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Genetic Analysis of Ancient Human Remains from the Early Bronze Age Cultures of the North Pontic Steppe Region

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GENETIC ANALYSIS OF ANCIENT HUMAN REMAINS FROM THE EARLY BRONZE AGE CULTURES OF THE NORTH PONTIC STEPPE REGION

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Abstract

During the Neolithic transition into the Early Bronze Age (EBA) in the North Pontic steppe region (NPR), people, cultures, and technologies were rapidly changing. Farming was on the decline and Indo-European languages were spreading through the region along with pastoralist way of life. In this study we used mitochondrial DNA (mtDNA) haplotyping to study the people living in the NPR during these times. Additionally, we used next-generation sequencing (NGS) technologies in attempts to develop novel methods to assess the degradation of ancient DNA (aDNA). We extracted ancient mtDNA from remains of 11 individuals belonging to late Neolithic and EBA populations of the NPR. Using single nucleotide polymorphisms (SNPs) as markers, we established mtDNA haplogroups of nine out of 11 individuals. Using our data, as well as mtDNA haplogroup frequencies from literature, we visualized genetic relationships among various Eurasian populations spanning the Mesolithic through EBA using principal component analysis (PCA). We then examined the changes in haplogroup frequencies through time using an F_{ST} analysis, comparing representatives of the Yamna (Pit Grave) and Catacomb groups, the main pastoralist EBA cultures of the NPR, and modern European populations. We found genetic evidence through mtDNA haplogroup frequencies and PCA linking the Catacomb people to hunter-gatherer populations from northern Europe and Russia. On the other hand, data on mtDNA haplogroup frequencies of individuals from the Yamna culture associated them with farming and pastoralist type populations from southwest and central Europe. An F_{ST} analysis of mtDNA haplogroup frequency distribution showed that the Yamna are most closely related to the Boyko group of ethnic Carpathian highlanders than to other modern European groups used in the study. The Catacomb people appeared genetically different from all other population groups in the F_{ST} analysis, including the

Yamna group, challenging the current understanding of the relationship between the Yamna and Catacomb populations. Further statistical analysis using an exact test of population differentiation confirmed genetic differences in mtDNA haplogroup frequencies between Yamna and Catacomb. The exact test also revealed a lack of genetic differentiation between the Yamna and the modern Ukrainian population, as well as Lemko, another group of Carpathian highlanders. Data gathered from the NGS aspect of the study was not informative in its original design. Modifications to the methods and techniques outlined in our NGS assay could provide useful information in building a more comprehensive understanding of DNA damage through time.

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Introduction

With the advent of modern molecular technologies for manipulation of DNA, population genetics has been enhanced with powerful tools to study the human evolutionary past (Nikitin et al., 2012, Brandt et al., 2013, and Lizaridis et al., 2013). Population genetic analyses, such as phylogeography, can attempt to answer questions we are no longer able to directly observe. Maternal DNA lineages, using mitochondrial DNA (mtDNA) single nucleotide polymorphisms (SNPs) as markers, give insight into the relatedness of people as they populated the Earth through time (Richards et al., 2006, van Oven and Kayser, 2009). In turn, this information can help resolve questions about where certain people came from, or how they genetically admixed with people from a different population. Cultures have already been attributed with the spread of technologies, such as the spread of language and the domesticated horse, through archaeological studies (Piazza et al., 1995). DNA sequencing now allows us to study the genetics of the cultures of people linked to these technologies, learning about the relatedness of these cultures. Moreover, we are now able to use molecular techniques to directly study the individuals that lived many thousands of years ago.

While useful for directly studying the genomes of the ancient past, such as constructing the sequence of a Neanderthal (*Homo neanderthalensis*), ancient DNA (aDNA) is not without its difficulties (Green et al., 2010). Without the living cell's DNA repair mechanisms, other chemicals in the environment, such as water, have the ability to damage and change the structure of the DNA (Lindhal, 1993). Ancient DNA is highly damaged and fragmented due to post-mortem degradation through hydrolysis, existing in fragment sizes typically no larger than 250 base pairs (Adler et al., 2011 and Fu et al., 2013). Damage to aDNA can also occur at each nucleotide as hydrolytic damage (deamination and fragmentation) and oxidative damage causing

cytosine to thymidine (C→T) and adenine to guanine (A→G) transitions in the DNA sequence (Lindhal, 1993). This damage effectively changes the natural variation occurring from mutation in the endogenous DNA sequence.

In addition to aDNA's fragile state, specimens are almost always contaminated with modern DNA from archaeologists, bacteria, animals, and many other sources. The presence of inevitable modern contamination often obscures endogenous aDNA within samples. Thus, there is a need for an effective method to discriminate between endogenous aDNA from modern contamination. Conventional aDNA processing techniques have relied on the sequencing of DNA fragments produced through multiple rounds of handling (amplification, cloning, etc.) where the difference between contamination and genuine aDNA can become obscured or vanish altogether.

One of the criteria to determine if an examined DNA molecule is genuine aDNA is to look for the presence of deaminated cytosines (Skoglund et al., 2014). Deamination, a hydrolytic reaction, chemically turns cytosine nucleotides into a uracil nucleotide. This modified nucleotide is then interpreted by DNA polymerase as thymine during downstream DNA amplification and sequencing. Current literature states that between 20-50% of cytosines in a genuine aDNA sequence should be deaminated (Fu et al., 2013, Skoglund et al., 2014). To view deamination rates in a particular aDNA sample, polymerase chain reaction (PCR) fragments are cloned into *E. coli* and sequenced to determine where genuine mutation variation (consistent in all reads) occurs and where deamination occurs (not consistent across all reads) in each of the amplified fragments of aDNA.

The highly degraded state of aDNA requires further procedures outside of deamination rate alone to authenticate endogenous ancient template DNA from modern contamination. Since aDNA is also highly fragmented, high sensitivity DNA quality control instruments, such as a Bioanalyzer (Agilent), can be used to determine the concentrations and fragment lengths of DNA within a given extraction. Samples which do not contain the expected size range of endogenous aDNA (<250bp) and are highly skewed to larger DNA fragment sizes may indicate more contaminant molecules in an extraction. With aDNA samples being inevitably contaminated by modern DNA, PCR bias also allows further authentication checks through fragment size as smaller fragments are preferentially amplified in a PCR reaction. Primer design becomes increasingly important for amplification of your locus of interest in an ancient sample. Due to the high fragmentation and low concentration of endogenous DNA, small fragment regions (between 60-200bp) are typically targeted (Nikitin et al., 2012). When using primers targeting DNA regions larger than 250bp, lower rates of successful amplification should be seen with genuine aDNA template.

Beginning with DNA extraction, ancient samples are typically prepared in a UV sterilized hood in a location separate from downstream DNA molecular biology to minimize the risk of modern DNA contamination (Cooper and Poinar, 2000, Adler et al., 2011). In further downstream applications, being able to replicate results over multiple DNA extractions, amplification, and sequencing events becomes imperative to the authentication of genuine aDNA (Cooper and Poinar, 2000). Low copy numbers of endogenous aDNA template molecules as well as small fragment size existing in a typical aDNA extraction, should mean that a PCR reaction may not amplify the target molecule in every reaction. Once successfully amplified, cloning is then required to parse out damaged nucleotide sites from endogenous nucleotide variation in a

particular sample (Cooper and Poinar, 2000). Recently, statistical methods using likelihood analyses have been used to combine authentication checks through post-mortem damage sites as well as fragment size to parse out modern DNA contamination from endogenous aDNA (Skoglund et al, 2014). To quantify authentication criteria including deamination rate, fragment size and modern contamination ratios, aDNA samples are sequenced using DNA sequencing technologies.

Currently within the field of archaeogenetics there exists two large overarching methods for directly studying the genetics of our ancient past. With one method, the whole-genome next-generation sequencing (NGS) approach, genetic studies have gained access to ever increasing amounts of data in comparison to older DNA sequencing technologies. However, the whole-genome NGS does not yet have the same power for population comparison across the field as the older method, sequencing only mitochondrial DNA (mtDNA), does. NGS offers exceedingly large amounts of data in comparison to older Sanger sequencing methods (Lazaridis et al., 2013, Brotherton et al., 2013, and Fu et al., 2013). While older capillary methods are limited to one run of ~800bp per sample, NGS technologies can retrieve DNA sequence from entire genomes with data of up to 60 gigabases in size (McCormick et al., 2013). Another stark contrast in data generation between these two technologies is the range across the genome that is possible to be covered by NGS. Specific targeting of the mtDNA through NGS may be achieved; using NGS to sequence only the targeted mtDNA genome negates the use for cloning in standard Sanger sequencing runs. The large amount of sequencing reads for NGS allows all possible mtDNA fragments to be sequenced in a single run. Furthermore, while Sanger sequencing runs are limited to one locus at a time per sample run, while NGS on the other hand can obtain DNA sequence for multiple loci or even the entire genome (Marguiles et al., 2005, McCormick et al.,

2013, Eid et al., 2008). Data from other loci across the genome can then be used in population genetic analyses to more clearly depict gene flow and is not limited to one locus inherited in one particular fashion. Specific areas within the genome may also be targeted and enriched for increasing the coverage and amount of data received for a particular locus of interest (Brotherton et al., 2013). In archaeogenetics, the mitochondria has been studied extensively for population genetic analyses and a significant amount of data for population comparison through time has been obtained (Brandt et al., 2013, Brotherton et al., 2013, Nikitin et al., 2012, Malmström et al., 2009 Hääk et al., 2008).

Chapter 1

Mitochondrial DNA (mtDNA)

Due to the degradation of DNA in ancient specimens, mtDNA has been used regularly by researchers in this field (Bramanti et al., 2009; Mälmstrom et al., 2009, Haak et al., 2005, 2010; Nikitin et al., 2010, 2012, Brandt et al., 2013). While each cell only contains two copies of nuclear DNA, each cell may carry multiple hundreds (100-1,000) of mitochondria each containing an mtDNA genome (Robin and Wang, 1988). Due to these properties, copies of the mitochondrial genome are more abundant in the cell than the nuclear genome. Human mtDNA is a maternally inherited, non-recombining, circular DNA sequence 16,569 base pairs (bp) in length and inherited separately from nuclear DNA (Andrews et al., 1999). Considering these properties of mtDNA, it becomes a useful molecule for tracking human lineages due to not recombining, meaning it is not greatly changed from generation to generation (Richards et al., 2000). Mutation motifs that occur within certain diagnostic regions of the mitochondrial genome are used to determine maternal lineages of ancient humans in population genetic studies (Richards et al., 2000 and references therein).

Within the human mitochondria, geneticists use SNP variation to group each individual into a haplogroup based on the mutation motif in each person's mitochondrial genome (Andrews et al., 1999). A combination of the SNPs located with the non-coding control region and the coding region form the mutation motif of each designated haplogroup (Andrews et al., 1999). The hypervariable region (HV1) is the non-coding region of the human mitochondrial genome ranging from base pair 16,000 to base pair 16,569. In the HV1 region, diagnostic mutations are noted and added to coding region mutations, together used to determine maternal lineage (Bramanti et al., 2009, Andrews et al., 1999). Mutation motifs are grouped into haplogroups

which are used to describe a maternal kinship of the ancient humans tested. There are two options when denoting mutation motifs for haplotyping when dealing with human genetic information, the revised Cambridge Reference Sequence (rCRS), and the reconstructed Sapiens Reference Sequence (rSRS) (Andrews et al., 1999 and Behar et al., 2012). The rCRS belongs to haplogroup H2 and is more commonly used as the baseline for determining mutations for haplogroup identification (Andrews et al., 1999). However, H2 is not the mtDNA sequence of the mitochondrial most recent common ancestor (MRCA), and due to the way in which mutations occur through time, researchers later constructed the rSRS (Behar et al., 2012). A human mitochondrial reference genome created with data from 8,000+ genomes, the rSRS provides increased resolution for haplogroup delimitation (Behar et al., 2012). Since the haplogroup of the rCRS, H2, is not the ancestral mtDNA sequence of the human MRCA, the rSRS was constructed to provide such a sequence allowing the quantity of mutations accumulated through time to be determined from a true ancestral human mtDNA sequence (Behar et al., 2012). To determine haplogroup calls, mutations are noted along the 16,569bp human mitochondrial genome, including both control region and coding region mutations. These mutations are then checked against the rCRS (or rSRS) with each mutation motif belonging to a specific mitochondrial haplogroup. For example, mutations at base pairs 16224 and 16311 in the HV1 segment of the mtDNA genome would give a haplogroup from the K clade when compared using the rCRS (Figure1 and Phylotree.org, mtDNA tree build 16, Feb. 19th 2014).

Human mtDNA Haplotypes

For humans, the MCRA of all maternal lineages coalesces to a lineage in Africa around 200,000 years ago denoted haplogroup L (Walker et al., 1987, Gonder et al., 2006). The L clade contains seven sub-clades, six of which stayed in Africa and one, L3, which migrated into the

Middle East around 70,000 years ago (Gonder et al., 2006). The L3 clade, through mutation and genetic drift, became the base for all genetic variation in maternal lineage outside of Africa (Richards et al., 1998). Once out of Africa, L3 split into two major clades, M and N, which, in turn, branched into the major haplogroups that populated the rest of the world outside of Africa (Maca-Meyer et al., 2001).

Once diverged from the L clade, carriers of haplogroup N moved into the Middle East (Torroni et al., 2006). From the major N clade, two major sub-clades diverged in N* and R (Andrews et al., 1999). In modern European populations, mitochondrial haplogroup H, a division of the R sub-clade, is the most frequent at around 40% (Brandt et al., 2012 and Brotherton et al., 2012). Haplogroup U, which also diverged from the R clade, is one of the oldest haplogroups in Europe (Fu et al., 2013, van Oven and Kayser, 2009). However, while U was prevalent in ancient Europe (before early Neolithic), modern European populations have a much lower frequency of U at 11% (Brandt et al., 2012 and Brotherton et al., 2012).

Once diverged from haplogroup L3, the M clade migrated from the Middle East into southern Asia (Gonzales et al., 2007). The M clade eventually gave raise to most of the Asian specific lineages including the C, E, G, Q and Z clades (van Oven and Kayser, 2009). Haplogroups C and Z share a common ancestor, with C originating around Lake Baikal in Russia around 27,000 years ago (Derenko et al., 2010). While rare in studies concerning ancient European populations, M clade individuals however have been identified previously in both modern and ancient populations of eastern and southeastern Europe (Nikitin et al., 2009, 2012; Newton, 2011; Guba et al., 2011).

Methods for aDNA Studies- Single Locus and Multi-locus Studies

While the study of mtDNA nucleotide variation can be essentially viewed as a single-locus analysis, which limits its scope, not enough characterization has been done with nuclear loci in aDNA to use for population comparison studies (Brandt et al., 2013, and Brotherton et al., 2013). Due to copy number and the probability of mtDNA remaining salvageable after thousands of years of chemical damage, mtDNA might be the only genetic information able to be retrieved from the majority of ancient specimens (Brandt et al., 2013, Brotherton et al., 2013, Adler et al., 2011, Cooper and Poinar, 2000). Tracking maternal lineage over paternal lineages (Y chromosomal markers) is more effective for understanding the migration of populations, as during ancient time periods maternal lineage movement is more likely associated to population migration instead of movement associated with war or hunting. Outside of the sex chromosomes, nuclear loci could give increased individual resolution at genes such as pigmentation or lactose persistence (Wilde et al., 2014, Burger et al., 2007). However, nuclear loci become less useful for population studies if insufficient ancient population data is available to compare different allele frequencies. Nuclear loci in this case would be effective for asking specific questions regarding an individual or specific population, such as determining if an ancient pastoralist (shepherd) population exhibited high allele frequencies of lactose persistence. In addition to its use for addressing different questions, nuclear loci are much less likely to survive thousands of years of DNA damage. In turn, studies able to sequence the whole genome of an ancient individual are severely limited (Fu et al. 2013, Keller et al., 2012, Green et al., 2010). Studying only the maternal inheritance will help the migration resolution over nuclear loci, directly showing maternal relatedness and movement through time. Genetic data outside of the mitochondria for ancient populations suffer from a lack of characterization in comparison,

and therefore peopling events and sociocultural relatedness information is less likely to have the same resolution through nuclear loci as it would currently through mtDNA (Brotherton et al., 2013).

When studying ancient DNA, degradation, damage, and innate modern contamination of the sample must be dealt with for accurate data analysis (Cooper and Poinar, 2000). Specifically when working with ancient humans, every person processing the bones from archaeologists to lab personnel, are possible contamination sources. Authenticating results for aDNA requires multiple rounds of PCR amplification and cloning checks to determine consistency in the determined SNP pattern such as haplogroup calls or damage sites (Cooper and Poinar, 2000, Brandt et al., 2013). To efficiently determine haplogroup calls, an assay commonly used in human haplogroup assignment, the GenCoRe22 assay (Hääk et al., 2010), checks for mitochondrial DNA mutations at diagnostic SNPs within the coding region of the mitochondrial genome (Brandt et al., 2013, Sarkissian, 2012). The combination of strict molecular methods to determine haplogroup calls, repetitive sequencing and cloning events and molecular assays all help determine the authenticity of aDNA (Brandt et al., 2013).

Once sufficient mitochondrial data is obtained and authenticated through aDNA methodology, it can be analyzed to determine haplogroups and their frequencies within the group studied (Cooper and Poinar, 2000, Brandt et al. 2013, Brotherton et al., 2013). Currently within the field, there has been a large focus on genetic discontinuity in haplogroup frequencies between the ancient peoples of Europe and the modern populations of Europe (Nikitin et al., 2012, Brotherton et al., 2013, Brandt et al., 2013, Wilde et al., 2014). Haplogroup frequencies can be used to compare among ancient populations and modern European populations as well, learning the most likely modern ancestors of these directly studied ancient populations

(Brotherton et al., 2013, Brandt et al., 2013, Wilde et al., 2014). Multidimensional analyses such as principal components analysis (PCA) can also use haplotype frequency information or allele frequencies to determine genetic relatedness among populations or individuals (Brotherton et al., 2013, Brandt et al., 2013). Using PCA can determine relationships among individuals or cultures by showing the underlying patterns within the data on a multidimensional scale. Setting up this analysis in a way to determine the relatedness of cultural groups during ancient time periods to other cultures existing around the world, could give insight into the mechanisms for the peopling of Europe across time (Brotherton et al., 2013, Brandt et al., 2013).

Population Characterization

Modern genetic diversity in European populations has shown discontinuity with ancient populations studied (Brotherton et al., 2013, Brandt et al., 2013). To understand this discrepancy in haplogroup frequencies between ancient human populations and modern human populations, population genetic analyses have been used to study ancient populations directly. Population dynamics of central and southwestern Europe have been characterized by other research in regards to the cultures living within those regions beginning in the Mesolithic through the Early Bronze Age (EBA) (Brotherton et al., 2013, Brandt et al., 2013). Prior to the Neolithic, hunter-gatherer populations across Europe were dominated by haplogroup clade U and its sub-clades (U4 and U5) (Brandt et al., 2013, Malmström, et al., 2009). However, beginning in the early Neolithic genetic evidence shows a drastic shift in the frequency of the U haplogroup in populations across Europe associated with the advancement of farming into central Europe, such as the Mittelebe-Saale region (Brotherton et al., 2013, Brandt et al., 2013). The Mittelebe-Saale region, and central Europe as a whole, has been primarily focused on for studies of maternal lineage discontinuity due to its consistent occupation by people from the Mesolithic through the

EBA (Brandt et al., 2013). Important cultures living in the Mittelebe-Saale such as the Bell Beaker, Linear Pottery, and Corded Ware cultures are associated with the spread of farming during these time periods (Brandt et al., 2013). Mitochondrial haplogroup H exists in high frequencies in modern European populations at ~40%, while prior archaeogenetic studies have shown a much lower frequency of haplogroup H in ancient European populations (Brotherton et al., 2013, Brandt et al., 2013) except for eastern and southeastern Europe (Nikitin et al. 2010, 2012). By using haplogroups as an indicator of genetic diversity changes across time, we may be able to understand the mechanism for the large shift in haplogroup frequencies between ancient populations and modern populations.

During the early Neolithic, cultures such as the Linear Pottery culture (LBK) and its descendants begin to see a large influx of haplogroup H, a clade typically associated with the expansion of farming during that time period (Brotherton et al., 2013). Due to genetic diversity based on F_{ST} comparisons with modern European populations, it has been hypothesized that the genetic variation existing in the modern H haplogroup is due to this influx of H during the Neolithic (Brotherton et al., 2013). As farming expanded from Anatolia beginning around 12,000 years ago the high frequencies of haplogroup U begin to diminish during the early Neolithic, transitioning into higher frequencies of N1a, T, and J clades also typically linked with the expansion of farming from the Anatolia region (Guba et al., 2011, Brandt et al., 2013).

The middle Neolithic in central Europe was mostly comprised of the Funnel Beaker culture and other smaller cultures associated with the Funnel Beakers in northern central Europe (Brandt et al., 2013). With frequencies of the H haplogroup on the rise from the influx of farming populations during the early Neolithic, hunter-gatherer populations are pushed to the outskirts of suitable farming land (Brandt et al., 2013). Hunter-gatherer haplogroup frequencies, typically

high in U (U4, U5) clades, begin having their numbers diminish as farming becomes more prevalent in central Europe (Brandt et al., 2013). Sub-H haplogroups from the early Neolithic seem to have become extinct or are at very low frequencies in modern central European populations (Brotherton et al., 2013). Middle to late Neolithic sub-H groups, however, are much more common in modern European populations (Brotherton et al., 2013). This could mean that the majority of the genetic diversity changes happened in the middle to late Neolithic and possibly into the EBA depending on the region. Based on this change in the genetic variation of the H haplogroup clade, other research has suggested that the main component in forming the modern genetic variation of haplogroup H came from the middle to late Neolithic (Brotherton et al., 2013).

During the late Neolithic and EBA, population dynamics begin to change rapidly across Europe. The Corded Ware culture (CWC) and the Bell Beaker cultures (BBC) predominate in central Europe with ever increasing frequencies of haplogroup H (H1 and H3) and other farming associated haplogroups such as T and J (Brotherton et al., 2013, Brandt et al., 2013). During this time, further influx of haplogroup H can be seen from the Iberian Peninsula in association with the Unetice culture complex, a culture in which the CWC and BBC eventually combine to form (Brotherton et al., 2013, Brandt et al., 2013). The presence of sub-H haplogroups, such as H1 and H3, have been associated with this influx of people from the Iberian Peninsula into central Europe during the middle to late Neolithic (Brotherton et al., 2013, Brandt et al., 2013). Haplogroup frequencies in central Europe become much more similar to modern day European populations during the Early Bronze Age, with differences in sub-clade frequencies being attributed to genetic drift and population migrations (Brotherton et al., 2013). However, while central and southwestern Europe have been extensively characterized through maternal lineages

and haplogroup frequencies, southeastern Europe remains understudied (Brotherton et al., 2013, Brandt et al., 2013).

Two previous studies have researched mtDNA haplotypes of individuals living in southeastern Europe during the Neolithic and into the Eneolithic, one studying the Neolithic hunter-gatherer Dnieper-Donets (DD) culture from the North Pontic region (NPR) and the other studying the Eneolithic farming Trypillian culture from eastern Carpathian Mountains (Nikitin et al., 2012, Nikitin et al., 2010). The Neolithic DD culture exhibited a rather dissimilar pattern of mtDNA haplogroup frequencies to central Europe. The DD culture had a higher frequency of haplogroup H than their Neolithic farming counterparts from central and southwest Europe, but lacking the H1 and H3 sub-clades commonly seen in central Europe (Brandt et al., 2013). The high frequencies of H in the DD culture were also accompanied by hunter-gatherer associated U clade haplotypes, as well as east Eurasian lineages of haplogroup C (Nikitin et al., 2012, Newton, 2011). The Eneolithic Trypillia culture from the region further northwest shows haplogroup H at high frequency comparable to DD (no H1 or H3 sub-clades), as well as including individuals with farming associated haplogroups belonging to T and J clades (Nikitin et al., 2010). Notably, the H clade haplogroups in the Neolithic NPR and Eneolithic Trypillia were not characterized by the same H1 and H3 sub-clades as were seen in southwestern and central Europe during this time. Since it remains unclear if southeastern European haplogroup frequencies influenced those of ancient central Europe, characterizing the populations of southeastern Europe can determine genetic relationships between these regions. Fitting southeastern Europe into the larger picture of haplogroup frequency distributions helps to clarify interpopulation genetic relationships as well as determine the source for the large shift in major haplogroups during the late Neolithic into the Early Bronze Age (Figure 2).

Cultures of the North Pontic Steppe Region

During the transition between the Neolithic (7,500-5,500 years before present (yBP)) and EBA (EBA, 4,100-3,700 yBP) a cultural and technological shift was taking place throughout Eurasia. The Holocene Climatic Optimum (HCO), beginning around 10,000 years ago shifted the climate in Europe to be much warmer and wetter (Schroder et al., 2004). These climate conditions made farming an effective way of life at areas much further north than previously possible. At the end of the HCO at around 4,200yBP this unusually warm period began to end returning climate to a cooler and drier environment (Schroder et al., 2004). Due to this shift in climate, farming cultures had to move south and southeast from central and northern Europe to find land that would sustain agriculture such as the North Pontic steppe region of Ukraine (NPR) (Kalis et al., 2003).

The NPR, located in modern day southern Ukraine was home to an important pastoralist culture, the Yamna, and to other pastoralist cultures during the Neolithic and EBA (Mallory, 1997). The Yamna (Pit Grave) culture is thought to have been a key component in the spread of proto-Indo-European language across this steppe region of Ukraine (Piazza et al., 1995) and beyond. Current archeological research suggests Yamna had been succeeded in the region by a culture known as Catacomb, based on the burial type used by the culture. It is also becoming increasingly clear that these cultures coexisted for an extended period of time (Wilde et al., 2014). The Catacomb people are thought to have borrowed some of the technologies from the Yamna culture, but it is unclear if they also exchanged genes. Studying the genetics of the people inhabiting the NPR during the Neolithic and EBA we may be able to see genetic evidence linking these cultures of the NPR to other cultures around Europe and Eurasia further clarifying the genetic story of Europe.

The current genetic story of Europe excludes southeastern Europe, but is yet it is hypothesized that Europe has been influenced by haplogroup frequencies from the North Pontic Steppe region (Brandt et al., 2013, Figure 2). Previous studies into other cultures of the NPR have shown haplogroup H occurring in individuals at relatively high frequency during the Neolithic, which has not been seen in central Europe (Newton, 2011, Nikitin et al., 2012). This previous characterization of the populations of the NPR also showed high frequencies of the Asian associated haplogroup C (Newton, 2011, Nikitin et al., 2012). Previous research has both hypothesized and shown evidence for genetic influence on central European populations through maternal lineages by the cultures existing in southeastern Europe (Nikitin et al., 2012, Nikitin et al., 2010, Brandt et al., 2013). Genetically characterizing NPR populations could further explain genetic variation existing in modern European human populations and further refine the view of the movement of people, cultures, and technologies during the late Neolithic and EBA.

Study Objectives

We extracted ancient human DNA from the Eneolithic and EBA people in the NPR to better understand population dynamics during the Neolithic through EBA in the steppe region of Ukraine. The objective of this part of the study was to use mtDNA haplogroups and their frequencies within the NPR to understand how the late Neolithic and Bronze Age individuals fit with other populations around Europe that have been studied to date. To analyze mtDNA data we used principal components analysis (PCA) on haplogroup frequencies of populations within our geographic area and that of the rest of ancient Europe and Eurasia ultimately showing maternal lineages and relatedness of the cultures inhabiting the NPR. To understand changes in population dynamics through time, we used F_{ST} and an exact test of population differentiation to test genetic

differentiation between our study cultures and between ancient individuals and modern European populations.

Methods - Haplotyping

Sample Collection and Preparation

Human remains of 11 individuals were gathered from burial mounds (kurgans) in the North Pontic Region of Ukraine, obtained courtesy of Dr. Svetlana Ivanova, Institute of Archaeology, Odessa, Ukraine. The remains dated from 5,500yBP to 3,000yBP (Table 1). Of the 11 samples, three individuals belonged to the Catacomb culture, three were from unidentified Eneolithic culture of the NPR region, three from the Yamna culture, and a final individual from the KMK culture (Table 1). To minimize risk of contamination prior to DNA extraction, all surfaces in the extraction lab were UV sterilized for up to 12 hours before extraction began. The extraction lab is separately located from the rest of the analytical labs as is standard practice when extracting DNA from ancient bones (Cooper and Poinar, 2000, Adler et al., 2011). Bones themselves were washed with bleach and UV sterilized on each side for one hour. Bones were then cut with a dremel tool to remove the outside layers of bone, which are the most exposed to outside contaminants, inside a laminar flow hood (Adler et al., 2011). Prior to extraction bones were ground with a sterile, bleached and autoclaved, mortar and pestle to obtain around 500mg of bone power to use in extraction.

Genetic Analysis

DNA was extracted using a QIAGEN QIAmp DNA Investigator Kit (Qiagen). DNA from the extract was then eluted in 20µl 18MΩ deionizedH_2O. Keeping with aDNA authentication procedures, each bone was extracted one to four times, depending on the amount

of starting tissue (Table 1).To obtain the HV1 region of the mtDNA for haplotyping of each sample, primers for four overlapping fragments (Nikitin et al., 2012 and Newton, 2011) were used and polymerase chain reaction (PCR) was run in replicates of three for each fragment to test low copy number and distinguish ancient DNA from modern contamination (Cooper and Poinar, 2000) (Table 1, Table 2). To overcome small aDNA fragment size (<250bp) we have subdivided the HV1 region into four fragments with a maximum size of 164bp for one set of primers and a maximum size of 84bp for another set (Table 2) (Nikitin et al., 2012 and Newton, 2011). For coding region SNPs, primers designed to flank restriction digest cut sites were used to check diagnostic SNPs for major clades H (7025) and U/K (12308) (Table 2) (Santos et al., 2004). PCR reactions to amplify the mtDNA coding and control regions were carried out using a QIAGEN FastCycling Kit with reaction volumes of 9.1µl H_2O, 10µl FastCycling Master Mix, 0.2µl of 10µM forward primer, 0.2 of 10µM reverse primer, and 0.5µL of template DNA. The thermocycler program was carried out as described in the FastCycling protocol with 50 cycles due to small amounts of template DNA (QIAGEN). After the original PCR, amplicons of the HV1 region of the mitochondria were cloned into *E. coli* (QIAGEN EZ competent cells) for further replication and damage determination. Much like obtaining DNA sequence for multiple alleles, only one aDNA template molecule is transformed into a bacterial plasmid. Since only one copy of the mtDNA template is present in each specimen, any discrepancies between sequences (such as C in some with T in others) at the same base pair, but not across all sequence reads from the same locus, can be identified. PCR was then carried out on successful clones, using T7 and SP6 bacterial primer pairs to amplify target DNA inserted into the *E. coli* using 30 cycles in the thermocycler based on the Genscript Green Taq protocol. These PCR reactions were done using 39.75µl H_2O, 5µl 10x Green Taq Buffer, 1µl of 2µM DNTPs, 1µl of 10µM T7,

1µl of 10µM SP6, 0.25µL of Green Taq and 2µL of template DNA(Genscript). Coding region SNP checks were directly sequenced, specifically to check for presence or absence of mutations at site 7028 (diagnostic for haplogroup H) or 12308 (diagnostic for U/K) in the mitochondria. PCR products were then cleaned using the ExoSap system to prepare for sequencing. Cleaned PCR products were sequenced using a BigDye terminator sequencing PCR and run on an ABI3130xl sequencer from Applied Biosystems (Life Technologies). Each sequence was then base-called using software from Applied Biosystems that is coupled with the sequencer. This base-calling software uses chromatogram quality information to determine the accuracy of each base called in the output DNA sequence. Once base called, sequences were edited using the program Sequencher (GeneCodes Corp. version 4.9).

To determine mutations and denote mtDNA haplogroups, sample sequences were aligned against the Cambridge Reference Sequence (rCRS), a reference sequence of the entire human mitochondrial genome, and mutations were determined using the program MEGA (version 5.2, Tamura et al., 2011). Approximate nucleotide deamination rates and rates of successful amplifications per fragment were used to determine authenticity of an ancient sample. Cytosine to Thymidine and Guanine to Adenine deamination damage has been shown to exist in 50% of the damaged sites of ancient DNA which can be seen through multiple amplification and cloning events (Lamers et al., 2009, Gilbert et al., 2003). Samples were assigned haplogroups based on nucleotide mutation motifs on phylotree.org (mtDNA tree build 16, Feb. 19th 2014). The entire process was repeated three separate times per fragment from different amplifications of the HV1 region and diagnostic control region to determine accuracy of haplogroup determination (Cooper and Poinar, 2000). In order to show low copy number of aDNA as well as distinguish contamination from authentic aDNA repeated extractions and amplifications of each fragment is

necessary (Cooper and Poinar, 2000). In addition to the standard methods for aDNA authentication, we also used a Bioanalyzer (Agilent) to determine DNA concentration and distribution of fragment sizes within a given extraction. Bioanalyzer data was gathered for all samples in this study excluding R3.7, R3.16 and the first extraction of K1.10. Due to typically low concentrations of DNA in a genuine aDNA sample, the Bioanalyzer was run using a high sensitivity assay to distinguish small changes in concentration and fragment size.

Statistics

To obtain genetic relationships among the individuals in this study when compared to the rest of the ancient world during the Neolithic through EBA, a PCA was run using haplogroup frequencies of cultures from various published datasets (Table 5). PCA, an Eigen vector based, multivariate, and non-parametric test that works to show the variance of the individual data points in an analysis by grouping them depending on the amount of variation explained by the vectors. The advantage of a PCA in the case of determining relatedness among populations is that none of the data on phylogenetic sense, geographic location, or relatedness of the individuals comprising a population influence the analysis. PCAs are known to reveal trends within the data without any prior knowledge of the data itself, making it an effective analysis for determining relatedness through grouping in haplogroup frequency analysis of ancient European and Asian populations (Brandt et al., 2013). Only haplogroup information from studies that followed strict aDNA protocols with results that make phylogenetic sense were used in the haplogroup frequencies dataset, such as we would not expect to see African specific lineages of the L clade in European datasets (Brandt et al., 2013). For a substantial number of populations to compare our data against, data of cultures haplogroup frequencies from around Europe and Asia from Brandt et al.2013 were used as well as their PCA methodology (Brandt et al., 2013). Data in our

study was also combined with genetic information from the same cultures from Wilde et al., 2014 as well as two individuals from the NPR Yamna population from Newton, 2011 to increase sample size thus enhancing the statistical power required for haplogroup frequency analysis. Combining the individuals studied here with other Yamna and Catacomb individuals from the Wilde et al. study and Newton, 2011 allowed the exploration of population based analyses. Once combined with Wilde et al. and Newton, 2011 data, the Yamna population was n=30 with the Catacomb at n=28. With individuals combined from this study, Wilde et al., 2014 and Newton, 2011, the undetermined Eneolithic culture population had n=13 samples. PCA on population haplogroup frequency data was performed with the R Statistics Package v3.0.2 with graphical output generated using the ggplot2 package within R.

To determine genetic affinities with modern European populations, the combined dataset containing Yamna and Catacomb haplogroup frequencies was compared with modern European population haplogroup frequencies through a pairwise F_{ST} analysis using Arlequin v3.5 (Excoffier and Lischer, 2010). F_{ST}, a measure of genetic diversity between subpopulations and the total population, provide a single number for characterizing genetic diversity to test the similarity and differences among populations based on allele frequency data (heterozygosity). The closer an F_{ST} value is to zero, the more genetically similar two subpopulations are, and a value closer to one, the more genetically different those subpopulations are. F_{ST} calculations were completed with 100 permutations to determine the significance of the differences between the cultures. Modern European population haplogroup frequency data was obtained from the literature for this comparison (Nikitin et al., 2009 and references therein) (Table 3). Data from this thesis was combined with Yamna and Catacomb individuals from Wilde et al., 2014 as well as, two individuals from the Yamna group included in Newton, 2011, for the ancient populations

in both the PCA and F_{ST} analyses. Modern European population data was gathered from the literature (Nikitin et al., 2009 and references therein) (Table 3).

Following the pairwise F_{ST}, an exact test of population differentiation was run to correct for the small sample size of the ancient population data. The exact test was run using the same Yamna, Catacomb, and modern European population haplogroup frequencies data from the F_{ST} analysis using Arlequin v3.5 (Excoffier and Lischer, 2010, Raymond and Roussett, 1995, Nikitin et al., 2009 and references therein). An exact test of population differentiation was chosen to more clearly interpret the genetic relatedness of the Yamna and Catacomb populations given their small combined sample size from this study, Wilde et al. 2014, and Newton, 2011 (Raymond and Roussett, 1995 and Waples, 1998). The exact test of population differentiation tests the hypothesis of panmixia, meaning that a significant *p*-value (<0.05) indicates population differentiation (Raymond and Roussett, 1995). In the case of small sample size and populations with high gene flow, the exact test of population differentiation can be an effective test for determining population differentiation (Waples, 1998). The exact test was run through a Markov chain method with 10,000 Markov chain permutations (Raymond and Roussett, 1995).

Results

Genetics

Of the eleven human remains tested for mtDNA haplogrouping, nine were able to be repeated and verified in this study over multiple (1-4 repeat extractions, see Table 1) extractions and sequencing events while meeting standard aDNA authentication criteria (Table 1). Due to the strict criteria for establishing genuine aDNA, two samples were unable to be used in data analysis, D1.10 and K2.1. Sequencing of D1.10 showed a consistent deamination pattern across

multiple extractions and sequencing attempts, but the SNP pattern showed some inconsistency in amplicons from the two separate extractions performed. However, when run on the Bioanalyzer, D1.10 showed the largest concentration of fragment sizes at greater than 1,000bp, again making it suspicious for a high degree of bacterial contamination. K2.1 showed large amounts of high fragment sizes (>10,000bp) on Bioanalyzer runs of all extractions performed for this specimen, as well as an absence of low molecular weight DNA (small fragments, <250bp). Sequencing attempts for K2.1 sample produced high numbers of chimeras composed of bacterial and human DNA, indicating high amounts of bacterial contamination.

Haplotyping information for all individuals in this study and the culture in which each individual belonged based on archaeological findings was used to characterize the Yamna, Catacomb and Eneolithic populations (Table 1).Of the three Catacomb individuals tested, our data included two individuals of the H clade and one belonging to U5. The three Yamna individuals haplotyped in this study all belonged to haplogroup U5. Haplogroup frequencies for the cultures tested with PCA were generated by combining relevant individual data into cultures from our study and comparing to mtDNA haplogroup frequencies from corresponding population groups of the same time period (Table 5). The Yamna (YAM) culture had high frequencies of haplogroups T and H, but, also contained individuals of the C clade, a group associated with cultures further east into Siberia and Asia. Catacomb (CAT) people had high frequencies of individuals belonging to the U and H clades, while the Eneolithic NPR people had highest frequencies of H among the three groups.

PCA and Population Relatedness

Axes 1 and 2 of the PCA explained a combined 31% of the total variance within the data. The Yamna (YAM) culture groups out with people belonging to the Trypillia culture, Corded Ware culture and other cultures belonging to farming as well as pastoralist populations in southwest and central Europe during the Eneolithic EBA (Nikitin et al., 2010, Brandt et al, 2013, Brotherton et al., 2013). The cultures located in this area of the PCA are characterized by haplogroups typically belonging to farming people during these time periods such as the H, T, N1a and J clades (Figure 3). Catacomb culture people (CAT) grouped together with hunter-gatherer type peoples from northern Russia and the Pitted Ware culture from Scandinavia. Cultures within this region of the PCA are characterized by the high frequencies of hunter-gatherer associated haplogroup clades such as U4 and U5 (Figure 3). The Eneolithic NPR population (ENE) groups out in-between the hunter-gatherers and the farming populations. Overall, the farming populations form two clusters in the top right quadrant of the PCA output. The top most cluster being formed by older central European farming cultures, and the bottom cluster consisting of younger central European farming populations as well as the Yamna culture from this study. Only three cultures fall out in the top left quadrant of the PCA, Neolithic Siberia (NSI), Bronze Age Siberia (SEBA), and the Alfold (ALF) populations. Populations here are characterized by East Eurasian haplogroups such as the D, A, G and C clades. At the bottom of the PCA chart, populations associated with hunter-gatherer type lifestyles and characterized by U clades group together.

F_{ST} with Modern European Populations

When comparing Yamna mtDNA haplogroup frequencies to that of modern European populations, we see non-significant *p*-values through pairwise comparison between Yamna and an isolated highlander population of Eastern Europe known as the Boyko (p=0.90090±0.0236) (Table 3). The Boyko are modern highlander population living in the Carpathian Mountains of Ukraine and Poland. Together with Hutsul and Lemko people living in the Carpathian region, the Boyko live in relative isolation from their lowland neighbors (Nikitin et al., 2009). Non-significant *p*-values are based on low F_{ST} values (closer to zero) meaning the populations are very similar. The remaining pairwise F_{ST} calculations between the Yamna and other modern European populations all had significant *p*-values (p<0.05) implying population differentiation given haplogroup frequencies. The Catacomb culture was significantly different from all other cultures in this analysis (*p*-values <0.05).

F_{ST} and Exact Test of Population Differentiation- Yamna and Catacomb

When directly comparing the mtDNA haplogroup frequency distribution in the Yamna and Catacomb populations, the F_{ST} value between them was 0.07882 implying little genetic substructure (panmixia, or the same population). At the same time, a significant *p*-value for this pairwise comparison was obtained (Table 3), implying genetic differentiation. To test the influence of low sample size on F_{ST} calculations, an exact test of population differentiation was run, correcting for the small sample size of the ancient population data through a Markov chain method (Raymond and Roussett, 1995). A significant *p*-value of 0.003 was obtained after 10,000 Markov chain permutations confirming that the Yamna and Catacomb populations are different in their mtDNA haplogroup composition (Table 4). The exact test also confirmed the lack of

differentiation between Yamna and Boyko, at the same time revealing a lack of genetic substructure between Yamna and Lemko, as well as the modern Ukrainian population (Table 4, respective *p*-values of 0.932, 0.243, and 0.051).

Discussion

Genetics of the Yamna and Catacomb Cultures

During the Neolithic and EBA important transitions were taking place in the people of the NPR. New people bringing the proto Indo-European language and new subsistence techologies were moving into the region (Mallory, 1997). Using maternal lineages and mtDNA haplogroup frequencies of cultures around the rest of Europe and Asia, we can begin to understand the population dynamics of the NPR during this critical time period for technological advancements in Europe. While maternal lineages only tell one side of the story, obtaining Y-chromosome or other autosomal marker information with aDNA is difficult due to preservation quality and its degraded state (Adler et al., 2011, Lamers et al., 2009). Using genetic information from other markers within the genome would increase the resolution for determining specific questions at the individual level, and increase marker numbers for resolution into mechanisms for cultural admixture during the Neolithic through EBA. For our study however, we focused on mtDNA and therefore our conclusions are only based on maternal lineage analysis.

Nine of the 11 total individuals in this study were able to be accurately characterized and haplotyped through mtDNA SNP markers (Wilde et al., 2014, Burger et al., 2007). Adhering to the strict authentication procedures in aDNA studies, the samples that were excluded for analyses (D1.10 and K2.1) were not able to be resolved accurately to a single haplotype through multiple extraction and amplification events. The addition in the use of the Bioanalyzer to this study also

helped resolve the authenticity of the samples allowing those which contained large amounts of high molecular weight DNA to be classified as at least mostly bacterial contaminant (fragment sizes >1,000bp) while also showing samples with small amounts of low molecular weight DNA.

Within the nine individuals of this study, high numbers of the U and H clades are not particularly surprising. Other research has shown that in central and southwestern European farming populations, especially during the EBA, begin to show high frequencies of the H clade and, in particular, haplogroups H1 and H3 (Brandt et al., 2013, Brotherton et al., 2013). At the same time, no H1 of H3 clade individuals have been unequivocally identified in southeastern European EBA specimens studied to date. The presence of the U clade haplogroups such as U4 and U5 are indicative of hunter-gatherer populations as typically seen in other studies as well, though, with the highest frequencies occurring before the EBA (Malmström et al., 2009, Brandt et al., 2013).

Comparing the Yamna in this study and Newton, 2011 (individuals from the NPR) to individuals from Wilde et al. (2014), we see differences in their haplogroup composition. The Yamna individuals haplotyped in this study all belonged to the U clade, which while present in the Wilde et al. data, is much less frequent than haplogroup H. Likely due to small sample size, the Yamna individuals haplotyped in this study also do not have individuals belonging to haplogroups X, T, W or J which are all present in the Wilde data. Haplogroups typically associated with farming in Anatolia such as N1a and I have been found in Yamna samples in the Wilde et al. dataset, possibly showing a link between the farming cultures in Anatolia and the influence of southeastern European haplogroups in central Europe (Wilde et al., 2014, Brandt et al., 2013). At the same time, in Newton, 2011 two individuals with east Eurasian specific haplogroup C were identified among representatives of the Yamna culture from the NPR. The C

lineage has not been found in EBA representatives studied elsewhere in southeast Europe (Wilde et al., 2014). Haplogroup C likely originated in south Siberia (Derenko et al., 2010) and the presence of this east Eurasian haplogroup in the NPR points at a genetic affinity of Yamna people with east Eurasian population groups. At the same time, the presence of haplogroup C in the Neolithic populations (Dnieper-Donets culture) of the NPR (Newton, 2011, Nikitin et al., 2012) could mean that the Yamna could have actually picked up the C's from the Neolithic NPR, rather than somehow directly acquiring these from the source of haplogroup origin. This would mean that Yamna may have local roots in the NPR.

Comparing the Catacomb people in our dataset to that of Wilde et al. we see similarities in the haplogroup distribution. In our sample, two Catacomb individuals belong to the H clade, and one belonging to the U clade (U5). Overall, the larger dataset in Wilde shows a higher frequency of hunter-gatherer U4 and U5 clades, while still containing H clade individuals.

Based on the grouping of the PCA output of mtDNA haplogroup frequencies, the Catacomb people seem to have a common origin with hunter-gatherer people from northern Europe and Russia (Malmström et al., 2009). Other research has already shown archaeological evidence in the similarity of burial practices found in the NPR to Scandinavian and northern European cultures (Nikitin et al., 2012). This study however, adds evidence of a genetic continuum between the hunter-gatherers of the north and the Catacomb people residing in the NPR. The high frequencies of the U clades, U4 and U5, isolate the Catacomb people from modern European cultures in the pairwise F_{ST} analysis. The lower frequencies of the U clade in modern European populations indicate that it is unlikely that the Catacomb and its nortern European hunter-gatherer counterparts were the main genetic contributors moving past the EBA and into modern human populations.

The Yamna people on the other hand, group together with mainly farming cultures from southwestern and central Europe (Nikitin et al., 2010, Brandt et al., 2013). Based on haplogroup frequencies it appears that the Yamna people were influenced by the advancement of European farmers into the NPR and admixing with the local population of the time. High prevalence of lineages associated with farming cultures, such as T and J, while also showing hunter-gatherer lineages such as U4 and U5 could be an admixture event in the NPR around 4,000 years ago as farming cultures and pastoralist type cultures met (Brotherton et al., 2013). While the F_{ST} value (0.07882) was low between the Yamna and Catacomb, humans overall are not highly genetically diverse, with some of the most diverse populations only having an F_{ST} value of ~0.2 between them (Nelis et al., 2009). Since human overall F_{ST} values are quite low between even the most genetically distinct populations, and our sample size for the Yamna and Catacomb populations was small, we ran an exact test of population differentiation to confirm the F_{ST} results. The significant *p*-value obtained from the exact text using the Markov chain method, correcting for small sample size, between the Yamna and Catacomb cultures from the EBA shows that these two cultures were unlikely to have admixed (Table 4).

Other research has suggested that the Catacomb people grew out of the Yamna culture and continued Yamna's burial practices and pastoralist way of living (Wilde et al., 2014). However, our data indicates the absence of demic introgression of Yamna into Catacomb, at least based on maternal genetic lineage marker analysis. While both cultures lived in the NPR during the same time period, it does not appear that they were genetically admixed to any great extent. A low F_{ST} value of 0.07882 between the Yamna and Catacomb cultures indicates genetically similar populations, however, significant *p*-values in both the F_{ST} and the exact test of population differentiation analyses show that these populations are genetically differentiated. Based on

burial type alone, there seems to be cultural exchange between these people as both used kurgan type burials with slight variation between them. Yamna using pit-graves dug straight into the kurgan mound and Catacomb people using pits with more of an L shape, giving a catacomb type burial in same kurgans erected by the Yamna people. Catacomb people seem to carry the same genetic signature during the Neolithic through EBA, while the Yamna pick up higher frequencies of farming maternal lineages such as T and J moving closer to the end of the EBA. In example, six of the 25 Yamna individuals haplotyped in Wilde et al. study belonged to haplogroup T, while Catacomb individuals in both this study and Wilde do not show any individuals belonging to T (Wilde et al., 2014). Overall, this could suggest that the Catacomb people retained their own distinct gene pool after being pushed to the outskirts of the steppe by farming type cultures, only taking in cultural and technological aspects from the Yamna instead of admixing with them genetically. The high frequencies of U4 and U5 in the Catacomb culture could also suggest that while they co-existed with the Yamna culture in the NPR, the Catacomb culture comprised of alleles from a different genetic pool than the Yamna. Statistical analyses presented in this report support this hypothesis. The PCA analysis presented in this study utilized mtDNA haplogroup frequencies from populations spanning a time period of thousands of years between the Mesolithic and EBA, and since the Catacomb people group together with hunter-gatherer cultures from five through ten thousand yBP (southern and northern European Mesolithic and Neolithic hunter-gatherers) we can assume based on haplogroup composition that their origins are similar. It is possible, since the Yamna origins exhibit different alleles than the Catacomb, and have higher frequencies of H, T and J haplogroups, they were associated with an influx of farming-associated gene lineages in the NPR.

While aDNA is typically difficult to work with, using it as a tool coupled with archaeology can help researchers further understand human population dynamics during the peopling of Europe and Asia. Since this study uses only maternal lineages to determine genetic relatedness, further research into other markers within the genome could show different population dynamics during the study period. Increasing the number of individuals studied from these important populations may also further resolve genetic affinities with other populations during this critical time period in the history of Europe.

Chapter 2 –Next Generation Sequencing

Sequencing Methodology

For archaeogenetic studies, the ultimate goal is to be able to sequence genuine aDNA and to confirm its authenticity for use in downstream population genetic analyses. Overall, there are three generations of sequencing technology that may be used to sequence the DNA in a particular sample, and each has their own specific application and methodology. Capillary sequencing using Sanger chemistry, is the oldest technology, next-generation sequencing was developed after Sanger in 2005, and the third generation of sequencing (PacBio) having been developed in 2009 (Sanger et al., 1977, Marguiles et al., 2005, Eid et al., 2009).

Sanger sequencing chemistry was developed in 1977 by Fred Sanger using DNA strand termination to sequence the molecule (Sanger et al., 1977). 3'-dideoxy nucleotide triphosphates (ddNTPs) are randomly incorporated into the growing DNA strand in place of standard deoxynucleotide triphosphates (Sanger et al., 1977). The addition of the ddNTPs terminates the newly synthesized DNA strand, theoretically creating DNA fragments of varying sizes and each ending at a specific and different base pair in the targeted sequenced region (Sanger et al., 1977). These different size fragments may in turn be separated through electrophoresis and visualized through dye staining on a gel or fluorescently tagged ddNTPs excited by a laser (Sanger et al., 1977, Lee et al., 1992). Each nucleotide in the sequence is then ordered by the size of the terminated fragments and the ddNTP that terminated the sequence is determined. Sanger sequencing chemistry using capillary electrophoresis produces one sequence read per reaction and averages around 700 base pairs per sequence.

Next-generation sequencing (NGS), first developed in 2005 through the Roche 454 pyrosequencing application, drastically changed the through-put of DNA sequencing technologies (Marguiles et al., 2005). Compared with the previous Sanger sequencing technologies, NGS increased the sequence read number per sample from one with Sanger to millions with NGS (Marguiles et al., 2005). Each run on a NGS machine typically contains hundreds of thousands of reactions simultaneously, with each of those hundreds of thousands of growing DNA strands visualized through the systematic addition of fluorescently labeled nucleotides (Marguiles et al., 2005). When a nucleotide is added to the growing DNA strand, a fluorescent tag is cleaved off the nucleotide, allowing the laser to pick up on the specific nucleotide that was added to each specific DNA strand. DNA sequence is determined by the systematic order in which each nucleotide is added to the reaction (Marguiles et al., 2005). The amount of sequencing reads and nucleotides sequenced using NGS technologies far surpasses Sanger methods allowing the sequencing of whole genomes within a single run on these machines (Marguiles et al., 2005).

In 2009 Pacific Biosciences developed a third generation of sequencing technology allowing the real-time visualization of polymerase kinetics as a sequence is being generated (Eid et al., 2009). This technology uses a SMRT bell adapter to anchor a single DNA molecule to a well in a micro-perforated chip with each well containing a DNA polymerase molecule (Eid et al., 2009). The SMRT bell adapter, added during the preparation of the sample for sequencing, creates a circular molecule using a bell shaped adapter on either side of the double stranded template DNA molecule. Circular consensus and sequence validation is obtained through the repeated circular replication of the DNA molecule in each of the micro-perforations (Eid et al., 2009) Polymerase kinetic information during the real-time sequencing of each molecule can

show polymerase stops and stalls while it replicates the DNA molecule, leading to information about methylation, DNA strand damage and secondary DNA structure (Eid et al., 2009). While the resolution at each of the sequencing reads is increased through the addition of polymerase kinetic information, the PacBio sequencer has very specific applications. The only limitation to sequence read length on the PacBio machine is polymerase exhaustion, and due to that, read lengths can be upwards of 15kB long far exceeding any other sequencing technology (Eid et al., 2009). However, the longer the sequenced read, the less number of times circular consensus can be achieved, lowering the quality score of each base pair in the sequence read.

Building a Method- NGS for aDNA Authentication

Taking aDNA authentication criteria into account, methodology and applications of NGS technologies could drastically increase the ability to determine genuine aDNA apart from its inevitable modern contamination in a sample. With the innate properties of endogenous aDNA damage and degradation, studying humans for aDNA studies has a unique problem; the researcher provides another possible source of contamination. While Sanger chemistries give one read of a targeted DNA sequence, NGS can sequence a much larger number of DNA strands in a sample. If a sample contained DNA sequence from multiple different species, as is common in aDNA contamination, NGS would be able to sequence all of those molecules. In turn, NGS technologies could have significantly increased resolution for studying aDNA and allow damage sites and contamination rates to be characterized for all DNA within a sample.

NGS offers a large increase in the amount of data on a particular DNA sample in a much shorter amount of time. Compared with a single read per run Sanger sequencing, NGS offers sequencing reads in the range of hundreds of thousands to hundreds of millions from one run

(Marguiles et al., 2005, Eid et al., 2009). Concentrations of genuine aDNA in an extract are likely to be small after thousands of years of degradation (Adler et al., 2011). However, since NGS technologies offer sequencing reads from loci across the genome, it is likely to sequence all of the aDNA in a sample as well as sequencing all of the contaminant molecules. When aligning NGS data to a reference genome, the source of the contamination in a particular aDNA extract becomes apparent. The determination of contamination ratios (aDNA:other), where sources of contamination are, and what DNA a sample is contaminated with (bacteria, plant, modern human, etc.) can all be pinpointed using NGS technologies.

Being able to view the rate of nucleotide damage (deamination and oxidation) is imperative in distinguishing aDNA from modern contaminant (Skoglund et al., 2014). Paired-end NGS applications offer the resolution to determine mutation variation from nucleotide damage without the need of the time consuming process of cloning and Sanger sequencing one clone at a time. Paired-end sequencing, while reducing overall genome coverage depth, increases the resolution of a sequencing run to both strands of DNA. Determining the mismatching in nucleotide pairing (T matched with G in the case of deamination) between paired-end reads from the same locus gives an accurate characterization of the damage rates across the sequenced portion of the genome (Skoglund et al., 2014). Recent research has used paired-end sequencing of ancient humans and a likelihood statistical model to use deamination rates and paired-end mismatches to pull genuine aDNA sequencing reads out of a pool of modern contaminants (Skoglund et al., 2014).

PacBio sequencing technology, the most recent incarnation of NGS, could allow the visualization of deamination and fragment size as they exist in un-amplified original DNA template, while increasing resolution of damage sites to oxidative damage, or possibly damage

types not yet identified in aDNA (Eid et al., 2009). Increasing resolution to entire single molecules of DNA in real time will allow studies of ancient genomes to have a more specific set of authenticity criteria to tell genuine aDNA apart from modern contamination. The PacBio sequencer uses polymerase kinetics to determine nucleotide modification in the template strand (ex: methylation) and could also show the cytosine to uracil switch in a deamination reaction (Eid et al., 2009, Fang et al., 2012). The circular consensus sequencing method for validation innately built into PacBio sequencing technologies could also show direct mismatches in nucleotides in real time on an unmodified (no library amplification) aDNA template molecule. However, the current state of the PacBio technology has a difficult time dealing with nucleotide damage sites as the DNA polymerase stalls or stops completely during replication (Eid et al., 2009, Fang et al., 2012, personal communication, Bob Lyons).

Duplex consensus sequencing (DCS), a method for detecting rare mutations and distinguishing them from PCR errors was developed for use in the medical field for cancer research and tumor sequencing (Schmitt et al., 2012). DCS uses a modified method of paired-end sequencing to determine the original template molecule from which an amplified cluster of paired-end reads originated (Schmitt et al., 2012). Through tags consisting of 12 random nucleotides and 5 static nucleotides as barcodes, called αβ tags, clusters of paired-end reads are grouped with their initial template molecule in downstream bioinformatics analyses (Schmitt et al., 2012). This allows determination of PCR error apart from natural sequence variation due to mutation in a particular sequenced sample (Schmitt et al., 2014). For aDNA, this could be used to distinguish deamination sites in the original template molecule and allow those deamination modifications to be validated against PCR error incorporated during NGS library preparation and amplification.

Using a combination of the different NGS technologies available today could allow the visualization of deamination, fragment size and contamination rates as they exist in an aDNA sample. Increasing resolution and understanding of endogenous aDNA in an ancient sample could significantly impact authentication criteria in determination of genuine aDNA apart from modern contamination. In a field where authentication of genuine aDNA is of utmost importance, NGS technology may offer a quality of information this field does not yet utilize (Paijmans et al., 2012).

Study Objectives

The second part of this study focused on the development of NGS technologies for use with aDNA authenticity. Using NGS technologies and modified methods for NGS sequencing runs, we sought to further understand aDNA damage patterns, contamination rates and types, and preservation status. From this information we planned on using NGS sequence data obtained from human aDNA samples in hopes to develop a method for more accurately identifying genuine aDNA and improving authentication criteria. The ultimate goal of this portion of the study was to quantify the extent and types of damage on a per molecule basis, linking that damage to a specific preservation and burial type which has not yet been done in aDNA research.

Methods- NGS Technology

All samples for analysis with NGS technologies were extracted using the same procedures as outlined above (p.26-27). Bones were processed and ground using a mortar and pestle in a UV sterilized laminar flow hood. DNA was extracted using a QIAGEN QiaAmp Investigator kit (Qiagen) and eluted in 20ul 18MΩ deionized H_2O. NGS technology requires

strict DNA concentrations for the preparation of the sequencing library. To determine which of our aDNA specimens were most appropriate for NGS analysis, the following criteria were used.

1) The sample must have detailed archaeological information such as burial environment.
2) The sample had to contain an appreciable concentration of DNA. NGS targets 1µg of DNA in an extract for library preparation and sequencing. Since aDNA is highly degraded and in small concentration, choosing an aDNA sample with the highest concentration of DNA was required.
3) The sample had to show the above concentration of DNA mostly in the low molecular weight region, increasing the chances for that DNA to be genuine ancient DNA existing in fragment sizes less than 250bp (Adler et al., 2011).

To determine the concentration of DNA in our samples, we used an Agilent Bioanalyzer running a high-sensitivity assay due to the known small concentrations of aDNA. This instrument allowed the visualization of the distribution of all DNA within the sample, specifically showing the concentration of DNA at each of the fragment sizes. Following the Bioanalyzer analysis of possible NGS samples, L8 was chosen due to the high concentration (1.6ng/µl, 40ng total DNA) of low molecular weight (small fragment size, median=311bp) DNA (Figure 4) as well as detailed archaeological information including the skull being covered with a red pigment. While this sample still had a relatively small concentration of DNA, out of all bones tested using the Bioanalyzer L8 had the highest concentrations of low molecular weight DNA. Since aDNA is very low in concentration and DNA damage and fragmentation is prevalent, a test run using an artificially fragmented PhiX DNA to simulate aDNA was sequenced. Running an artificially generated sample to mimic aDNA allowed us to test the DCS adapter tagging, low concentration, and innate small DNA fragments that are typical for aDNA.

Following sample selection, the entirety of the L8 extract was sent to the University of Michigan sequencing core for library preparation and sequencing on the Illumina HiSeq 2500 platform (Illumina). Library preparation for this sample was completed using Illumina TruSeq adapter sequences as well as αβ tags from the DCS method following the Illumina TruSeq DNA protocol (Illumina). The addition of αβ DCS tags were constructed into the library to allow increased resolution in determining the original aDNA template molecule from which each sequencing read came from. In turn, this method could allow us to specifically determine deamination sites, SNP variation, and PCR errors from library amplification of this sample. The Illumina HiSeq 2500 platform was chosen for this process due to the still small DNA concentration in the L8 sample, as well as, for issues PacBio sequencers have when dealing with highly damaged DNA molecules (personal communication, Bob Lyons).

After sequencing, raw NGS data was cleaned and then analyzed using bioinformatics software. First, to clean raw sequence data, Illumina TruSeq adapter sequences were removed from each of the sequencing reads and high quality sequencing reads were determined using the web-based software FastQC (Bahbraham Bioinformatics). NGS raw sequence files include quality based scores coupled with each base pair in a given sequence, much like Sanger based methods. In this analysis, any sequence read with an average quality score of less than 30 was removed from downstream analysis. Once sequence reads were cleaned, reads were separated into two files, one file for single-end sequencing reads (not paired with opposite strand) and one for paired-end sequencing reads (both strands paired back together). Single-end and paired-end cleaned sequencing data files were constructed using the SAMtools v1.19 software package (Li et al., 2009). Finally, both single-end and paired-end sequencing read files were aligned to the human hg19 reference genome using the BWA v0.7.9 software package (Li and Durbin, 2009)

and visualized using the Integrative Genomics Viewer (IGV) v2.3 **(**Thorvaldsdóttir et al.,2012). For alignment purposes, it was expected that genuine aDNA would have a higher number of variable sites due to deamination. To be able align reads to the reference genome, up to 5 nucleotide differences were allowed for each sequencing read.

Results

Next Generation Sequencing

Once completed, the NGS run on the Illumina HiSeq 2500 for the L8 specimen had 198,000,000 sequencing reads. Once trimmed of Illumina TruSeq adapters as well as the created DCS 12-nucleotide tags, the number of reads drops to 7 million containing a an insert of DNA above 15bp (single-end reads). A total read length cutoff of 15bp for each sequence was used to avoid random alignment of small DNA fragments to the reference genome in downstream genome alignments. Overall the 7 million single-end reads had a tri-modal distribution of fragment sizes with peaks at 15-25bp, 35-40bp, and finally at 90-100bp. Pairing those 7 million reads back to their partner (αβ tag from DCS) the number of reads drops to around 2.5 million paired end reads. Out of the original 7 million single end reads 40% mapped to the hg19 reference genome and all of the mapped reads were the 15-25 nucleotide insert reads.

Once fragments were mapped to the hg19 reference genome further checks were run to determine if the mapped fragments were human specific. The 7 million single end reads were also mapped to the mouse reference genome (*Mus musculus*). We chose to use another mammal species to see if conserved areas of the genome were being sequenced and mapped to the same regions in both humans and mice. We found that the same reads that mapped to humans, also mapped to mice and again were only the 15-25bp insert reads. However, while these reads

mapped to both mouse and human, they did not map to conserved areas across the two genomes. To examine the sequencing reads with 90-100bp inserts, a BLAST search was run on all 7 million sequencing reads using an in-house constructed program at the University of Michigan. Sequence reads for *Actinoplanes* bacteria genome had BLAST hits from within our data. Alignments for the 7 million single end sequence reads to the *Actinoplanes* genome (Accession Number: NC_021191) produced 28,488 matches to the reference genome (Figure 5).

Discussion

Next-Generation Sequencing Methodology- Improving aDNA Authentication Protocols

Development of NGS technologies for use of aDNA authentication in this study was unable to address the original question of aDNA damage characterization or contamination rates. While this aspect of the study was unable to answer our questions, improving this method could allow for increased ability to study aDNA in future studies using NGS technologies.

Concentrations of DNA become increasingly important for sequencing using NGS machines. Typically, when the library is created for sequencing around 1µg of total DNA is required. Due to the highly degraded state of aDNA it becomes difficult for one sample and one extraction to obtain concentrations high enough for this step of the protocol. In attempts to increase the concentration of DNA in the extractions used in this study, many of the bones were re-extracted using the protocol outlined in the methods above and multiple extractions of the same bone were combined to increase DNA amounts. However, when these samples were analyzed using the BioAnalyzer there was a much larger concentration of high molecular weight DNA, indicating a higher presence of bacterial contamination (Figure 4). While L8 was chosen as our best sample, it only contained ~100ng of total DNA, much lower than the suggested 1µg

suggested for DNA sequencing on NGS machines. Research into the extraction method for ancient specimens has shown that premade kits, such as the one used in this study, use lysis buffers which may be too harsh for the already compromised cells and degraded DNA (Rohland and Hofreiter, 2007). Using this silica-based extraction method may improve the starting material and allow higher concentrations of genuine aDNA in an extraction (Rohland and Hofreiter, 2007).

Paired-end sequencing and DCS provides an increased depth of coverage at each sequenced locus in a NGS run. However, while providing increased depth and resolution, the use of paired-end sequencing and DCS reduces overall genome coverage. The adapters and tags added to each of the template DNA strands in a paired-end DCS library preparation significantly reduces the number of base pairs between the adapters that are sample sequences. Due to the smaller fragment size of the sample being sequenced, less of the overall genome retains the coverage of sequencing reads. On top of that, due to the small fragment inserts between the adapter sequences, sequencing into the adapter sequence commonly occurs in paired-end sequencing runs. When cleaned of adapter sequences in this studies data, the number of sequencing reads dropped from 198 million down to 7 million. The remaining sequence between the two flanking adapter sequences in most cases was too small to accurately align to any genome (below 15bp), significantly reducing the quality and coverage across the alignment to the reference genome. In further studies, using a single-end sequencing run and using statistical methods to parse out contamination and identify deamination could lead to better genome coverage (Skoglund et al., 2014).

Modification to PacBio methodology could give increased information about aDNA template molecules in real-time. Watching the DNA polymerase as it replicates the aDNA

template molecule could shed light on nucleotide damage sites not previously seen in aDNA research. Currently, the polymerase stalls too frequently along highly damaged template molecules during sequencing runs on the PacBio machine. In most studies this has been rectified by using DNA repair enzymes such as using uracil-DNA glycosylase (UDG) repairing uracil nucleotides in the template sequence (personal communication, Bob Lyons). However, this defeats the purpose for aDNA applications as the presence of deaminated cytosine nucleotides into uracil is a hallmark indication of genuine aDNA template (Skoglund et al., 2014).

While method development in this study was unable to address aDNA damage and contamination rates, further research and method changes could provide an increased resolution for distinguishing aDNA authenticity. Changes to the way DNA was extracted, library preparation, sequencing run type, and possibly NGS platform type could have the impact needed to improve this method.

Conclusions

In this study nine of eleven ancient individuals belonging to pastoralist cultures from the North Pontic steppe region of southeast Europe during the late Neolithic and EBA were accurately haplotyped using mtDNA. Relatedness analyses in the form of PCA of mtDNA haplogroup frequencies of ancient cultures through time, F_{ST} with modern European populations and an exact test of population differentiation showed the genetic affinities of the Yamna and Catacomb cultures studied in this thesis. Haplogroup information based on the HV1 and coding sequence of the human mtDNA genome revealed that the Yamna and the Catacomb people living in the NPR during the EBA appear to have not been genetically admixed. The Yamna people have shared east Eurasian maternal lineages with the Dnieper-Donets culture, who had previously occupied the NPR during the Neolithic time period, likely indicating deep local roots of the Yamna population in the NPR. After pulling our mtDNA data together with data from literature, it became apparent that the Catacomb population, while also living in the NPR at the same time as the Yamna (late Neolithic through EBA), do not share the same mtDNA haplogroup frequencies as the Yamna. While the representatives of the Catacomb culture featured predominantly mtDNA lineages of the H and U haplogroup, of which U is characteristic of pre-farming hunter-gatherer populations of Europe, the Yamna representatives displayed a greater variety of mtDNA lineages characteristic to the Anatolian demic influx typically associated with the advancement of farming technologies. Unlike the Catacomb, the Yamna culture was genetically similar to the modern day Ukrainian population, as well as to two populations of Carpathian highlanders, Boyko and Lemko, showing that the gene pool of ancient farming type cultures more significantly influenced the modern European population structure than ancient hunter-gatherer populations. Catacomb people, on the other hand, seem to have been

more influenced by hunter-gatherer mtDNA lineages and their genes are not as significantly represented in modern European populations.

In this thesis method development for the authentication of aDNA using NGS technologies was also attempted. While this method produced data unable to answer the original question of aDNA damage characterization during this study, important information was gathered for the furthering of this method in future aDNA studies using NGS technologies. Changes to the way aDNA is extracted as well as downstream sequencing methodology may increase the ability for these new technologies to help understand ancient material.

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Figure 1: A network representation of human mtDNA haplogroups from Newton, 2011. This diagram shows the mutations separating the major clades of the human mtDNA haplogroups as compared with the rCRS human reference mtDNA genome. Mutations, in red, starting with 16,000 are from the HV1 region of the mtDNA. Mutations marked with restriction enzymes denote coding region mutations typically distinguished using restriction cut sites at that locus.

Figure 2: A modified map from Brandt et al., 2013 Supplementary Information. In this figure, the movement of the H haplogroup clades from southwest Europe and the movement of U clades are shown. The North Pontic Steppe Region, the location of this study, is circled in blue.

Table 1: All specimens tested for mitochondrial DNA haplotype in the North Pontic steppe region of Ukraine in this study. Superscript numbers next to sample names designate the number of repeat extractions from the same bone. Subscript β indicates samples checked on the Bioanalyzer for further aDNA quantification. Coding region mutations based on RFLP checks are in blue where information is available. Individuals are broken down into their corresponding culture based on archaeological findings (Yamna, Catacomb or Eneolithic). Samples in red were not able to be resolved over multiple replications and therefore not used in further analysis.

Table 2: Primer information for all primer sets used in mitochondrial DNA haplotyping of people from the North Pontic steppe region in this study. Lower PCR product length primers (<84) were used to check for amplified fragment lengths and ratios to find endogenous aDNA template molecules. Primer sets H7025 and H12308 were used as mtDNA coding region checks for haplogroups H and U respectively.

Table 3: Pairwise F_{ST} values for between modern European populations and the Yamna and Catacomb ancient populations. F_{ST} values are listed below the diagonal. All F_{ST} calculations were run using haplogroup frequencies for the cultures listed. Cells shaded green have a non-significant *p*-value (>0.05) meaning those population pairs are statistically similar. Cells above the diagonal are *p*-values reported for the $F_{ST.}$

Table 4: Exact test of population differentiation between the Yamna, Catacomb, and modern European populations. Significant *p*-values (*p*-value <0.05), in white cells, indicate genetic differentiation between population pairs. Green shaded cells indicate non-significant *p*-values (>0.05) and show populations that are genetically similar or are panmictic.

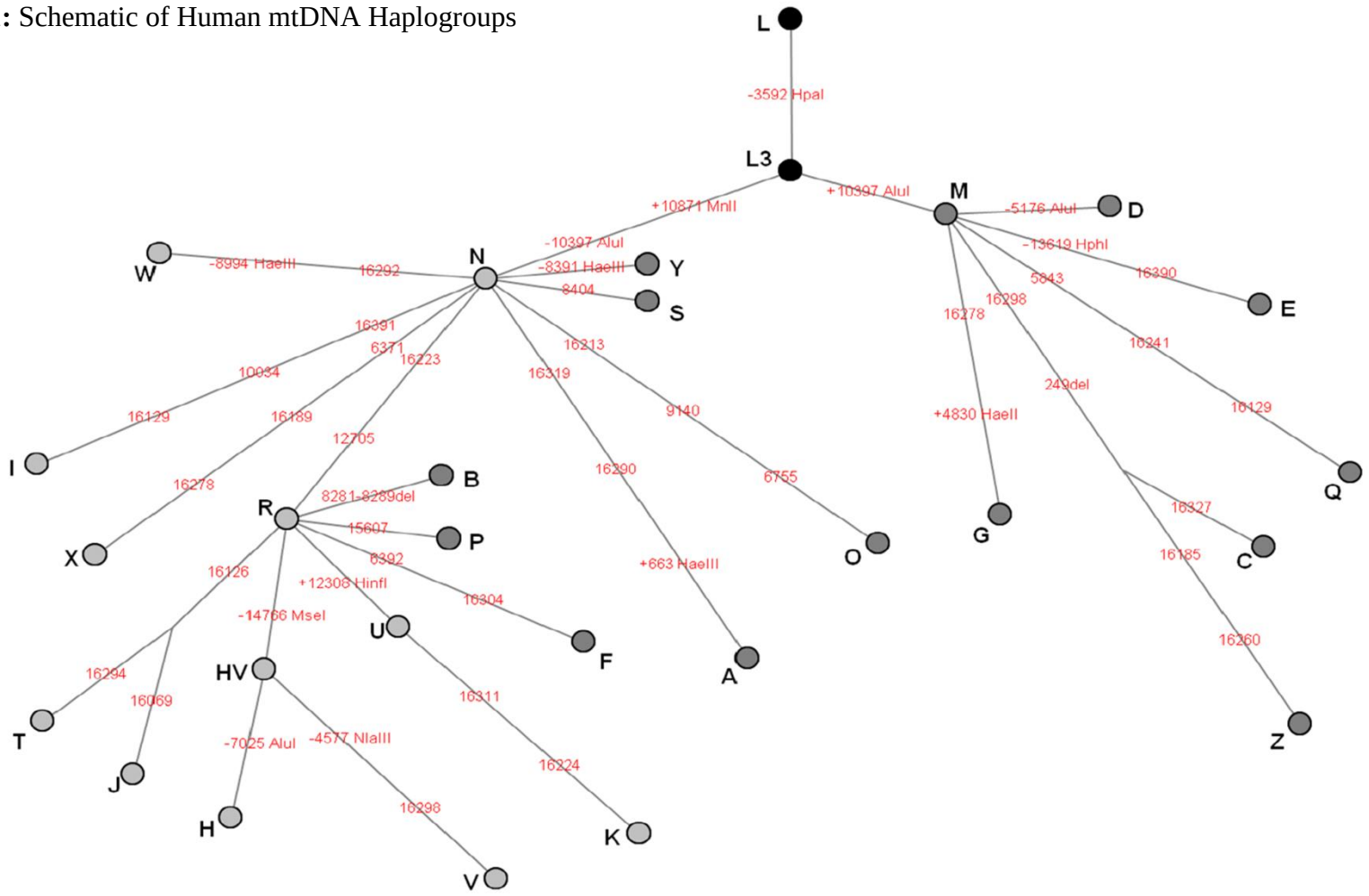
Figure 3: Principal Component Analysis of haplogroup frequencies of various cultures of different geographic regions during the Mesolithic through the Early Bronze Age. The Yamna (YAM) and Catacomb (CAT) cultures are abbreviated in the figure itself. More information about the specific cultures is presented and the studies from which the corresponding data originate is shown in table 5. Polygons describing the clustering of the cultures focused on in this study are labeled in blue.

Figure 4: An example Bioanalyzer output for the L8 sample. DNA concentrations are shown at the bottom of the output. For this sample, a concentration of 1,594 picograms per microliter was obtained. The total distribution and concentration of the varying DNA fragment sizes are shown on the graphical output.

Figure 5: BWA and IGV genome alignment of the 7 million single-end sequencing reads generated in the sequencing of the L8 specimen in this study. The top picture shows an area where sequences are giving overlapping consensus coverage for a particular region of the *Actinoplanes* reference genome. The bottom picture shows the BWA output of the number of sequences aligned to the *Actinoplanes* reference genome. Colored dashes in the IGV alignment show variants between the mapped reads and the reference genome.

Table 5: Culture haplogroup frequency data represented in the PCA analysis of Figure 1. Cultures are identified, and the abbreviations used in the PCA graph are given. Since data from the literature was combined for this analysis, the source of each cultures mtDNA haplogroup data is also listed in this table.

Figure 1: Schematic of Human mtDNA Haplogroups



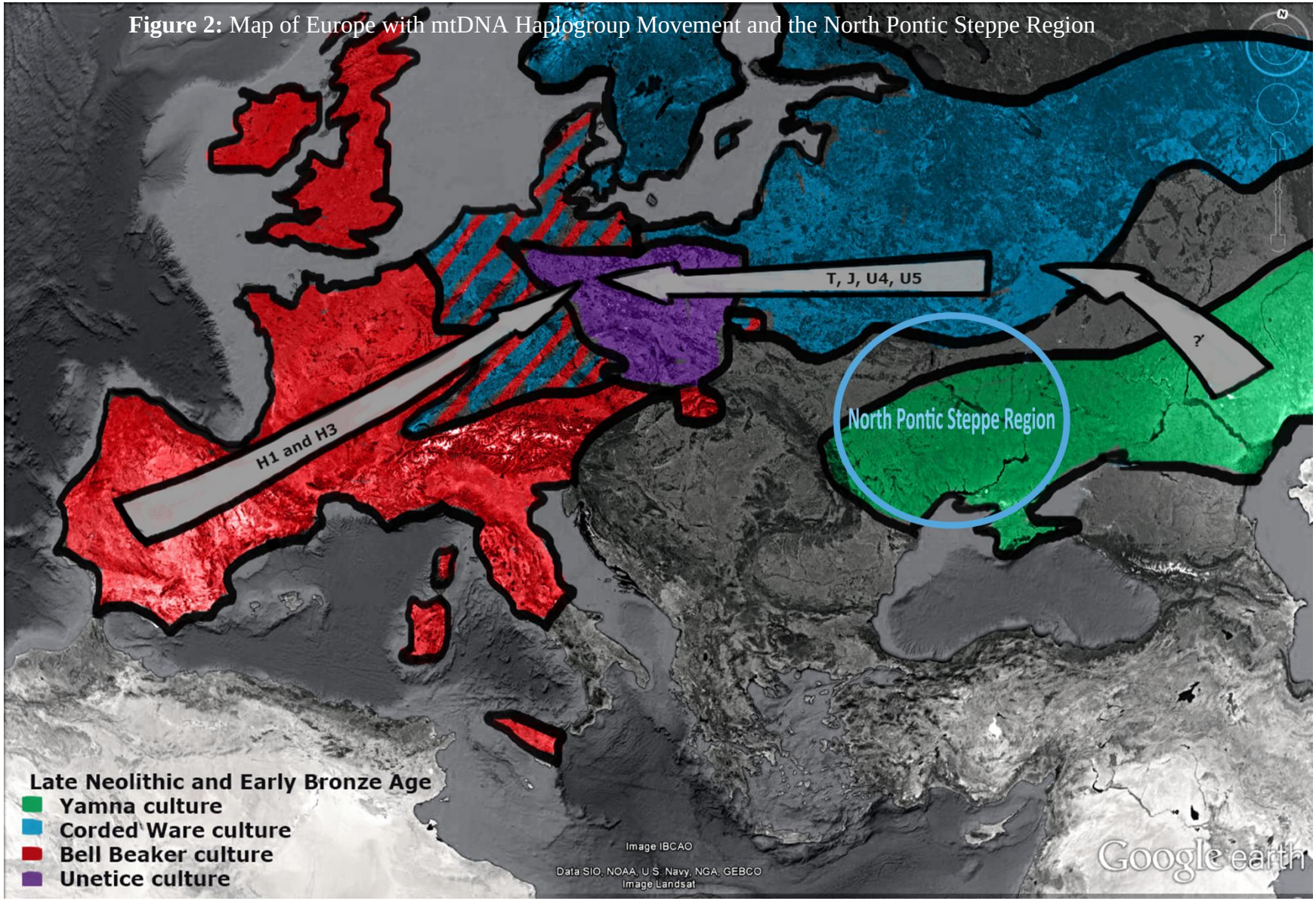


Figure 2: Map of Europe with mtDNA Haplogroup Movement and the North Pontic Steppe Region

Table 1: Specimen data for individuals in this study

Specimen ID	Culture	Age (BP)	Coding and HV1 SNPs	Halogroup
R3.13[3]	Catacomb (Ingul)	3,940±60	16270T	**U5**
D1.12[2]	Catacomb	3,900±80	rCRS	**H**
D1.11[2]	Catacomb (Ingul)	3,720±70	rCRS, 7028C	**H**
R3.19[3]	Eneolithic (main burial)	5,450±80	16356C, 12308G	**U4**
K1.10[2]	Eneolithic (main burial)	4,950±70	rCRS	**H**
L11[2]	KMK	3,230±70	16067T, 16192T	**HV1a2**
L19[1]	Yamna	4,030±60	16241G, 16270T, 12308G	**U5**
R3.7[2]	Yamna	3,910±60	16192T, 16256T, 16270T	**U5a**
R3.16[2]	Yamna	4,135±60	16174C-A, 16311C, 12308G	**U (U5b1c?)**
D1.10[2]	Catacomb	N/A	16179T, 16224C, 16265G, (16270T?), 16295Ains, 16311C	**K / Contamination**
K2.1[4]	Eneolithic (main burial)	4,270±90	Inconsistent pattern	**Not Determined**
Lab personnel 1			16134C-T, 16356T-C	**U4a1**
Lab personnel 2			16134C(ins), 16224T-C, 16311T-C	**K**
Lab personnel 3			16007A-G, 16134C-T, 16234C-T, 16356 T-C	**U4a1c**
Lab personnel 4			16126T-C, 16163A-G, 16186C-T, 16189T-C, 294C-T	**T1**
Lab personnel 5			16068T-C, 16126T-C, 16235A-G, 16265A-C	**R***
Lab personnel 6			16304T-C	**H5a**
Archeologist 1			rCRS	**H**

Table 2: Primers used for mtDNA Haplotyping

Primer Name	Primer Sequence	Product length	Region of Coverage (mtDNA)	Primer Source
HV1-1F	CAAGCAAGTACAGCAATCAACC	64	16201-16264	This Study
HV1-1R	GAGGGGTGGCTTTGGAGT			This Study
HV1-2F	CACATCAACTGCAACTCCAAA	63	16234-16296	This Study
HV1-2R	GGGTGGGTAGGTTTGTTGGT			This Study
HV1-3F	CCCTCACCCACTAGGATACC	73	19260-16333	This Study
HV1-3R	TGTACGGTAAATGGCTTTATG			This Study
HV1-4F	CAAACCTACCCACCCTTAACA	84	16282-16365	This Study
HV1-4R	GGGACGAGAAGGGATTTGAC			This Study
L15993	ACTCCACCATTAGCACCCAA	142	15994–16092	Nikitin et al., 2012 and Newton, 2011
H16093	GGTGGCTGGCAGTAATGTACGAA			Nikitin et al., 2012 and Newton, 2011
L16085	TGACTCACCCATCAACAACCGC	145	16086–16188	Nikitin et al., 2012 and Newton, 2011
H16189	CTTGCTTGTAAGCATGGGGA			Nikitin et al., 2012 and Newton, 2011
L16163	ACTTGACCACCTGTAGTACATAA	161	16164–16277	Nikitin et al., 2012 and Newton, 2011
L16265	GTTAAGGGTGGGTAGGTTTGTTGG			Nikitin et al., 2012 and Newton, 2011
H16278	GCAACTCCAAAGCCACCCCTCA	164	16266–16385	Nikitin et al., 2012 and Newton, 2011
H16386	GATGGTGGTCAAGGGACCCCTA			Nikitin et al., 2012 and Newton, 2011
7025H-F	CCGTAGGTGGCCTGACTGGC	123	6950–7051	Santos et al., 2004
7025H-R	TGATGGCAAATACAGCTCCT			Santos et al., 2004
12308U-F	CACAAGAACTGCTAACTCATGC	123	12217–12308	Santos et al., 2004
12308U-R	ATTACTTTTATTTGGAGTTGCACCAAGATT			Santos et al., 2004

Table 3: Pairwise F_{ST} with Yamna and Catacomb against modern European human populations.

	Boyko	Hutsul	Lemko	Hungary	Poland	Romania	Belorussia	Croatia(Mainland)	Czech	Russia	Ukraine	Yamna (Ancient)	Catacomb (Ancient)
Boyko		0.01802 +-0.0121	0.20721 +-0.0451	0.00901+ -0.0091	0.01802 +-0.0121	0.09910+ -0.0252	0.03604+- 0.0148	0.00901+- 0.0091	0.02703+- 0.0139	0.13514+- 0.0365	0.08108+- 0.0286	0.90090+- 0.0236	0.00000+- 0.0000
Hutsul	0.0758		0.05405 +-0.0201	0.59459+ -0.0364	0.34234 +-0.0354	0.54955+ -0.0417	0.17117+- 0.0316	0.81081+- 0.0304	0.62162+- 0.0438	0.18919+- 0.0344	0.56757+- 0.0651	0.00901+- 0.0091	0.00000+- 0.0000
Lemko	0.00921	0.01664		0.00901+ -0.0091	0.00901 +-0.0091	0.22523+ -0.0244	0.04505+- 0.0203	0.00901+- 0.0091	0.13514+- 0.0412	0.05405+- 0.0201	0.17117+- 0.0286	0.03604+- 0.0148	0.00000+- 0.0000
Hungary	0.08505	-0.0063	0.0336		0.12613 +-0.0278	0.13514+ -0.0339	0.12613+- 0.0278	0.29730+- 0.0490	0.09910+- 0.0252	0.07207+- 0.0297	0.26126+- 0.0394	0.00000+- 0.0000	0.00000+- 0.0000
Poland	0.04337	0.00039	0.01622	0.00394		0.09009+ -0.0235	0.13514+- 0.0244	0.09910+- 0.0212	0.55856+- 0.0425	0.65766+- 0.0385	0.67568+- 0.0668	0.00000+- 0.0000	0.00000+- 0.0000
Romania	0.03294	-0.0025	0.0028	0.00783	0.00271		0.00000+- 0.0000	0.19820+- 0.0379	0.57658+- 0.0609	0.25225+- 0.0402	0.48649+- 0.0364	0.00000+- 0.0000	0.00000+- 0.0000
Belorussia	0.04079	0.01196	0.02787	0.00839	0.00493	0.01589		0.07207+- 0.0227	0.10811+- 0.0227	0.27928+- 0.0438	0.45045+- 0.0407	0.03604+- 0.0148	0.00000+- 0.0000
Croatia(Mainland)	0.06247	-0.0086	0.02434	0.00145	0.00356	0.0042	0.01179		0.26126+- 0.0566	0.02703+- 0.0139	0.76577+- 0.0455	0.00000+- 0.0000	0.00000+- 0.0000
Czech	0.04085	-0.0047	0.00893	0.00539	-0.0015	-0.0024	0.01	0.00114		0.48649+- 0.0474	0.50450+- 0.0546	0.00000+- 0.0000	0.00000+- 0.0000
Russia	0.02968	0.00561	0.0113	0.00732	-0.0012	0.0013	0.0009	0.00672	-0.0004		0.64865+- 0.0446	0.00000+- 0.0000	0.00000+- 0.0000
Ukraine	0.03276	-0.0057	0.01691	0.00405	-0.0039	-0.0006	-0.0009	-0.00639	-0.0039	-0.0025		0.02703+- 0.0139	0.00000+- 0.0000
Yamna(Ancient)	-0.0224	0.08078	0.02349	0.09219	0.05685	0.05068	0.03639	0.07202	0.05651	0.04203	0.03899		0.00000+- 0.0000
Catacomb (Ancient)	0.13197	0.14082	0.13838	0.13538	0.13114	0.14026	0.07997	0.12288	0.13778	0.11885	0.10811	0.07882	

Table 4: Exact Test of Population Differentiation for Yamna and Catacomb against modern European human populations.

	Boyko	Hutsul	Lemko	Hungary	Poland	Romania	Belorussia	Croatia(Mainland)	Czech	Russia	Ukraine	Yamna (Ancient)	Catacomb (Ancient)
Boyko													
Hutsul	0.01034+-0.0021												
Lemko	0.31218+-0.0086	0.26804+-0.0101											
Hungary	0.00019+-0.0002	0.06234+-0.0059	0.00016+-0.0002										
Poland	0.00755+-0.0019	0.06202+-0.0047	0.00310+-0.0007	0.05411+-0.0048									
Romania	0.10425+-0.0075	0.51781+-0.0121	0.07727+-0.0063	0.01171+-0.0012	0.00461+-0.0014								
Belorussia	0.02308+-0.0022	0.06305+-0.0056	0.00407+-0.0010	0.06405+-0.0086	0.38251+-0.0143	0.00991+-0.0019							
Croatia(Mainland)	0.00237+-0.0007	0.46556+-0.0077	0.00002+-0.0000	0.00222+-0.0007	0.00000+-0.0000	0.00689+-0.0019	0.00000+-0.0000						
Czech	0.00881+-0.0018	0.50363+-0.0129	0.05498+-0.0032	0.00658+-0.0018	0.36067+-0.0090	0.23781+-0.0153	0.11766+-0.0086	0.00343+-0.0007					
Russia	0.01776+-0.0029	0.04225+-0.0057	0.00558+-0.0015	0.02436+-0.0032	0.54953+-0.0139	0.06819+-0.0077	0.57428+-0.0115	0.00000+-0.0000	0.20376+-0.0154				
Ukraine	0.08986+-0.0080	0.28263+-0.0100	0.02802+-0.0030	0.02189+-0.0018	0.11401+-0.0084	0.46261+-0.0092	0.09001+-0.0050	0.90393+-0.0047	0.20180+-0.0081	0.22343+-0.0139			
Yamna(Ancient)	0.93196+-0.0028	0.00615+-0.0009	0.24317+-0.0062	0.00000+-0.0000	0.00673+-0.0022	0.00806+-0.0020	0.02977+-0.0031	0.00013+-0.0001	0.00674+-0.0018	0.00606+-0.0014	0.05061+-0.0032		
Catacomb (Ancient)	0.00198+-0.0005	0.00575+-0.0016	0.00024+-0.0002	0.00026+-0.0002	0.00000+-0.0000	0.00003+-0.0000	0.00811+-0.0015	0.00146+-0.0008	0.00000+-0.0000	0.00026+-0.0001	0.00326+-0.0011	0.00364+-0.0012	

Sources of Population Data for F_{ST} and Exact Test of Population Differentiation:

Boyko: Nikitin, A et al.. (2009).
Hutsul: Nikitin, A et al.. (2009).
Lemko: Nikitin, A et al.. (2009).
Hungary:Semino et al (2000).
Poland:Malyarchuk et al. (2002)
Romania:Bosch et al. (2005)
Belorussia:Belyaeva et al. (2003)
Croatia (Mainland):Pericic et al. (2005)
Czech:Malyarchuk et al. (2006)
Russia:Malyarchuck and Derenko (2001)
Ukraine: Malyarchuck and Derenko (2001)
Yamna (Ancient): This study, Wilde et al., 2014 and Newton, 2011.
Catacomb (Ancient): This study, Wilde et al., 2014.

Figure 3: PCA of Ancient Culture Haplogroup Frequencies

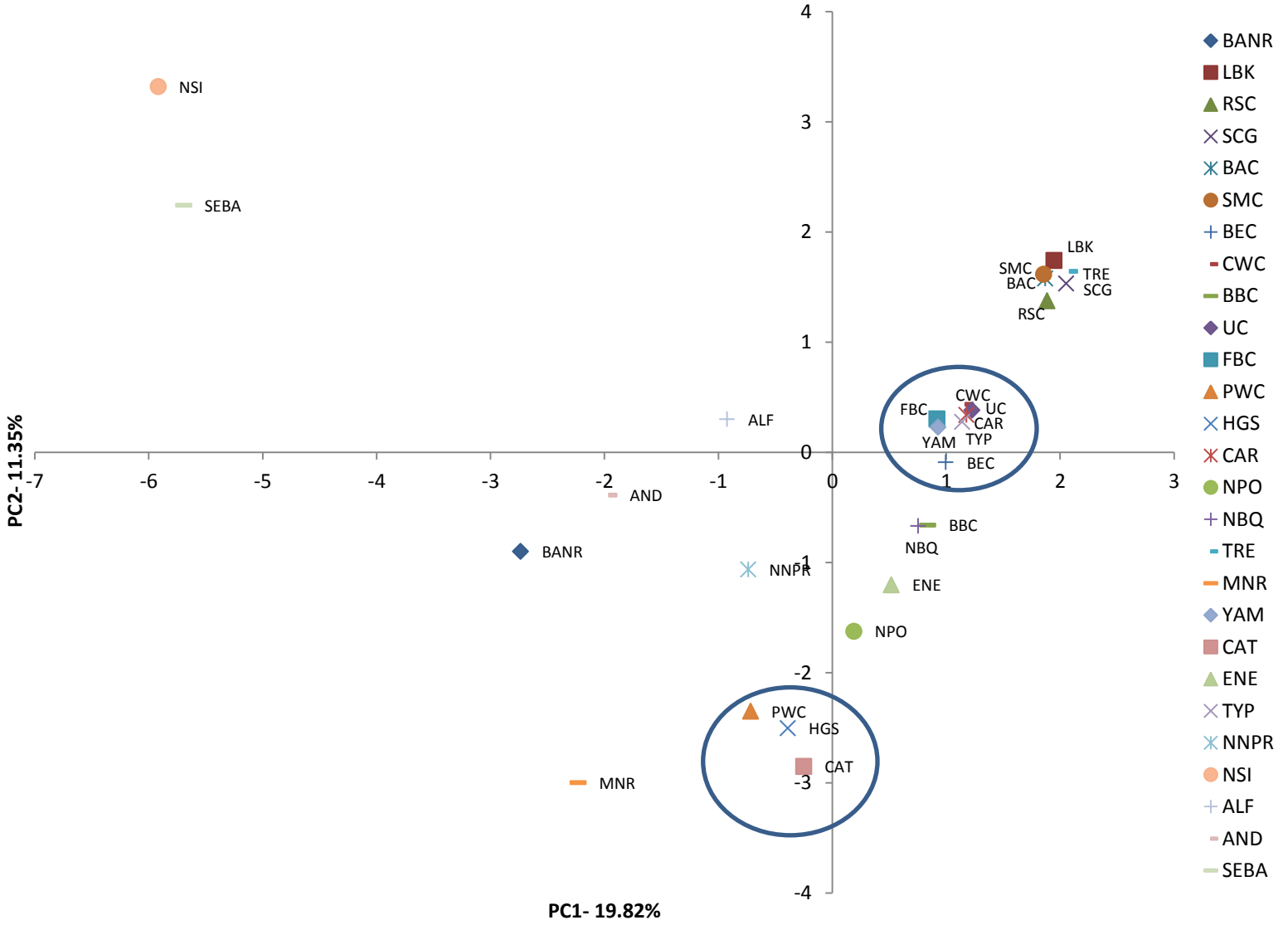


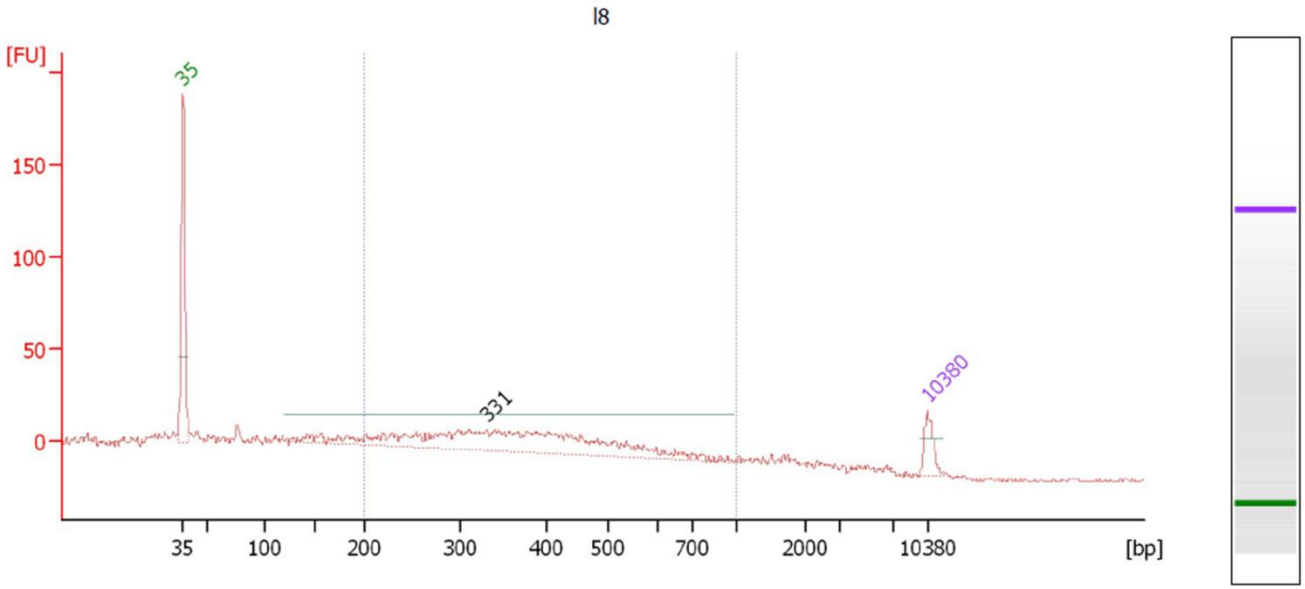
Figure 4: L8 Bioanalyzer Output

Ancient_DNA_High Sensitivity DNA Assay_DE72901290_2013-03-08_17-24-20.xad Page 7

Assay Class: High Sensitivity DNA Assay
Data Path: C:\...gh Sensitivity DNA Assay_DE72901290_2013-03-08_17-24-20.xad

Created: 3/8/2013 5:24:
Modified: 3/11/2013 10:38:

Electropherogram Summary Continued ...



Overall Results for sample 6 : l8

Number of peaks found:	1	Corr. Area 1:	416.2
Noise:	0.6		

Peak table for sample 6 : l8

Peak	Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	331	1,345.59	6,164.7	
3	10,380	75.00	10.9	Upper Marker

Region table for sample 6 : l8

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	416.2	67	404	36.1	1,594.07	7,035.1	

Figure 5: Example IVG and BWA *Actinoplanes* Alignment

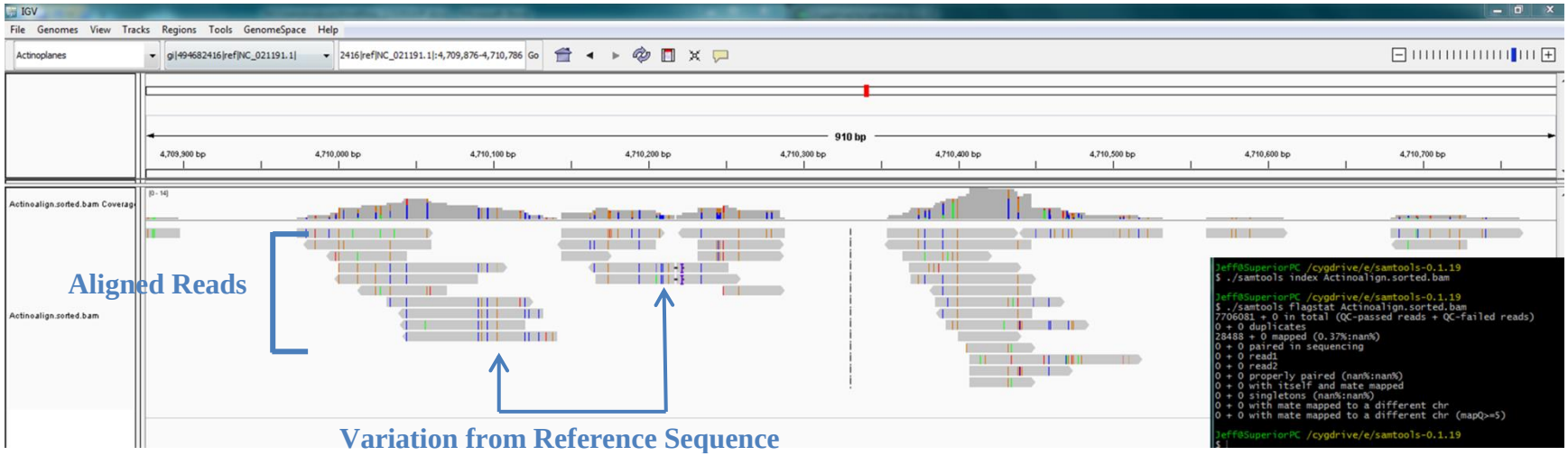


Table 5: Source data and culture abbreviations for PCA.

Population	abbr.	Papers
Bronze Age Northern Russia	BANR	Der Sarkissian 2013 dissertation
Linear Pottery culture	LBK	Haak et al. 2005, Haak et al. 2010, Brandt et al. 2013
Rössen culture	RSC	Brandt et al. 2013
Schöningen group	SCG	Brandt et al. 2013
Baalberge culture	BAC	Brandt et al. 2013
Salzmönde culture	SMC	Brandt et al. 2013
Bernburg culture	BEC	Brandt et al. 2013
Corded Ware culture	CWC	Haak et al. 2008 , Brandt et al. 2013
Bell Beaker culture	BBC	Brandt et al. 2013
Unetice culture	UC	Brandt et al. 2013
Funnel Beaker culture	FBC	Malmström et al. 2009, Skoglund et al. 2012 , Bramanti et al. 2009
Pitted Ware culture	PWC	Malmström et al. 2009, Skoglund et al. 2012
Hunter-Gatherer south	HGS	Chandler 2003, Chandler et al. 2005 , Hervella 2010, Hervella et al. 2012 , Snchez-Quinto et al. 2012
(Epi)Cardial	CAR	Gamba et al. 2011 , Lacan 2011, Lacan et al. 2011b
Neolithic Portugal	NPO	Chandler 2003, Chandler et al. 2005
Neolithic Basque Country & Navarre	NBQ	Hervella 2010, Hervella et al. 2012
Treilles culture	TRE	Lacan 2011, Lacan et al. 2011a
Mesolithic Northern Russia	MNR	Der Sarkissian et al. 2013
Yamna	YAM	This Study, Wilde et al. 2014, Newton 2011
Catacomb	CAT	This Study, Wilde et al. 2014, Newton 2011
Eneolithic	ENE	This Study, Wilde et al. 2014, Newton 2011
Trypillia	TYP	Nikitin 2010
Neolithic NPR	NNPR	Nikitin 2012
Neolithic Siberia	NSI	Mooder et al., 2010.
Alfold	ALF	Guba 2011, Burger 2007
Bronze Age Siberia- Androvo	AND	Molodin et al., 2012, Keyser et al. 2009.
Siberia-Early Bronze Age	SEBA	Molodin et al. 2012, Mooder et al., 2010.