti. The conquering Incas did not, however, force their subjects to discard their own religious beliefs, which produced an interesting cultural melting pot

and set the stage for further cultural admixture with the advent of its later conquest and colonization by the Spanish (Klaus, 2013; Ortiz et al., 2017; Quilter, 2014).

On the northern coast (Figure 1), the Late Formative Period (1500 - 650 BCE) involved the Cupsinique culture, followed by the Gallinazo (200 BCE - 100CE), the Moche (100 - ~800CE), and the Sicán (900 - 1375CE). The Sicán were eventually conquered by the Chimu Empire, who reigned from (900 – 1470CE), and were in turn conquered by and incorporated into the Inca Empire around 1470CE (Klaus & Alvarez-Calderon, 2017a; Quilter, 2014; Shimada et al., 2005*).*

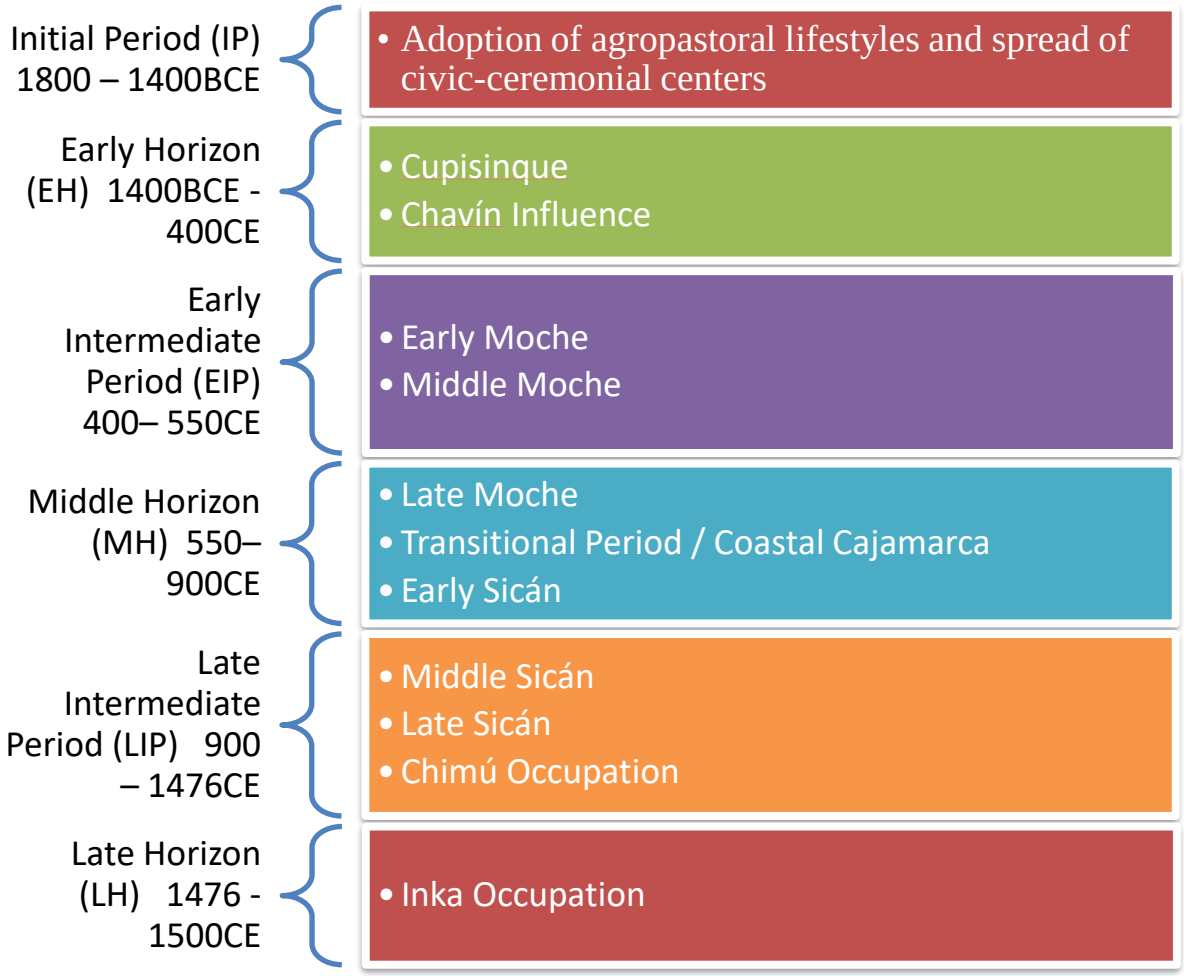

Figure 1Timeline showing the Cultural Changes in the Northern Coast of The Andean Region.

1.2 The Effects of the Spanish Colonization on Andean Societies

Most of what is known about the fall of the Inca Empire is through the accounts of Spanish ecclesial or high-level colonial administrative accounts and communications. We are, therefore, provided with a biased account of a seemingly inevitable conquest by the will of God (Cummins, 2002; Ramirez, 1996). The story began with Pizzaro's successful entrance of Cajamarca, which resulted in the fall of the Inca Empire. This, of course, was not the case. The Inca managed to resist Spanish colonizers for many decades after their invasion. That was until,

with the help of former Inca subjects who were unhappy with their forceful incorporation into the Empire, they managed to conquer and colonize polity-by-polity with the Spanish (Quilter, 2014).

The Spanish distorted or transformed existing economic and sociopolitical systems. They knew enough about how the Inca Empire functioned and attempted to use their existing system involving dispersed polities overseen by Native elite leaders or *curaca.* This, however, was enforced from their own European perspective. Power and wealth to the Inca were measured by the number of subjects one has and thus the amount of labor and product that can be produced from them. This is opposed to the European concept of wealth and power, which is based on the accumulation of material goods. Therefore, once Spanish colonizers received their *encomiendas* or gifted polities, including their native leader or *curaca* and his subject laborers, the siphoning of local resources commenced (Ramirez, 1996). Native *curaca* were, with the passage of time, systematically be replaced by Spaniards, profoundly changing the lives of their subjects, essentially gentrifying their *encomiendas* and making them an extension of their mother land.

Once established, the Spanish would destroy old Inca polities or completely refurbish them to suit their forceful Catholic indoctrination of Indigenous Andean communities. The once dispersed Native populations found themselves forced into planned colonial towns called *reducciones* and made to internalize, or at least perform, C atholic teaching and European morals. This was enforced by the emphasis on separated homes built for nuclear families and sexual shame by separating generations (and especially daughters) into private rooms within each house (Cummins, 2002). Indigenous communities under *encomiendo* rule were forced to produce tribute, which was in the form of lists of goods that had to be handed to the Spanish in exact quantities once a year, under threat of violent punishment (Ramirez, 1996).

As the Spanish colonization progressed, a steady influx of Spanish colonizers began to arrive to both replenish the dead male conquistadores and establish Spanish families in the Andes. Spanish women in the Viceroyalty of Peru were outnumbered by Spanish men by 10:1 during the mid-1500s. Spanish men in the Andes would regularly consort (consensually and often non-consensually) with Andean women as well as enslaved African women, often producing *mestizo* and *mullato* children. According to Lockhart (1968), Native women were considered far more important and incorporated within the Spanish colonizers' lives than men; as they were household servants, mistresses, and wives and would often benefit from relations with Spaniards by becoming enmeshed within the Spanish society by association (Lockhart, 1968). *Mestizo* children of high-status Spaniards were usually taken by the fathers' household, away from their mothers, and raised with his legitimate children, becoming privileged and educated (in the case of males) individuals within Spanish colonial society. Conversely, *mestizo* children of lower-status Spaniards would sometimes be provided for by their fathers but often completely ignored, their mothers included (Lockhart, 1968). By the early 1600s, a caste system was employed by the Spaniards due to the thriving slave markets in the Andean region. Large numbers of enslaved Africans were sold as commodities. Native Andeans occupied the lower and middle classes, while the Spanish holding the upper echelons. This caste system placed illicit *mestizo* children in an awkward position between master and laborer (Klaus, 2012; O'Toole, 2015). Unfortunately, ideas surrounding the Spanish racial castes still shape the individual identities of modern Andeans; specifically, many modern *mestizos* value their Spanish heritage and look down on indigenous and mixed Afro-indigenous peoples as inferior (Ruiz-Linares et al., 2014). The imposition of Spanish colonial, bloodline-based caste system, and subsequent production of mixed identities is an important component in the story of Spanish colonial period

Indigenous and mestizo lived experiences. The typical method in the biological anthropology toolkit of metric and non-metric measurements of skeletal traits cannot be used to explain the complexities of these mixed identities. Therefore, there is a need for ancient DNA analyses of individuals that lived during the Spanish colonial period to estimate the amount of continental admixture in addition to other genetically based relationships.

1.3 Purpose of the Study

There are many questions yet to be asked about the experience of Native populations under Spanish colonial rule and how it related to their population genetics. I propose an umbrella question to form a foundation for this study: *What affects did the Spanish colonization of Peru (between 1532 and 1824CE) have on population genetics of indigenous peoples that lived on (or were relocated to) the northern coast of Peru?*

In the quest for answers to this broad question, I will focus my attention on the following, specific question: *How did Spanish colonization of the northern coast of Peru Affect maternal inheritance in the Lambayeque town of Eten?*

This study is the first of its kind within the geographical and temporal context of Spanish Colonial Eten. I have, therefore, designed it as a pilot study to first assess the DNA sequencing viability of the Spanish Colonial samples, and second, perform a precursory mitochondrial DNA (mtDNA) haplogroup estimation study of the viable samples.

In the following chapter, I will discuss Ancient DNA (aDNA), how it can be accessed and analyzed, and its relevance to this study.

2 ANCIENT DNA RETRIEVAL, ANALYSIS, AND RELEVANCE

In this chapter I discuss the application of aDNA analyses in bioarchaeological and anthropological research. Followed by a section discussing what is known so far regarding Andean population genetics. To end this chapter, I discuss the possible uses of aDNA analyses to probe for continental admixture among individuals living during the Spanish colonial period.

2.1 Bioarchaeology and Ancient DNA

The destructive nature of aDNA methods poses a major ethical issue with regard to ancient human samples. The methods of DNA extraction from ancient material always begin with the removal, decontamination, and powdering of the sample. Therefore, care must be taken when assessing the need to apply aDNA analysis upon scarce ancient human samples, and this is doubly important when such samples are not sufficiently preserved for efficient and productive aDNA analysis (Kaestle & Horsburgh, 2002). With the advent of PCR and NGS techniques, aDNA analysis has become increasingly feasible and reliable, providing stringent and dedicated methodology.

Ancient DNA research has been used to answer bioarcheological questions from species, individual, familial/local, and population perspectives. From the species perspective, questions revolving around human origins and our relation to other hominids have been investigated; examples of which are those involved in the sequencing of Neanderthal and Denisovan DNA (Hawks, 2017; Prüfer et al., 2014; Rogers et al., 2017; Slon et al., 2017) and their relationship to modern humans (Caldararo, 2016; Vernot et al., 2016). From the individual perspective, aDNA investigations allow biological sexing of sub-adult, fragmented and undetermined skeletons (Faerman et al., 1998), the estimation of a minimum number of

individuals, and reassociation of disarticulated remains via individual genome sequencing (Kaestle & Horsburgh, 2002), the estimation of the presence of infectious or genetic disease (Roberts C. & Ingham S., 2008) as well as the identification of ancient individuals with a known descendant line (Gill et al., 1994; Lalueza-Fox et al., 2011). From the familial/local perspective; aDNA investigations allow the building of pedigrees and genealogies (Gerstenberger et al. 1999), Identifying patterns of inheritance (Chilvers et al., 2008; Sampietro et al., 2005; Tishkoff et al., 2007) and residence (Baca et al., 2014; Brewer, 2016). From the population perspective, ancient DNA can be used to understand population-level genetic change or continuity (Adcock et al., 2001; Heinz et al., 2015; Lindo et al., 2017; Valverde et al., 2016). It has also been used to investigate population movement and replacement (Baca et al., 2014; Shinoda et al., 2006; Valverde et al., 2016) as well as the peopling of regions and continents (Fuente et al., 2018; Matisoo-Smith, 2015; Scheib et al., 2018).

2.2 Ancient DNA and Anthropological Theory

Ancient DNA (aDNA) has been used as part of a multidisciplinary approach to understanding social activities, practices, and perceptions. Most of such studies have focused on elite (an assumption made based on apparent lavish grave goods) burials and used ancient mitochondrial DNA, which is inherited maternally, to understand their kinship patterns (Shimada et al., 2005; Shinoda et al., 2006). Many investigations to date that attempt to answer questions about identity, class, agency, and ethnicity have used biodistance analysis, as it is non-invasive and does not require expensive equipment (Klaus & Tam, 2010). The addition of aDNA analyses to the bioarcheologists toolkit has provided a more accurate and finer definition, the idea of biological affinities within and without groups and allows the estimation of definite biological, familial relationships at a one-to-one scale.

One possible avenue involving hypotheses of identity, in the sense of both personal and public perception of individuals within populations, is through juxtaposing the genetic analysis of ancient populations with ancestral genetic analysis of modern populations that populate the same geographical areas. Comparing the ancient and modern populations of a single geographic region within archaeological and historical contexts could provide insights about genetic change or continuity in a temporal milieu.

2.3 Ancient DNA Sequencing Methods

Historically, endogenous DNA damage and exogenous contamination have proved to be the two chief hurdles to successful aDNA analysis. Therefore, methods used to prepare and analyze aDNA samples are far more stringent than modern DNA methods and must be performed within dedicated laboratories (Fulton, 2012).

Within living cells, DNA molecules are faced with constant and spontaneous damage. As a result, they require tight regulation of dedicated enzymes that, under homeostatic conditions, have the ability to continuously detect and repair damage within DNA molecules (Lindahl, 1993). Once an organism dies, however, its cells become subject to autolytic enzymes, which have the ability to completely degrade the dead cells in their entirety (Campana et al., 2013). DNA damage can also be a result of organisms that feed on macromolecules, including insects, fungi, and bacteria, as well as the chemical composition of the surrounding environment of a decaying organism (Eglinton et al., 1991). DNA can be preserved after cellular death under circumstances that arrest the taphonomic activity of autolytic enzymes, and these circumstances include desiccation and low temperatures (Pääbo et al., 2004). Consequently, DNA that had previously been incorporated within the hard tissues of bone and teeth, and keratinous structures such as hair and nails, possess a greater probability of remaining intact for long periods of time

until their protective outer layers become compromised (Campos & Gilbert, 2012; Lindahl, 1993). Source tissues/organic material selection is a major factor regarding aDNA yield and preservation. The most reliable source tissue is the petrous portion of the temporal bone, and more specifically, in the cochlea (Gaudio et al., 2019; Hansen et al., 2017). In the absence of the petrous portion of the temporal bone, studies have shown that teeth are an acceptable source of aDNA, providing an intact cementum and/or enamel layer (Adler et al., 2011; Hansen et al., 2017; Hollund et al., 2015) Dentine and cementum have been found to be the most reliable aDNA sources from teeth, due to their cellular makeup and close contact to the vascular system (Hollund et al., 2015). Of the two dental layers, cementum has been shown to provide larger yields and less damaged DNA overall (Adler et al., 2011; Hansen et al., 2017; Higgins et al., 2013; Hollund et al., 2015).

Samples of ancient biological materials must be selected based on an evaluation of preservation of their source material, environment and storage conditions (Adler et al., 2011). Correlations have previously been made between visual assessments of the histological integrity of sample tissues and their performance as aDNA source materials (Hansen et al., 2017; Hollund et al., 2015). This means that the better preserved the source material, the higher the yield of its endogenous DNA, and the less likely that damage occurred.

Ancient DNA is highly susceptible to contamination with modern DNA. When not controlled for contamination of modern DNA, the ancient DNA is unfit for further analysis (Campana et al., 2013; Kaestle & Horsburgh, 2002; Kirsanow & Burger, 2012; Yang & Watt, 2005). This is highly problematic for ancient human DNA studies as modern human DNA is in constant supply throughout the processes of its collection and preparation and laboratory analysis (Yang & Watt, 2005). As ancient DNA is generally more degraded and found in lower copy

numbers than modern DNA, the use of highly sensitive polymerase chain reaction or PCR, a laboratory technique used to amplify nucleic acid sequences through cycles of high and low temperatures in the presence of heat-stable polymerase enzyme and nucleotides, would preferentially amplify modern DNA if not stringently controlled (Fulton & Stiller, 2012; Kirsanow & Burger, 2012; Yang & Watt, 2005).

In many cases, aDNA investigations are done years after biological material have been collected from archaeological sites, often where the excavation was carried out with no intention of performing such studies. This means that there is an increased risk of contamination from modern DNA from human handling. In such cases, samples must be decontaminated prior to any attempts of aDNA extraction (Hummel et al., 1994; Hummel, 1994; Kirsanow & Burger, 2012; Yang & Watt, 2005). This is done in the cases of teeth and bone samples by washing the samples with bleach followed by DNA free water, ultraviolet irradiation, or scraping/abrading the outer surface of the sample using disposable scalpels or handheld drills with disposable cutting discs to avoid cross-sample contamination (Barnett & Larson, 2012; Brown & Brown, 2011; Hummel, 2003). After the removal of a thin outer layer, the sample can be powdered in the case of bone and whole teeth or could be scraped in the case of cementum and powdering the internal surface of the tooth if dentine is required (Adler et al., 2011; Barnett & Larson, 2012; Hummel, 2003).

Once prepared the DNA is extracted from the samples by releasing the cell contents, stabilizing DNA molecules, digestion of proteins, phase separation, and DNA concentration. To date there are several different protocols established that have yielded decent results. The first step involves using a chelation buffer containing Ethylenediaminetetraacetic acid (EDTA) which reacts with calcium ions of the cell wall and stabilizes DNA; In the case of bone and enamel, samples will highly react with the calcium of the apatite mineral (Barnett & Larson, 2012; Hummel, 1994).

A negative extraction control is also prepared during this step containing only the chelating buffer. Second, after centrifugation of the samples and control the resultant pellet is subjected to a digestion buffer containing SDS and proteinase K which solubilizes lipids, denatures proteins, and breaks proteins down to their forming amino acids respectively. Previously, the most effective method involved the use of phenol and chloroform (Hummel, 2003). Recently, however, there has been a move to using silica columns or magnetic beads to perform the final molecular separation step (Dabney et al., 2013; Rohland et al., 2018).

As mentioned above, PCR is a well-defined targeted nucleic acid amplification technique. In order to amplify DNA, a pH buffered solution of potassium chloride, magnesium chloride, and Tris containing the DNA sample, primers, nucleotides, and heat-stable DNA polymerase is prepared. This solution is then placed within a thermocycler, which when programmed, will facilitate the three steps: 1) Denaturation of the sample DNA into single-stranded target DNA, 2) Annealing of the primers to the target DNA and, 3) Elongation of the primers to form a complement strand to the target DNA sequence via DNA polymerase. When amplifying DNA from fresh tissues, the reaction efficiency of optimal PCR is usually between 80-90%. Due to the degraded nature of aDNA, and its possible accompaniment of other PCR inhibitors, such as tannins, humic acids, fluvic acids, Maillard products (components of decaying plant material and is often found in soil), and collagen, PCR reaction efficiency is generally between 40-70%. The lower reaction efficiency in turn results in a low yield of effective amplified DNA. A compensation of low reaction efficiency can be attained by increasing the number of amplification cycles. Within every amplification cycle temperatures and durations must be programmed into the thermocycler for each of the three steps of the cycle. More important to the success of the PCR reaction is the use of correct temperatures for the

denaturation, annealing, and elongation stages at 94˚C, 50-60˚C, and 62-72˚C respectively. Another important factor to a successful PCR reaction is the use of an adequate primer design. Primers are DNA nucleotide sequences that provide a starting point for a specific target sequence amplification (Fulton & Stiller, 2012; Hummel, 2003).

As a control measure, the amplified DNA samples should be assessed for errors induced by temporal/environmental damage of the DNA, presences of numts -nuclear copies of mitochondrial DNA (Hazkani-Covo et al., 2010)-, as well as the ratio between endogenous and exogenous DNA (Cooper & Poinar, 2000; Fulton & Stiller, 2012). Conversely, the extracted DNA could be directly sequenced according to the method published by Fulton (2012). Although not the norm, cloning has not been systematically proven to enhance the quality of ancient DNA sequencing data (Winters et al., 2011).

Once authenticated, amplicons can then be further analyzed with DNA sequencing techniques. The first of its kind, developed in 1975 was the Sanger sequencing method. Its main premise is to create an environment in which a target DNA sequence can replicate in the presence of predominantly chemical analogs of deoxynucleotides (dNTPs) and a small number of radio-labeled dideoxynucleosides (ddNTPs), which lack the 3’ hydroxyl group necessary for the continuation of the strand’s elongation. Whenever a ddNTP is incorporated into the sequence it is forced to terminate. This technique when performed in four parallel reactions, one for each ddNTP, will result in every possible fragment length terminating in each individual nucleotide. When run on a polyacrylamide gel and visualized via autoradiography, a chain of terminating nucleotides can be inferred from the labeled fragment reads for the targeted DNA sequence (Heather & Chain, 2016; Linderholm, 2016). Next-generation sequencing (NGS) techniques follow a similar premise with the main difference in its ability to sequence millions of reads in

parallel as opposed to the one strand per run produced by Sanger sequencing. The use of NGS as mentioned above minimizes the cost and time spent on collecting sequence data (Linderholm, 2016; Palencia-Madrid & de Pancorbo, 2015). Collected sequence data must then be analyzed using specialized programs within bioinformatics and statistical software, in order to test hypotheses posed by aDNA investigators (Kircher, 2012). The results are then authenticated via NGS if needed. According to Palencia-Madrid & de Pancorbo's study (2015), the ion semiconductor sequencing of amplicons (an NGS method) proved to be more advantageous than cloning as an authentication method. Such next-generation sequencing (NGS) authentication methods are generally cheaper, and less time-consuming. In addition to their ability to produce a larger number of sequences from each amplicon than cloning does. Which allows for easier detection of errors, as mentioned above (Palencia-Madrid & de Pancorbo, 2015).

2.4 Current Understanding of Population Genetics in the northern Central Andes

Ancient DNA based genetic population investigations in the north-eastern Andes are sparse and have mainly focused on Pre-contact sites and tested ancient mtDNA in search of markers (single nucleotide polymorphisms, SNPs) to estimate maternal lineages, and their categorization as haplogroups A, B, C, D and Other as noted previously. These markers of SNPs manifest within a non-coding region of the mitochondrial genome between positions 16001-16568 known as the D-loop. Haplogroups A, B, C, and D are associated with Native American groups. In this section I will present ancient DNA (aDNA) studies, followed by modern DNA studies of Andean populations residing within the modern borders of Peru.

Ancient DNA Studies performed by Shimada et al. (2005). In their study they classified fifty-three individuals, from mortuary contexts of Middle Sicán (900 -1350CE) sites; Huaca Loro (19 individuals), Huaca Las Ventanas (4 individuals), Huaca Rodillona, and Huaca Sialupe (1

individual each), based on differences between their sequence into sixteen haplotypes. Fifteen of the individuals were estimates as sub-groups of the founding Native American Haplogroups (A, B, C, and D). The remaining individual's haplogroup estimation is either an error (which is most likely) or belongs to an unknown founding haplogroup, to which, Shimada et. al (2005) designated the name 'Other'. They also sequenced haplotypes of 16 Mochica individuals from the Moche site of Sipán (AD ~100-800) and classified them into six haplotypes all of which fell within the known founding haplogroups of the Americas. From their results they were able to extrapolate kinship and class relationships in the Mochica site of Sipán and suggest a southward migration pattern of Sicán people into the Lambayeque region (Shimada et al., 2005). In another study of Middle Sicán (AD 900 -1350), 43 individuals were analyzed and found to have differential proportions of the four Native American haplogroups and others. The 'elite' were found to possess higher frequencies of Haplogroups A and B with instances of Haplogroup C, while the commoners were found to possess higher frequencies of Haplogroup D and Other and an absence of Haplogroup C. A major issue in this study was the blanket assumption that some individuals were deemed "elite" based on the presence of lavish grave goods and mortuary architecture. And because higher frequencies of Haplogroups A and B were estimated within these "elite" graves, the respective buried individuals were hypothesized to originate from farther south of the Lambayeque region (Shimada et al., 2005). These two assumptions do not account for regular movement of peoples around the Andean regions and implies that lavish graves could only be for so called elites.

A study performed by Shinoda et. al. (2006) compared haplogroup frequencies between highland and coastal regions of northern Peru. They concluded that Pre-Spanish Invasion coastal

populations of Peru were more closely related to each other genetically, than to the highland's populations (Shinoda et al., 2006).

Another example of mtDNA population analysis was performed by Fehren-Schmitz et al. (2010) on 172 individuals from 6 sites within the Palpa region of the southern coastal Andes. north of the Rio Grande de Nasca drainage, in addition to 44 individuals from three sites outside the Palpa region. These individuals were buried over a large chronological expanse attributed to Paracas, Nasca, Middle Horizon and Late Intermediate Period. Once assessed alongside ecological and archaeological contexts the results of their mtDNA Hypervariable Region I sequence data brought them to three main conclusions. First, since low genetic distance was observed between Paracas individuals of inner and outer regions of the Palpa were biologically related as well as culturally. Second, that there is evidence of genetic continuity within the Palpa region between the Paracas and Nasca periods as well as Nasca and Middle Horizon (600-100CE). Third, a degree of genetic difference was observed between urban and rural Nasca individuals which is assumed to be due to dominance on local elite individuals rather than foreign influence (Fehren-Schmitz et al., 2010). The authors noted a necessity to analyze aDNA data on the continental level to reveal any population genetic relationships (Fehren-Schmitz et al., 2010).

Ancient DNA (aDNA) studies performed on samples from Morrope dating to the Late Horizon (1400-1532CE), and from San Jose and Eten dating to the Middle Horizon (600-100CE) in the Lambayeque valley were performed by Wester et. al (2020): In the Lambayeque Valley; haplogroup C was the most common estimation in Pre-Spanish Invasion populations (Wester et al., 2020).

Another interesting example is a study by Baca et al. (2014), was performed on samples from the Arequipa region of southern Peru. Twenty-three individuals from Acchaymarca and Puca dating the Late intermediate Period (900 – 350CE) and the Late Horizon (1400-1532CE) were sampled. Both their mtDNA and their nuclear DNA was extracted for Hypervariable Region I (HV1) and autosomal STR and sex estimation analyses. They supplemented their results with previously studied Pre-Contact populations genetic data including D-loop sequences for nine, Haplogroup frequency data for fifteen and microsatellite profiles for three populations. As a result, they concluded that the pattern of Haplotype sharing suggested gene flow between Arequipa and the Palpa region possibly a result of Pilgrimage to the site and its long history of occupation. The least diverse population was that of the Tompullo 2 site belonging to a group of isolated llama herders. Individuals of the Puca site are distinct in both their mtDNA results and their architecture and shows similarity with the Chen and Azapa Valleys of southern Peru and norther Chile, respectively. As a result of their sex-based dispersal analysis they were also able to conclude that community organization in the western central Andes was most likely a result of patrilocality (Baca et al., 2014).

In their study of modern populations in Peru, Shimada et al. (2005 and 2008) found the following. Haplogroup A was found in higher frequency in northern latitudes, Haplogroup B was mostly found within lower Central America and northern Andes, Haplogroup D is prevalent in in the Central Andes and particularly the highlands, and haplogroups C and D are found in high frequencies in southern latitudes.

Cabana et al. (2014) presented a comprehensive study of population genetic variation using samples, collected between 2001 and 2005, of 611 individuals from 17 traditional populations, residing in 9 regions, four of the populations sampled were from coastal areas, six were from

lowlands, the remaining populations were from highlands. Three of the coastal populations reside to the north of Cajamarca (Caleta Santa Rosa, Catacaos, Islilla), while the fourth coastal population was to the south of Cajamarca (Trujillo). The concluded the following: Highland and coastal populations differ in mtDNA genetic structure, that Inca and Spanish population structure policies alone do not explain the Peruvian Central Andean demography, And that there is evidence of female-biased gene flow in highland groups (Cabana et al., 2014).

Messina et al. (2018) performed Autosomal short tandem repeats (STR) and mitochondrial DNA (mtDNA) haplogroup estimation studies on ten Moche identifying,132 urban Peruvian and ninety Native Amazonian individuals. Their autosomal STR data highlighted that 67% of urban Peruvian individuals have a strong similarity to the Amazon Native population, 22% showed similarity to African populations and only ~1% to European populations. The estimation of maternal lineage continuity was in favor of a strong (~90%) Native American contribution, and much lower frequencies of African (~6%) and European (~3%) haplogroups (Messina et al., 2018)

2.4 Ancient DNA Analysis and Spanish Colonial Admixture

Little is known about the native Peruvian experience of Spanish colonialism. Even less is known about how underprivileged and forgotten *mestizos*, described in chapter 1, viewed themselves, and how they were viewed by their native mothers, relatives and neighbors. On the population level, there have not been any investigations, as of yet, of Spanish admixture within Colonial Peruvian cemetery contexts. Such investigations would shed light on the extent of Spanish genetic admixture in a manner that cannot be obtained by biodistance analysis. Such bioarcheological Spanish colonial investigations should however be performed on the

encomienda or *reduccion* level, as sweeping generalizations proclaiming the homogeneous or heterogeneous nature of ethnic groups would only skew and confuse their resultant data.

3 RESEARCH DESIGN

In this chapter I will present my research design including the context surrounding the individuals sampled in this study and my hypothesis.

3.1 Eten, Lambayeque Valley, Peru

On the northern coast of Peru lies the Lambayeque Valley Complex, containing the River Valleys of Zaña, Roque, Lambayeque, La Leche, and Motupe, shown in Figure 1 (Klaus & Alvarez-Calderon, 2017). Despite its current aridity, the Lambayeque valley complex once boasted the largest area of arable land as well as a third of the population of all Peru. This Valley complex has experienced the rise and fall of several complex societies starting in the Late Formative Period (1500 - 650 BCE) with the Cupsinique, followed by the Gallinazo (200 BCE - 100CE), the Moche (100 - ~800CE), and the Sicán (900 - 1375CE). The Sicán were conquered by the Chimu Empire, who reigned from (900 – 1470CE), and were in turn conquered by and incorporated into the Inca Empire around 1470CE (Klaus & Alvarez-Calderon, 2017a; Quilter, 2014; Shimada et al., 2005).

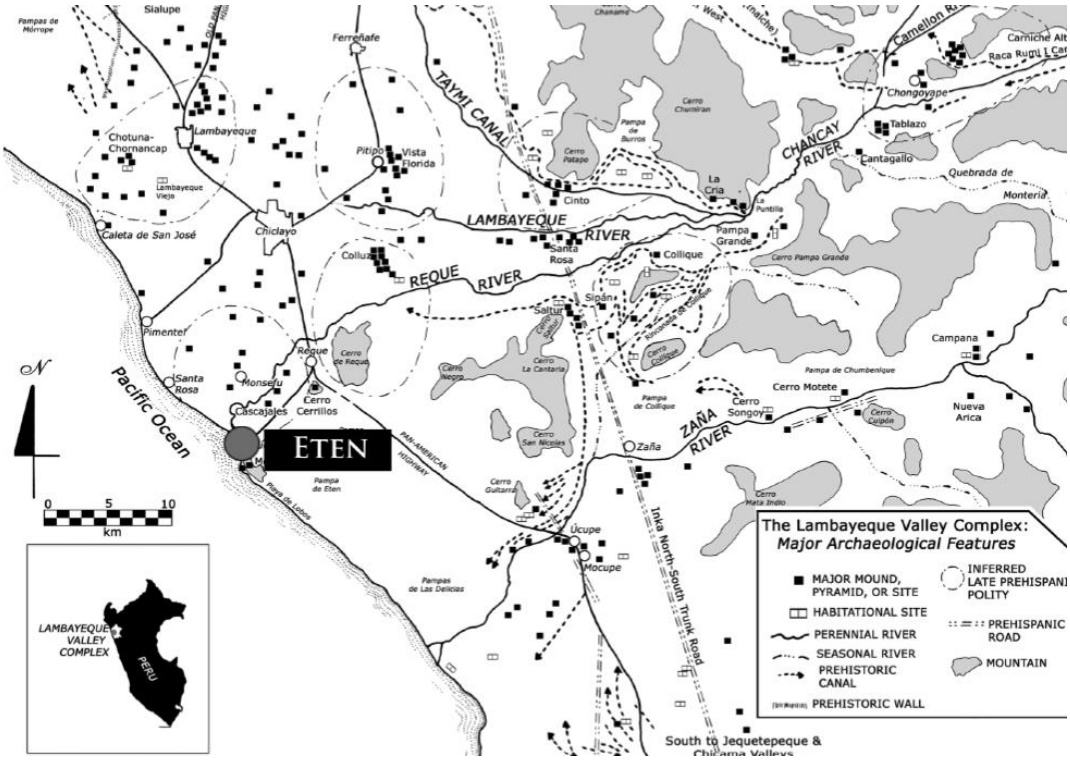

Figure 2 Map showing the position of Eten, Lambayeque Valley, Peru. Map from:(Klaus and Alvarez-Calderon 2017)

One of the factors making Lambayeque a prime locus of complex polities and imperial states is its strategic and economical value. In addition to a pleasantly sunny and warm climate and rich agricultural and maritime resource base, the Lambayeque Valley is also well situated for seaports. These conditions quickly caught the eye of Spanish colonizers and became the site of one of the first *reducciones* in the town of Mórrope.

The Lambayeque Biohistory Project is a multi-decade, multifaceted and regional study of the Lambayeque Valley, including two Spanish Colonial Period archaeological sites; including the Spanish colonial site surrounding the Capilla de San Pedro de Mórrope as the project's first excavation (Klaus, 2012), followed by the Spanish colonial cemeteries in the town of Eten further south.

Eten is a town located on the Reque River in the southern part of the Lambayeque Valley Complex. Historical information about this area is limited to oral traditions of its Mochica and tell the story of the establishment of a mission church by a Franciscan missionary in 1533 and changed the name of the once fishing village of Ätim to Santa Maria Magdelena de Eten. Once the local populace grew out of the missionary church around the early 1600s, a second grander church was erected by the same name as the village: The Church of Santa Maria Magdelena de Eten (CSMME).

Apparitions of the Divino Niño (the Divine Child) were confirmed at the site in 1649 by church officials positioned in the town of Chicalyo, elevating its status to a pilgrimage site even after the town's abandonment between 1740 and 1760. Another apparition of the Divino Niño was seen aiding a wracked ship a little off the shore. To commemorate the miracle Miguel Castillo, the captain of the saved ship, built the Chapel of the Niño Serranito (CNS) which served as a local religious monument until its abandonment in 1900 even though it was never consecrated by the Catholic authorities. Excavation at the CNS site showed that it was built over the remains of a small missionary church dating to the Early/Middle Colonial period between 1530-1620CE and is believed to be the original missionary church. This original church was later replaced with the CSMME in the Middle/Late Colonial period (1620- 1750 CE), shown in Figure 2.

The excavation of the two churches and the buried town in between revealed subfloor cemeteries under both churches. A total of 253 burials were found at the CNS and 256 were found at the CSMME (Klaus & Alvarez-Calderon, 2017a). Burial patterns within the two sites were mostly oriented north-south reminiscent of Pre-Hispanic rituals, instead of the Catholic east-west orientation.

Archaeological evidence collected at the Chapel of San Pedro de Mórrope indicated the infliction of continual systemic violence, including widespread living and nutritional disparity on top of forceful religious indoctrination into the Catholic faith (Klaus, 2012). Unlike the Chapel of San Pedro de Mórrope site, no clear evidence of systemic violence and resistance in the osteological record or burial treatment were found within the Eten cemeteries. This indicates marked regional differences regarding the experiences of Spanish colonialism in Peru (Klaus & Alvarez-Calderon, 2017a).

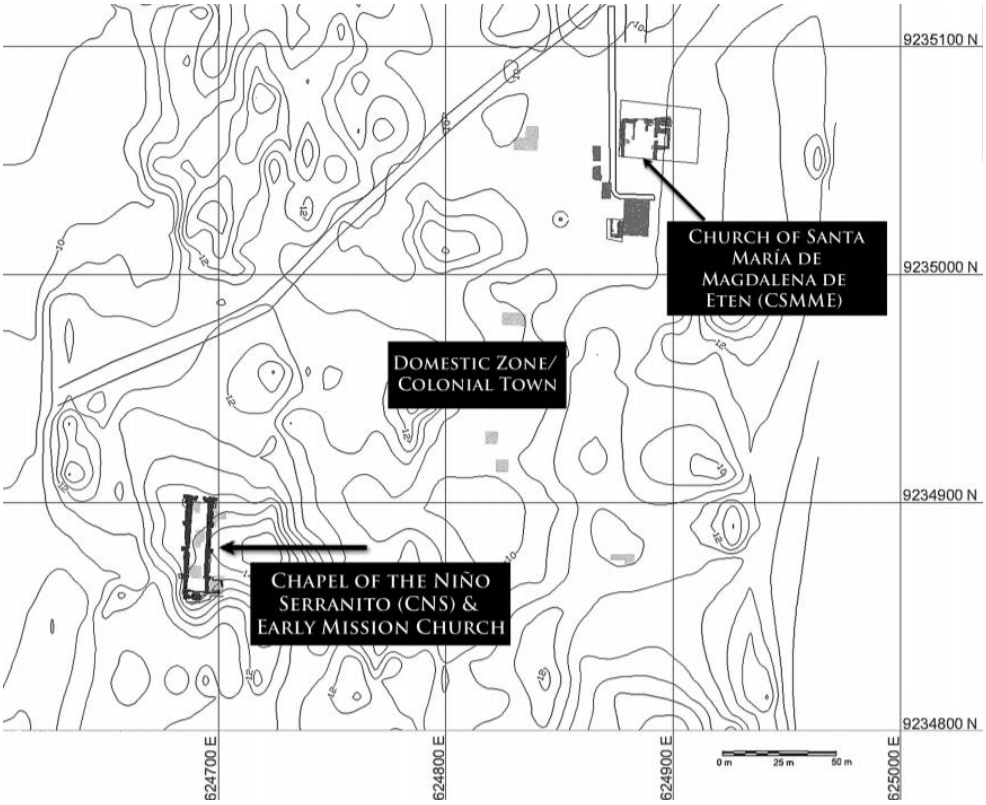

Figure 3 Map showing the positions of CSMME and CNS. Map from:(Klaus & Alvarez-Calderon, 2017a)

3.2 The Enduring Muchik Ethnic Identity

Ethnohistoric and colonial accounts state that there is a long tradition of social ranking and stratification on the northern coast of Peru. *Parcialidad,* the Spanish colonial term meaning

part of a whole, is attributed to the hierarchical social and economic system that governed north coast societies since the Late Moche period in the late 8th century AD. *Parcialidades* were defined and organized based on economic specialization and kinship. At the peak of this hierarchical system is the lord or *curaca* followed by increasingly divided and ranked pairs of lower and middle-class individuals, ending with the working class, including farmers, camelid herders, fishers, and specialized craftspeople (Klaus, Shimada, et al., 2017). According to Klaus et al. (2017), mortuary analysis of the Middle Sicán sites resulted in evidence of a social hierarchy in which elites of different stratifications would possess certain metal alloys such as gold or silver commoners would only have copper-arsenic alloys, if any. Said burials were categorized into four hierarchical tiers; High elite, Low elite, Commoner, and those with no grave goods assumed to be undesirables or captives (Klaus, Shimada, et al., 2017; Shimada et al., 2015). The Moche, Middle Sicán, and Chimu cultures were all organized hierarchically. Their mortuary practices differ in some ways and show continuity in others. In broad strokes, all three societies juxtapose extravagant "elite" burials in contrast to humble "commoner" burials. All of which show their status archaeologically using location, architecture, and type/quality of grave goods. The lived experiences of those variably buried individuals can be hypothesized using bioarchaeological analyses related to patterns of biological stress, diet, trauma, physical activity and genetic variation between the elites and non-elite individuals (Klaus, Shimada, et al., 2017). In fact, most non-elites in the Lambayeque region appear to be descendants from local groups that lived during the Moche and Gallinazo periods.

Klaus and others discuss the apparent occurrence of ethnogenesis along the northern coast during the Early and Middle Moche periods as non-elite groups found that their participation in Moche material and ritual culture was to their advantage. Muchick was the

resultant ethnic identity that embodied a resilient and persistent and adaptive cultural foundation, atop which dominant societies come and go with their political organizations and material cultures (Turner & Klaus, 2020). Evidence of Muchik ethnic identity can be followed through time. The most striking of which can be found in their mortuary practices, from funeral rites to ritual killings and the usage of specific iconographic media.

As Turner and Klaus (2020) eloquently noted: "they knew they were Muchik, and they used it in the creation, maintenance, and negotiation of group solidarity and goals especially in times when they were a subordinate or subaltern social group. Further, this Muchik phenomenon seems most tenacious and clearly conveyed in Lambayeque Valley Complex. As a cultural substratum, ethnic Muchik peoples represented a unique persistence of a local identity, cultural memory, and practical consciousness that outlived all later macropolitical systems including the Sicán and the Inka."

3.3 Hypotheses

As mentioned above those buried at the town of Eten did not seem to have suffered systematic violence (or at least, was not visible on skeletal remains), by Spanish colonizers. The inhabitants of Eten were likely under the constant and watchful eye of the church due to the apparitions of the Divino Niño and, at least to the Spaniards, appeared to completely assimilate into Catholic doctrine. The inhabitants could have also successfully assimilated as a result of biological and cultural admixture as a population containing a sizable amount of *mestizo* individuals. This is a highly likely scenario as Eten was a cosmopolitan sea-port town. No biodistance analysis has been done on the two Eten sites. Therefore, molecular investigations of

these two sites should provide useful information with regard to kinship, biological affinity, sex estimation and Spanish (European) admixture.

I hypothesize that mtDNA haplogroup estimates of Colonial Period individuals from CSMME and CNS would be proportionally comparable with pre-Hispanic Andean populations than to modern Andean populations. Based upon previous studies that showed a stronger continuity within maternal haplogroups in the region (Cabana et al., 2014), I also expect that any evidence of European admixture within mtDNA haplogroups from CNS and CSMME would be rare if seen at all. If present, however, I expect a higher frequency of Spanish-European and Sub-Saharan African mtDNA haplogroups in the Mid/Late Spanish colonial period Cemetery (CSMME) than in the Early/Mid Spanish colonial period cemetery (CNS)

4 METHODS

In this section, I will the methods I used to perform ancient DNA extractions, DNA amplification, Sanger sequencing, and Bioinformatic analyses used for Haplogroup estimation.

4.1 Sampling

I collected a total of twenty-nine dental samples (each sample representing an individual) that were house in Dr. Bethany Turner's Bioarcheology Lab at GSU. Nineteen of which belonged to the Early/Mid Spanish colonial site CNS, the remaining ten samples belonged to the Mid/Late Spanish colonial site CSMME as displayed in Tables 1 and 2. Teeth were chosen based on their morphological appearance; minimal wear, preserved enamel and/or cementum layer. All subsequent steps took place in Dr Sloan Williams dedicated Ancient DNA and modern DNA laboratories (housed in different buildings) at the University of Illinois in Chicago (UIC) under the guidance of Drs Sloan Williams and Leland Rogers. The individuals included in the study sample from each cemetery are summarized in Tables 1 and 2.

Table 1 List of Collected Samples Belonging to Early Colonial Period CNS

Site	Individual	Accession #
CNS	N/A	KGSU 151
CNS	N/A	KGSU 153
CNS	N/A	KGSU 154
CNS	N/A	KGSU 156
CNS	N/A	KGSU157
CNS	U2-21	KGSU 158
CNS	N/A	KGSU 159

CNS	N/A	KGSU 164
CNS	N/A	KGSU 167
CNS	N/A	KGSU 179
CNS	N/A	KGSU 183
CNS	N/A	KGSU 191
CNS	N/A	KGSU 193
CNS	N/A	KGSU 199
CNS	N/A	KGSU 217
CNS	N/A	KGSU 218
CNS	N/A	KGSU224
CNS	N/A	KGSU 230
CNS	N/A	KGSU 235

Table 2 List of Collected Samples Belonging to Mid-Late Colonial Period CSMME

Site	Individual	Accession #
CSMME	E25	KGSU 62
CSMME	5D-04	KGSU 93
CSMME	5E_30	KGSU 114
CSMME	5D_78	KGSU 137
CSMME	5E_09	KGSU 138
CSMME	5E_06	KGSU 139
CSMME	5E_28	KGSU 142
CSMME	N/A	KGSU 145

CSMME	N/A	KGSU 148
CSMME	5E_27	KGSU 149

4.2 Sample Preparation and Ancient DNA Extraction

Collected samples were prepared using a modified Dabney (Dabney et al., 2013) protocol extraction. The samples were decontaminated by submersion in 6% bleach solution and washed off with Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water, they were then left to dry completely. Since ancient DNA analysis has never been performed on any samples from individuals at the two cemeteries, a destructive method was chosen to ensure maximum extraction yield of the degraded DNA. Once surface-decontaminated, the sampled teeth were powdered using a decontaminated hammer and UVd (15min on each side) weigh boats. Once powdered, approximately 120mg of each tooth was measured and treated with a proteinase K and EDTA mixture at 37°C overnight. This step allows the enclosed cells to release their contents into the solution and degrade unwanted proteins.

DNA extraction was performed using freshly prepared silica suspension and elution buffer with Roche High Pure Extender Assembly kits. Resultant extractions were stored at -20°C in the Ancient DNA Lab at UIC. Extractions were performed in sets of five samples. Each set included a negative extraction control (no DNA). Two Extractions were performed for each sample with the same protocol mentioned above. The resultant aDNA extracts were then prepared for amplification.

4.3 Mitochondrial DNA Amplification of the Hypervariable Region 1

Hypervariable regions (HVR) 1, 2, and 3 are located on the D-loop portion of the mitochondrial genome and encompass most single nucleotide polymorphisms (SNPs) used for

maternal ancestry estimation on the mitochondrial genome (Bandelt, 2006; Brown & Brown, 2011; Mann & Kaur, 2015).

For this experiment, I focused on sequencing the HVR1 on the mitochondrial genome. This was done to serve as an indicator of the ability to successfully extract degraded human DNA from the individuals sampled. In addition, it initiates the process of estimating maternal inheritance of haplogroups for each individual. Primers were designed to target HVR1.

Primers shown in Table 3 were designed to create four overlapping sequences in four separate reactions: A, B, C and D, in order to obtain the most complete and accurate HVR1 sequences as possible. Some instances required further modification of primers, specifically in parts A and C.

Table 3 Primers used in the mtDNA amplification for this study

	Primer	Sequence (5'-3')	Bp	I ength
Part a	HVR1a1 Forward	GCT AAG ATT CTA ATT TAA ACT ATT CT	15995-16020	26
	HVR1a1 Reverse	GGA TTG GGT TTT TAT GTA CTA C	16174-16153	22
	HVR1a2 Forward	TCT TTA ACT CCA CCA TTA GCA CC	15968-15998	23
	HVR1a2 Reverse	ACT ACA GGT GGT CAA GTA TTT ATG	16157-16133	25
Part b	HVR1b Forward	TGC CAG CCA CCA TGA ATA TTG TA	16105-16127	23

	HVR1b Reverse	GCT TTG GAC TTG CAG TTG ATG TGT	16256-16233	24
Part c	HVR1c1 Forward	TGC TTA CAA GCA AGT ACA GCA A	16195-16216	22
	HVR1c2 Forward	AGC AAG TAC AGC AAT CAA CC	16203-16222	20
	HVR1c Reverse	GAG AAG GGA TTT GAC TGT AAT GT	16360-16338	23
Part d	HVR1d Forward	CCT CAC CCA CTA GGA TAC CAA CA	16261-16283	23
	HVR1d Reverse	GCG GGA TAT TGA TTT CAC GGA	16429-16409	21

The PCR mix for each sample contained; 13.13µl of Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water, 2.5µl of PCR Enhancer, 2.5µl of 10x PCR Buffer, 1.5µl of Magnesium Chloride ($MgCl_2$), 0.52µl Bovine Serum Albumin, 0.4µl dNTPs, 0.4µl Forward Primer, 0.4µl Reverse Primer, 0.4µl Platinum Taq polymerase, and 0.25µl of AmpErase® Uracil N-Glycosylase (UNG). Once each sample was prepared, they were moved to the Applied Biosystems GeneAmp PCR System 9700 located in the modern DNA Laboratory at the Department of Anthropology, UIC.

As mentioned in section 2.3, The successful amplification of DNA relies on the usage of the correct denaturing, annealing and elongation temperatures for each primer pair. The samples were amplified according to the general program detailed in Table 4.

Table 4 General PCR amplification program used for all primers in this study.

PCR Stage	Temp ℃	Time	Cycles
Hold	95℃	2min	-
Denature	94℃	20s	20 or 10 cycles for interval start temperature. 40 cycles for interval end temperature.
Anneal	Touchdown Interval*	40s	
Extend	72℃	20s	
Hold	72℃	7min	-

* 1 explained in the following passage.

Specific differences between each of the primers used in this study regarding their touchdown temperature intervals and number of cycle repetitions can be viewed in Table 5. In these processes I employed the use of touchdown temperature intervals which are used to maximize the probability of the primers annealing to the correct sequence of DNA. The touchdown temperature interval allows the annealing stage to cyclically decrease its temperature, in increments of 0.5℃, between the highest possible to the most optimal annealing temperatures.

Table 5 Touchdown temperature intervals and number of cycles needed for the successful amplification for each primer used in the study.

Primer	Touchdown Start Temperature	Number of cycles	Touchdown End Temperature	Number of cycles
HVR1a1	**57℃**	**20**	**47℃**	**40**
HVR1a2	**53℃**	**10**	**47℃**	**40**
HVR1b	**61℃**	**20**	**56℃**	**40**
HVR1c1	**57℃**	**10**	**52℃**	**40**
HVR1c2	**57℃**	**10**	**52.5℃**	**40**
HVR1d	**59℃**	**10**	**55℃**	**40**

Extraction sets were amplified in strips of 8 PCR microtubes: including their respective negative extraction control in addition to two negative amplification controls (No extracted DNA in PCR mix).

The following section will explain how amplified samples were confirmed and subjected to quality control.

4.4 Polyacrylamide Gel Electrophoresis: Confirmation and Quality Control

I performed Polyacrylamide gel electrophoresis to confirm the presence or absence of amplified aDNA for each sample. Each sample and control within PCR microtubes were run in a separate well on the polyacrylamide gels.

The active components of polyacrylamide, Acrylamide and bis-acrylamide are neurotoxic in nature which is an unfortunate danger when using polyacrylamide gels. Since this procedure requires a finer grain of separation for small double-stranded DNA molecules (~100-200bp) than what Agarose can provide, polyacrylamide gels were used.

I prepared a mini 12% gel for each set of amplifications including 5 different samples, and 3 controls. A 12ml polyacrylamide gel stock solution would contain the following ingredients for a 12% gel: 4.8ml of a 30% polyacrylamide solution (29g Acrylamide, 1g Bis-acrylamide, and 100 mL double-distilled water), 1.2ml of 10x TBE (a mixture of Tris Base, Boric acid and EDTA), 200µl of a 10% solution of APS (Ammonium persulphate solution), 6ml of double distilled water, and 10µl of TEMED (Tetramethylethylenediamine, an important polymerization catalyst for polyacrylamide gels). I then carefully poured the gel between two prepared mini gel electrophoresis glass plates (to avoid trapped bubbles), taped on three edges to secure the gel between them. Wells were then made using a comb, then poured gels were left to solidify for 10-15 minutes. I would the set up a vertical electrophoresis rig connect it to a power supply and add the electrophoresis buffer of 1x TBE. The buffer used in the rig comprises of 89mM Tris (pH 7.6), 89mM boric acid, 2mM EDTA prepared in double distilled water. The tape and combs were then removed from the gel plates. The plates were then placed in the electrophoresis rig and submerged in 1x TBE buffer. A set of five samples and 3 controls were then each mixed with loading dye, separately on a piece of parafilm and loaded into predetermined wells. Before running the electrophoresis rig, a DNA ladder was added to aid in size estimation of the amplicons. The gels were then run at 125-150 V for ~45-30min or until the dye front nears the bottom of the gel. The gel is subsequently removed from its encasing plates, submerged in a vessel containing a mixture of 1x TBE buffer and 5µl of SYBR™ SAFE DNA gel stain for ~20min. Once the staining procedure ends, I viewed the gels with a UV transilluminator. For each set, I first affirm that the two negative amplification and one negative extraction controls indeed negative (no band on the gel). Because the loaded DNA was previously amplified the expected result is a singular bright band around 100-200bp long. I then take note of any samples

that produced a single band within the expected range of nucleotide length and considered them to be successful amplifications. This process was repeated a minimum of four times to collect the complete set of four parts (a, b, c, and d) making up the HVR1.

When a sample is missing one of the four parts, the amplification process for that part is repeated on the second aDNA extraction of that sample to eliminate the possibility that the first extraction was faulty. I noticed that some parts proved to be fickler than others during the amplification step (particularly parts a and c). This is most likely due to the large number of tandem repeats within their primer annealing sequences that can confuse the amplification process. Additional primers were designed to attach to earlier or later locations of the HVR1 while still encompassing the whole region. If a complete set of four parts were then obtained using the new primers, those samples were cleared for the next step.

4.5 Sequencing the Amplified HVR1

Successfully Amplified samples and their respective primers were prepped in the modern DNA lab at UIC and were sent to the Genome Research Core in the Research Resources Center at UIC for Sanger sequencing (https://rrc.uic.edu/cores/genome-research/genome-research-core/). At the Genome Research Core, the samples were prepped for sequencing and loaded into their Sanger sequencer (Life Technologies 3730xl Analyzer). The resultant sequences were then sent back to the ancient DNA lab at UIC.

4.5 Bioinformatic Analysis

Ancient DNA and other fragmented DNA must be put through several quality control measures. The first is to clean the sequences by removing primers and other noise around the targeted region. Second, the forward and reverse sequences of each HVR1 segment are aligned,

if alignment is not satisfactory, resequencing is required. Third, the aligned sequences are manually edited for oddities and ambiguities. I used Geneious Prime 2019 to view and perform the quality control measures above for each of the 4 segments (see Table 3) from each sample. Out of the original 29 samples, the HVR1 of 14 samples were successfully sequenced and passed quality control. A consensus sequence was built for each of the successful samples by overlapping the sequences from each segment (A, B, C, and D) to obtain maximum coverage of HVR1.

The consensus sequence of each sample was compared to the revised Cambridge Sequence of human mitochondrial DNA (rCRS) using the BLAST (Basic Local Alignment Tool) on NCBI (National Center for Biotechnology Information) and was manually scanned for the presence of SNPs (Altschul et al., 1990).

Haplogroups for the 14 sequence samples were estimated using a combination of two free haplogroup classification tools, Haplogrep2 (Weissensteiner et al., 2016), Empop (Parson & Dür, 2007; Röck et al., 2013) followed by a comparison against Phylotree build 17 (van Oven & Kayser, 2009).

As this study represents a preliminary endeavor, I sequenced one of the three hypervariable regions (HVR1), and will, therefore, conservatively estimate mtDNA haplogroups for the CNS and CSMME samples, as many diagnostic SNPs are located in HVR2 and HVR3. Since my ultimate sample size (N=14; CNS=8, CSMME=6) was small, I compiled data from several sources (Table 4) to compare haplogroup distributions through time within what is now the borders of Peru.

5 RESULTS

Through this project, I verified the viability of samples taken from two cemeteries within the colonial context of Eten for ancient DNA studies. I then began to estimate the maternal haplotypes of some of the samples mentioned above using Sanger sequencing. Through statistical analysis I conducted a preliminary comparison in maternal inheritance between the two cemeteries. I then compared my data with previous ancient DNA maternal inheritance data from past studies of more ancient samples located within the territory that is now the northern coast of Peru. Finally, I take the first steps toward a method to probe for Spanish colonial admixture within two colonial Catholic cemeteries within the town of Eten in the Lambayeque Valley of Peru and connect those findings with what is already known about cultural admixture within the mortuary assemblages, their relation to the persistent Mochik identity of the buried individuals.

Of the original N=29 samples (CNS N=19, CSMME N=10), I successfully amplified all four parts of HVR1, for N=14 samples (CNS N=8 , CSMME N=6). For the remaining N=15 samples, at least one of the four parts from all 29 samples were successfully amplified. Samples that did not successfully amplify all four parts of HVR1, however, were not included in the sanger sequencing step.

In this chapter I present the results of my ancient mtDNA analysis of the HV1 sequence from each of the N=14 successfully amplified samples. I then estimate how Haplogroups A, B, C and D are distributed through time.

5.1 Mitochondrial HV1 Sequence Results

In this section I compiled exploratory mtDNA haplogroup data from studies spanning the last 20 years with a particular focus on ancient DNA within modern Peru, summarized in Table 7. Table 8 displays the relative frequency of haplogroup estimations pooled by time-period and

graphed as percent/per period in figures 3, 4, 5, and 6 (R Core Team, 2021; Wickham, 2020; Wickham et al., 2020).

Table 6 Haplogrep and Empop Results for CNS samples (Early Spanish Colonial Period)

Sample ID	Observed SNPs	Haplogrep2	EMPOP	Diagnostic SNPs
154	16223T 16362C	M6	D, G	16223T 16362C 16519Y*
159	16223T 16320T 16325C 16362C	G1a1	D1, G1a1	16223T 16325C 16362C
167	16223T 16298C 16309G 16325C 16327T	C1	C1	16223T 16298C 16325C 16327T 16519Y*
183	16223T 16298C 16320T 16325C	M8	C1	16223T 16298C 16325C 16327T 16519Y*
191	16093C 16223T 16311C 16325C 16362C	D1d2	D1d2	16093C 16223T 16325C 16362C 16519Y*
217	16223T 16298C 16325C 16327T	C1	C1	16223T 16298C 16325C 16327T 16519Y*
230	16196A 16209T 16223T 16268G 16325C 16362C	G1a1	C1, C1b8a	16223T 16298C 16325C 16327T 16362Y 16519Y*

** 2 The Y in 16519Y indicates an unspecified pyrimidine (C, T, U).*

A combination of Haplogrep2 and EMPOP were used initially used to estimate haplogroups of the sequenced HVR1s. I then provide an explanation of the unexpected haplogroup estimations in Table 7.

Table 7 Haplogrep and Empop Results of CSMME samples (Middle-Late Spanish Colonial Period)

Sample ID	Observed SNPs	Haplogrep2	EMPOP	Diagnostic SNPs
62	16223T 16325C 16362C	G1a1	D1, G1a1	16223T 16325C 16362C 16519Y*
93	16223T	N	L3	16223T 16519Y*
138	16223T 16320T 16325C 16362C	G1a1	D1, G1a1	16223T 16325C 16362C 16519Y*
142	16223T	N	L3	16223T 16519Y*
145	16092T 16223T 16325C 16362C	G1a1	D1, G1a1	16223T 16325C 16362C 16519Y*
149	16223T 16361A	N	R	16223T 16519Y*

** 3 The Y in 16519Y indicates an unspecified pyrimidine (C, T, U).*

Tables 6 and 7 display for each sample: The observed single nucleotide polymorphisms (SNPs), their Haplogrep2 and EMPOP haplogroup estimations and the diagnostic SNPs for each estimation. Several unexpected haplogroups were estimated by both Haplogrep2 and EMPOP. To make sense of these unexpected results, I searched for the most probable lineage for each deviance with relation to Native American haplogroups (A, B, C, D and X) using Phylotree Build 17. A Summary of which is displayed in Table 8.

Table 8 Unexpected haplogroup estimations from Haplogrep2 and EMPOP and their most probable lineage related to Native American Haplogroups.

Haplogroup Estimation	Lineage regarding Native American haplogroups according to Phylotree build 17
M6	L3 > N > M > M6 (no possible branch to A, B, C, D or X)
M8	N > M >M8 > CZ > C
N	N > R > A, X
G, G1a1	N > M > M12'G > G >G1 >G1a > G1a1 (no possible branch to A, B, C, D or X)

The haplogroup estimations from Haplogrep2 and EMPOP were then adjusted manually via Phylotree build 17 to estimate the most likely haplogroup based on HVR1 SNPs as detailed in Table 9.

Table 9 List of MtDNA Haplogroup SNPs (bp15968-16409) Adjusted based on Phylotree Build 17

Sample ID KGSU	**Cemetery**	**Haplogroup**	**16092**	**16093**	**16129**	**16196**	**16209**	**16298**	**16309**	**16311**	**16320**	**16325**	**16327**	**16361**	**16362**
		rCRS	**T**	**T**	**G**	**G**	**T**	**T**	**A**	**T**	**C**	**T**	**C**	**G**	**T**
62	CSMME	D	-	-	-	-	-	-	-	-	-	C	-	-	C
93	CSMME	D	-	-	-	-	-	-	-	-	-	-	-	-	-
138	CSMME	D	-	-	-	-	-	-	-	-	T	C	-	-	C
142	CSMME	D	-	-	-	-	-	-	-	-	-	-	-	-	-

145	CSMME	D	C	-	-	-	-	-	-	-	-	C	-	-	C
149	CSMME	C	-	-	-	-	-	-	-	-	-	-	-	A	-
154	CNS	D	-	-	-	-	-	-	-	-	-	-	-	-	C
159	CNS	D	-	-	A	-	-	-	-	-	-	-	-	-	C
167	CNS	C	-	-	-	-	-	C	G	-	-	C	T	-	-
183	CNS	C	-	-	-	-	-	C	-	-	T	C	-	-	-
191	CNS	D	-	C	-	-	-	-	-	C	-	C	-	-	C
193	CNS	D	C	-	-	-	-	-	-	C	-	C	-	-	C
217	CNS	C	-	-	-	-	-	C	-	-	-	C	T	-	-
230	CNS	C	-	-	-	A	C	-	-	-	-	C	-	-	C

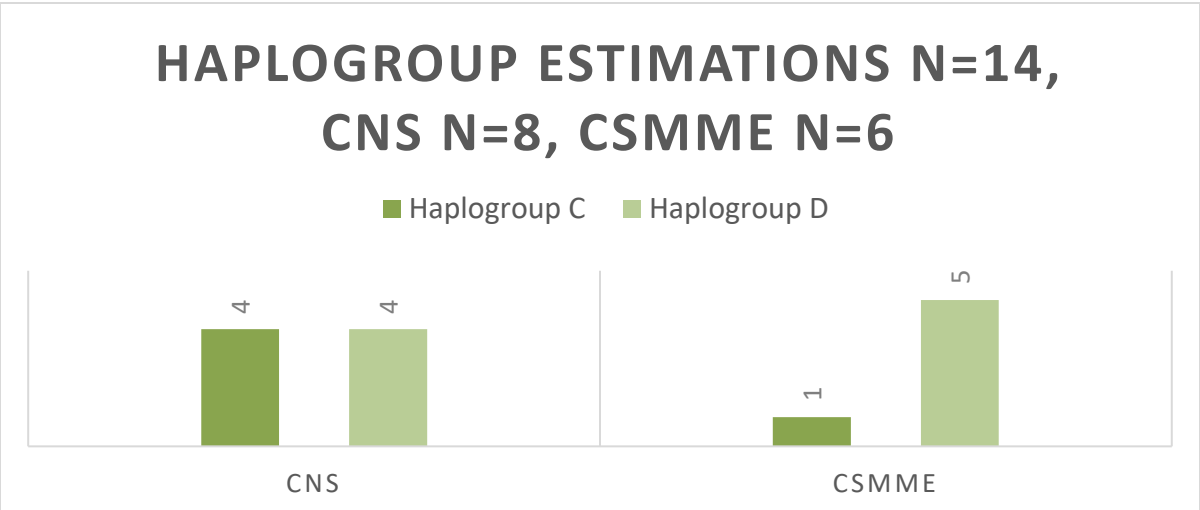

Figure 4 Bar graph displaying the haplogroup estimation results for both the Early/Mid CNS(N=8) and Mid/Late CSMME(N=6) Spanish colonial cemeteries.

Summary

In this section I displayed the results to each step of the haplogroup estimation process. The best match estimations are as follows; For the eight (N=8) early Spanish colonial period samples of CNS sequenced, four (N=4) were estimated as haplogroup C, the remaining four (N=4) were estimated as haplogroup D. The six (N=6) Mid to late Spanish colonial samples of CSMME were mostly estimated as haplogroup D (N=5), and a single sample as haplogroup C (N=1).

5.4 Relative Frequency Comparisons of Compiled Data

In this section I compiled exploratory mtDNA haplogroup data from studies spanning the last 20 years with a particular focus on ancient DNA within modern Peru, summarized in Table 10 and Figure 5. Table 11 displays the relative frequency of haplogroup estimations pooled by time period and graphed as percent/per period in figures 6,7,8,9, and 10 (R Core Team, 2021; Wickham, 2020; Wickham et al., 2020).

Table 10 List of Compiled Haplogroup Data and Their Sources

Time Period	Source	Sample N (total = 773)
Initial Period (IP)	Gómez-Carballa et al. 2018	22
Early Horizon (EH)	Gómez-Carballa et al. 2018 Fehren-Schmitz et al., 2010	9 37
Early Intermediate Horizon (EIH)	Gómez-Carballa et al. 2018	19
Middle Horizon (MH)	Gómez-Carballa et al. 2018 Shimada et al 2005	9 35

	Fehren-Schmitz et al., 2010 Lewis et al. 2005 Wester et al., 2020	93 33 12
Late Intermediate Horizon (LIP)	Gómez-Carballa et al. 2018	16
Late Horizon (LH)	Shinoda et al 2006 Wester et al., 2020	36 15
Spanish-Colonial Period (SCP)	This study Gómez-Carballa et al 2018	14 6
Modern Period (M)	Gómez-Carballa et al 2018 Messina et al. 2018 Fuselli et al. 2003 Cabana et al. 2014	210 116 91 611

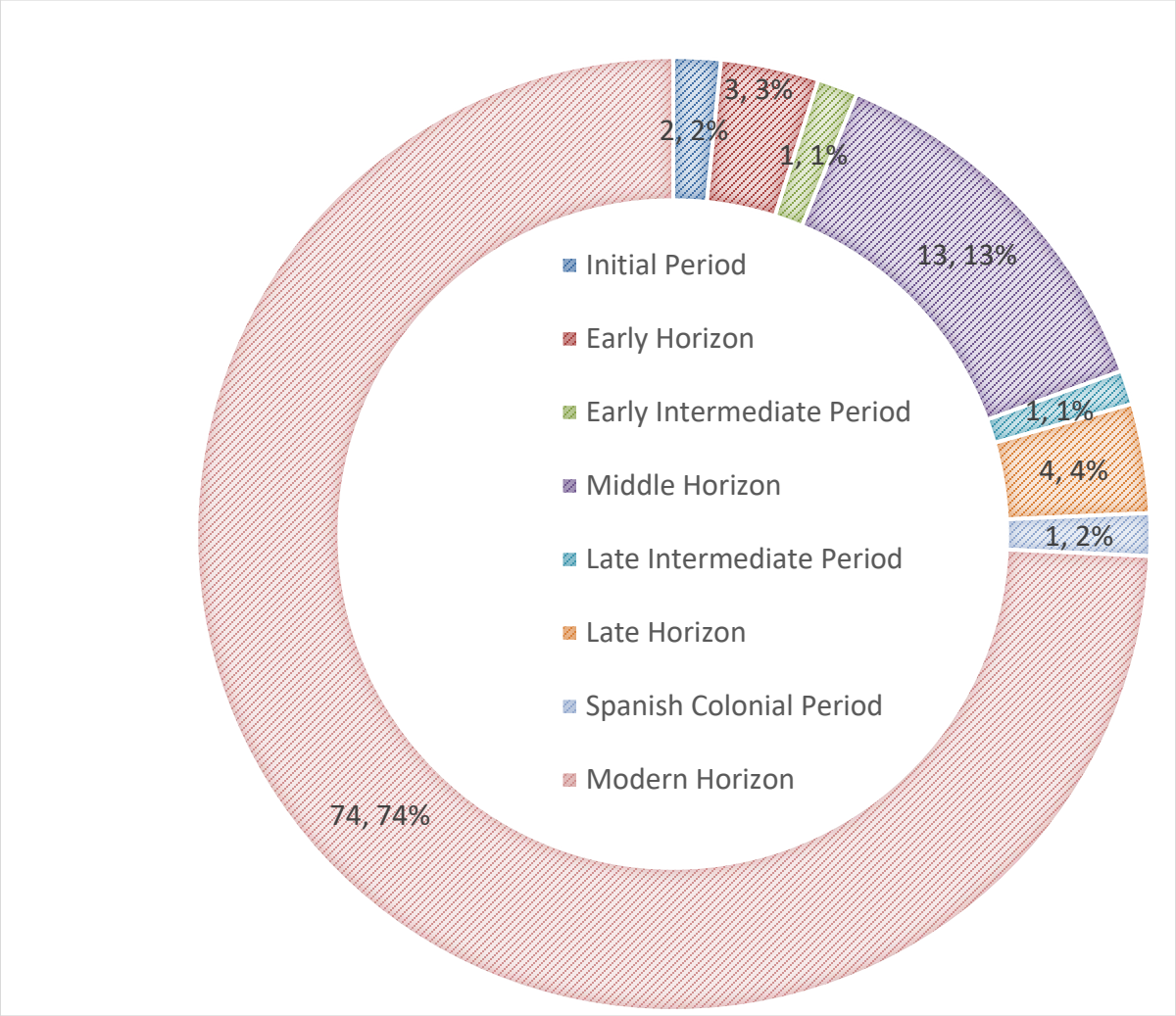

Figure 5 Graph displaying the percentage of samples which represent their respective time periods.

Table 11 Relative frequencies of Haplogroups A, B, C and D by Time Period

Horizon/Period	Approx. Years (BCE/CE)	N=	Relative frequencies				
			A	B	C	D	Other
Initial Period (IP)	1600 – 800BCE	22	0.273	0.409	0.273	0.0454	0
Early Horizon (EH)	900BCE -0CE	46	0.0435	0.109	0.196	0.652	0
Early Intermediate Horizon (EIH)	0 – 650CE	19	0.5	0.11	0.04	0	0.3
Middle Horizon (MH)	600 – 1000CE	182	0.0435	0.109	0.196	0.652	0
Late Intermediate Horizon (LIP)	900 – 350CE	16	0	0.562	0.375	0.0625	0
Late Horizon (LH)	1300 -1500CE	51	0.05	0.6	0.36	0.02	0
Spanish-Colonial Period (SCP)	1500-1800CE	20	0	0.25	0.45	0.3	0
Modern Period (M)	1800CE-Present	417	0.140	0.516	0.164	0.145	0.034

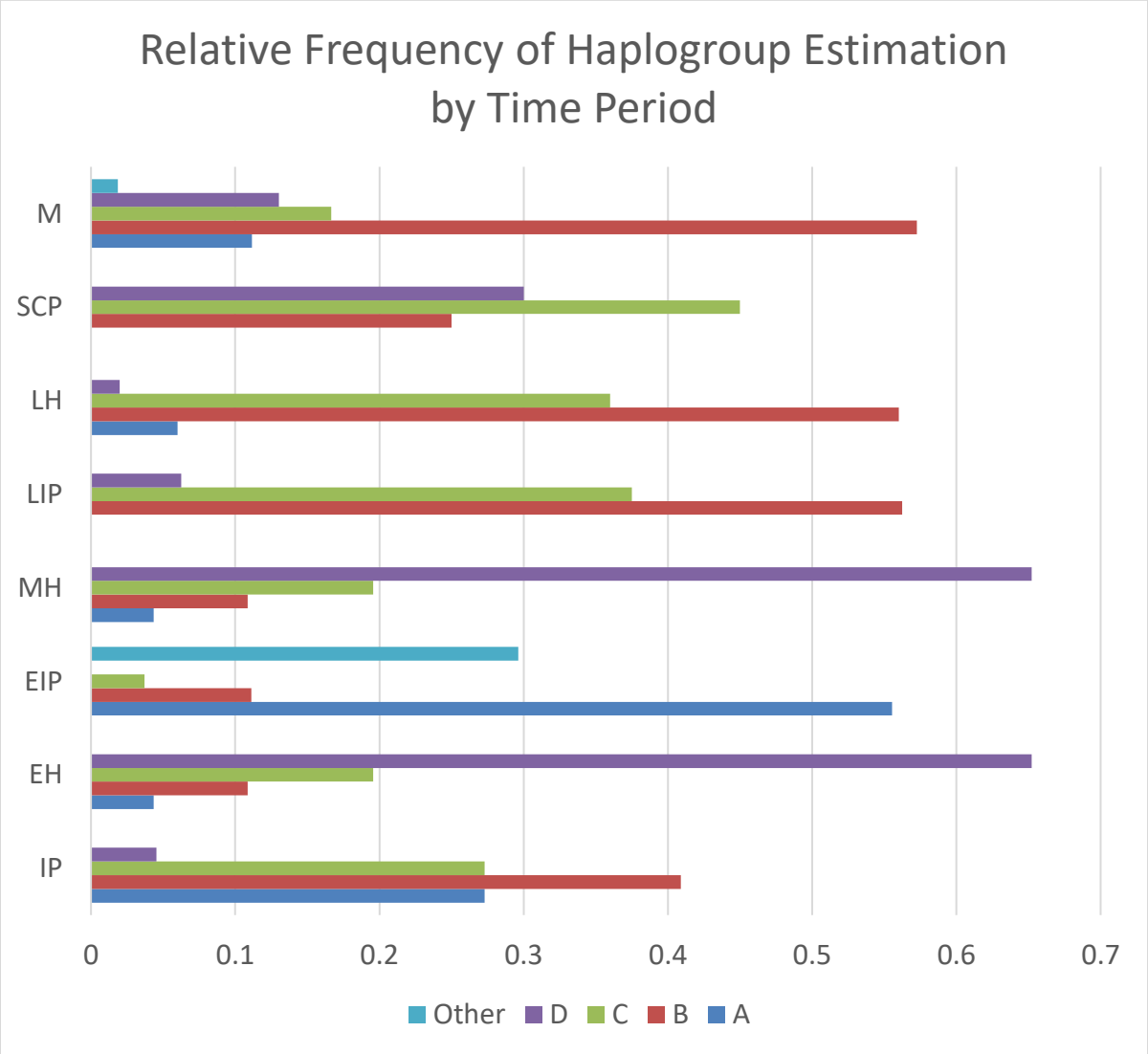

Figure 6 Graph summarizing the relative frequencies of haplogroup estimations by time-period.

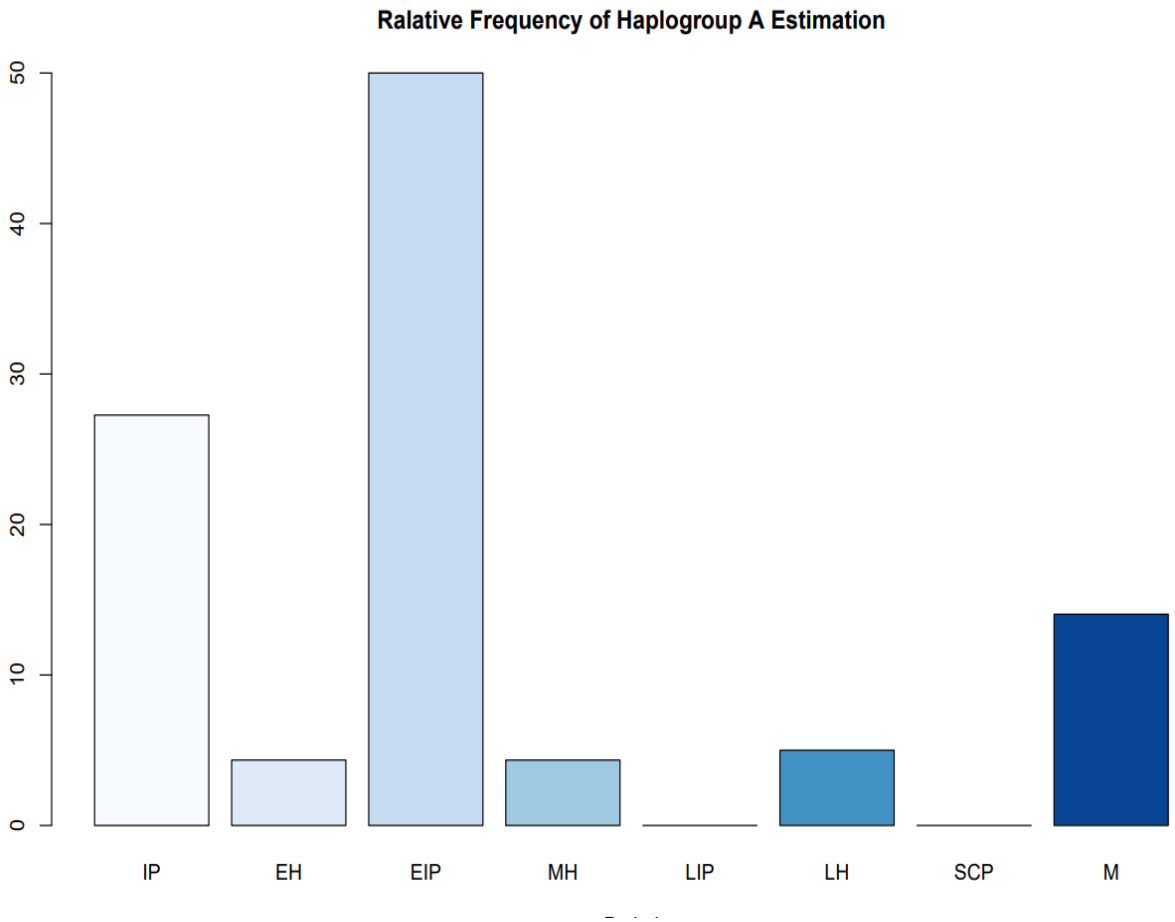

Figure 7 Bar chart displaying the relative frequency of haplogroup A through time.

Haplogroup A appears to be estimated in greater frequencies (>20%) within Initial Period and Early Intermediate Period contexts, and absent in Late Intermediate and Spanish-colonial periods (Figure 7).

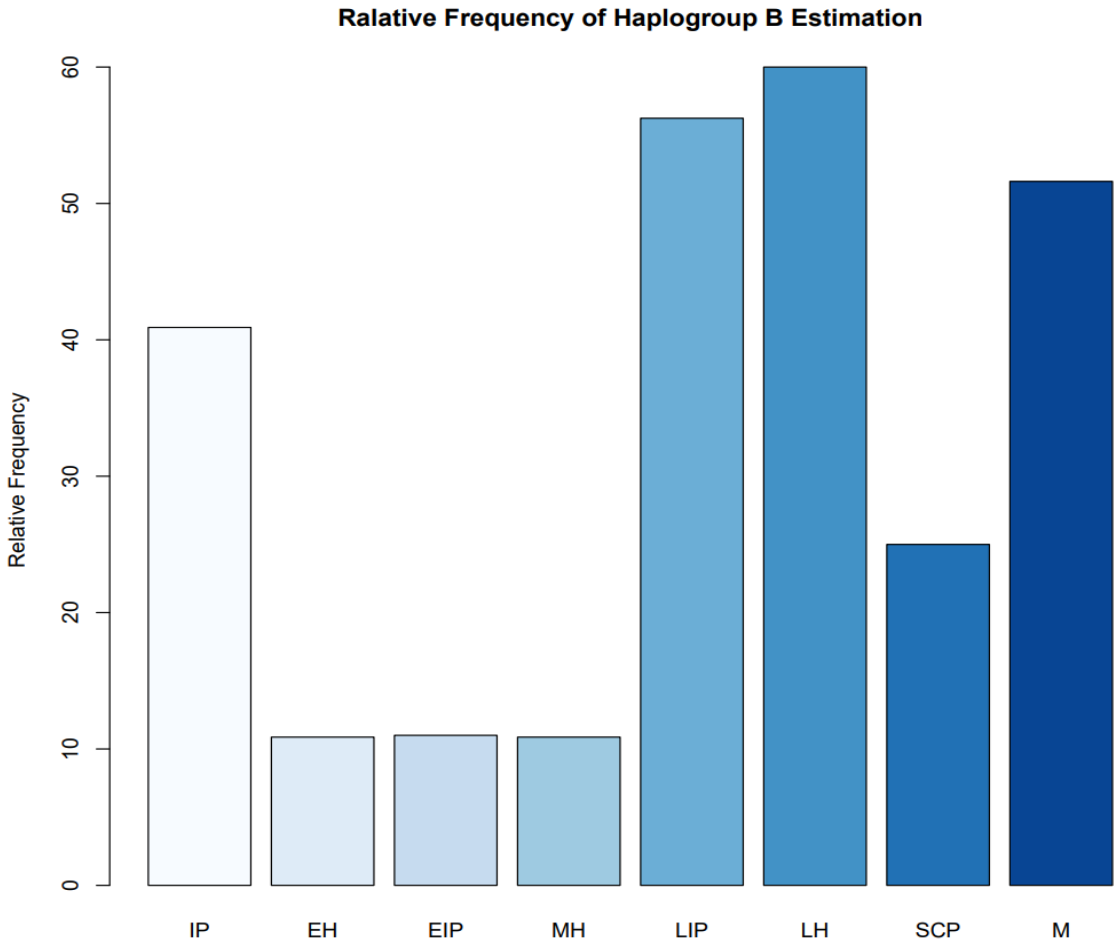

Figure 8Bar chart displaying the relative frequency of haplogroup B through time.

Haplogroup B was estimated in high frequencies (>40%) within Initial Period, Late Intermediate Period, Late Horizon and Modern contexts (Figure 8).

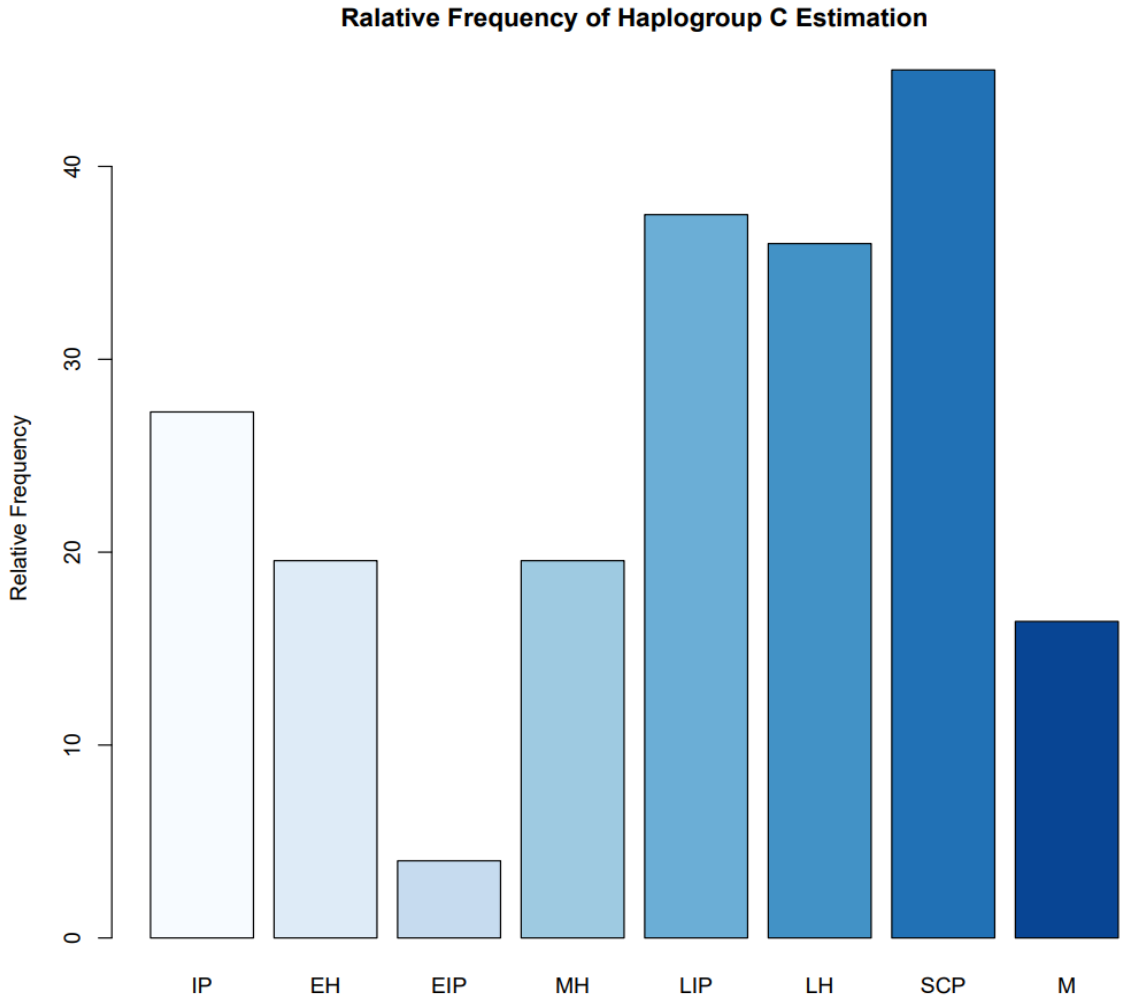

Figure 9 Bar chart displaying the relative frequency of haplogroup C through time.

Haplogroup C was estimated at a higher frequency (<30%) within Late Intermediate, Late Horizon and Spanish-colonial contexts (Figure 9).

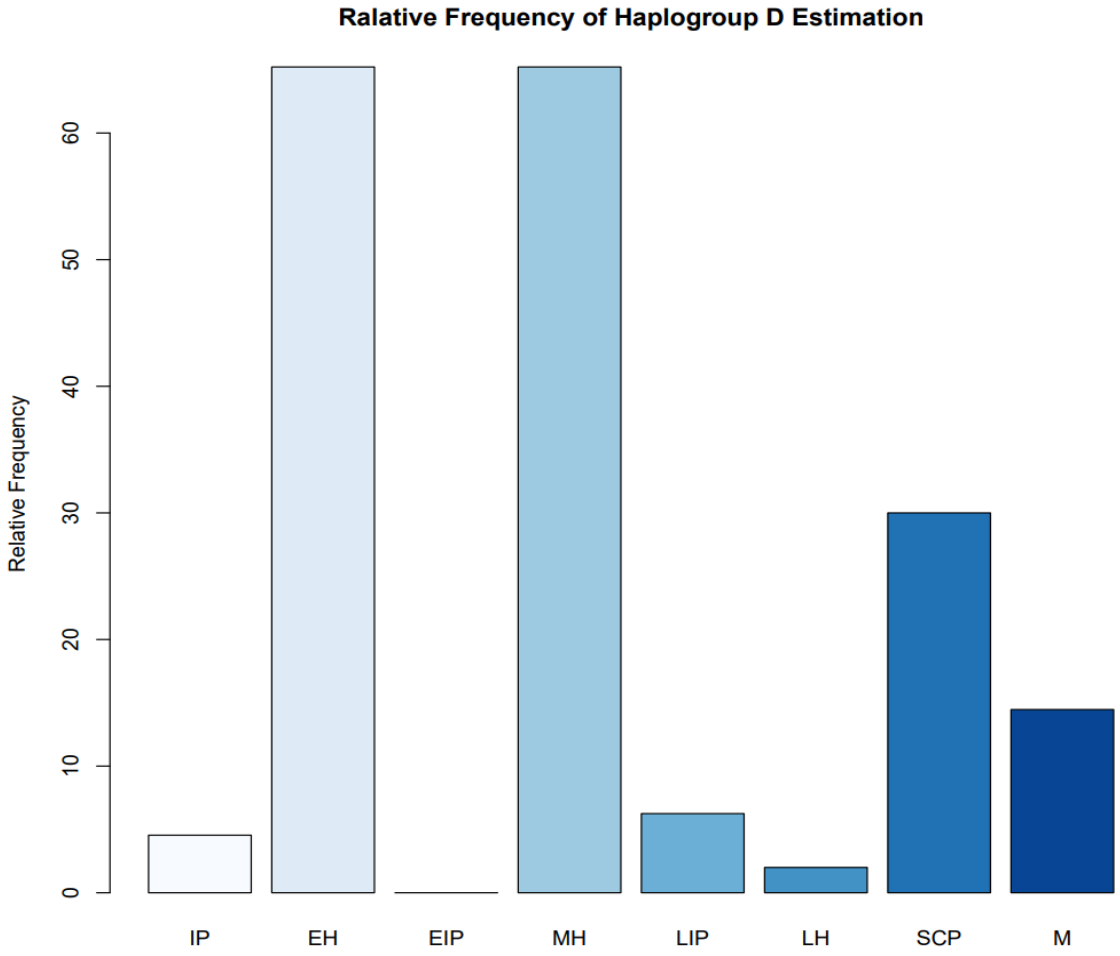

Figure 10 Bar chart displaying the relative frequency of haplogroup D through time.

Haplogroup D was estimated in higher frequency (>60%) within Early Horizon and Middle Horizon contexts, and moderately (~30%) in Spanish-colonial contexts as seen in Figure 10.

6 DISCUSSION

Through the Lambayeque Biohistory Project regional study of the Lambayeque Valley, a total of three Spanish colonial period cemeteries were excavated, including the site surrounding the Capilla de San Pedro de Mórrope (Klaus, 2012), and the Spanish colonial cemeteries in the town of Eten south of Mórrope; the Early/Mid Spanish colonial period (1530-1620CE) Chapel of the Niño Serranito (CNS), and the Mid/Late Spanish colonial period (1620- 1750 CE) Church of Santa Maria Magdelena de Eten (CSMME). A total of 253 burials were found at the CNS and 256 were found at the CSMME (Klaus & Alvarez-Calderon, 2017a). Variation regarding bioarchaeological evidence of the lived experience between the sites at the Chapel of San Pedro de Mórrope and the Eten sites CNS and CSMME was stark. Archaeological evidence collected at the Chapel of San Pedro de Mórrope indicated the infliction of continual systemic violence, including widespread living and nutritional disparity on top of forceful religious indoctrination into the Catholic faith (Klaus, 2012). No clear evidence of systemic violence and resistance in the osteological remains or burial treatment was found within the Eten cemeteries. This indicates marked regional differences regarding the experiences of Spanish colonialism in Peru (Klaus & Alvarez-Calderon, 2017a).

The Muchik ethnic identity appears to be in a process of constant adaptation. According to Klaus et. al (2017), Muchick was the resultant ethnic identity that embodied a resilient and persistent and adaptive cultural foundation, atop which dominant societies come and go with their political organizations and material cultures (Turner & Klaus, 2020). Evidence of Muchik ethnic identity can be followed through time. The most striking of which can be found in their mortuary practices, from funeral rites to ritual killings and the usage of specific iconographic media.

Hyper variable region 1 (HVR1) is one of two hypervariable regions located on the mitochondrial genome and is located between bases 16001 and 16568. Sequencing the HVR 1 allows some estimation of maternal haplogroup inheritance at a “low resolution”. A “fine resolution” or a better understanding of maternal haplogroup inheritance, however, will form once the complete mitochondrial genomes are sequenced and analyzed (Översti et al., 2017).

Through this study I endeavored to answer the question: *How did Spanish colonization of the northern coast of Peru Affect maternal inheritance in the Lambayeque town of Eten?*

I did so by testing the following hypotheses:

1. mtDNA haplogroup estimates of colonial period individuals from CSMME and CNS would be proportionally comparable with pre-Spanish colonial Andean populations, more than to modern Andean populations.
2. Previous studies that showed a stronger continuity within maternal haplogroups, therefore, I expect that of any estimation of European admixture within mtDNA haplogroups from CNS and CSMME would be rare if seen at all.
3. If present, however, I expect a higher frequency of Spanish-European and Sub-Saharan African mtDNA haplogroups in the mid/late Colonial Period Cemetery CSMME

In this chapter, I will discuss my interpretations of the results from the HVR1 sanger sequencing, haplogroup estimations, and relative frequencies of haplogroup distributions through time.

6.1 A peak into Haplogroup Distribution Colonial Eten

The initial goal for this project was to identify molecular evidence of European and African admixture within the Mochik population of Eten. Of the samples collected from the Spanish-Colonial cemeteries in Eten (CNS and CSMME), I was able to go as far as building a “low

resolution" haplogroup estimation data frame for 14 of the 29 samples, using HVR1 sequences and their frequencies within CNS and CSMME.

This study began with twenty-nine samples until the PCR amplification step, at which 15 of the 29 samples were disregarded because they did not result in a complete set of four (parts a, b, c and d) amplicon sequences that make up the HVR1. Fourteen of which were successfully amplified all four parts of the HVR1 of fourteen samples all four parts of the HVR1were successfully amplified and subsequently sequenced.

Overall, for the fourteen individuals (N=14), haplogroups C and D were the most likely estimates based on HVR1 sequences as seen in Table 7. The samples of CNS (N=8) were estimated as haplogroup C (N=4), and haplogroup D (N=4). The samples from CSMME (N=6), were estimated as haplogroup C (N=1), and haplogroup D (N=5). It appears that there is a pattern of genetic continuity at least on the mtDNA haplogroup level between individuals interred in CNS (1536-1640CE) and individuals interred in CSMME (1640-1750CE). Further sequencing of these same samples to the level of haplotype would provide a much better understanding of maternal lineage variation within the two cemetery populations.

If I were to suggest a targeted model that could be applied to the Lambayeque Biohistory project, I would suggest that at least in Eten, the surrounding natural and depositional environment allow for approximately 50% viable aDNA samples that can be successfully amplified and sequenced.

Through this study, I have shown that KGSU dental samples from both CNS and CSMME are viable for aDNA analysis. Their successful HVR1 sequencing has now opened the door to perform a deeper analysis of not only the mtDNA of the sampled individuals, but also shows promise for sequencing autosomal DNA from the same individuals. Because mtDNA

haplogrouping is only a small part of the admixture formula, Y chromosome and autosomal aDNA analysis would be the next logical step to probe for European and/or African admixture within the sampled individuals from both cemeteries. In combination with autosomal and Y chromosome haplogroup data, we could estimate continental/Atlantic admixture from both parental lineages as well as identify congenital diseases afflicting kin groups.

6.2 Relative frequencies of Haplogroup Distributions Through Time

Several studies have described mitochondrial haplogroup and, haplotype frequencies. Some of these studies were comparing haplogroup and/or haplotype frequencies in relation to geographic location (eg. Highland, coastal proximity, North and South) (Cabana et al., 2014; Fehren-Schmitz et al., 2010; Gómez-Carballa et al., 2018; Shinoda et al., 2006). Other studies would relate haplogroup/type frequencies to populations that belong to indigenous language families (Cabana et al., 2014; Lewis et al., 2005; Wester et al., 2020). Few studies have related frequency data comparing pre-Spanish invasion, during the Spanish colonization period and modern population in Peru (Fehren-Schmitz et al., 2010; Gómez-Carballa et al., 2018; Wester et al., 2020). In this project I collected datasets from some of these studies with an emphasis on haplogroup frequencies through time, and a focus on Native American Haplogroups (A, B, C and D). I organized those datasets, along with my own haplogroup estimates, based on established archaeological time periods, mentioned in chapters one and five, followed by the Spanish Colonial period into the modern or post-Spanish Colonial period. As a contextual part of this study, I compiled haplogroup data to find a cursory baseline of Native American haplogroups through time and within the modern borders of Peru.

Previous studies of Andean population genetics provided this study with a factual foundation atop which I could build my own hypotheses. To summarize the important points of

previous ancient DNA studies: A study by Shinoda et al. 2006 concluded that Pre-Spanish Invasion coastal populations of Peru were more closely related to each other genetically, than to the highland's populations. Fehren Smith et. al, 2010 noted a necessity to analyze aDNA data on the continental level to reveal any population genetic relationships. In their study, Wester et.al 2020 found that in the Lambayeque Valley; haplogroup C was the most common estimation in Pre-Spanish Invasion populations.

A summary of relevant points from modern DNA studies is as follows: Shimada et al. (2005 and 2008) found that haplogroup A was found in higher frequency in northern latitudes, Haplogroup B was mostly found within lower Central America and northern Andes, Haplogroup D is prevalent in in the Central Andes and particularly the highlands, and haplogroups C and D are found in high frequencies in southern latitudes. In a study by Cabana et al. (2013), the noted that highland and coastal populations differ in mtDNA genetic structure, Inca and Spanish population structure policies alone do not explain the Peruvian Central Andean demography, and there is evidence of female-biased gene flow in highland groups. Messina et al. (2018) concluded in their study of autosomal STR data indicated that 67% of urban Peruvian individuals have a strong similarity to the Amazon Native population, 22% showed similarity to African populations and only ~1% to European populations. Conversely, their estimation of maternal lineage continuity was in favor of a strong (~90%) Native American contribution, and much lower frequencies of African (~6%) and European (~3%) haplogroups

2.4.2 6.2.1 Andean Haplogroup Estimations

Haplogroup A is found was to be estimated in greater frequencies (>20%) within Initial Period (1600 – 800BCE) and Early Intermediate Period (0 – 650CE) contexts, absent in Late Intermediate (900 – 350CE) and Spanish-colonial period (1500-1800CE) and low frequencies

(<20%) in the remaining periods (Figure 7 & 8). It is interesting that a significant change in the relative frequencies of haplogroup A happened during intermediate periods as opposed to horizons. This could indicate that there was an influx of people carrying haplogroup A into the Andean region during the IP and EIP and a marked decrease during the LIP and SCP.

Haplogroup B, specifically the subclade B2 (sometimes referred to as B4b), is predominant in the Andean regions of Peru, Boliva and Argentina. Particularly, within some Qechua and Ayamara speaking populations, haplogroup B2 makes up around 70%-90% of estimated haplotypes in their respective populations (Gómez-Carballa et al., 2018). Maternal haplogroup data collected by Shinoda et.al (2006) and Baca et.al. (2014) show that a similar pattern appears in the Peruvian highlands and reaches back in time, before the Spanish invasion of Peru. Frequencies of haplotype B2 reported in those studies were between 42% - 75% (Baca et al., 2014; Shinoda et al., 2006). Modern Urban Peruvian haplogroup frequencies as reported by Messina et.al. (2018) had 51.5% of haplogroup B estimations within their sample. Conversely, haplogroup B was found in much lower frequencies to the north of Peru.

Haplogroup C was present in each of the time-periods discussed in this study. Haplogroup C was estimated at a higher frequency (<30%) within Late Intermediate period (900 – 350CE), Late Horizon (1300 -1500CE) and Spanish-colonial period (1500-1800CE). The persistence of Haplogroup C through time is further evidence of indigenous maternal lineage continuity as described by Cabana et. al (2014)

Haplogroup D was estimated in higher frequency (>60%) within Early Horizon (900BCE -0CE) and Middle Horizon (600 – 1000CE) contexts, moderately (~30%) in Spanish-colonial contexts (1500-1800CE) and absent in Early Intermediate period (0 – 650CE). Unlike Haplogroup A, Haplogroup D appears to increase in relative frequency within horizons rather

than intermediate periods. This could indicate that relative environmental, political and social stability within the Andean region allowed for an increase in Haplogroup D estimations, perhaps due to firmly defined cultural and ritual processes.

7 CONCLUSION

In this section I will discuss the limitations of this study and future directions for related research projects.

7.1 Limitations

This project was designed as a pilot study to assess the viability of aDNA extraction form the Spanish colonial period cemeteries CNS and CSMME and probe for non-Native American maternal admixture. Because this was a pilot study, I decided that Sanger sequencing of the HVR1 would be a more cost-effective way to test both the above the goals mentioned above than to seek out next-generation sequencing (NGS). This choice was a trade-off between the low-cost and low-resolution Haplogroup estimations using Sanger sequencing, and the high-cost, high-resolution NGS in order to effectively utilize the funding a resource available to me.

The small effective sample size analyzed in this study (N=14) creates two issues: Sample selection bias and lack of investigative depth. Delgado-Rodriguez (2004) define sample selection bias as "The error introduced when the study population does not represent the target population" which is an inevitable pitfall of small sample sizes. As a result of sample selection bias, we may only scratch the surface of what our samples can interrogate and explain our hypotheses.

Additionally, specific high resolution geographic context, a very important dimension to the study of population genetics, was not available with much of the data compiled and was therefore not used as a parameter in this study. Although many population genetics studies have been performed on the southern coast of modern day Peru, Genetic sampling within the northern coast of Peru has been minimal at best (Nakatsuka et al., 2020; Urban & Barbieri, 2020).

7.2 Future Directions

This study serves as evidence that samples excavated from CNS and CSMME are at least 50% viable for aDNA analyses. This opens the possibilities of future research to many intriguing directions.

The most obvious direction is to widen the scope from which we would probe for Continental admixture. The first is to sequence HVR2 and adjust or confirm haplogroup estimations that were based on HVR1 alone. Second, since there is a great deal of evidence temporal continuity of Indigenous Andean maternal lineages, Y chromosome haplogroup estimations will be more likely to present evidence continental admixture. Additionally, NGS of autosomal DNA will allow the collection of demographic information including biological sex, presence or absence of congenital diseases as well as kinship and biological group affinity studies.

Through the Lambayeque Biohistory Project we could build an ancient DNA sequence database including demographic, trauma, diet, and mortuary context information for each archaeological site. This would be a great boon not only to the study of Spanish colonial Andean contexts but to other European colonial contexts in the Americas and beyond.

In closing, this project is the first attempt at any direct genetic understanding of the colonial period within the Lambayeque Valley complex. I was able to extract ancient DNA from Spanish colonial period samples, amplify and sequence the HVR1 for N=14 samples, and edit and analyze the resultant sequences to us in mtDNA haplogroup estimation software. The haplogroup estimates for the Spanish colonial period cemeteries at Eten appear to fit neatly within the overall patterns found in my comparative study of Native Andean haplogroup frequencies through time.

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