Maternal and Paternal Polymorphisms in Prehistoric Siberian Populations of Lake Baikal

by

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Abstract

The study of Ancient DNA (aDNA), DNA recovered from archaeological and historic post mortem material, has complemented the study of anthropology and archaeology. There are several challenges in the retrieval and analysis of DNA from ancient specimens including exogenous contamination with modern DNA, polymerase chain reaction (PCR) inhibitors and DNA damage because of environmental factors. Despite all the obstacles, the extraction of aDNA is still possible through reliable extraction methods and highly sensitive PCR-based technologies that facilitated the use of aDNA analysis in revealing the maternal and paternal backgrounds of ancient populations. This dissertation examines prehistoric hunter-gatherer populations that inhabited Siberia, Russia, several thousand years ago. The Lake Baikal of Siberia was home to two temporally distinct populations from Early Neolithic, EN (8000-6800 cal BP) to Late Neolithic-Early Bronze Age, LN-EBA (5800-4000 cal BP). The EN group was separated from the LN-EBA group by a 1000-year gap (hiatus). Several cemeteries have been excavated as part of an international Baikal Archaeology Project (BAP). These include one EN cemetery (Shamanka II) and two LN-EBA cemeteries (Kurma XI and Khuzhir-Nuge XIV). Maternally inherited mitochondrial DNA (mtDNA) has been examined previously for two EN cemeteries (Lokomotiv and Shamanka II) and one of the LN-EBA cemeteries (Ust'-Ida). mtDNA has not been analyzed before from the Kurma XI cemetery. This dissertation hypothesis focused on the examination of mtDNA from Shamanka II and Kurma XI cemeteries and examination of Y-chromosomal DNA from the four excavated cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI) to identify genetic discontinuity and/or continuity between and within the EN and LN-EBA of prehistoric populations. The project aims were; first, modification of published methods for sample preparation, DNA extraction and PCR amplification for aDNA research. Second, interpretation of mtDNA haplogroup distribution from Kurma XI in the context of other Lake Baikal cemeteries. Third, compare the genetic affinities of the prehistoric populations with the contemporary populations of the area through the maternal lineage. Finally, comprison of mtDNA and Y-chromosomal haplogroup distributions to determine maternal and paternal genetic affinities. Four different mtDNA haplogroups were found in Kurma XI individuals including A, D, F and Z. mtDNA haplogroup Z was not represented before in Lake Baikal's

prehistoric populations. In addition, six extra samples from Shamanka II were analyzed to reveal that Shamanka II and Lokomotiv did not share the same maternal background as was previously suggested. New mtDNA results from Kurma XI and Shamanka II suggested that each of the EN cemeteries and LN-EBA cemeteries had a different maternal origin; however, Kurma XI shared a similar maternal origin with Lokomotiv and also with Shamanka II. Through SNaPshot multiplex PCR amplification, Y-chromosomal haplogroups were obtained from male individuals in the four cemeteries. Individuals from Lokomotiv and Shamanka II were found to possess haplogroups K, R1a1 and C3, and individuals from Ust'-Ida and Kurma XI were found to belong to haplogroups Q, K and unidentified SNP (L914). For those individuals belonging to haplogroup Q, further experimentation to examine sub-haplogroups of Q revealed that these individuals belong to sub-haplogroup Q1a3. There was significant heterogeneity in the males from the Lokomotiv cemetery when compared to the other three cemeteries. Furthermore, the Y-chromosome results showed a discontinuity between the EN and the LN-EBA populations of Lake Baikal. Combining the maternal and the paternal results from the prehistoric populations of Lake Baikal suggested a patrilocal post-marital residence pattern, where females moved to their husbands' birthplace after marriage. This research highlighted the utility of DNA analysis as an archaeological tool in conjunction with burial practices and artifacts in making inferences about the prehistoric population structure.

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List of Abbreviations

aDNA	Ancient DNA
BAP	Baikal Archaeology Project
bp	Base Pair
BSA	Bovine Serum Albumin
cal BP	Calibrated years before present
EN	Early Neolithic (8000-6800 cal BP)
HV1	Hypervariable region 1
HV2	Hypervariable region 2
LN-EBA	Late Neolithic- Early Bronze Age (5800-4000 cal BP)
Mb	Megabases
MITOMAP	A Human Mitochondrial Genome Database
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NE	Northeast
NGS	Next-Generation Sequencing
NRY	Non-recombining region on the Y-chromosome
PARs	Pseudoautosomal regions
PC	Principal Component
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RFUs	Relative Fluorescence Units
SNPs	Single Nucleotide Polymorphisms
Sr	Strontium
SRY	Sex-determining Region on Y-chromosome
SW	Southwest
YBP	Years Before Present
YCC	Y Chromosome Consortium
Y-STRs	Y-chromosomal Short Tandem Repeat

Chapter 1

❖

Literature Review

1. Introduction

The majority of North Asia comprises Siberia, which extends from the Pacific Ocean in the east to the Ural Mountains in the west and between the Arctic Ocean in the north to China, Mongolia and Kazakhstan in the south (Fig. 1.1). Siberia has provided a crucial geographical connection between Asia and North America since the Paleolithic era. Siberia played a major role as a transitional region for human migration towards the Americas and Japan; therefore, it is essential that the history and the genetic affinities of people inhabiting this area is understood in studying the migratory history of humans to the Americas and Japan and in particular, the role of the Siberian people in the formation of the New World population (1). Northern Asia was occupied from more than 40,000 years ago with the first evidence of human settlement found in the Altai region of Siberia. Archaeological data suggest the occupation of the Lake Baikal area in southern Siberia since the Upper Paleolithic (40,000 years) (2-5). However, there is insufficient information available to indicate whether there was a biological continuity between the current populations and the ancient population from around 40,000 years ago (4).

Figure 1.1. Map representing Siberia, Russia (6).

Adapted with permission from [Population affinities of Neolithic Siberians: A snapshot from prehistoric Lake Baikal. K.P. Mooder,T.G. Schurr,F.J. Bamforth,V.I. Bazaliiski,N.A. Savel\'ev. American Journal of Physical Anthropology. Copyright © 2005 John Wiley and Sons] (License number 3581560282120).

Previously, reconstructing the human history of this region depended mainly on archaeological, anthropological and linguistic research. However, this was not enough to understand the genetic affinities and social structure of ancient and extinct populations and to link them to modern populations. Thus, including genetic research with other research areas was essential in allowing reconstruction of historical genetic events that formed modern populations.

The analysis of ancient DNA (aDNA), defined as DNA recovered from archaeological and historical post mortem material (7), has complemented other fields such as anthropology and archaeology. Studying prehistoric populations' genetic signature unveiled several important aspects that other conventional methods could not. Many aDNA studies, worldwide, have been conducted and published revealing information about human migration patterns, kinship between individuals buried in the same grave, marriage patterns, social organization and more (8-14).

2. Genetic markers

Each nucleated human cell carries two genomes. The one inside the nucleus is the nuclear genome, and the one inside the mitochondria is the mitochondrial genome.

2.1. Mitochondrial genome

The mitochondria are the powerhouse of cells. They provide much of the cell's energy requirement through OXPHOS (oxidative phosphorylation) where hydrogen ions derived from carbohydrate and fat metabolism are oxidized by breathed oxygen (15) with the generation of adenosine triphosphate (ATP).

Mitochondrial DNA (mtDNA) is a small circular genome within the mitochondria found in the cytoplasm and is able to replicate independently from the nuclear DNA (nDNA). mtDNA has only 16,569 base pairs and consists of the L strand (light strand), which is the sense strand, and the H strand (heavy strand), which is the anti-sense strand. mtDNA has no intronic sequences and has very few non-coding bases, which are found in the D-loop (Displacement Loop), or the control region (CR), which has two hypervariable regions, HV1 and HV2 (16) (Fig. 1.2). Each cell has a high copy number of mtDNA that can reach hundreds to thousands of copies per cell (17). mtDNA has a high mutation rate and accumulates mutations about 10 times more rapidly than

nDNA genes of similar function (18). mtDNA is not repaired with the same efficiency as nDNA and has an increased error rate during replication (19), leading to the accumulation of population-specific polymorphisms. Unlike nDNA, mtDNA undergoes almost no recombination and has a rapid base substitution rate (15,20). One of the key characteristics of mtDNA is its maternal inheritance (17). The maternal inheritance feature allows the study of population migration through the maternal lineage (20). Because of its high copy number, mtDNA was the first genetic markers that could be reliably analyzed in aDNA laboratories and is considered highly valuable for anthropologists because of its maternal inheritance (6,21-26).

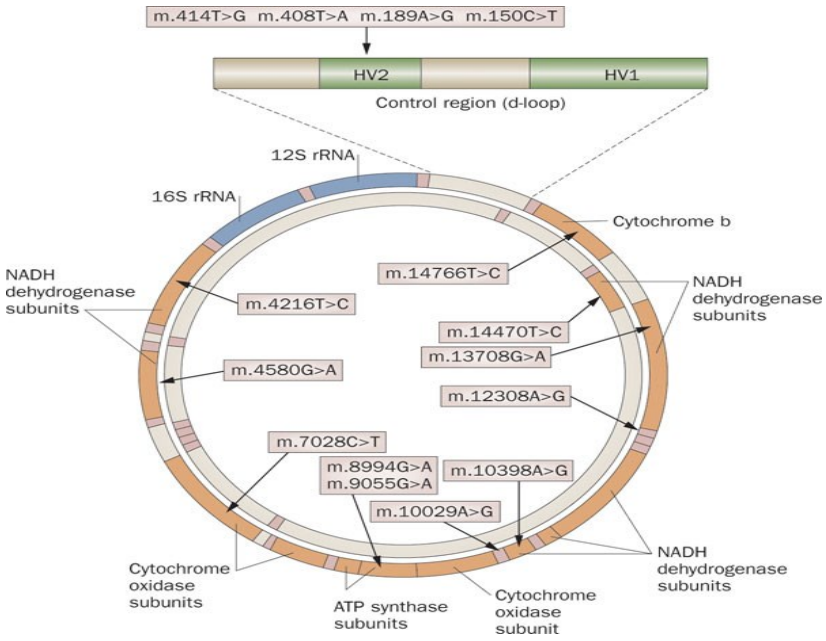

Figure 1.2. Mitochondrial DNA (mtDNA) structure featuring the control region with its two hyper-variable regions HV1 (16024-16383), HV2 (57-327). The arrows pointing at the common polymorphic sites on mtDNA identifying several mtDNA haplogroups (27).

2.1.1. Methods for typing mtDNA

2.1.1.1. Restriction Fragment Length Polymorphisms (RFLPs)

Polymorphisms found in mtDNA generate specific haplotypes, which in combination form haplogroups that are unique to each population (20). A haplogroup is an assembly of specific polymorphic variants inherited together, which reflect an individual's evolutionary background. There are several examples in the literature of mtDNA haplogroups defined by RFLPs. The first demonstration of mtDNA polymorphisms in different ethnic groups was done by digesting mtDNA with *HpaI* a restriction enzyme that cuts the DNA sequence at a specific site known as the restriction site (28), (restriction site: GTT/AAC) to show variation in mtDNA sequence in Caucasian, Asian and African individuals. These individuals showed different *HpaI* restriction sites in mtDNA (29). It was later shown, using RFLP analysis, the greatest variation in mtDNA sequences was in Africa, with the constructed mtDNA phylogenetic tree dating back to 100, 000 years before present (YBP) (30). RFLP analysis then became a powerful tool in identifying variation in mtDNA sequence and thus identifying different mtDNA haplogroups (31) in human population evolutionary history (32-37). The subsequent development of the polymerase chain reaction (PCR) with amplification and direct sequencing of the hypervariable 1 region (HV1) facilitated mtDNA haplogroup analysis at much greater resolution in both prehistoric and modern populations. It became possible to link mtDNA haplogroups in prehistoric individuals with modern populations (6,38-40).

2.1.1.2. Direct sequencing of hypervariable I region (HV1) by Sanger sequencing

The Sanger sequencing principle is based on sequencing a specific single-stranded DNA segment through repetitive annealing of a DNA primer, and extension with a DNA polymerase while incorporating deoxynucleotidetriphosphates (dNTPs). The sequencing reaction is terminated with a modified dNTP called the dideoxynucleotidetriphostphate (ddNTP). ddNTPs lack the 3'-OH group needed for binding with another nucleotide to form the phosphodiester bond, and the incorporation of a ddNTP during sequencing ceases the polymerase from extending the DNA sequence (41).

Direct sequencing, or Sanger sequencing, of the HV1 region, which contains the majority of mutations for specific mtDNA lineages, has greatly expanded the scope of mtDNA haplogroup

analysis. Direct sequencing of the HV1 region is usually combined with data from RFLP typing in assigning mtDNA haplogroups (6,38-40).

Sequencing of the control region (D-loop) (Fig. 1.2), containing both HV1 and HV2 regions has been used in many aDNA research studies. One of the early examples was the analysis of mtDNA from an Egyptian cemetery located in the Dakhleh oasis in the western desert (the cemetery dates back to 100-450 AD (1565-1915 YBP)), with the aim of proving that there are significant differences between the modern and ancient populations that inhabited this area (21). Another example came from the mtDNA HV1 region of the one of the most celebrated frozen mummies that was discovered in 1991 in the Eastern Alps near the Austro-Italian border, the 5,000 year old Tyrolean "Ice Man". The mtDNA HV1 region was sequenced to reveal that the "Ice Man" is related to the contemporary European population (42).

2.1.1.3. Other sequencing techniques for mtDNA genome

Complete sequencing of the mtDNA genome has greatly improved the mtDNA phylogenetic tree allowing definition of sub-haplogroups and has provided enhanced insight into the mtDNA haplogroups associated with populations and their geographic distributions (43-47). High-throughput sequencing technology, or next-generation sequencing (NGS), allows the sequencing of thousands or even millions of sequences in parallel. NGS sequencing technology is one of the methods applied for complete sequencing of the mtDNA genome. NGS platforms show that a complete sequence of the mtDNA genome can be obtained rapidly and efficiently (47). These platforms have been applied to several aDNA studies of mtDNA. NGS was conducted on one of the most famous mummies "Ice Man" (as described above) (48). Another study analyzed a 700 year old first human New Zealander entering New Zealand, which is considered the last major part of the globe to be inhabited by humans after their dispersal across the world from Africa (49).

2.1.2. Distribution of mtDNA haplogroups

Figure 1.3 illustrates the human mtDNA tree representing the mtDNA lineages with the core branch, formed by haplogroup L originating in Africa. The African L3 lineage is shared with the rest of the world, where it is the only lineage outside of Africa, forming the other main branches of the mtDNA tree, but the rest of the L branches are found exclusively in the African

population (50-54). The remaining world population belongs to two main lineages, M and N, derived from L3. The Asian population is derived from the two main mtDNA lineages, M (51) and N (55). Despite inaccuracy in figure 1.3, some mtDNA haplogroups might not be represented in the Asian population (e.g. haplogroups Q and S).

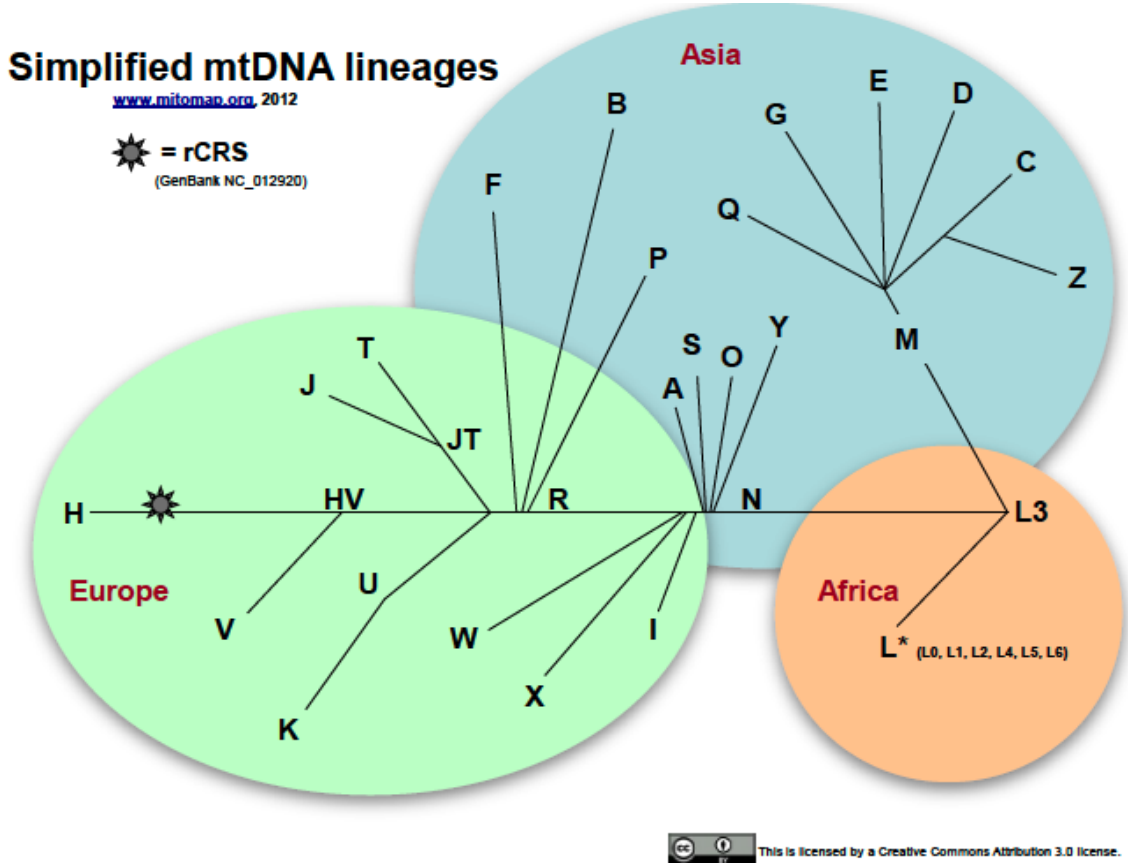

Figure 1.3. Simplified mtDNA Tree for Europe, Asia, Africa illustrating the divergence of the Afrian lineage L3 to Asia and Europe forming the other two main lineages M and N and their sub-lineages (56).

Adapted from (URL: http://www.mitomap.org/pub/MITOMAP/WebHome/simple-tree-mitomap-2012.pdf) licensed by Creative Commons Attribution 3.0 license that allows sharing and adaption for any purpose, even commercially (http://creativecommons.org/licenses/by/3.0/deed.en_US).

Figure 1.4 Illustrates the mtDNA haplogroup distribution and the estimated time of human migratory events around the globe.

The focus of this research was the Siberian population, occupying the majority of north Asia. The dispersal of Asian mtDNA haplogroups varies widely across Asia. The main sub-lineages of M detected in the Asian populations are C, D, G, E and Z (34), and the sublineages of N include A, B, F and Y (34). All these haplogroups are formed as a result of specific mutations in the mtDNA HV1 region. Several studies have defined the mutations in the HV1 region (e.g. (32,34,36,57,58)). According to MITOMAP (A Human Mitochondrial Genome Database) (56), haplogroup C is defined by base substitutions in the HV1 region at 16223, 16298 and 16327. Haplogroup D defined by two base substitutions at 16223 and 16362, and G has three base substitutions at 16223, 16278 and 16362. Haplogroup E defined by two base substitutions at 16362 and 16390, and the last Asian haplogroup derived from the main M mtDNA lineage is haplogroup Z, defined by base substitutions at 16185, 16223, 16260, and 16298. For the cluster of haplogroups under the main N lineage, haplogroup A is defined by base substitutions at 16223, 16290, 16319 and 16362. Haplogroup B is defined by three base substitutions at 16183, 16189 and 16217. Haplogroup F, according to MitoMap, is defined by one base substitution at 16304. The last haplogroup found in the Asian population, haplogroup Y is defined by one base substitution at 16126 (56). Haplogroups and their base substitutions, either transitions or transversions, are summarized in Table 1.1.

The distribution of the mtDNA haplogroups diverges widely between the northern and southern Asian populations. Haplogroups B and F are widely distributed throughout the south Asian population; however, this population generally lacks other Asian haplogroups, A, C, D, G, E, Y and Z, which are found predominantly in the north Asian population (15,59).

Most of the north Asian haplogroups (A, C, D, G, Y and Z) are represented in the Siberian populations (32,35-37,57), but Siberian population lacks haplogroup B (36). The native Siberian population is genetically heterogeneous with the respect to mtDNA haplogroup distribution (36). A number of studies have been conducted to define the connection between the Siberian and Native American populations through the analysis of mtDNA haplogroup distribution (36,37,60-64). These analyses demonstrated the links between the Asian mtDNA haplogroups

and the contemporary populations inhabiting the New World and the Americas. There are four main mtDNA haplogroups, A, B, C and D, found in Native Americans. Haplogroups A, C and D are found in the Siberian population with high frequency suggesting their early arrival to the New World approximately 35,000-25,000 years before present (YBP). However, haplogroup B might indicate a second later wave of migration events to the New World (60,65) as the estimated time for its divergence in the New World is 17,000-13,000 YBP, which is much more recent than haplogroups A, C and D (36).

Many questions about the origin of the New World population and the number and timing of the migratory events that formed the Native American population remain unanswered. Studying prehistoric Siberian populations through mtDNA analysis may help to address some of the unsolved mysteries of the origin of the New World population.

Table 1.1. Asian mtDNA haplogroups and the HV1 base substitutions defining them created with reference to the MITOMAP database (56).

Asian mtDNA Haplogroups	HV1 Base Substitutions
M	
C	16223 (C>T), 16298 (C>T) and 16327 (C>T)
D	16223 (C>T) and 16362 (T>C)
G	16223 (C>T), 16278 (C>T) and 16362 (T>C)
E	16362 (T>C) and 16390 (G>A)
Z	16185 (C>T), 16223 (C>T), 16260 (C>T) and 16298 (C>T)
N	
A	16223 (C>T), 16290 (C>T), 16319 (G>A) and 16362 (T>C)
B	16183(A>C), 16189 (T>C), and 16217 (T>C)
F	16304 (T>C)
Y	16126 (T>C)

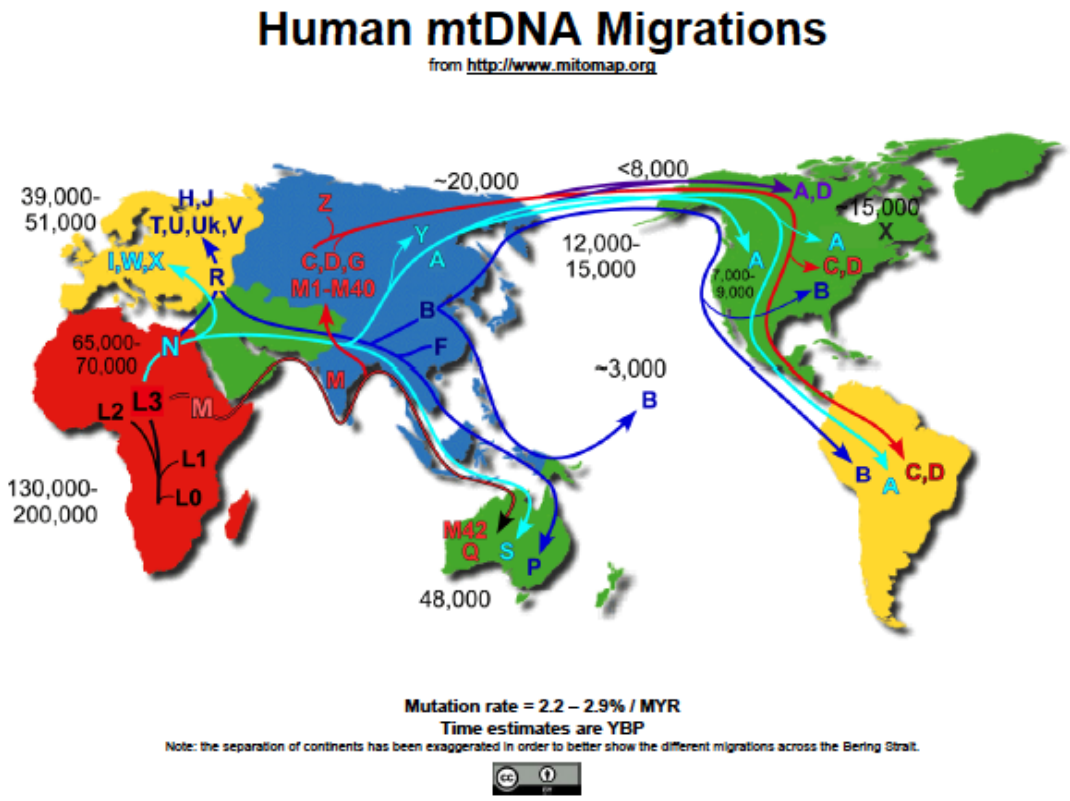

Figure 1.4. Human mtDNA migrations through the globe with the first evidence of migration from Africa with an estimated timing of the event, plus the distribution and the estimated timing of the migratory events of various mtDNA haplogroups around the world (updated March 2013) (56).

Adapted from (URL: http://www.mitomap.org/pub/MITOMAP/MitomapFigures/WorldMigrations2013.pdf) licensed by Creative Commons Attribution 3.0 license that allows sharing and adaption for any purpose, even commercially (http://creativecommons.org/licenses/by/3.0/deed.en_US)

2.2.Nuclear genome markers

Unlike mtDNA, autosomal nDNA is limited to two copies per cell found in the nuclear chromosomes, which makes its retrieval from ancient human skeletal remains more challenging because of its low copy number.

2.2.1. Autosomal microsatellites or short tandem repeats (STRs)

Microsatellites, also known as STRs, are DNA sequences of variable length and number of repeat units at a given locus (66). Each repeat element varies in length from two to six bases and is widely used in population genetics (67). Variation in repeat number in these microsatellites occurs because of slippage during DNA replication that results in different repeat sizes (68). Ninety percent of mutations in microsatellites result from addition or deletion of one repeat unit, which creates the variation in repeat number (69). The rate of mutation in the STRs is much higher than in single nucleotide polymorphisms (SNPs), sequence variants that appear on average every several hundred bases all through the human genome (70). It is estimated at 10^{-3} per locus per generation whereas the SNP mutation rate is 10^{-8} mutation per generation (69,71-73). Because STRs have multiple alleles (usually more than 5) while SNPs almost always have only two alleles, they provide more information per marker (74). STRs can be detected on both autosomes and sex chromosomes. Y-chromosomal STRs will be discussed later. Table 1.2 summarizes the differences between SNPs and STRs.

Autosomal STR analysis provides a well-established marker system that has been used by several archaeological, forensic and aDNA studies to determine genetic kinship between closely related individuals by biparental loci, because autosomes are inherited from both parents (22,75-77). Autosomal STRs have numerous characteristics, facilitating their utility in analysis of highly degraded aDNA (78), including their abundance and high frequency throughout the genome, wide range in the number of repeats per locus, and short amplicon length (78). Autosomal STRs used for forensic studies are either on separate chromosomes or are far apart from each other on the same chromosome to maximize linkage disequilibrium, non-random association between alleles on different loci (79).

Table 1.2. A comparison between SNPs and STRs

Points of Comparison	SNPs (Single Nucleotide Polymorphisms)	STRs (Short Tandem Repeats)
Number of alleles	Two alleles only [(a)]	Multiple alleles (usually more than 5) [(a)]
Mutation rate	10^{-8} mutation/generation	10^{-3} per locus/generation
Frequency throughout the genome	More frequent (occur every several hundred bases)	Less frequent (occur every several bases)
Amplicon size	Can be less than 100 bp	Mostly between 100 to 450 bp
Recovery of information from degraded DNA	Possible because mostly only a single nucleotide need to be measured [(b)]	Hard because an array of nucleotides (hundreds) need to be measured [(b)]
Power of discrimination	Not as powerful as STRs (40-60 SNPs needed to be equivalent to 13-15 STR loci) [(b)]	Powerful because they are more polymorphic than SNPs [(b)]
Databases	dbSNP-NCBI (www.ncbi.nlm.nih.gov/projects/SNP/)	ATCC STR Database (www.atcc.org)

- (a)= (74)
- (b)= (80)

2.2.2. Amelogenin locus

Amelogenin, a gene involved in dental enamel formation, is found on both the X and the Y chromosomes (81). The amelogenin locus can be used to identify the sex of individuals because of a 6 base pair (bp) deletion polymorphism unique to the X-chromosome (82). PCR primers for amelogenin amplification, in most of the world populations, give 106 bp products for females and 106/112 bp product for males (83).

A commercial application, the AmpFlSTR® Identifiler® kit (Applied Biosystems, NY, USA) has been applied to several aDNA studies that focused on identifying autosomal STRs and the amelogenin locus (22,75). The AmpFlSTR® Identifiler® kit, amplifies 15 STR loci together with the variable amelogenin locus in one single multiplex reaction.

2.2.3. The Y-chromosome

The study of the human Y-chromosome is of importance in tracing human evolution through the paternal lineage. The Y-chromosome is exclusively inherited from the father, meaning each male carries the same Y chromosome as his father, paternal grandfather, brothers, paternal uncles, and any male individual related to his father (84). Y-chromosome analysis allows researchers to trace the evolution and human migration patterns from the paternal lineage while the maternally inherited mtDNA traces the evolution and human migration patterns from the maternal lineage.

2.2.3.1. Y-chromosome structure

The Y-chromosome is the second smallest chromosome after chromosome 21 (Fig. 1.5). It is an acrocentric chromosome about 60 megabases (Mb) in length. It consists of two parts, the non-recombining region (NRY) and the pseudoautosomal regions (PARs). About 35 Mb of the NRY is euchromatic DNA, and the rest is a block of heterochromatic DNA. The PARs, about 5% of the sequence, are found at the telomeres of the Y-chromosome. The PARs are the only parts on the Y-chromosome that recombine with the PARs on the X-chromosome during meiosis in order to maintain their attachment on the spindle fibers during cell division and act as regular autosomes (85). The NRY part of the Y-chromosome has specific genes that are mostly related

to conferring male phenotype, and the PARs have diverse genes acting as autosomal genes (i.e. expressed on both the X and the Y chromosome).

The NRY region comprises about 95% of the human Y chromosome, and it was previously believed that this area of the Y chromosome was just "a waste land" without any genes (84). However, in 1997 a paper was published in Science by Lahn and Page identifying 12 genes within the human Y-chromosome NRY (86). They divided these 12 genes into two groups. The first group has five genes, which are considered housekeeping genes. These five Y-chromosome NRY genes have homologues on the X-chromosome, which encode proteins similar to the ones on the X-chromosome but not identical, and have functions in different tissues and there is mostly only one copy of each gene on the Y-chromosome. The second group consists of seven genes that have multiple copies in the NRY region, appear to be specifically expressed in the testis, and are associated with the male phenotype and reproductive fitness. The *SRY* (Sex-determining region on Y) gene is one of the second group of genes, and is responsible for sex determination. *SRY* is expressed in the testis but there is only one copy of this gene unlike all the other genes in the second group, as they have multiple copies (86,87).

There are two PARs on the human Y chromosome (PAR1 and PAR2). PAR1 is on the tip of the short arm (Yp) of the Y chromosome and about 2.7 Mb in physical length. PAR2 is on the tip of the long arm (Yq) and about 0.33 Mb in length. PARs were named "pseudoautosomal" regions because pairing and crossover between the X and the Y chromosomes take place at these loci. They act as autosomal genes where they have similar gene density and structure in regard to the presence of exons and introns (88,89). Currently, there are known to be 24 genes in PAR1 and only five genes in PAR2 (90). Loss of the PAR1 region is associated with male infertility, which indicates that the presence of PAR1 is essential for X-Y homologous pairing and proper segregation during meiosis (91). Mapping genes on the PARs can be done through both physical mapping techniques and genetic linkage techniques and because the PARs act as autosomes, the recombination rate can be determined between their genes (90).

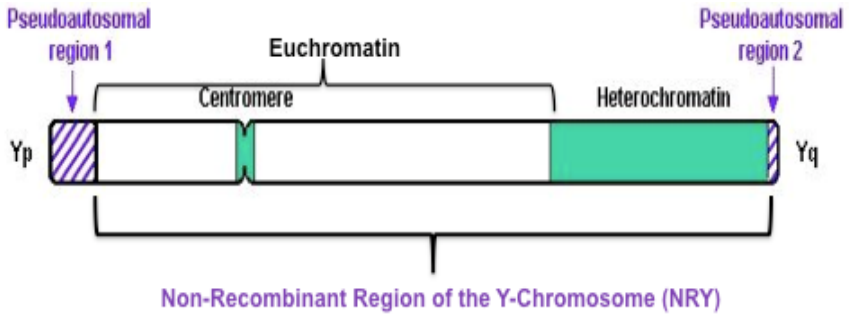

Figure 1.5. Y-chromosome structure featuring the Non-Recombinant Region (NRY) and the pseudo-autosomal regions on the Y-chromosome, which act as autosomes and undergo recombination during meiosis (84).

2.2.3.2. Y-chromosomal markers

During the last few years many DNA polymorphisms had been described specific to the Y-chromosome. Many DNA polymorphisms among different human populations that can be found on Y-chromosomal DNA have led to a dramatic increase in studies of the paternally inherited Y-chromosome and its significance in evolutionary and population genetics (92-98). These polymorphisms include single nucleotide substitutions, small and large deletions, inversions and duplications. These polymorphisms have different mutation rates (99). Some loci have low mutation rates, which provide the opportunity to use them in differentiating between ancestral branches on the human evolutionary tree (94,95,100,101). Other loci with high mutation rates are used for the analysis of more recent evolutionary events (96,102-104). There are two types of markers on the Y-chromosome, binary markers and Y-chromosomal STRs, and both will be discussed in more detail below.

2.2.3.2.1. Y-chromosome binary markers

There are two main groups of polymorphic markers on the Y-chromosome. The first group consists of rare (unique) event polymorphisms (UEPs) characterized by a low mutation rate (10^{-8} mutations per generation). These mutation events generate biallelic or binary markers because a mutation event at a locus takes place only once, and only two alleles of this mutated locus will be established in the population. The biallelic markers include single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) (69,105-107). Binary markers are exceptionally useful because of their low mutation rate, making them appropriate for identifying stable paternal lineages, which can be tracked back in time for thousands of years (108). Before 1997 only a few Y-chromosomal biallelic markers had been described, but the technique of denaturing high performance liquid chromatography (DHPLC) facilitated the discovery of hundreds more Y-chromosomal SNPs (Y-SNPs) by a group of researchers at Stanford University led by Peter Underhill (109,110). By 2002 there were 245 binary markers described (108), and by 2008 the number of binary markers reached 600 (111).

2.2.3.2.2. Y-chromosomal STRs

Y-chromosomal STRs (Y-STRs) represent the second group of Y-chromosomal polymorphic markers. The Y-STRs are used widely in the forensic field because of their typing simplicity and high level of diversity. Analyzing STRs requires simple PCR amplification techniques. In addition, STR typing techniques can be performed on degraded DNA samples (99).

DYS19, also known as DYS394, was the first Y-STR identified (112). DYS19, a tetranucleotide repeat, contains a $(TAGA)_3$ TAGG$(TAGA)_n$ repeat unit. In the Brazilian population, five different alleles (A-E) were found with sizes ranging from 186 to 202 base pairs (113). Later more alleles were described for the DYS19 microsatellite (114). More Y-STR markers have been described recently because of "…the availability of DNA sequence information from the Human Genome Project and improved bioinformatics tools for searching DNA sequence databases" (115). The European forensic community in 1997 established a group of Y-STR markers called the "minimal haplotypes" which includes DYS19, DYS389I/II, DYS390, DYS391, DYS392, DYS393, and DYS385a/b including YCAIIa/b as an optional marker. The "minimal haplotypes" are the most used Y-STR markers to examine specific population polymorphisms. These markers were used to create most of the Y-chromosome databases using Y-STRs (116-118).

2.2.3.3. Methods of typing Y-chromosomal markers

Several techniques have been applied for typing Y-chromosomal markers (Y-SNPs and Y-STRs), and other new technologies have been developed. The LightCycler (Real-Time PCR system) (Roche Molecular Biochemicals, Mannheim, Germany), an ultra fast thermal cycler that allowed researchers to monitor the amplification of the PCR product in real time, was used to analyze Y-SNP marker in singleplexes or duplexes only (119), and multiplex analysis was used for analyzing more than one Y-SNP marker at a time (120). One recent innovation was the primer extension technique using the SNaPshot kit supplied by Applied Biosystems (NY, USA) (74). SNaPshot is a simple method; it consists of a single base extension of a primer, chosen to amplify the SNP of interest. The primer is unlabeled and it anneals one base upstream of the SNP of interest. During extension, a fluorochrome-labeled dideoxynucleotide (ddNTP) binds to the SNP site, and the fluorescence color can be detected by capillary electrophoresis (121). The SNaPshot assay was used not only for the analysis of Y-chromosome SNPs (122-126), but also

with mitochondrial DNA (127-130) and autosomes (131,132). These studies suggest that the SNP typing method is not only robust but also highly sensitive. The SNaPshot technique can even be used with degraded and aged DNA including aDNA. However, aDNA primers need to be designed to amplify small amplicons because of the degraded conditions of aDNA (121). Multiplex PCR amplifications for Y-STR markers allow for the examination of more than one STR marker at a time. The multiplex approach saves effort and time for researchers and also minimizes sample quantity required for more than one PCR amplification especially from precious samples (133). The crucial part of the multiplexing assay is designing the primers (133-135). The primer annealing conditions used in multiplexing several STRs must have compatible conditions, and must not interfere with one another (135,136). There are several commercial kits for Y-STR multiplexing assay; ReliaGene has produced two types of commercial kits for typing Y-STR markers: one amplifies six Y-STR makers (Y-PLEX^{TM} 6), DYS19, DYS389II, DYS390, DYS391, DYS393 and DYS385a/b, and the second multiplex examines five Y-STR markers (Y-PLEX^{TM} 5), DYS389I/II, DYS392, DYS438 and DYS439. Using both kits allows analysis of the "minimal haplotypes", including DYS438 and DYS439 (74).

Multiplexing several Y-STR markers can also be used with aDNA skeletal materials with primers designed to fit the DNA degraded conditions. A group of researchers (137) attempted to amplify four Y-STR markers (DYS19, DYS389I/II, and DYS390) from archaeological samples of human skeletal remains (250-3000 years old). Not all the Y-STR markers yielded results in each amplification reaction because of the degraded DNA conditions. With highly degraded material, the system that amplifies the largest product would tend to fail first, which is why it is essential to design primers to amplify short products to increase the chance of a successful amplification. Still, Y-STR markers are not highly discriminatory because of the linkage properties of the Y-chromosome that can be demonstrated through a group of males carrying identical haplotypes (e.g. (137)), which means that two males can carry the same Y-STRs and yet they are not paternally related.

2.2.3.4. Distribution of Y-chromosomal haplogroups based on binary markers

In 2002, only 245 mutational events were described on the NRY region, giving rise to 153 haplogroups and their sub-haplogroups using binary markers on the Y-chromosome such as

base substitutions and insertions/deletions. The YCC (Y Chromosome Consortium), a group of researchers collaborating to study the genetic varations in the NRY of the human Y-chromosome, constructed a single parsimonious tree for the 153 haplogroups (108). The main root of the tree, the position of the oldest common ancestor, drops between two main haplogroups. One defines haplogroup A, the first haplogroup above the position of the root, with two mutations (M91 and P97), and the other is described by a group of markers (SRY $_{10831a,}$ M42, M94 and M139) (Fig. 1.6). Haplogroup A is an African haplogroup (138,139). In 2002, there were only 18 Y-chromosomal haplogroups (A-R) (108). Because more binary markers have been discovered and added to the single parsimonious tree created by the YCC, a unified update was required. Thus, by 2008, when the number of binary markers reached 600, and 311 haplogroups with two new major haplogroups (S and T), a group of researchers updated the YCC tree to include all the new markers and haplogroups (111). Sub-haplogroups under each major haplogroup are designated through a number following the alphabetic designation of the major haplogroup (e.g. haplogroup E has three basal haplogroups assigned numbers E1, E2, and E3). Also, there are paragroups, marked by a star (*), where the group is related to the main haplogroup and not to the sub-haplogroups (e.g. E* underived paragroup belongs to E major haplogroup but neither to E1, E2 or E3). Nested haplogroups under each major one are named using the same system of alphabet but with lower-case letters instead of capitals (e.g. R1a1 haplogroup where the major clade is R). The other way of defining a haplogroup is to designate each haplogroup with the mutation that describes it (e.g. haplogroup G* is the same haplogroup as G-M201*) (108). Formerly, it was mentioned that the focus of this study is on Siberia. Therefore, it is important to describe the distribution of Y-chromosomal haplogroups in Asia. Haplogroup C might have originated in Asia, after the modern human migration out of Africa about 200,000-130,000 YBP. It exists with a high frequency among the Asian population (138-141). Sub-haplogroups of C (C4, C5 and C6) are found in South and Central Asia (111). Haplogroup D is found specifically in Asia and rarely outside of Asia, which suggests an Asian origin. Haplogroup D is a common lineage between Central Asian and Japanese populations and less common in Southeast Asian population (140,142). Sub-haplogroup D3 is found exclusively in Tibet, the Altai and Mongolia (111). Haplogroup E, which holds the highest degree of

diversity among all the major Y-chromosomal haplogroups, is detected occasionally in Central and South Asia (95,138,143). The J lineage has a common occurrence in Central Asia (138,139), and haplogroup L is also detected in Central Asia (138,143). The O lineage is a major haplogroup in East Asia and also occurs in Central Asia (138,140). One of the major haplogroups widely distributed in North Eurasia and specifically in Siberia, is haplogroup Q (144). In addition, haplogroup Q is a founder lineage among the Native American population with the Q1a3a exclusively limited to the Native American population (145). Figure 1.7 illustrates the Y-chromosomal haplogroup distribution in the world.

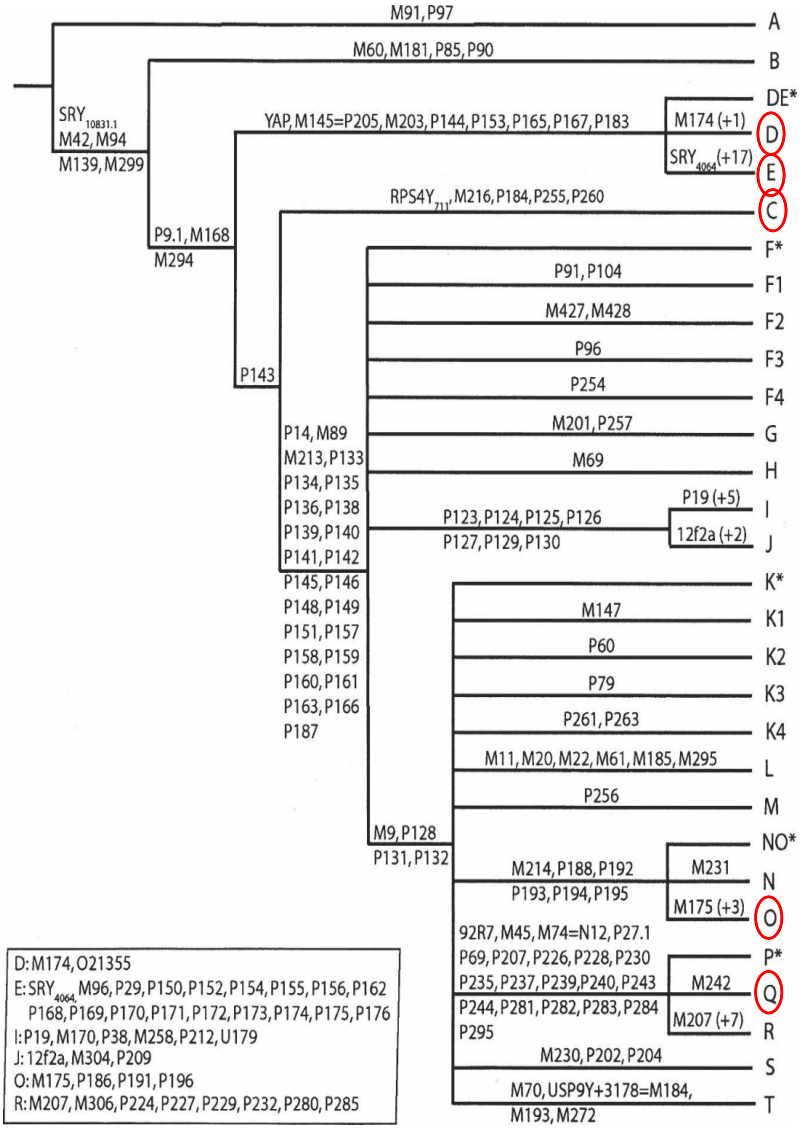

Figure 1.6. Y-chromosomal parsimony tree showing all the defined haplogroups from A to T with the Asian haplogroups circled (111).

Adapted with permission from [New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. Karafet,T.M.; Mendez,F.L.; Meilerman,M.B.; Underhill,P.A.; Zegura,S.L.; Hammer,M.F. Genome Research. 18(5). Copyright © 2008 Cold Spring Harbor Laboratory Press]. Genome Research is an open access journal, which permits non-commercial use, distribution, and reproduction in other forums, provided the original authors and source are credited.

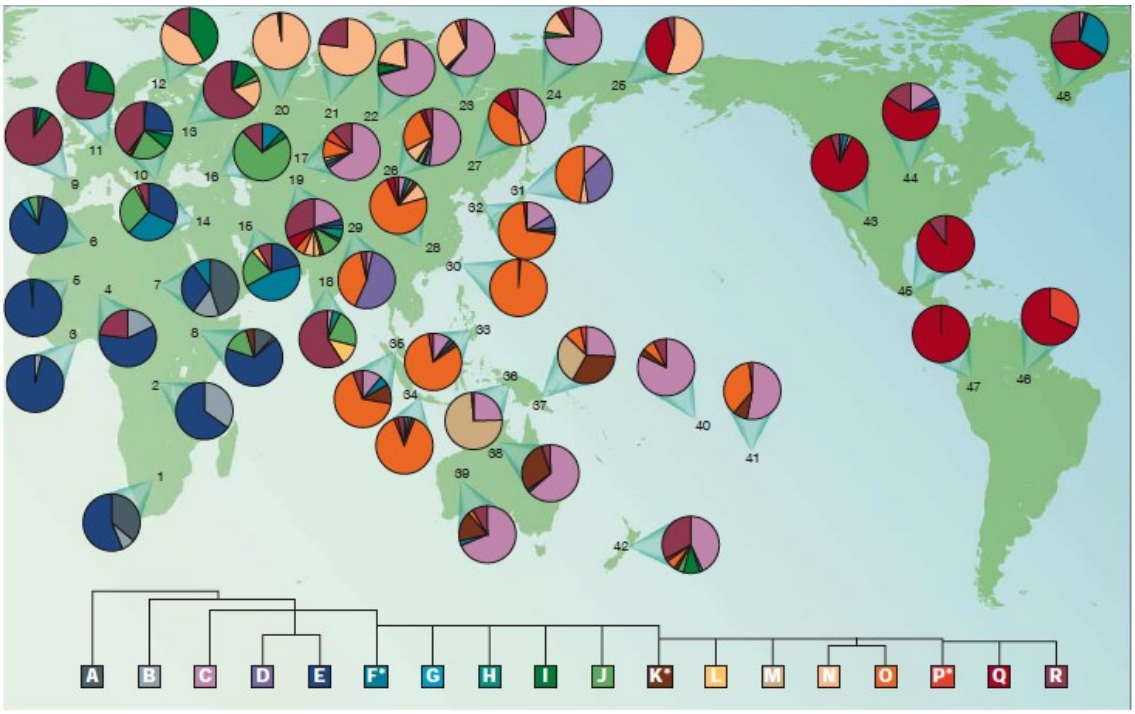

Figure 1.7. Y-chromosomal haplogroup distribution around the world representing haplogroups A to R (143).

Adapted with permission from [The human Y chromosome: an evolutionary marker comes of age. Mark A. Jobling and Chris Tyler-Smith. Nature Reviews Genetics. 4(8). Copyright © 2003 Nature Publishing Group] (License number 3584550608180).

3. Genetic markers and the Baikal Archaeology Project (BAP) multidisciplinary research initiatives

The Lake Baikal region of Siberia has provided a number of large mortuary complexes dated back to several thousand years ago. Canadian and Russian archaeologists have recovered a large number of well-preserved skeletal remains from discrete burial sites in the area, dating from 8000 to 4000 YBP (146,147). The area was home to two temporally distinct populations, the Kitoi, 8000-6800 YBP, (Early Neolithic, EN) and the Serovo-Glazkovo, 5800-4000 YBP, (Late Neolithic-Early Bronze Age, LN-EBA). The EN group was separated from the LN-EBA group by an approximate 1000-year gap (hiatus) during which large mortuary sites are entirely absent in the area. Several cemeteries have been excavated as part of an international multidisciplinary initiative, the BAP. These include one EN cemetery (Shamanka II) and two LN-EBA cemeteries (Kurma XI and Khuzhir-Nuge XIV). One EN cemetery (Lokomotiv) and one LN-EBA cemetery (Ust'-Ida) were excavated prior to and independently of BAP. BAP seeks to reconstruct the lifestyle of hunter-gatherers who lived around Lake Baikal through the application of research methods from archaeology, human osteology, bone chemistry, human genetics, and environmental studies (147). Archaeological data suggest cultural discontinuity between the EN and LN-EBA groups with differences in mortuary practices, diets and mobility patterns (147,148). The cemetery burials provide a unique opportunity to study prehistoric population genetics, in contrast to making inferences about prehistoric populations extrapolated from modern DNA studies. This is because: 1) Lake Baikal lies directly on the west-east Paleolithic human migration route from Europe to the New World, 2) There are an unusually large number of well-preserved specimens from which DNA population specific polymorphisms can be examined to determine population origins, genetic affiliations within and between burials,and 3) Differences in burial practices (e.g. single and multiple graves, presence or absence of ornaments, fish hooks) facilitate the use of DNA polymorphisms to make inferences about social structure, family groupings and marriage patterns within each cemetery.

3.1. Significance of the project

The significance of the project lies in the utility of DNA analysis as an archaeological tool used in conjunction with more traditional investigative methods (e.g. tools, burial practices, artifacts) in

order to make inferences about the prehistoric population structure. In addition, the relatively large number of well preserved skeletal remains at this location is unusual and strengthens any statistical analysis of population genetics.

3.2. Aim of the project

The aim of this project was to trace the maternal and the paternal lineages of the EN and LN-EBA populations by examination of mitochondrial DNA (mtDNA) and Y-chromosomal DNA polymorphisms. The degree of genetic continuity and/or discontinuity between and within the EN and LN-EBA cemeteries was established by DNA analysis and results interpreted within the archaeological context of other BAP research findings (146-150). Previous researchers have demonstrated that mtDNA and Y-chromosomal polymorphisms in archaeological and historic post mortem specimens (22,75,151) can give information on population origin, migration patterns, and the affinities between contemporary and prehistoric populations.

3.3. Ancient DNA

aDNA is DNA recovered from archaeological and historical post mortem material (7). While there are many techniques available for amplifying and examining genetic markers (e.g. mtDNA and Y-chromosomal DNA), their application to ancient degraded samples rather than modern samples can be challenging. These techniques require modification to make them suitable for short degraded sequences of aDNA.

aDNA, retrieved either from soft or hard tissues, is highly fragmented and degraded with environmentally induced mutations (e.g. temperature, humidity, pH of the soil, radiation, and microorganism activity) (152-154). It may also contain inhibitors to PCR. aDNA quality is marginal and aDNA quantity is minimal. aDNA amplification protocols must be accurate and sensitive (155-157). Avoidance of contamination with exogenous modern DNA is crucial and authenticity of the results must be established through multiple analyses of the same sample (158). Limitations in studying aDNA will be discussed in more detail throughout this thesis and specifically in Chapter 4, which illustrates the unique challenges in accurate analysis of aDNA from human remains.

3.4. Previous aDNA research on Lake Baikal's prehistoric populations

Previous researchers in the BAP project have examined mtDNA polymorphisms from skeletal samples from EN and LN-EBA cemeteries, by both RFLP analysis and direct sequencing of the HV1 region from bp 16191 to 16367, an area containing most of the Asian specific haplogroups (6,23,159-163). Previous work suggests possible discontinuity between the EN and LN-EBA populations, represented by the different mtDNA haplogroup distributions (6,159,160). mtDNA analysis from the Lokomotiv, Shamanka II (EN) and Ust'-Ida (LN-EBA) cemeteries indicated that the mtDNA haplogroup distribution in Lokomotiv and Shamanka II cemeteries is similar (159). Both demonstrated a high frequency of haplogroups D and F and low frequency of haplogroups A and C (6,162). However, there is a significant difference in mtDNA haplogroup distribution between the Lokomotiv /Shamanka II cemeteries and the Ust'-Ida cemetery sample. Ust'-Ida showed a high frequency of haplogroup A and C and low frequency of haplogroups D and F (159,162). These findings are illustrated in the pie chart (Fig. 1.8).

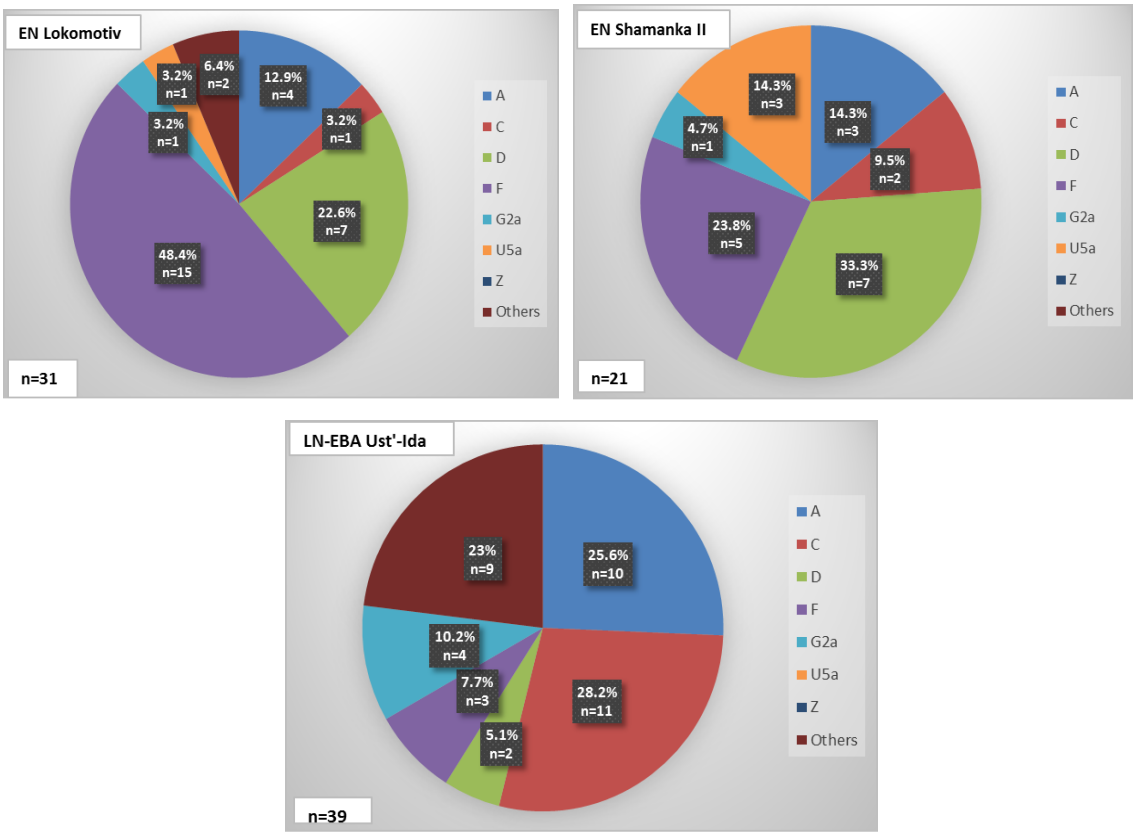

Figure 1.8. Pie charts illustrating the mtDNA haplogroup frequencies of the three Cis-Baikal cemeteries (Lokomotiv and Shamanka II (EN) and Ust'-Ida (LN-EBA)) (created from (6,159,160,162)).

3.5. Novel aDNA research on Lake Baikal's prehistoric populations

My research focused on four main aspects:

1. A detailed review of the previous mtDNA haplogroup distribution within and between the previously analyzed Cis-Baikal cemeteries (Lokomotiv, Shamanka II, and Ust'-Ida) is outlined in Chapter 2 of this thesis.

2. No previous mtDNA analysis had been undertaken on a second LN-EBA site, Kurma XI. DNA analysis from Kurma XI burials has strengthened our understanding of the genetics of LN-EBA populations, as Ust'-Ida was the only LN-EBA cemetery examined to date. More details about the Kurma XI cemetery are discussed in Chapter 2 of this thesis.

3. Examination of the paternal lineage through Y-chromosomal polymorphisms is a novel approach for BAP and facilitated the assessment of the paternal continuities and/or discontinuities within and between the EN and the LN-EBA groups, and complemented the maternal data (Chapter 3). Based on the reliability, sensitivity and robustness of the SNaPshot assay (121), we decided to determine Y-chromosomal haplogroup distribution of individuals from Lokomotiv and Shamanka II (EN), Ust'-Ida and Kurma XI (LN-EBA) cemeteries through the analysis of Y-chromosomal SNPs. Modification of the published methods and the significance of the results is discussed in more detail in Chapter 3.

4. Technical difficulties during sample preparation are addressed; DNA extraction, and PCR amplification, and methods used for optimization in Chapter 4.

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Chapter 2

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Genetic and Archaeological Background of the Kurma XI Cemetery in the Little Sea Micro-region of Cis-Baikal, Siberia

1. Introduction

The Cis-Baikal region in Siberia, Russia (52°–58° north latitude, 101° –110° east latitude) is characterized by numerous large mortuary complexes belonging to Middle Holocene foragers. Extensive archaeological research of these sites suggests that the area was home to two temporally distinct populations from Early Neolithic, EN (8000-6800 cal BP) to Late Neolithic-Early Bronze Age, LN-EBA (5800-4000 cal BP). The EN group was separated from the LN-EBA group by about a 1000 year gap (hiatus) during which large mortuary sites are entirely absent (1). In addition to temporal changes, variation in lifeways is also visible between the area's four micro-regions: South Baikal, Angara River Basin, Lena River Basin and Little Sea (or Ol'khon) region along the Lake's northwest coast (Figure 2.1: REGIONAL MAP). Research by the Baikal Archaeology Project (BAP) over the last 15 years has endeavored to explain this unique culture history through extensive multidisciplinary work, focusing on the synthesis of biological, environmental, and archaeological data.

Ancient DNA (aDNA) analysis has provided a powerful new tool for archeological research. An array of new techniques has greatly improved analytical sensitivity and has facilitated analysis of the genetics of prehistoric human populations (2,3), while increasing knowledge of the genetics of modern human populations that allowed researchers to compare prehistoric and modern human populations (4,5). Rigorous attention to field excavation techniques, laboratory design, and use of experimental conditions to maximize sensitivity and to avoid contamination from extraneous DNA has increased the robustness and reliability of the analysis (2,3,6,7). More specifically, analysis of mitochondrial DNA (mtDNA) in prehistoric human populations is facilitated by its high copy number, high mutation rate and pattern of maternal inheritance leading to the accumulation of population-specific polymorphisms referred to as haplogroups. This allows the study of population origins and movement patterns through the female lineage (8,9). Dependable methods for Y-chromosomal analysis were developed several years after mtDNA analysis. The haploid Y-chromosomal non-recombinant region (NRY) loci, which accounts for 95% of the Y-chromosome (10), is paternally inherited and reflects the male lineage (11).

Ancient DNA studies were first conducted in the Cis-Baikal by Russian scholars (e.g. (12,13)). The Russian scholars studied mtDNA for 19 individuals found in the Ust'-Ida cemetery, and they compared the mtDNA results to the modern populations of Siberia, Mongolia and the Urals. They found that the Baikal Neolithic populations are ancestral to the modern contemporary Siberian populations (12, 13). More recent analysis by BAP of mtDNA haplogroup distribution in individuals from the various cemeteries (Lokomotiv, Shamanka II and Ust'-Ida) has suggested that the EN population is genetically distinct from the LN-EBA groups (5,14-17). In addition, it appears that at least some attributes of mortuary practices, such as spatial arrangement of graves and body treatment, may be related to maternal kinship lineages (15). These genetic data have also been used to evaluate large scale regional and temporal patterns of population affinity across Siberia and Inner Asia (e.g. (16, 18)). However, all previous ancient DNA work in this region has been limited to the analysis of mtDNA, which only provides insights into maternal lineages, thus excluding paternal contributions. This focus on mtDNA is a limitation because we know that female and male migration patterns can be quite different (19). In addition, previous work has been geographically limited to cemeteries located in the South Baikal and Angara River Basin, and all previous attempts to extract DNA from bones and teeth from sites located on Baikal's Little Sea (or Ol'khon) micro-region along the northwest coast of the Lake, have failed due to poor preservation of skeletal material (e.g. (20)). Therefore, existing genetic data for the Cis-Baikal may not be representative in a spatial, temporal, or cultural sense. This is especially the case for the post-hiatus material, since the entire LN-EBA genetic dataset comes from only a single site (Ust'-Ida), which contains both LN and EBA graves, located in the Angara Valley, and it is rather doubtful that this cemetery is representative of the contemporary human genetic landscape in the region. These biases prevent us from fully investigating the extensive behavioral patterning identified from mortuary, biological and geochemical data, or from evaluating the genetic relationships between these various groups (21).

Significant recent technological advances in ancient DNA research now provide us the opportunity to address some of the limitations with previous ancient DNA research in the Baikal

region by evaluating both maternal (mtDNA) and, for the first time in this region, paternal (Y-chromosomal DNA) lineages of individuals.

In this study, ancient DNA from the prehistoric cemetery Kurma XI, which contains both EN and EBA graves, has been examined. Kurma XI is located in the previously unrepresented Little Sea micro-region. Therefore, these data will expand the existing ancient DNA evidence from the Cis-Baikal both geographically and temporally.

2. Background

Since 1996, an international collaboration of researchers, BAP, has attempted to reconstruct the lifeways of ancient foragers from the Lake Baikal region through the application of several groups of research methods including archaeology, human osteology, bone chemistry, and environmental studies (1). This work has demonstrated that the populations on either side of the hiatus differed in terms of diet, health, mobility patterns, demography, spatial distribution, social organization, and mortuary protocols. Genetic evidence from four sites has contributed to this analysis: the EN cemetery Shamanka II, located at the southwestern tip of Lake Baikal; the EN cemetery Lokomotiv, located on the Angara river in what is now the city of Irkutsk, the LN-EBA cemetery Ust'-Ida, also located on the Angara river, downstream of Lokomotiv, and LN-EBA cemetery Khuzhir-Nuge XIV on the northwest coast of the Little Sea area of Lake Baikal and 15 km southwest of the Kurma XI cemetery (Fig. 2.1).

Human skeletal and dental remains from all four sites were examined initially by restriction fragment length polymorphism (RFLP) and later, as new methods were developed, by direct sequencing of the mtDNA hypervariable 1 region (HV1) from base pair (bp) 16191 to 16367, which contains most of the Asian specific haplogroups (15-17,22). Human skeletal and dental remains from Khuzhir-Nuge XIV were visibly poorly preserved and despite numerous attempts, DNA extraction from 44 bone samples and 34 teeth samples was unsuccessful (20). However, a recent attempt using modified aDNA techniques has identified mtDNA haplogroups in two teeth samples belonging to one EBA individual (K14_1997.011) from the Khuzhir-Nuge XIV cemetery (unpublished data).

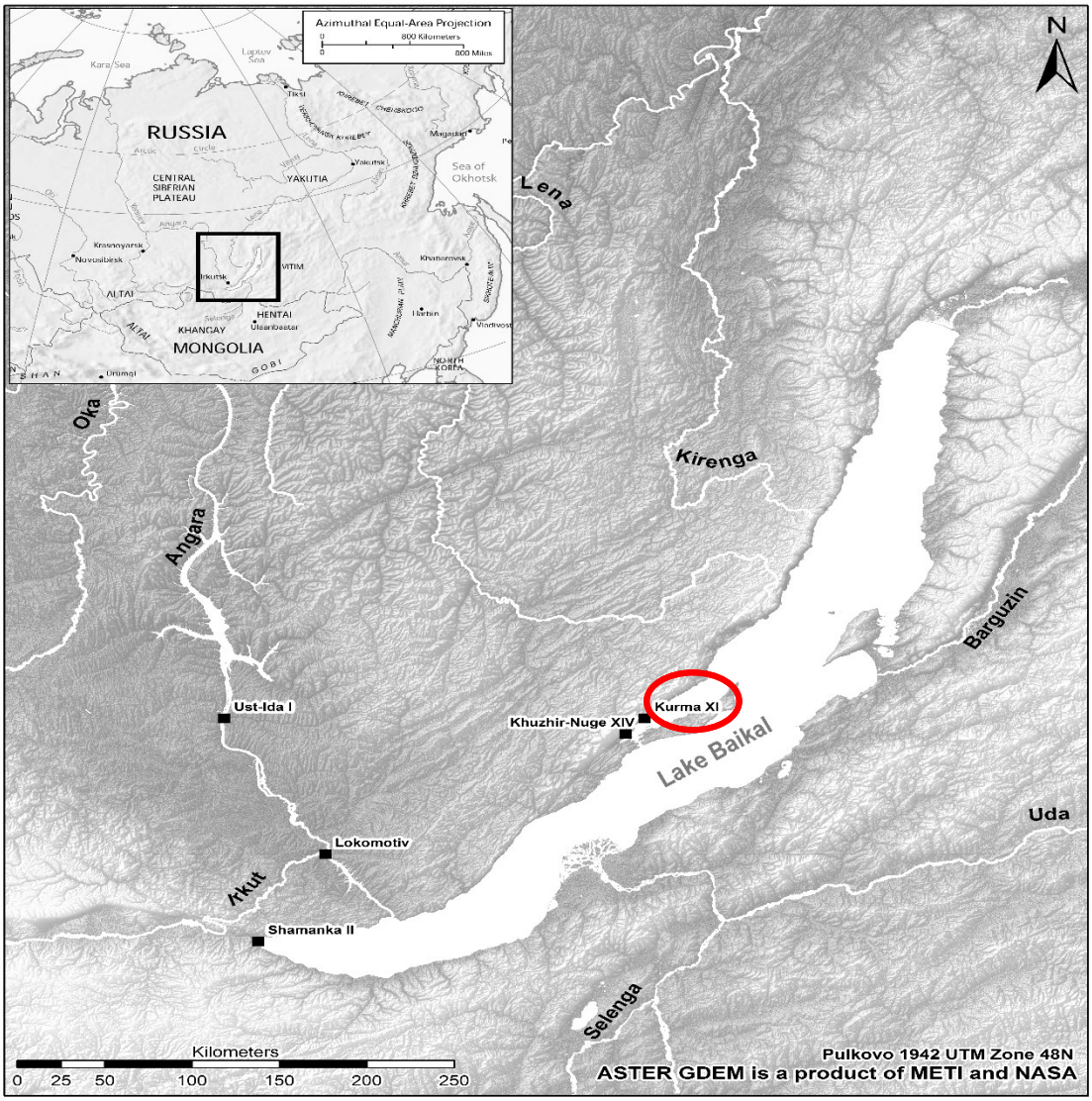

Figure. 2.1. Kurma XI cemetery (circled) location on Lake Baikal (33).

Adapted with permission from [Point taken: An unusual case of incisor agenesis and mandibular trauma in Early Bronze Age Siberia. A.R. Lieverse, I.V. Pratt, R.J. Schulting, D.M.L. Cooper, V.I. Bazaliiskii, A.W. Weber. International Journal of Paleopathology. 6. Copyright © 2014 Elsevier] (License number 3571161002487).

However, successful mtDNA analysis in samples from the remaining three sites suggests possible discontinuity between the EN and LN-EBA populations, as evidenced by the different frequencies of mtDNA haplogroups (5,14,16). Previous DNA analysis of individuals from two EN cemeteries (Lokomotiv and Shamanka II) was successful in identifying six east Eurasian mtDNA haplogroups (A, C, D, F, G2a, U5a) in 31 out of 40 skeletal remains from Lokomotiv and 21 out of 28 samples from Shamanka II (5,15,17). The mtDNA haplogroup distribution was similar in the two burial sites with F and D accounting for 38.5% (n=20/52) and 27% (n=14/52) of haplogroups at Lokomotiv and Shamanka II, respectively. Using principal component (PC) analysis, Mooder et al. (2010) demonstrated that the EN population at Lokomotiv, because of the high prevalence of mtDNA haplogroups F and D, did not cluster with Sojots, Buryats and Tuvinians who currently occupy the broader region (5). In contrast, mtDNA analysis from 39 out of 42 individuals (29 LN and 10 EBA) from the LN-EBA cemetery, Ust'-Ida, demonstrated the same six haplogroups but with significantly different frequencies relative to the Lokomotiv ($p=0.001$; (16)) and Shamanka II haplogroups ($p=0.008$). Haplogroups A and C accounted for 54% (21/39) of mtDNA haplogroups at Ust'-Ida, and PC analysis demonstrated affinities with modern Siberians (Buryats and Yakuts) living close to Cis-Baikal (5,14).

Because of methodological restraints, all previous ancient DNA work on the cemeteries discussed has been limited to mtDNA analysis, which provides insights into maternal lineages but excludes paternal contributions. In addition, as noted, all of the cemeteries are located in the south Baikal (Shamanka II) or Angara (Lokomotiv and Ust'-Ida) micro-regions, and only one of these cemeteries comes from the post-hiatus period. Thus, the ancient DNA data currently available may not be representative of the genetic background of all hunter–gatherer groups living in the entire Cis-Baikal and during all relevant archaeological periods. Furthermore, the number of individuals from whom ancient DNA has been examined are only a small proportion of individuals interred in graves (29). Samples have been selected based on quantity of sample, availability to the researcher, and degree of preservation. Ancient DNA results have been obtained from only 16% (n=31/194) of the individuals buried in Lokomotiv, 11.3% (n=21/186) of individuals buried in Shamanka II, 22.8 % (n=29/127) of LN individuals buried in Ust'Ida, and 5% of EBA individuals buried in Ust'-Ida (29). To address this problem of under-representation,

both mtDNA and Y-chromosomal haplogroup analysis were performed from individuals at the Kurma XI cemetery, which is located in the previously unrepresented Little Sea micro-region and contains individuals from both EN and EBA periods. Before selecting individuals for Y-chromosomal analysis, molecular sexing analysis was undertaken to make sure that males from the Kurma XI sample were selected for examination (Chapter 3). In addition, to expand the dataset of samples from the region, mtDNA from a further six EN Shamanka II individuals was analyzed, and four EN Shamanka II individuals, previously studied (17), were re-analyzed. This re-analysis is discussed in more detail in Chapter 4. All the mtDNA results from the EBA Kurma XI and the additional results from EN Shamanka II were compared statistically with the data previously obtained from the EN cemetery (Lokomotiv) and the LN-EBA cemetery Ust'-Ida to examine the effect of the new data on the maternal discontinuity concept between EN and LN-EBA prehistoric populations of Cis-Baikal.

2.1. The Kurma XI cemetery

Kurma XI is located on the northwest coast of Lake Baikal's Little Sea (Russian: *Maloe mor'e*). The 26 graves within the cemetery are distributed along the southeastern slope of a small hill approximately 500m from the lake's shoreline. Eighteen graves, all EBA, were arranged in a roughly linear ~200m long distribution along the base of the hill (6-16m above the lake), while eight graves (6 EN and 2 EBA) were arranged on small terraces approximately 18-32m above the lake. These two main clusters largely, although not entirely, correspond to the two different periods of cemetery use: EN and EBA.

Based on both typological and radiocarbon information, it seems that EN foragers first interred their dead at Kurma XI between ~6500-5800 cal BP[1], and that these initial six graves were built exclusively on the upper terraces (graves No. 20-24, 27). Owing to the small number of burials, the poor preservation of skeletal material, and the paucity of grave inclusions, relatively little can be said about the six graves that comprise the EN component at Kurma XI except that they appear to resemble contemporaneous graves from the Little Sea micro-region, more than those from the Upper Lena, and much more than those in Angara valley or on South Baikal (23).

[1] Recent examination of the radiocarbon evidence from this and other middle Holocene cemeteries in Cis-Baikal shows that these graves may be significantly older than previously believed and belong to the Late Mesolithic period (about 8300–7500 cal BP) rather than the EN (54).

Following a gap of at least ~2800 years in mortuary use of the Kurma XI cemetery, EBA foragers arrived at the site at ~4430 BP, and began to inter their dead (24). Two of the individuals examined for their ancient DNA (EBA KUR_2003.025, EBA KUR_2003.026) were interred on the upper terraces alongside the EN burials. Analysis of the ^{14}C dates at Kurma XI reveals that the site had two episodes of EBA use (24). The first phase (EBA-1), starting ~4770-4730 cal BP and ending ~ 4750-4690 cal BP, is characterized by considerable diversity – including both males and females ranging from older adolescents (17–19 years) to old adults (50+ years) who received a range of different burial protocols in all areas of the cemetery (e.g., two different body positions, single and double burials, some covered in ochre, diverse grave disturbance patterns, unique artifact inclusions, and variation in number and diversity of grave inclusions). During the second phase (EBA-2), starting at ~4560-4470 cal BP and ending ~4490-4400 cal BP, in contrast, there seems to be more homogeneity. This latter group of burials is dominated by males, all interred in single graves in extended-supine position in the southwest portion of the site, with no evidence of the use of ochre (with the exception of Burial No. 19 (KUR_2003.019), unusual also for its sitting body position and spatial location at the eastern end of the site). Artifact assemblages are generally larger than those from the EBA-1, and a number of artifact types are exclusive to this phase. What this shift to increasingly standardized and formalized burials with a narrower demographic profile indicates is, at present, unclear, and will likely require more extensive understanding of the broader cultural context of the EBA, including comparison with other, neighboring sites. On the basis of the more restricted demographic (no children) and large artifact assemblages with rare goods, McKenzie (2011) has suggested that Kurma XI might have acted as a 'specialized' or exclusive burial ground in comparison to other Little Sea sites such as the neighboring Khuzhir-Nuge XIV, which seem to have more of a 'community' representation (25). A 'community' cemetery is a cemetery where a broader proportion of the community is entitled to be buried in it, and this is represented in the variety of mortuary treatment and the diversity of demographic profile (25).

As a part of the archaeological research, estimation of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope signatures in human bone is considered the most direct and reliable method to determine diet and subsistence strategies of prehistoric populations (26). In BAP, the values of

$\delta^{13}C$ and $\delta^{15}N$ isotopes from the individuals' osteological remains were compared to $\delta^{13}C$ and $\delta^{15}N$ isotopes' ratios of local fauna and flora in different Cis-Baikal micro-regions to create a thorough descriptive reconstruction of prehistoric populations' diet and subsistence practices since the EN period to the LN-EBA period (27-29). The Little Sea hunter-gatherer ancient populations, during the EBA period, appear to have a heterogeneous source of diet (24,30,31). There are "… two different diets drawing on different food groups and perhaps on two different ecosystems" (32). The Kurma XI population was divided into two groups of diet; game-fish-seal (GFS) and game-fish (GF) according to the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures obtained (Table 2.1) (32).

As noted above, no ancient DNA data exist from Kurma XI, Khuzhir-Nuge XIV, or any other site in the Little Sea region. DNA analysis from Kurma XI would strengthen the previously published genetic evidence from the other regions and contribute to a better understanding of the genetic background from the Cis-Baikal as a whole, and specifically from the EBA population. New mtDNA data would allow comparison with the contemporary EBA data from the Angara valley.

3. Material and Methods

3.1. Contamination control

Contamination by modern DNA is one of the most difficult challenges that face researchers extracting aDNA from prehistoric specimens. aDNA is highly degraded, and the current extremely sensitive PCR techniques preferentially amplify intact contaminant modern DNA (34). Rigorous excavation and laboratory protocols were essential to detect and eliminate contamination sources. Published criteria for verifying authenticity of aDNA results have been established through multiple analyses of the same sample and were followed for all laboratory procedures in this study (6).

A laboratory at the University of Alberta was dedicated to aDNA analysis and was used to process, extract and amplify aDNA specimens. Researchers dressed in protective suits, booties, sterile sleeves, masks and goggles before entering the laboratory. All equipment and reagents were decontaminated either by bleaching, UV irradiation and/or autoclaving before entering the laboratory depending on the suitability of the material. All equipment and bench surfaces

were cleaned with a 10% (v/v) bleach solution. Sterile filtered tips were used for each analysis. Negative controls (reagents with no added DNA) were introduced during key extraction and PCR amplification steps. Positive modern DNA controls were analyzed only in the dedicated Post-PCR laboratory.

3.2. Samples

Vertebral bone or tooth samples were available from 4 EN and 13 EBA Kurma XI individuals for aDNA analyses (Table 2.1). Vertebral bones were selected in our study for two main reasons. Firstly, vertebrae contain a high proportion of spongy bone tissues, which have a higher DNA yield compared with other types of bone tissue (35). Secondly, because multiple vertebrae are available from one individual, destruction of tissue for DNA extraction and analysis is seen as more justifiable. Teeth are a preferred source of DNA relative to bones. Teeth are embedded within bone, where they are largely protected from environmental factors that might accelerate the DNA decomposition process (36), and their composition makes them less prone to contamination with extraneous DNA (37). Moreover, the enamel, an acellular tissue covering the tooth crown and considered to be the hardest tissue in the human body (38), protects the tooth pulp, a cellular tooth tissue that is the richest source of DNA in teeth (39). Molars were the preferred tooth samples because they have more than one root available per tooth for DNA extraction. Multi-rooted teeth provide more DNA than single rooted teeth (40) due to the larger amount of pulp in them (41). There were 30 tooth samples and five vertebral bone samples available for aDNA analysis from the Kurma XI cemetery. Three Kurma XI individuals had both bone and tooth samples available for aDNA analysis (KUR_2002.013, KUR_2002.014 and KUR_2002.015), and the rest of the Kurma XI individuals had more than one tooth sample available for analysis.

The 6 newly examined Shamanka II individuals had vertebral bone samples available for analysis (Table 2.2).

Table 2.1. Summary of archaeological, osteological and demographic data for Kurma XI individuals' (42) used in ancient DNA analyses.

No.	Master ID	Sample	Age	Morphological Sex	Burial Type	Period of Use	Sector	Diet
1	KUR_2002.001	Tooth	25-30	Male	Single	EBA-2	SW	GF
2	KUR_2002.007.01	Vertebra	20+	Undetermined	Double	EBA-1	SW	GFS
3	KUR_2002.007.02	Vertebral	20-29	Male	Double	EBA-1	SW	GFS
4	KUR_2002.010	Tooth	18-25	Probable Male	Single	EBA-2	SW	GFS
5	KUR_2002.012	Tooth	20+	Undetermined	Single	EBA-2	SW	GF
6	KUR_2002.013	Tooth	40+	Male	Single	EBA-2	SW	GFS
7	KUR_2002.014	Tooth & Vertebra	30-39	Female	Single	EBA-1	NE	GFS
8	KUR_2002.015	Vertebra	17-18	Probable Male	Single	EBA-1	NE	GF
9	KUR_2002.016	Vertebra	20-30	Probable Female	Single	EBA-1	NE	GFS
10	KUR_2003.017	Tooth	20+	Probable Male	Single	EBA-1	NE	GFS
11	KUR_2003.018	Tooth	17-19	Probable Female	Single	EBA-1	NE	GFS
12	KUR_2003.019	Tooth	20-30	Probable Male	Single	EBA-2	NE	GF
13	KUR_2003.021	Tooth	20-35	Probable Female	Single	EN	N	m.d.
14	KUR_2003.022	Tooth	50+	Probable Female	Single	EN	N	GF
15	KUR_2003.024	Tooth	20-35	Probable Female	Single	EN	N	GFS
16	KUR_2003.026	Tooth	35-50	Probable Male	Single	EBA-1	N	GFS
17	KUR_2003.027	Tooth	20-35	Undetermined	Single	EN	N	m.d.

- EBA = Early Bronze Age, EN = Early Neolithic
- EBA-1 = Early Bronze Age Phase 1, EBA-2 = Early Bronze Age Phase 2, EN = Early Neolithic
- SW = Southwest sector, NE = Northeast sector, N = North sector
- GFS = game-fish-seal diet, GF = game-fish diet
- m.d. = missing data

Table 2.2. The six newly analyzed Shamanka II samples with the individuals' age and morphological sex (42).

No	Master ID	Sample	Age	Morphological Sex	Period
1	SHA_2004.052.01	Vertebra	20-24	Probable Male	EN
2	SHA_2004.044.02	Vertebra	20+	Undetermined	EN
3	SHA_2005.059.01	Vertebra	35-39	Male	EN
4	SHA_2006.083.01	Vertebra	20-22	Male	EN
5	SHA_2007.090	Vertebra	18-20	Male	EN
6	SHA_2007.096.02	Vertebra	30-35	Female	EN

3.2.1. Sample preparation and decontamination

Decontamination protocols for the vertebral and tooth samples differed slightly. A sterile saw was used to cut the processes away from the body of the vertebra, and the root from the tooth crown, preserving the crown for other types of analysis. The outer surface of the vertebra was removed using a sterile scalpel. The vertebra was immersed in 16.7% (v/v) bleach for five minutes, and the root was immersed in 50% (v/v) bleach for 10 minutes to destroy any contaminating surface DNA, then samples were rinsed in HPLC grade water. The sample surface was exposed to UV light for one hour to destroy any remaining surface DNA and then left to dry in a sterile container for three days. The bleach concentration was optimized according to published protocols (43). The samples were frozen in liquid nitrogen while in the sterile containers, and then pulverized using sterile mortar and pestle. The bone or tooth powder was collected with a sterile spatula and stored in a sterile container at -20 degrees C until further analysis.

3.2.2. DNA Extraction from bones and teeth

The silica-guanidium thiocyanate extraction protocol was adapted from a previously published method (2), with some minor modifications. For reagent preparation, the extraction buffer was filtered using a sterile PVDF filter (0.22 µM, Millex- GV, Billerica, MA, USA). The extraction, the binding (before adding the guanidium thiocyanate), and the washing buffers (before adding the ethanol), were autoclaved for 20 minutes at 121 degrees C. All centrifugation times were doubled from the published method to assure a good separation between the precipitate and the supernatant. The last elution step was repeated twice to avoid transferring a large amount of silica, which can inhibit PCR amplification.

3.3. Mitochondrial DNA haplogroup analysis

mtDNA was amplified as previously described by Mooder et al. 2005 (15) with slight modifications. The PCR amplified the HV1 region of the mtDNA (bp 16191 to 16367), where most Asian specific polymorphisms were found (18,44). L16211 and H16346 primers (15) were designed to amplify a 176 bp sequence of mtDNA in a 40-cycle PCR amplification reaction. PCR amplification was performed using Eppendorf AG Thermocycler (Hamburg, Germany). Each 25µl PCR consisted of 1X PCR buffer (Invitrogen, NY, USA), 1.5 mM $MgCl_2$ (Invitrogen, NY, USA),

0.2 mM of each dNTP (Invitrogen, NY, USA), 0.4 mg/ml BSA (Roche, IN, USA), 1.0 µM of each primer (Invitrogen, NY, USA), and 1.25 U of Platinum *Taq* DNA Polymerase (Invitrogen, NY, USA). The amplification cycle started with a denaturing step at 95 degrees C for 2 minutes, and 40 cycles of 95 degrees C for 1 minute, 56 degrees C annealing temperature for 1 minute and 72 degrees C for 1 minute. For PCR clean-up, the ExoSAP-IT Clean-up Kit (GE Healthcare, Life Sciences, NY, USA) was used at The Applied Genomic Core (TAGC, University of Alberta). Then, sequencing was performed using the BigDye® Terminator Kit v3.1 (Life Technologies, NY, USA) at TAGC (University of Alberta) with the same PCR amplification primers (16). For post-reaction clean-up of unincorporated dyes, nucleotides and the primers, the magnetic beads Agencourt CleanSEQ (Beckman Coulter, California, USA) was used on an automated Biomek 3000 work station (TAGC). Samples were loaded on a 3130xl Genetic Analyzer (Life Technologies, NY, USA) and analyzed using Sequence Scanner v1.0 (Applied Biosystems, NY, USA). Sequence data were compared with the Cambridge reference sequence (GenBank number: NC_012920) (45,46), and base substitutions in the HV1 region were used to assign mtDNA haplogroups.

3.4. Molecular Sex Determination

Molecular sex determination was done as part of the Y-chromosome analysis. The sex of the Kurma XI samples was assigned by amelogenin analysis. Amelogenin, a gene involved in the formation of dental enamel, is found on both X and Y chromosomes (47). PCR primers for a region of intron 1 in the amelogenin gene gave a 106 bp product for females and 106/112 bp product for males (48). The PCR amplification was performed as stated above for mtDNA but with a modification of the annealing temperature to 62 degrees C. The amelogenin PCR amplification product was visualized via the Gel Doc™ EZ System (Bio-Rad, Ontario, Canada) after gel-electrophoresis on 12% polyacrylamide gel and ethidium bromide (10 mg/ml) staining.

3.5. Y-Chromosome Single Nucleotide Polymorphisms (SNPs) analysis

Specific Y-chromosomal SNPs representative of East Asian and Siberian paternal lineages were selected for this study. Y-chromosomal SNPs were analyzed through the SNaPshot Multiplex kit (Life Technologies, NY, USA) via the ABI PRISM 3130xl Genetic Analyzer (Life Technologies, NY, USA) using POP-4® (life Technologies, NY, USA). The method was optimized for aDNA (Moussa et al., 2015) by adaptation of a published protocol (49). The 13 SNP markers were examined via

two multiplex PCR amplification reactions. The modified PCR amplification was performed using an Eppendorf AG Thermocycler (Hamburg, Germany). Each 25µl PCR consisted of 1X PCR buffer (Invitrogen, NY, USA), 4.0 mM $MgCl_2$ (Invitrogen, NY, USA), 400 µM of each dNTP (Invitrogen, NY, USA), 1.0 mg/ml BSA (Roche, IN, USA), 2.0 U of Platinum *Taq* DNA Polymerase (Invitrogen, NY, USA), and the two multiplex primers' mixtures (Invitrogen, NY, USA) (49). The ExoSAP-IT PCR clean-up kit (GE Healthcare, Life Sciences, NY, USA) was selected for PCR clean-up. The SNaPshot results were analyzed on GeneMapper 4.0 software (Life Technologies, NY, USA). Y-chromosomal haplogroups were assigned according to the updated human Y-chromosomal haplogroup tree (50).

3.6. Y-chromosomal Q sub-haplogroup analysis

Haplogroup Q is identified by the M242 mutation (50) and is widely distributed among different groups in Siberia (51). While there are several sub-haplogroups of haplogroup Q, only three sub-haplogroups are represented in the Siberian population (Q1a*-MEH2, Q1a2-M25, Q1a3-M346) (52). Primers suitable for amplifying the SNP sites were designed (Chapter 3) using Primer3Plus software (53) (Table 2.3). Amplicon size was less than 155 bp to match the criteria used with the SNaPshot multiplex PCR reaction. PCR amplification was prepared and performed as stated above for mtDNA with only a modification of the annealing temperature to 58 degrees C.

Table 2.3. Primers designed to amplify sub-haplogroups of Q-M242.

SNP Markers (Sub-haplogroups)	PCR primer sequence (5'→3')		Amplicon size (bp)	Annealing Temperature
	Forward	Reverse		
MEH2 (Q1a*)	CAAATTTTGAGTAAGCCATCACC	TGGAAACACAACTGTTTGAAAAT	150	58°C
M25 (Q1a2)	CACCCAGAGACACACAAAACA	TGTTGTAAGAATTCAGTAGGATTGATG	107	58°C
M346 (Q1a3)	TTTGTCTCTGAGCTGACAAGGA	TCCACTCACTCTGCCTACCTG	125	58°C

3.7. Evaluation of Authenticity

Avoidance of contamination with exogenous modern DNA is crucial, and authenticity of results was established through several criteria. First, each sample was subjected to multiple analyses (6). In this paper results were reported only if they were reproducible either by extracting DNA twice from the same sample (n= 8) or from two different samples belonging to the same individual at two independent occasions (n= 6). Samples that did not show consistent results (n= 1) or from which no DNA was obtained (n= 2) were excluded from the further analysis. Because only the East Siberian population were studied and their mtDNA (18,44) and Y-chromosomal haplogroups (50) any result apparently indicating a non-Asian haplogroup would have been excluded, although there were no samples in this category. Fortuitously, during this study, only females were working in the ancient DNA laboratory thus minimizing the risk of contamination by male DNA. All students or researchers working with the samples gave consent to have their mtDNA haplogroups assigned to facilitate early detection of contamination

3.8. Statistical analysis

Only EBA Kurma XI individuals (n=12) were included in the statistical analysis. The other two individuals were older than previously believed and belong to the Late Mesolithic period (about 8300–7500 cal BP) and were not ~~be~~ combined with the EBA individuals (54). The newly obtained mtDNA haplogroups from the EN Shamanka II individuals (n=6) and the re-analyzed EN Shamanka II individuals (n=4) were included in the statistical analysis.

The mtDNA haplogroup distributions from the four prehistoric cemeteries (EBA Kurma XI, LN Ust'-Ida and EBA Ust'-Ida, EN Lokomotiv and EN Shamanka II) were compared to each other

using Fisher's exact test with a two-by-two contingency table. The table extended to the size described by the number of populations and the number of haplogroups examined in the study. Fisher's exact test is analogous to two-way contingency chi-square test, but is always chosen when dealing with a small sample size as in this study. The two-way contingency chi-square test requires that any given cell have a minimum frequency of five (55), which was not obtainable in this study for some haplogroups.

Fisher's exact test was performed to estimate the genetic relationship, similarity or difference, between the four Cis-Baikal prehistoric populations separately. In addition, the relationship in genetic diversity between the Cis-Baikal prehistoric populations in the three different periods (EN, LN and EBA) was estimated in relation with the modern Siberian populations living in or around Lake Baikal region. Therefore, the LN-EBA cemetery sample from Ust'-Ida was divided into two groups, LN Ust'-Ida individuals (n=29) and the EBA Ust'-Ida individuals (n=10). Fisher's exact test was re-evaluated after obtaining the results from the newly analyzed EBA Kurma XI and EN Shamanka II individuals to assess thier effect on the estimation of genetic discontinuity between EN and LN-EBA populations and to compare it to Mooder's et al. (2006 & 2010) results (5,16). Fisher's exact test was performed using the **SAS/STAT®** software.

Biological distances were predicted from mtDNA haplogroup frequencies between modern and prehistoric (EN, LN and EBA) Siberian populations using Nei's pairwise G_{ST} estimate (56). To calculate pairwise G_{ST} estimate, it is essential to have a large sample size (n>20) to produce a reliable estimate of differentiation (57-60) and to reduce the bias created by a small sample size (61). The small sample sizes at both EBA Kurma XI (n=12) and EBA Ust'-Ida (n=10) prevented us from comparing these two populations' genetic differentiation independently to the indigenous Siberian population without pooling their data together. However, EN Lokomotiv (n=31) and EN Shamanka II (n=27) were compared both independently, because the sample sizes allowed, and with pooling the EN Lokomotiv with the EN Shamanka II dataset to estimate the genetic relationship between the prehistoric EN populations of Cis-Baikal and the modern Siberian populations. The LN Ust'-Ida (n=29) was compared separately to the modern Siberian population because it is the only dataset representing Cis-Baikal's prehistoric population during

the LN period. According to Nei (1973) "G_{ST} is equivalent to Wright's F_{ST}, and is often referred to as the coefficient of gene differentiation" (56):

$$G_{ST} = D_{ST} / H_T$$

$$H_T = 2x\,(1 - x)$$

and $$D_{ST} = 2\sigma^2_x$$

where x is the mean of the frequency of an allele among subpopulations, and σ^2_x is the variance of the frequency of an allele in subpopulations. mtDNA haplogroup frequencies of several indigenous Siberian populations and two prehistoric populations (Fig. 2.2) were incorporated for comparative analysis with Lake Baikal's prehistoric populations (Table 2.4). Pairwise G_{ST} estimate was calculated using the **SAS/STAT®** software, and results from pairwise G_{ST} estimate were plotted on a two-dimensional PC plot using **SAS PROC PRINCOMP** software.

Figure 2.2. A map representing the geographic distribution of modern and prehistoric Siberian populations used for comparative analysis in this study. KRK= Koryak, ITL=Itel'men, YKT=Yakut, BUR=Buryat, EVK=Evenkis, KET=Kets, TOF=Tofalar, TDJ=Todjins, TUV=Tuvanians, ALT=Altaians, EYG=Egyin-Gol, KRG=Kurgan, LOK=Lokomotiv, SHA=Shamanka II, KUR=Kurma XI, UID=Ust'Ida.

Table 2.4. Mitochondrial DNA haplogroup frequencies (%) of prehistoric and modern Siberian populations.

	n	A	C	D	F	G2a	U5a	Z	Others	References
Prehistoric groups										
Lokomotiv (EN)	31	12.9	3.2	22.6	48.4	3.2	6.4	0	3.2	(15)
Shamanka II (EN)	27	18.5	18.5	40.7	11.1	7.4	3.7	0	0	(17) &This study
Ust'-Ida (LN)	29	24.1	13.8	6.9	10.3	13.8	3.4	0	27.6	(16)
Lokomotiv (EN)/ Shamanka II (EN)	58	15.5	10.3	31	31	5.2	5.2	0	1.7	(15) /(17) &This study
Kurma XI (EBA)/ Ust'-Ida (EBA)	22	18.2	31.8	27.3	13.6	0	0	9.1	0	This study/ (16)
Egyin-Gol	46	17.3	13	41.3	8.6	2.1	4.3	0	13	(99)
Kurgan	26	0	7.6	0	3.8	3.8	7.6	3.8	73	(64)
Modern groups										
Tofalars	58	5.1	62	0	0	1.7	0	0	31	(83)
Altaians	110	0	19	15.4	8.1	0.9	16.3	0	40	(83)
Tuvinians	90	1.1	47.7	17.7	2.2	2.2	3.3	0	25.5	(83)
Todjins	48	4.1	47.9	4.1	2	0	6.2	0	35.4	(83)
Buryats	91	2.1	28.5	32.9	1	14.2	1	0	19.7	(83)
Evenks	79	3.7	48.1	26.5	1.2	2.5	0	0	17.7	(83)
Yakuts	62	0	41.9	27.4	1.6	1.6	0	0	27.4	(83)
Kets	38	7.8	15.7	2.6	23.6	0	5.2	2.6	42.1	(73)
Koryaks	155	5.1	36.1	1.2	0	41.9	0	5.8	9.6	(18)
Itel'men	47	6.3	14.8	0	0	68	0	6.3	4.2	(18)

4. Results

mtDNA results were obtained from 14 out of 17 individuals (12 EBA individuals and 2 EN individuals) from Kurma XI (82% success rate; Table 2.5). Four mtDNA haplogoups (A, D, F, Z) were assigned to 14 individuals. Six individuals belong to haplogroup D (base substitutions at 16223 'C>T'), five belong to haplogroup F (base substitutions at 16232 'C>A', 16249 'T>C', 16304 'T>C', and 16311 'T>C'), two belong to haplogroup Z (base substitutions at 16223 'C>T' and 16260 'C>T'), and one belongs to haplogroup A (base substitutions at 16223 'C>T', 16290 'C>T', and 16319 'G>A'). One haplogroup D individual additionally carries a 16224 'C>T' transition, which has not been found previously to be associated with haplogroup D in the prehistoric population of Cis-Baikal. Results are summarized in Table 2.5.

All mtDNA results from Shamanka II are summarized in Appendix 2.1. Of the four re-analyzed samples, one belongs to haplogroup A (base substitutions at 16223 'C>T', 16290 'C>T', and 16319 'G>A'), two belong to haplogroup D (base substitution at 16223 'C>T'), and one belongs to haplogroup G2a (base substitutions at 16223 'C>T', 16227 'A>G', and 16278 'C>T'). Of the six newly analyzed Shamanka II samples, two belong to haplogroup D (same base substitution as stated above), three belong to haplogroup C (base substitutions at 16223 'C>T', 16298 'T>C' and 16327 'C>T') and one belongs to haplogroup A (same base substitutions as stated above).

Molecular sex using amelogenin analysis in 11 Kurma XI individuals identified six males and five females. In nine individuals molecular sex was concordant with the morphological sex. For one individual (KUR_2003.018), molecular sex assignment was a male discordant with morphological sex, which identified the individual as a probable female. One individual (KUR_2002.007.01) with undetermined morphological sex, and interred in the only double grave in Kurma XI, was female through molecular sexing (Table 2.5). In cases where discordance was found between molecular and morphological sexing, we relied on the molecular sex for further Y-chromosomal haplogroup analysis. For the three individuals (KUR_2002.010, KUR_2002.013 and KUR_2003.026), where molecular sex assignment was not successful, morphological sex assignment (probably male or male) was assumed to be correct in selecting samples for Y-chromosomal analysis.

Y-chromosomal haplogroup analysis was successful in five males. Two males were assigned to haplogroup Q-M242 through SNaPshot analysis. Further testing of the Q-M242 sub-haplogroups was not possible because of inadequate sample quantity. Three males were assigned to sub-haplogroup Q1a3-M346 by SNaPshot and sub-haplogroup analyses. Q-M242 and/or its sub-haplogroup Q1a3-M346 were the only haplogroups represented in the males of Kurma XI. Figure 2.3 illustrates the spatial distribution of mtDNA and Y-chromosomal DNA haplogroups plus the molecular sex assignment across the Kurma XI cemetery site map.

Table 2.5. Summary of the mtDNA haplogroups and their base substitutions, Y-chromosomal haplogroups and molecular sex assignment for Kurma XI individuals.

No	Master ID	mtDNA Haplogroup	HV1 Variants +16000	Molecular Sex	Morphological Sex	Y-Chr Haplogroup
1	KUR_2002.001	D	223 319	XY	Male	m.d.
2	KUR_2002.007.01	D	223	XX	Undetermined	
3	KUR_2002.007.02	D	223 319	XY	Male	Q1a3
4	KUR_2002.010	Z	223 260	m.d.	Probable Male	Q1a3
5	KUR_2002.012	m.d.	m.d.	m.d.	Undetermined	m.d.
6	KUR_2002.013	A	223 290 319	m.d.	Male	m.d.
7	KUR_2002.014	F	232A 249 304 311	XX	Female	
8	KUR_2002.015	Z	223 260	XY	Probable Male	Q1a3
9	KUR_2002.016	F	232A 249 304 311	XX	Probable Female	
10	KUR_2003.017	D	223	XY	Probable Male	Q
11	KUR_2003.018	D	223 224	XY	Probable Female	Q
12	KUR_2003.019	F	232A 249 304 311	XY	Probable Male	m.d.
13	KUR_2003.021	m.d.	NA	m.d.	Probable Female	m.d.
14	KUR_2003.022	F	232A 249 304 311	XX	Probable Female	
15	KUR_2003.024	F	232A 249 304 311	XX	Probable Female	
16	KUR_2003.026	D	223	m.d.	Probable Male	m.d.
17	KUR_2003.027	Inconsistent Results	m.d.	m.d.	Undetermined	m.d.

- m.d. = Missing data, no data obtained from the marked individuals

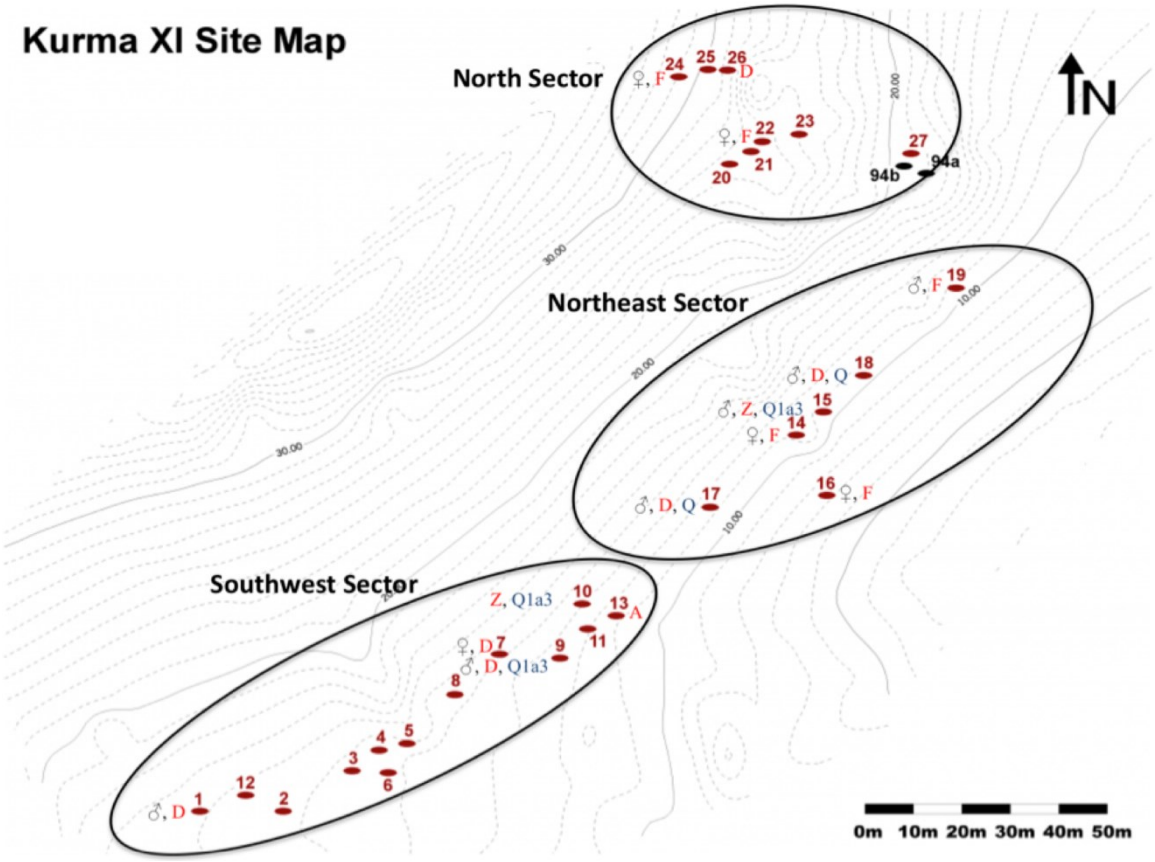

Figure 2.3. Distribution of graves in Kurma XI cemetery and its different sectors with mtDNA (red) and Y-chromosomal (blue) haplogroups assigned to the individuals with their molecular sex (black).

5. Discussion

This is the first study providing genetic data from the Kurma XI cemetery. It gives information on the genetic background of the EN/EBA cemetery in the Little Sea micro-region in Lake Baikal and a comparison with mtDNA analysis from previously studied cemeteries in the Cis-Baikal with the newly and re-analyzed samples from Shamanka II.

5.1. A closer look at mtDNA haplogroup distribution at Kurma XI samples

In addition to the comparisons described above, two EBA individuals from Kurma XI belong to haplogroup Z, the first time this haplogroup has been identified in the Cis-Baikal prehistoric population. Haplogroup Z is a subcluster from the macro-haplogroup M (62). Its sister clade, haplogroup C, has higher frequency and diversity in eastern Eurasian populations (63). Haplogroup C is present in the Cis-Baikal prehistoric populations as described above (16,17). Both haplogroups C and Z are represented in the ancient south Siberian Kurgan people (Bronze Age), with frequencies at 7.6 % (n=2/26) and 3.8 % (n=1/26), respectively (64). Haplogroup Z is considered to be one of the dominant haplogroups in the northern East Asian populations (65). Haplogroup Z is widely distributed throughout the northern Lake Baikal region.

In modern populations, haplogroup Z is found in northeast Asia (Itel'mens, 6.3%, n=3/47) and (Koryaks, 5.8%, n=9/155) (18), but is also present in south Siberian populations, for example, the Altai-Sayan population (range between 1.1 to 6.5%) (66). It is widespread among numerous Finnic- and Turkic-speaking people in the Volga-Ural region (e.g. Udmurts, 5%, n=5/101, Maris, 2.9%, n=4/136, and Komis, 1.6%, n=1/62) (67,68) Haplogroup Z also appears in other Siberian populations: the Evens, Yukaghirs and Dolgans and in the Northern Yakuts and Evenks (69). The age of sub-haplogroup Z1a, about 9,400 years, would imply its existence in the northern Siberian region predating the Neolithic era (69). Haplogroup Z is also present in the European population (e.g. Scandinavian-Saami, 1.3%, n=6/445), which might be due to its recent expansion (70). With respect to the only double grave in Kurma XI, both individuals (one was identified as a XY (KUR_2002.007.02, age 20-29) and one as a XX (KUR_2002.007.01, 20+) through molecular sex) belong to haplogroup D. However, they are not related through the maternal lineage because each individual has different SNP markers identifying haplogroup D (Table 2.5). Both individuals in the double grave had the same diet GFS (game-fish-seal) (32).

Neither of the two individuals had undergone any specific burial treatments such as staining with ochre or burning. However, a greater number of artifacts was associated with the male burial (KUR_2002.007.02) (e.g. different shapes of arrowheads, a leaf-shaped bifacial knife, a copper/bronze knife) (71). These two individuals might have been a husband and wife, but we do not have evidence to prove that.

Two EN individuals from Kurma XI, (KUR_2003.022 and KUR_2003.024) were both found to belong to mtDNA haplogroup F. These two EN individuals were assigned XX (female) through molecular sexing. Haplogroup F is found also in EBA Kurma XI (25%, n=3/12), and the other Cis-Baikal cemeteries, LN Ust'-Ida (10.3%, n=3/29), Lokomotiv (48.4%, n=15/31) and Shamanka II (11.1%, n=3/27), but was absent in EBA Ust'-Ida.

5.2. mtDNA results from Cis-Baikal and comparison with modern Siberian populations

Table 2.6 illustrates the matrix of genetic distance between the modern and prehistoric Siberian populations using pairwise G_{ST} estimate, and Figure 2.4 represents the PC plot generated by the results from G_{ST} genetic distances of Table 2.6. The first two components from the PC plot explain 22.12% of the total variance appearing from the G_{ST} estimates of the mtDNA haplogroup frequency distribution in modern and prehistoric Siberian populations. From the PC plot we can deduce several useful points. Firstly, the PC plot shows that Tuvinians are an outlier, while the remaining groups cluster together. Secondly, the EBA Kurma XI_EBA Ust'-Ida group is in close affinity with the Itel'men population, which was not shown previously (16). This close genetic affinity between the EBA Kurma XI_EBA Ust'-Ida and the Itel'men can be due to the increase in haplogroup C frequency, which comprises 70% (n=7/10) of the available EBA Ust'-Ida population. The frequency of haplogroup C in EBA Kurma XI_EBA Ust'-Ida is 31.8% (n=7/22) compared to 14.8 % (n=7/47) in the Itel'men population (18). Also, the novel appearance of haplogroup Z, more frequent in northeastern Siberian populations (18,72), gives the EBA Kurma XI_ EBA Ust'-Ida group a closer affinity with some northeast Siberian populations such as the Koryaks and Itel'men. Furthermore, because of the presence of haplogroup Z, the EBA Kurma XI_ EBA Ust'-Ida population now clusters more closely with Kets than previously described by Mooder et al. (2006) (16); haplogroup Z is present in 9.1%

(n=2/22) of the EBA Kurma XI_ EBA Ust'-Ida populations and 2.6% (n=1/38) in the Kets (73) (Table 2.4).

Thirdly, LN Ust'-Ida appears in close association with Buryats, which was previously suggested by Mooder et al. (2010) (5). EN Shamanka II associate closely to the Egyin Gol ancient Mongolian population, and this close affinity can be due to the fact that both of EN Shamanka II and the Egyin Gol have a high frequency of haplogroup D (40.7%, n=11/27, and 41.3%, n=19/46, respectively). EN Lokomotiv is not in a close affinity to any particular modern Siberian population. However, Lokomotiv_Shamanka II pooled data are close to the Todjins, which could be due to the presence of haplogroup U5a in both populations (5.2%, n=3/58 in Lokomotiv_Shamanka II, and 6.2%, n=3/48 in Todjins).

mtDNA haplogroups A and D, in EBA Kurma XI (8.3%, n=1/12, and 50%, n=6/12, respectively) and EN Shamanka II (18.5%, n=5/27) and 40.7%, n=11/27, respectively), are both present in the modern Siberian population. Haplogroup A is present in several Siberian populations including Kets (7.8%, n=3/38), Itel'men (6.3%, n=3/47) and Koryaks (5.1%, n=8/155) (18,73), but reaches a higher frequency in the northeastern Siberian populations (18,74-79). Haplogroup D is significantly represented in the eastern Siberian population from the west at the Yenisey River and as far east at the Bering Sea (18, 80-82).

Haplogroup F, the second most common haplogroup represented in EBA Kurma XI (25%, n=3/12) is found in several Siberian populations surrounding Lake Baikal area, including Kets (23.6%, n=9/38)(73), Altaians (8.1%, n=9/110), Tuvinians (2.2%, n=2/90), Todjins (2%, n=1/48) and Buryats (1%, n=1/91) (83). The presence of haplogroup F in EBA Kurma XI and the other Cis-Baikal EN (e.g. Lokomotiv, 48.4%, n=15/31, and Shamanka II, 11.1%, n=3/27) and LN Ust'-Ida cemeteries (10.3%, n=3/29) (5,14-17,22) indicates that this haplogroup existed for some time in the prehistoric and indigenous southeastern Siberian populations (84).

Despite the small sample size of EBA Kurma XI, the mtDNA signature is of importance in understanding the maternal background of the area. The obvious difference between mtDNA haplogroup distribution at EBA Kurma XI, LN Ust'-Ida and EBA Ust'-Ida, and the similarities in the mtDNA profile between EBA Kurma XI, EN Lokomotiv and EN Shamanka II individuals might indicate that the maternal background of EBA Kurma XI is in closer genetic affinity to the EN Cis-

Baikal populations, and both LN Ust'-Ida and EBA Ust'-Ida are outliers. However, it has been suggested that Kurma XI is an 'exclusive' or a 'specialized' cemetery with some unique burial practices and small size (23,25). Therefore, the Kurma XI population might not be representative of the entire population that inhabited the Little Sea micro-region during the EBA. It is unfortunate that DNA analysis has to date been unsuccessful from Khuzhir-Nuge XIV, the largest LN-EBA 'community' cemetery in the Little Sea micro-region (25,85,86). mtDNA haplogroup analysis from this cemetery would confirm one of three hypotheses: first, if Khuzhir-Nuge XIV were similar to either LN Ust'-Ida or EBA Ust'-Ida it would suggest that EBA Kurma XI might be an outlier; second, if Khuzhir-Nuge XIV were similar to EBA Kurma XI, it might suggest that both LN Ust'-Ida and EBA Ust'-Ida were outliers; and third, if Khuzhir-Nuge XIV showed a different haplogroup distribution to EBA Kurma XI, LN Ust'-Ida and EBA Ust'-Ida it would indicate that LN-EBA prehistoric population was heterogeneous with respect to maternal origins or that the cemeteries were being used by different population subsets, as it was shown with the new obtained Shamanka II data that the EN prehistoric population is heterogeneous with respect to maternal origin.

The improvments to the ancient DNA techniques, performed in our laboratory, have allowed us to determine that mtDNA of one individual from the Khuzhir-Nuge XIV cemetery (K19_1997.011) belonged to haplogroup D, and further analysis might allow us to evaluate these hypotheses.

Table 2.6. Matrix of G_{ST} genetic distances predicted by mtDNA haplogroup frequencies between modern and prehistoric Siberian population. Pairwise G_{ST} estimate was calculated using the **SAS/STAT®** software.

	LOK	SHA	LOK_SHA	LN_UID	EBA_KUR EBA_UID	Egyin_ Gol	Kurgan	Tofalars	Altaians	Tuvinians	Todjins	Buryats	Evenks	Yakuts	Kets	Koryaks	Itel'men
LOK	0	0.06563	0.0148	0.08114	0.07097	0.06918	0.24902	0.22937	0.10987	0.14918	0.17453	0.10912	0.14491	0.14172	0.08749	0.16865	0.23399
SHA		0	0.01868	0.0603	0.01672	0.00778	0.23943	0.16128	0.08549	0.07912	0.12091	0.03148	0.05823	0.06438	0.10759	0.10941	0.18496
LOK_SHA			0	0.05517	0.02933	0.02423	0.22446	0.17904	0.08163	0.09967	0.13196	0.05704	0.08807	0.08902	0.08021	0.12396	0.19178
LN_UID				0	0.05496	0.04811	0.10311	0.10173	0.03623	0.06307	0.0615	0.04671	0.07219	0.06389	0.02758	0.06746	0.13206
EBA_KUR EBA_UID					0	0.02719	0.22817	0.10671	0.07966	0.05006	0.08201	0.03606	0.03486	0.04684	0.08885	0.09172	0.1913
Egyin_ Gol						0	0.19059	0.15509	0.05888	0.07208	0.1067	0.02591	0.05743	0.05308	0.08257	0.12266	0.20183
Kurgan							0	0.20004	0.0616	0.15782	0.12481	0.15879	0.19753	0.15377	0.05966	0.22034	0.32039
Tofalars								0	0.09084	0.02378	0.01204	0.08975	0.04357	0.04784	0.10321	0.10416	0.26524
Altaians									0	0.042	0.03957	0.0408	0.05983	0.03843	0.02023	0.10905	0.20469
Tuvinians										0	0.0115	0.02681	0.00584	0.00532	0.0678	0.07839	0.20969
Todjins											0	0.05723	0.03198	0.02603	0.05426	0.09132	0.22909
Buryats												0	0.01972	0.01499	0.07349	0.06466	0.1508
Evenks													0	0.0055	0.09033	0.08321	0.21282
Yakuts														0	0.06945	0.09146	0.21635
Kets															0	0.11954	0.21213
Koryaks																0	0.04694
Itel_men																	0

- Population abbreviations: LOK=Lokomotiv, SHA=Shamanka II, EBA_KUR= Early Bronze Age_Kurma XI, LN_UID= Late Neolithic_ Ust'-Ida, EBA_UID= Early Bronze Age_Ust'-Ida.

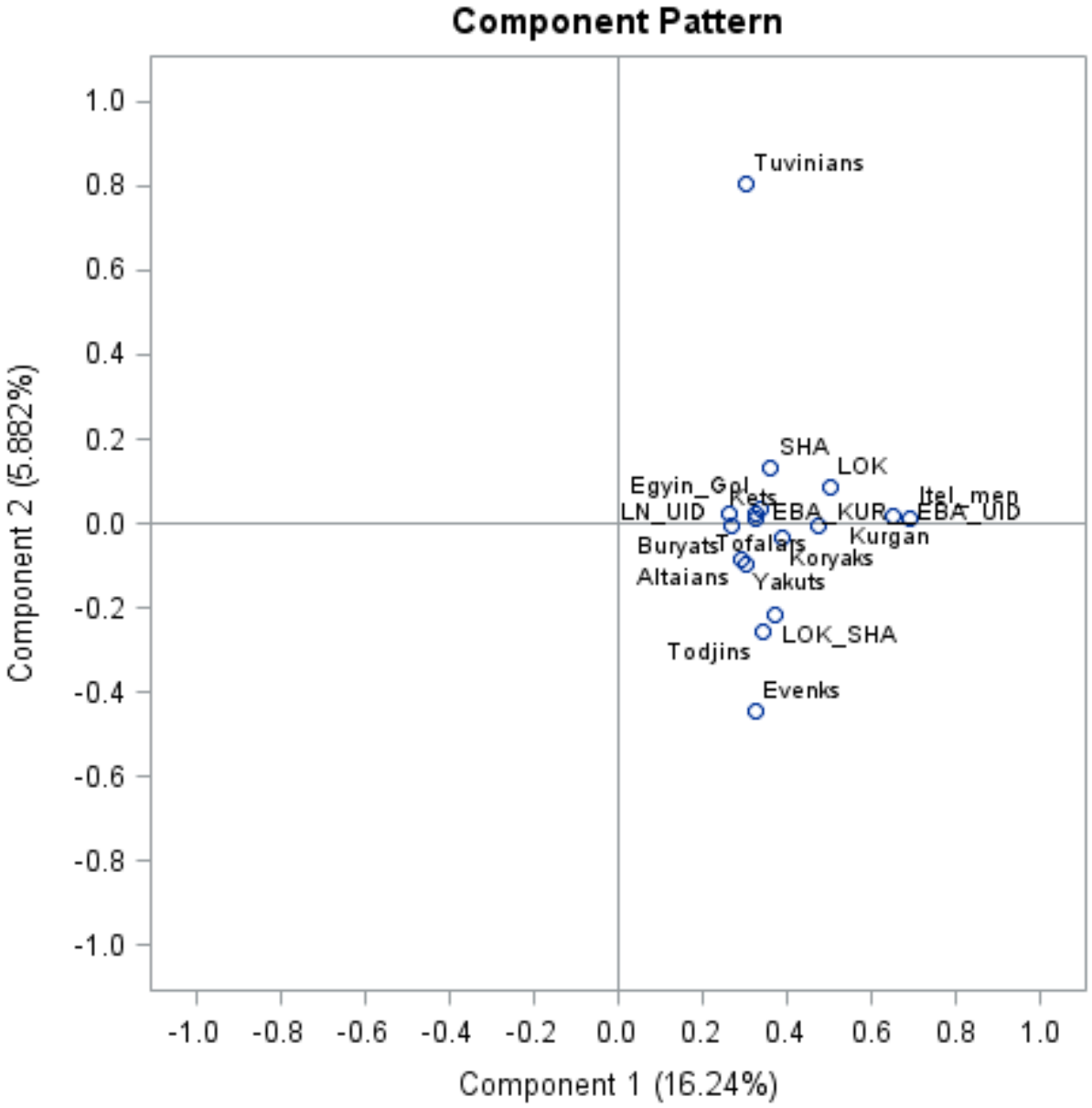

Figure 2.4. Principal component plot of G_{ST} estimates created by **SAS PROC PRINCOMP** software.

5.3. Y-chromosome results from Kurma XI and comparison with modern Siberian populations

Unfortunately, Y-chromosomal haplogroup analysis was only available from five EBA Kurma XI males, all belonging to haplogroup Q-M242, which is also the dominant Y-chromosomal haplogroup in the Ust'-Ida samples (Chapter 3).

The approximate age of this haplogroup is about 17,700 $\pm$ 4,800 years. Haplogroup Q-M242 is widely distributed across Siberia (51), Central to South Asia, West Eurasia and northern East Asia, which might be indicative of Q-M242 expansion through northern Eurasia as a migratory route (87). Individuals carrying haplogroup Q-M242 might have migrated from Siberia via the Altai/Baikal area to the Americas (88-90), which suggest the appearance of this haplogroup in the area just after the Last Glacial Maximum (52). Haplogroup Q-M242 is found in the Sel'kup and Ket populations of Siberia, and also in the Altai people and other Northeast Siberian groups, for example, the Yukagirs and Koryaks. The Q-M242 sub-haplogroup Q1a3a-M3 (Q-M3), the most frequent haplogroup in the Native American population (91), is not found in the Kurma XI samples. However, sub-haplogroup Q1a3-M346, which is a sister sub-haplogroup to Q1a3a-M3 (52), is identified in three of five males in Kurma XI samples. A study of several northern East Asian groups by Malyarchuk, B. et al. (2011) examining specific sub-haplogroups of Q-M242, showed that Q1a3-M346 is more frequent than other sub-haplogroups (e.g. Q1a*-MEH2, Q1a2-M25 and even Q1a3a-M3) in the Siberian modern populations. Q1a3-M346 is found in the Altaians (25.8%, n=23/89), Todjins (38.5%, n=10/26) and Tuvinians (38%, n=41/108), and also distributed in Khakassians (6.3%, n=4/64) and Sojots (7.1%, n=2/28), and even in Kalmyks (1.7%, n=1/60) population (52). Sub-haplogroups Q1a*-MEH2 and Q1a2-M25 were not found in the Kurma XI males.

The Y-chromosomal findings from the Kurma XI available males would support ancient links between the Siberian and the Native Americans.

5.4. Overall evaluation of mtDNA and Y-chromosomal DNA haplogroup distribution in EBA Kurma XI

Summing the mtDNA and the Y-chromosomal DNA haplogroups results from the Kurma XI cemetery shows interestingly that all five males from whom Y-chromosomal haplogroups were obtained belonged to haplogroup Q-M242 or its sub-haplogroup Q1a3-M346 and all carried mtDNA haplogroup either D or Z. This could lead us to a potential scenario for the social structure during the prehistoric time in Lake Baikal.

Patrilocality, when women move to their husbands' residence after marriage and men stay in their birthplace, is the norm in about 70% of the world's populations (92). According to Kelly, R.L. (1995 and 2013), patrilocality is the most common form of post-marital residence, found at 65%, in hunter-gatherer societies (93,94). Patrilocal societies are usually characterized by a high level of diversity in mtDNA haplogroups and low level of diversity in Y-chromosomal haplogroups within the same group with the reverse situation between groups (95-98). The Kurma XI results are suggestive of a patrilocal post-marital residence pattern. Also, the high level of diversity in mtDNA haplogroups and low level of diversity in Y-chromosomal haplogroups within the Kurma XI prehistoric population could be suggestive of an exogamy marriage pattern, when men marry women from outside the group. The genetic analysis in this study shows that the Kurma XI population carried four different mtDNA haplogroups (A, D, F and Z), while only one Y-chromosomal haplogroup Q-M242 and/or its sub-haplogroup Q1a3-M346 were observed. Studies of the Y-chromosomal haplogroups at Lokomotiv, Shamanka II and Ust'-Ida are in progress and may elucidate social structure in those groups.

5.5. Previous vs. new mtDNA haplogroup distribution from Cis-Baikal cemeteries

mtDNA results have been obtained previously from two EN cemeteries (Lokomotiv, n= 31, and Shamanka II, n= 21) and one LN-EBA cemetery (LN Ust'-Ida, n=10, and EBA Ust'-Ida, n=29). mtDNA analysis from EN Lokomotiv individuals showed a higher distribution of haplogroup F (48.4 %, n=15/31) and a lower distribution of haplogroup C (3.2%, n=1/31) (15).

Previous data from the EN cemetery (Shamanka II) showed a higher distribution of haplogroups D and F (28.6%, n=6/21, and 23.8%, n=5/21, respectively) and lower distribution of haplogroup G2a (4.8%, n=1/21) (17). Prior analysis of 29 individuals from LN Ust'-Ida showed a higher

distribution of haplogroups A (24%, n=7/29) and “Others” (27.6%, n=8/29). The designation “Others” is assigned when a group of reproducible single nucleotide polymorphic sites on the mtDNA HV1 region do not relate to a specific haplogroup (63). The ten individuals from EBA Ust’-Ida showed a higher distribution of haplogroup C (70%, n=7/10) than haplogroup A (30%, n=3/10) and the absence of any other haplogroups (16). Previously, it was demonstrated that the two EN cemeteries, Lokomotiv and Shamanka II, were similar to each other (p=0.600) but both were statistically different from the LN-EBA cemetery Ust’-Ida (p=0.001 and p=0.008, respectively) (5,16).

Comparison of the EBA Kurma XI population and the newly obtained results from EN Shamanka II individuals, together with the division of the LN-EBA Ust’-Ida population into LN and EBA groups, gives us new insight into the maternal background of the Cis-Baikal prehistoric population. The mtDNA haplogroup distribution between the two EN cemeteries and the LN, EBA Ust’-Ida and EBA Kurma XI are all significantly different. Table 2.7 summarizes all the Fisher’s exact test results between all the cemetery samples with $p<0.05$ as the statistical significance level. However, EBA Kurma XI mtDNA haplogroups are not significantly different from the EN Lokomotiv and Shamanka population (Table 2.7). EBA Kurma XI shows a higher distribution of haplogroup D (50%, n=6/12), a lower distribution of haplogroups A (8.3%, n=1/12) and Z (16.7%, n=2/12) (not previously found in Cis-Baikal prehistoric population), and the absolute absence of haplogroup C (Fig. 2.5).

Interestingly, the two EN cemeteries Lokomotiv and Shamanka II (including the new results) were found to be statistically different, contradicting previous findings (e.g. (5)). The new Shamanka II dataset shows a higher distribution of haplogroup D (40.7%, n=11/27) and a lower distribution of haplogroup U5a (3.7%, n=1/27) than previously indicated (e.g. (5)) (Fig. 2.5).

The new mtDNA data from EBA Kurma XI and Shamanka II and the new statistical analysis results question the previous finding of a discontinuity between EN and LN-EBA populations (5,16). The EN populations of Cis-Baikal are not homogeneous in their maternal origin because Lokomotiv and Shamanka II are now shown to have different mtDNA haplogroup distributions. However, both these EN cemeteries, share a common maternal background with the EBA Kurma XI population but all are significantly different from the LN and EBA Ust’-Ida.

The LN Ust'-Ida and EBA Ust'-Ida differ significantly in haplogroup distribution. The low haplogroup diversity in EBA Ust'-Ida is striking and has been previously discussed by Mooder 2006 (16).

In summary, the analysis of Kurma XI and additional material from Shamanka II now suggest that all the Cis-Baikal are genetically distinct with respect to their maternal origin. However, the individuals analyzed from all Cis-Baikal cemeteries are small and may be unrepresentative of the entire prehistoric populations that lived in the Baikal area. Therefore, studying more individuals from all the Cis-Baikal prehistoric cemeteries is essential to make inferences about the social structure of the prehistoric population and their migration and marriage patterns.

Table 2.7. Summary of Fisher's exact test results between all of Cis-Baikal's cemeteries populations at different periods (EN, LN, EBA).

Population (n= number of individuals)	Fisher's exact test results ($p<0.05$)
EBA KUR (n=12) and EBA UID (n=10)	$p<0.0001$
EBA KUR (n=12) and LN UID (n=29)	$p=0.0003$
EBA KUR (n=12) and EN LOK (n=31)	$p=0.1079$
EBA KUR (n=12) and EN SHA (n=27)	$p=0.1515$
EBA UID (n=10) and LN UID (n=29)	$p=0.0239$
EBA UID (n=10) and EN LOK (n=31)	$p<0.0001$
EBA UID (n=10) and EN SHA (n=27)	$p=0.0151$
LN UID (n= 29) and EN LOK (n=31)	$p=0.0007$
LN UID (n=29) and EN SHA (n=27)	$p=0.0066$
EN LOK (n=31) and EN SHA (n=27)	$p=0.0174$

- $p<0.05$: the statistical significance level
- KUR= Kurma XI, UID= Ust'-Ida, LOK= Lokomotiv, SHA= Shamanka II

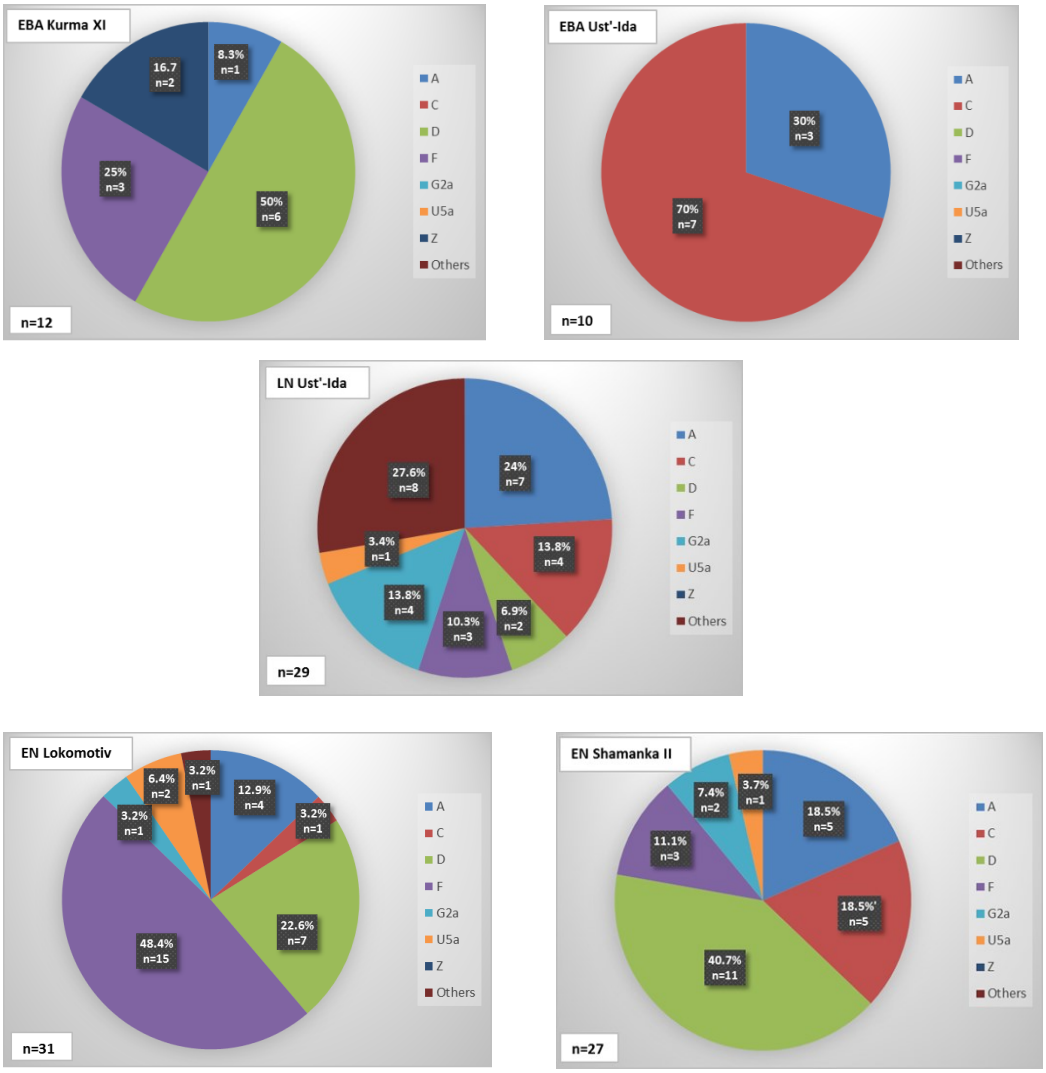

Figure 2.5. Pie charts representing mtDNA haplogroup frequency distribution for EBA Kurma XI (this study), LN Ust'-Ida and EBA Ust'-Ida (16), EN Lokomotiv (15) and EN Shamanka II (This study and (17)).

5.6. Summary of the Y-chromosomal haplogroup distribution from the four Cis-Baikal cemeteries

Y-chromosomal haplogroup analysis was obtained from the two EN cemeteries, Lokomotiv (number of males with positive results, n=7/ 17, and from Shamanka II, n=9/9), to show that Lokomotiv males are more heterogeneous than the Shamanka II males. Lokomotiv males show a high frequency of Y-chromosomal haplogroup K, and low frequency of haplogroup C3, but all of the Shamanka II males belong to haplogroup K. The EN Lokomotiv population shows the highest degree of heterogeneity in the Y-chromosomal haplogroup distribution when compared to all the other studied cemeteries' populations from the Cis-Baikal region. The high frequency of haplogroup K in the Lokomotiv population and the dominance of haplogroup K in Shamanka II population suggest that the two EN populations have a common paternal background.
The other LN-EBA cemetery, Ust'-Ida (n=14/16), has a high frequency of haplogroup Q-M242 and its sub-haplogroup Q1a3-M346 and low frequency of haplogroup K. When comparing paternally LN-EBA Ust'-Ida with the EBA cemetery Kurma XI, both share a high frequency of haplogroup Q-M242/Q1a3-M346. The high frequency of haplogroup Q-M242/Q1a3-M346 in both LN-EBA cemeteries' populations, Ust'-Ida and Kurma XI, suggests a common paternal background for these LN-EBA populations; however, it is different than the EN populations' paternal background. The Y-chromosomal haplogroup distributions in the Cis-Baikal region propose a paternal discontinuity between the EN and LN-EBA populations, and the male migration patterns differ from the female migration patterns during prehistoric times. More details about Y-chromosomal analyses from all four cemeteries can be found in Chapter 3.

6. Conclusion

In conclusion, mtDNA results from Kurma XI raise many questions about the maternal background of the EBA population of Lake Baikal. Also, the new data obtained from Shamanka II and the new statistical analyses conducted on all the cemeteries after adding the EBA Kurma XI individuals and Shamanka II individuals to the analyses gave a different perspective about the maternal origin of the prehistoric populations in Cis-Baikal. The two EN cemeteries (Lokomotiv and Shamanka II) do not share the same maternal origin because they are significantly different in their mtDNA haplogroup distributions. However, since the number of individuals analyzed

were really small and there are still quite a few Lokomotiv and Shamanka II individuals to be analyzed, the chances are high that the presepective that Lokomotiv and Shamanka II have a different maternal origin might change once again. The LN Ust'-Ida and EBA Ust'-Ida also do not share the same maternal origin, which might indicate that the cemetery has been used by two different groups of people belonging to different populations. The Kurma XI mtDNA haplogroup distribution differs from the only other LN-EBA cemetery, Ust'-Ida for which mtDNA data are available. However, EBA Kurma XI mtDNA distribution is similar to the EN cemeteries of Lokomotiv and Shamanka II.

A significant limitation is the small size of EBA Kurma XI and the suggestion that it is 'exclusive' might mean that it is not representative of the entire population inhabiting the micro-region during the EBA period. Y-chromosomal DNA was a novel addition to the genetic study of Lake Baikal prehistoric populations and provides an insight into the contribution of the prehistoric population of Lake Baikal in the formation of the Native American population.

For future direction, the analysis of mtDNA and Y-chromosomal polymorphisms from the large Khuzhir-Nuge XIV population would elucidate the genetic signature by estimating the influence of social norms on their genetic diversity.

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Chapter 3

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Y-Chromosomal DNA Analyzed for Four Prehistoric Cemeteries from Cis-Baikal, Siberia

1. Introduction

1.1. Background

Archaeological data indicate that the Cis-Baikal region of Siberia has provided a several large prehistoric cemeteries probably since the Early Upper Paleolithic period (1,2). Cis-Baikal area has four major micro-regions: the Angara River Valley, the upper Lena River Valley, the Little Sea (Ol'khon) on the northwest coast of the Lake, and the South Baikal region (Figure 3.1) that have been investigated by several Russian and Canadian scholars since 1990. The Baikal Archaeology Project (BAP), an international multidisciplinary initiative, aims to reconstruct the lifestyle of the hunter-gatherer groups inhabiting the area and buried in formal cemeteries during the Neolithic and Bronze Age periods. The area was home to two temporally distinct populations from Early Neolithic (EN) (Kitoi culture), 8000-6800 cal BP, to Late Neolithic-Early Bronze Age (LN-EBA) (Serovo-Isakovo-Glazkovo culture), 5800-4000 cal BP. The EN group was separated from the LN-EBA group by a 1000-year gap (hiatus) during which large mortuary sites are entirely absent (3).

The EN (Kitoi) culture had formal cemeteries "…as an area used repeatedly and more or less exclusively for disposal of the group's dead" (e.g. (4)). The EN people practiced hunting, fishing and sealing, the population was large and unevenly distributed, had differential mobility and substantial social differentiation (5).

The LN-EBA (Serovo-Isakovo-Glazkovo) culture had formal cemeteries, practiced hunting, fishing and sealing, were larger and evenly distributed populations, and had moderate mobility and social differentiation (5).

Earlier Russian craniometric studies suggested that the EN and the LN-EBA populations are genetically distinct (6-9). Measuring the biological differentiation between the two cultures can also be achieved through their genetic signatures, which would give a strong verification of their genetic relationships.

Ancient DNA (aDNA) research of several hunter-gatherer individuals from Cis-Baikal was first conducted by Russian researchers analyzing mitochondrial DNA (mtDNA) composition (10,11). aDNA analysis was continued by several researchers, who examined mtDNA polymorphisms in skeletal samples from different mortuary sites. These mortuary sites include the EN cemetery

(Lokomotiv) and LN-EBA cemetery (Ust'-Ida) (12), the EN cemetery (Shamanka II) (13) and the LN-EBA cemetery (Khuzhir-Nuge XIV) (14). Unfortunately samples from Khuzhir-Nuge XIV were poorly preserved and mtDNA analysis was not possible. In my project a third EBA cemetery (Kurma XI) was investigated, six new EN Shamanka II samples were also analyzed, and another four EN Shamanka II samples were re-analyzed (Chapters 2 &4). Each of these cemeteries is located in a different micro-region of Lake Baikal and provides a unique mtDNA signature.

1.2. Archaeological context

As a part of the archaeological research, estimation of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope signatures in human bone is considered the most direct and reliable method to determine diet and subsistence strategies of prehistoric populations (15). In BAP, the values of $\delta^{13}C$ and $\delta^{15}N$ isotopes from the individuals' osteological remains were compared to $\delta^{13}C$ and $\delta^{15}N$ isotopes' ratios of local fauna in different Cis-Baikal micro-regions to create a thorough descriptive reconstruction of prehistoric populations' diet and subsistence practices since the EN period to the LN-EBA period (5,16,17). Examination of different samples from Cis-Baikal micro-regions and the different periods provided clear evidence for the consumption of freshwater foods (e.g. fish and seal- in groups from Lake Baikal), still "there is significant spatio-temporal variability within and between micro-regions" (5).

Mobility strategies are correlated with several aspects including social, cultural and environmental variables in hunter-gatherer societies. Mobility patterns can be addressed through dietary evidence, as carbon and nitrogen stable isotope ratios, and also through Strontium (Sr) isotope signatures, which is considered a more considerable method to measure mobility than carbon and nitrogen stable isotope signatures (5).

Sr, a natural element of earth, is integrated into the skeletal tissues through the ingestion of food and water. The ratios of ^{87}Sr to ^{86}Sr detected in the skeletal tissues of a human or an animal is a reflection of the $^{87}Sr/^{86}Sr$ signatures found in the environment and directly related to the food and water consumed by the human or the animal from a specific region. 99% of the total amount of Sr in a living organism's body is found in bones and teeth (18). The bone tissue undergoes a constant remodeling every 7 to 10 years during the individual's lifetime, but the tooth enamel development stabilizes after infancy and childhood (19). Thus, any difference in

the Sr isotope ratios between bones and teeth would reveal the migratory history of an individual throughout a different geographical area, which might differ from the individual's residential location during childhood. Therefore, $^{87}Sr/^{86}Sr$ ratios were measured from bones and teeth of some individuals from Lokomotiv and Ust'-Ida cemeteries (20).

1.3. Burial sites

1.3.1. EN cemeteries

a) <u>Lokomotiv cemetery</u> is the largest Neolithic cemetery identified in northern Asia. It was exposed during the construction of the Trans-Siberian Railway in the 1800s (21,22). The cemetery is located in the junction between the Irkut and the Angara rivers (in the Angara River Valley micro-region) in a park in Irkutsk city (23).

b) <u>Shamanka II cemetery</u> located at the southwestern tip (on the South Baikal micro-region) of Lake Baikal is also considered a large cemetery (23).

Mortuary rituals and grave structure demonstrate similarities between the two EN cemeteries. For example, the usage of red ochre, as a mortuary ritual, is observed at both Lokomotiv and Shamanka II (23). Several burials in both cemeteries have missing crania. Both cemeteries have single, double, triple, and communal graves (13,21,22,24,25). The extended-supine body position is common in both Lokomotiv and Shamanka II (26). However, there is some variation in mortuary rituals. Many graves in Shamanka II cemetery had bear skulls, mandibles, canines and molars indicating an interesting bear mortuary ritual (23), but none were found in Lokomotiv (21). Also, fire rituals were documented in Shamanka II "…suggested by the presence of fire pits that often disturbed the graves" (23).

Pendants were present in all Shamanka II graves except one (13), but many graves at Lokomotiv had no grave assemblages (21). Different types of ornaments were represented in both cemeteries including different types of pendants (red deer pendants, oval bone pendants, mother-of-pearl pendants), and mother-of-pearl rings. Small art forms represented by elk heads carved in antler, antler spoons with elk head shaped handles, and images of Baikal seal represented on a talc object (e.g. Lokomotiv) and on an L-shaped bone artifact (e.g. Shamanka II) (26).

1.3.2. LN-EBA cemeteries

a) Ust'-Ida cemetery is situated on the Angara River (on the Angara River Valley micro-region) with graves scattered parallel to the river. The Ust'-Ida cemetery was discovered after a flood from a river dam caused the erosion of 11 graves (27).

b) Kurma XI cemetery located on the northwest coast of Lake Baikal's Little Sea micro-region, is smaller than the other cemeteries. After a test excavation on a Kurma XI grave, the cemetery was dated to the EBA (28). However, six graves out of 26 were found to be EN according to archaeological and radiocarbon data (29).

The use of fire was evident at Ust'-Ida with occasional burials showing red ochre treatment (27,30). However, at Kurma XI, the use of fire mortuary ritual was completely absent, but still three burials were covered in red ochre (31).

Extended-supine body position is common in Ust'-Ida with the head oriented to the north, or sometimes the head was facing south and bodies either extended-supine or sideways (12,27,32). Also, two body positions were found at Kurma XI, one mostly extended-supine with the head pointing southwest, and the other was a sitting position (three cases only) (33,34).

Other mortuary variability include, single, double, triple and multiple graves in Ust'-Ida cemetery (12,27,32). At Kurma XI, all of the graves were single except for one double grave (35). Few rich graves ", with a variety of objects placed in each hand of the deceased individuals" (26), were found in the Ust'-Ida cemetery. Only two burials at Ust'-Ida can be considered rich. At these two burials different objects were placed in each hand of the deceased individuals including "…points made of elk metapodial bones, double-sided composite inset points, harpoons, and a variety of other points carved from long bones" (26). Kurma XI had a number of grave inclusions including axes/adzes, knives made of green nephrite, disks of white nephrite and calcite, lithic arrowheads, red deer canine pendats, spoons, harpoons and metal knives and needles (34). Many artefacts found in the Kurma XI graves were considered to be unique finds to the entire Cis-Baikal region (36). For example, one EBA grave at Kurma XI had a large copper/bronze medallion featuring an anthropomorphic figure inside a circular frame directly associated with the burial (Grave no. 1) (37). Another EBA grave had a silver ring object directly associated with the burial (Grave no. 15) (37).

1.4. Previous DNA analysis

Previous studies on DNA from EN Lokomotiv (28 individuals), LN-EBA Ust'-Ida (29 LN and 10 EBA) and EN Shamanka II (21 individuals) have examined restriction fragment length polymorphisms (RFLP) and/or direct sequencing of the hyper-variable 1 region (HV1) of mtDNA to identify mtDNA population specific polymorphisms (haplogroups). Several Eurasian mtDNA haplogroups were identified with varying frequencies in the different cemetery samples (12,13,22,38).

mtDNA analysis from the three cemeteries showed that individuals from the two EN cemeteries (Lokomotiv and Shamanka II) both had higher frequencies of haplogroups D and F and lower frequencies of haplogroups A and C, suggesting that Lokomotiv and Shamanka II people were maternally related (39). The LN-EBA cemeteries (Ust'-Ida) illustrated different mtDNA haplogroup frequencies. LN Ust'-Ida shows higher frequency of haplogroup A and lower frequencies of haplogroups D and F, and EBA Ust'-Ida shows higher frequency of haplogroup C, lower frequency of haplogroup A and the absence of haplogroups D and F (38).

As part of this project, mtDNA was analyzed, through direct sequencing of the HV1 region, for 12 EBA Kurma XI individuals and two EN Kurma XI individuals. In addition, mtDNA was examined from six additional EN Shamanka II individuals, and mtDNA was re-analyzed from four EN Shamanka II individuals. EBA Kurma XI shows a high frequency of haplogroup D, a low frequency of haplogroup A, the absence of haplogroup C, and the appearance of haplogroup Z, which was not found in other Cis-Baikal cemeteries. Statistical analysis (Fisher's exact test) shows that EBA Kurma XI is maternally different from both LN Ust'-Ida and EBA Ust'-Ida (Chapter 2). Also, the updated Shamanka II mtDNA data show that Shamanka II is maternally different from Lokomotiv, which contradicts the previous findings (Chapter 2). Interestingly, Kurma XI has a similar haplogroup distribution to the two EN cemeteries, Lokomotiv and Shamanka II. The new results from EBA Kurma XI and EN Shamanka II question the notion of maternal continuity/ discontinuity between EN, LN and EBA populations of Cis-Baikal. Thus, the new mtDNA results suggest that the two EN cemeteries (Lokomotiv and Shamanka II) have a different maternal origin; however, they both might share the same maternal origin with the EBA Kurma XI individuals. In addition, the mtDNA haplogroup distribution from EBA Kurma XI

individuals suggest that those individuals have a different maternal origin from the LN Ust'-Ida and even the EBA Ust'-Ida individuals.

Therefore, investigating the populations of the two EN cemeteries and the two LN-EBA cemeteries through the patrilinear lineage using Y-chromosomal polymorphisms is crucial to verify discontinuity or continuity between the EN and LN-EBA populations.

1.5. Y-chromosomal analysis

The aim of this study is to explore the paternal lineages of the Cis-Baikal prehistoric populations through the examination of Y-chromosomal polymorphisms. Examination of the patrilineal lineage is a novel and essential approach to BAP not only to complement the matrilineal lineage data, but also to elucidate patrilineal continuities and/or discontinuities within and between the EN and the LN-EBA groups.

The Y-chromosome consists of two main regions, the non-recombining region (NRY) and the pseudoautosomal regions (PARs). Loci on the Y-chromosome's NRY are haploid, inherited paternally and have been shown to be useful in tracking the male lineage in populations (41). The PARs are the only parts on the Y-chromosome that recombine with the PARs on the X-chromosome during meiosis in order to maintain their attachment on the spindle fibers during cell division and act as regular autosomes (42). Many DNA polymorphisms among different human populations that can be found on Y-chromosomal DNA, have led to a dramatic increase in studies of the paternally inherited Y-chromosome and helped in highlighting the important role of Y-chromosomal polymorphisms in evolutionary and population genetics (43-49). These polymorphisms include single nucleotide substitutions, small and large deletions, inversions and duplications. These polymorphisms have different mutation rates (50). Some loci have low mutation rates, which provide the opportunity to use them in differentiating between ancestral branches on the human evolutionary tree (45,46,51,52). Y-chromosomal SNPs (Single Nucleotide Polymorphisms) have a low mutation rate (10^{-8} mutation/generation), making them appropriate for identifying stable paternal lineages that can be tracked back in time for thousands of years (53). About 600 SNPs belonging to 20 Y-chromosomal haplogroups have been identified to date from the modern populations of the world (54). To determine the Y-chromosomal haplogroup distribution of individuals from EN and LN-EBA cemeteries, we

examined Y-chromosomal SNPs. The results would give us insight not only on paternal lineage of individuals but also on marriage and post-marital residence patterns practiced by males when compared to published data of modern haplogroup distribution in the same region (55,56). Certain Y-chromsomal SNPs were examined in this study that define Y-chromosomal haplogroups (e.g. C-M216, C3-M217, F-M89, K-M9, N3-Tat, O-M175, P-M45, Q-M242, Q3-M3, R1-M173 and R1a1-M17) distributed among East Asian populations and Siberian populations (57). Regarding analyzing Y-chromosomal sub-haplogroups, haplogroup Q-M242 is identified by M242 SNP mutation (54) and is widely distributed among different groups in Siberia (58). There are several sub-haplogroups under haplogroup Q-M242. Only certain sub-haplogroups from the haplogroup Q-M242 tree are represented in the Siberian population (Q1a*-MEH2, Q1a2-M25 and Q1a3-M346) (59).

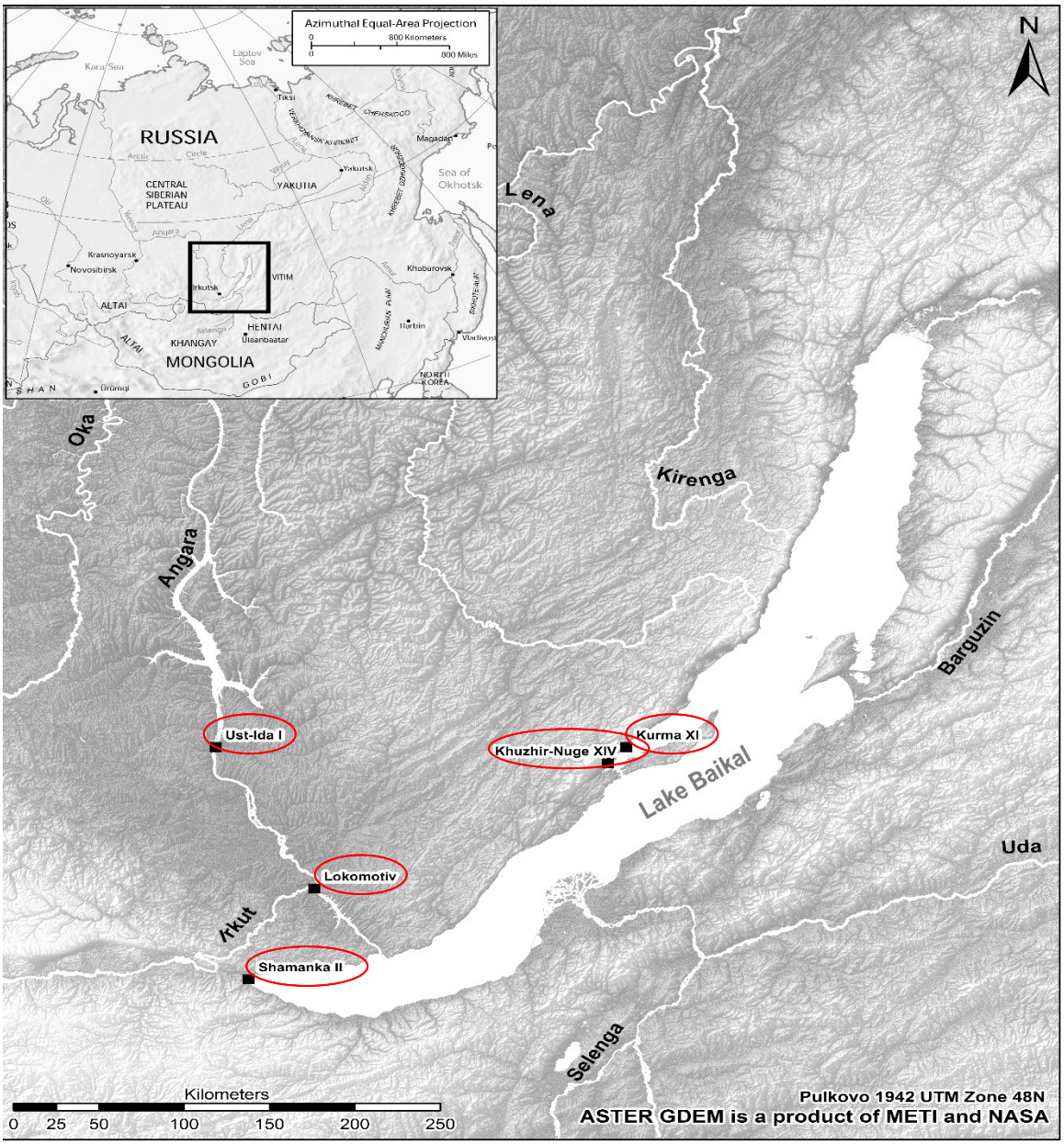

Figure 3.1. Cis-Baikal cemeteries' and their locations-circled in red (Shamanka II and Lokomotiv-EN) and (Ust'-Ida, Khuzhir-Nuge XIV and Kurma XI-LN-EBA) (60).

Adapted with permission from [Point taken: An unusual case of incisor agenesis and mandibular trauma in Early Bronze Age Siberia. A.R. Lieverse,I.V. Pratt,R.J. Schulting,D.M.L. Cooper,V.I. Bazaliiskii,A.W. Weber. International Journal of Paleopathology. 6. Copyright © 2014 Elsevier] (License number 3571161002487).

2. Materials and Methods

2.1. Contamination controls

Rigorous measures are required when working with ancient DNA to minimize the risk of exogenous contamination with modern DNA. Published recommendations (61) were followed in our laboratory. During DNA extraction and PCR amplification negative controls were introduced to detect any probability of contamination. All sample preparations, DNA extractions and preparation for PCR amplifications were conducted in a dedicated laboratory (clean room). Prior to entering the clean room, researchers dressed in gowns, booties, sterile sleeves, masks and goggles. After entering the clean room another set of gloves was worn, by researchers, and sprayed with 30% (v/v) bleach (sodium hypochlorite). The clean room was equipped with a separate workstation for sample preparation, and with a class II type A2 biological safety cabinet (Thermo Scientific™, USA) for all other procedures. The bone box was cleaned with undiluted industrial strength bleach (100% v/v) between each sample to avoid cross contamination. The biological safety cabinet was cleaned with 70% ethanol before and after usage and exposed to UV light for four hours after each usage. Racks, pipettes, and containers were cleaned with 10% (v/v) bleach before and after each single use. Sterile pipette tips (Rose Scientific, AB, Canada) were used. Reagents were decontaminated either by bleaching, UV irradiation and/or autoclaving prior to use depending on the nature of the material. PCR amplifications and data analysis were carried out in duplicate on two separate occasions. All personnel working in the laboratory gave consent to have their mtDNA and Y-chromosomal haplogroups determined. Establishment of the authenticity of results is discussed in section 2.5.

2.2. DNA Sampling

2.2.1. Sample selection from Lokomotiv, Ust'-Ida, Shamanka II and Kurma XI

Male samples confirmed by molecular sex analysis were selected for analysis from the four main cemeteries in the Cis-Baikal area, except for one Kurma XI individual, whose molecular sex could not be determined but has a morphological sex of probable male (KUR_2002.010). This Kurma XI individual was added only to increase the possibility of obtaining more Y-chromosomal results from Kurma XI cemetery. Two cemeteries (Lokomotiv and Shamanka II) belong to the EN period and two belong to the LN-EBA period (Ust'-Ida and Kurma XI).
The molecular sex of Lokomotiv and Ust'-Ida samples was determined previously through amelogenin analysis (22). The male samples were selected for further Y-chromosomal analysis. The reliance was mostly on molecular sex results rather than morphological sex because there was occasionally discordance between molecular and morphological sexing (38). For the analysis, 26 vertebral bones and/or teeth samples were available from 17 males from the Lokomotiv cemetery and 21 vertebral bones and/or teeth samples from 16 males from the Ust'-Ida cemetery. All male samples from Lokomotiv belong to the EN period. Fourteen males samples from Ust'-Ida belong to the LN period except for two EBA samples and one unknown (Table 3.1). Nine male samples were available for Y-chromosomal analysis from the Shamanka II cemetery. The molecular sex of five samples was previously determined by Thomson (2005) (13), and the remaining four samples were analyzed as part of this study (Table 3.1). The male samples from Shamanka II belong to the EN period (62). Sixteen vertebral bone and tooth samples from Kurma XI, belonging to seven confirmed males through amelogenin analysis (Moussa et al. n/d), were available for Y-chromosomal analysis. All Kurma XI males belong to the EBA period (62). For bone samples, vertebral bones were preferred for two main reasons. Vertebrae contain a high proportion of spongy bone tissues, which has a higher DNA yield compared with other types of bone tissue (63), and because multiple vertebrae are available from one individual. Molars were the preferred teeth samples because there is more than one root available for DNA extraction in each tooth.

Table 3.1. Male individuals available for Y-chromosomal analysis from the four studies cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI) with the individuals' sample type, and IDs, age of the individuals, period, morphological sex and number of individuals in grave (62).

No	Cemetery & Master ID	Sample No	Type of sample	Morphological Sex	Age of individual (Years)	Archaeological Age
Lokomotiv (EN)						
1	LOK_1980.004	2009.002.	Bone	Female	25-35	EN
2	LOK_1980.006	1995.100.	Bone	Male	20+	EN
3	LOK_1980.010.02	2009.007, 2001.504	Bone and Tooth	Male	20-25	EN
4	LOK_1980.010.03	2009.008.	Bone	Female	50+	EN
5	LOK_1980.012	2009.010.	Bone	Female	18-22	EN
6	LOK_1981.013	2009.023, 2001.492	Bone and Tooth	Male	25-35	EN
7	LOK_1980.014.03	2009.011.	Bone	Immature	10- 11	EN
8	LOK_1980.016	2009.014, 2001.509	Bone and Tooth	Male	45-55	EN
9	LOK_1980.017	2009.015.	Bone	Male	35-50	EN
10	LOK_1980.022.02	2009.020, 2009.021	Bone	Male	20+	EN
11	LOK_1981.024.01	2009.024.	Bone	Immature	11.5-15	EN
12	LOK_1984.027	2009.027, 2001.527, 2001.529	Bone and Teeth	Male	15-18	EN
13	LOK_1985.031.02	2009.032, 2001.412	Bone and Tooth	Male	25-30	EN
14	LOK_1988.038.01	2009.036.	Bone	Female	50+	EN
15	LOK_1990.042	1995.130, 2009.041, 2001.549	Bone and Tooth	Male	40-50	EN
16	LOK_1990.044.01	2009.042.	Bone	Male	35-39	EN
17	LOK_1990.044.02	2009.043.	Bone	Male	30-39	EN
Shamanka II(EN)						
1	SHA_2001.012	2009.060.	Bone	Undetermined	25-35	EN
2	SHA_2001.013.03	2009.061.	Bone	Probable Female	18-19	EN
3	SHA_2002.021.02	2002.257.	Tooth	Male	25-30	EN
4	SHA_2002.021.03	2009.070.	Bone	Undetermined	16-18	EN
5	SHA_2002.023.04	2001.221.	Bone	Undetermined	20+	EN
6	SHA_2004.052.01	2004.131.	Bone	Probable Male	20-24	EN
7	SHA_2004.044.02	2004.031.	Bone	Undetermined	20+	EN
8	SHA_2005.059.01	2004.058.	Bone	Male	35-39	EN
9	SHA_2006.083.01	2009.109.	Bone	Male	20-22	EN

Table 3.1. (Continued…)

No	Cemetery & Master ID	Sample No	Type of sample	Morphological Sex	Age of individual (Years)	Archaeological Age
	Ust'-Ida (LN-EBA)					
1	UID_1987.005	2009.177	Bone	Immature	7- 9	LN
2	UID_1987.009	2009.179, 2001.546	Bone and Tooth	Immature	4-7.5	LN
3	UID_1987.012	2001.490	Tooth	Male	50+	LN
4	UID_1988.016.01	2009.182	Bone	Male	25-35	LN
5	UID_1988.016.02	2009.183	Bone	Male	50+	LN
6	UID_1989.020.01	2009.185, 2001.418	Bone and Tooth	Male	18-24	LN
7	UID_1989.026.01	2009.190, 2001.547	Bone and Tooth	Immature	13-15	LN
8	UID_1989.029	1995.157, 2001.489	Bone and Tooth	Male	50+	EBA
9	UID_1989.030	2009.194	Bone	Female	50+	LN
10	UID_1990.033.01	2009.196, 2001.513	Bone and Tooth	Male	12- 15	LN
11	UID_1991.038	2009.199	Bone	Male	45-60	LN
12	UID_1993.043	2009.201	Bone	Male	Mature	LN
13	UID_1993.044.03	2001.574	Tooth	Immature	11- 12	LN
14	UID_1994.048	2009.202	Bone	Male	50+	EBA
15	UID_1994.053.02	2009.204	Bone	Immature	4- 6	LN
16	UID_1994.055.02	2009.205	Bone	Male	15-18	LN
	Kurma XI (EBA)					
1	KUR_2002.001	2002.106	Teeth	Male	25-30	EBA
2	KUR_2002.007.02	2002.116	Bone	Male	20-29	EBA
3	KUR_2002.010	2003.099	Tooth	Probable Male	18-25	EBA
4	KUR_2002.015	2002.134	Bone	Probable Male	17-18	EBA
5	KUR_2003.017	2003.013	Teeth	Probable Male	20+	EBA
6	KUR_2003.018	2003.003	Tooth	Probable Female	17-19	EBA
7	KUR_2003.019	2003.008, 2003.010	Tooth	Probable Male	20-30	EBA

2.2.2. Specimen preparation and decontamination

Bones and teeth were cleaned to ensure a full decontamination of the surface of the samples from exogenous modern DNA or bacterial DNA that would affect the final results. The method was a modification of that described by Mooder et al. (2004) (12). Slightly different methods were used for decontaminating vertebral and molar teeth samples.

Vertebral samples: A sterile saw was used to remove the processes from the body of the vertebra. The outer surface of the vertebra was removed using a sterile scalpel. The vertebra was immersed in 16.7% (v/v) bleach for five minutes. The bleach concentration was optimized according to a published protocol (64).

Teeth samples: A sterile saw was used to separate the root from the crown. The root surface was removed using a sterile scalpel. The root was immersed in 50% (v/v) bleach for 10 minutes. Following decontamination, all samples were rinsed in HPLC grade water. The sample surface was exposed to UV light for one hour and then left to dry in a sterile container for two days in the Dead Air Box workstation. The samples were then frozen in liquid nitrogen, and pulverized using a sterile mortar and pestle. The powder was collected with sterile spatulas and stored in sterile containers at -20 degrees C until further analysis.

2.2.3. DNA extraction from bones and teeth

A modified silica-guanidium thiocyanate extraction protocol targeted for optimal extraction of DNA from ancient bones and teeth was used (65). The DNA extraction protocol was adapted from the published method (65) (Figure 3.2) with modifications to reduce the risk of contamination.

All glassware and spatulas used in the preparation of extraction reagents were washed with concentrated bleach (100% v/v) and detergent, rinsed several times with distilled water then twice with Milli-Q water and autoclaved for 30 minutes at 121 degrees C.

2.2.3.1. Procedure for DNA extraction (Figure 3.2)

Day 1: Approximately 0.5 g of bone or tooth powder was incubated in 10 ml extraction buffer, rotating (MyLab Intelli-Mixer, Rose Scientific, AB, Canada) overnight at low speed. Five samples plus a negative control (contained extraction buffer only) were processed per extraction.

Day 2: Samples were centrifuged (Eppendorf AG, Centrifuge 5430R, Hamburg, Germany) for 4 minutes at 5000*g.* The supernatant was transferred to a 50 ml polypropylene tube containing 100 µl silicon dioxide suspension and 40 ml binding buffer. The silicon dioxide suspension was UV irradiated before use. The pH was adjusted to ~ 4.0 with 10N HCl. The tubes were rotated for 3 hours in the dark at room temperature before centrifuging for 4 minutes at 5000*g*. The supernatant was discarded. The silica pellet was washed once with 1 ml binding buffer. The re-suspended silica and the binding buffer were transferred to a 2 ml Eppendorf tube, and centrifuged for 30 seconds at 16,000*g*. The silica pellet was washed twice with 1 ml washing buffer. After each wash the pellet was re-suspended in the buffer, and centrifuged for 30 seconds at 16,000*g.* After the final wash, the silica pellet was allowed to dry, with an open lid, in the biological safety cabinet for 15 minutes. The pellet was re-suspended in 50 µl TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), and rotated at very low speed for 10 minutes. The TE buffer was UV irradiated before use. The mixture was centrifuged at 16,000*g* for 4 minutes; the supernatant removed and stored in a sterile Eppenddorf 1.5 ml tube. The last elution step was repeated twice to avoid transferring silica, which can inhibit PCR amplification. Finally, the DNA extract was stored at -20 degrees C until further analysis.

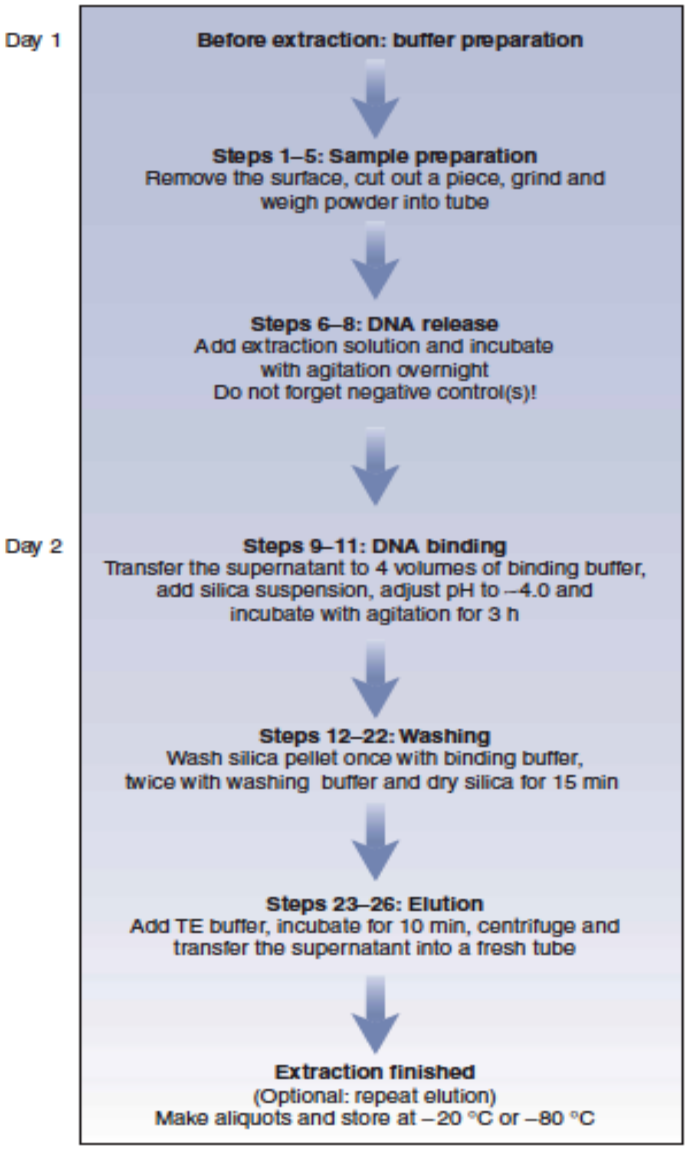

Figure 3.2. A summary of the ancient DNA extraction protocol from bones and teeth (65).

2.3. Molecular sex assignment

Molecular sex of the Lokomotiv, Ust'-Ida and most Shamanka II samples had been determined previously by other researchers (Table 3.1.) (12,13). The sex of the Kurma XI samples and four Shamanka II samples was assigned by amelogenin analysis as part of this study. Amelogenin, a gene involved in the formation of dental enamel, is found on both X and Y chromosomes (66). PCR primers for amelogenin intron 1 amplification give a 106 bp product for females and a 106/112 bp product for males (67). The PCR amplification reaction was performed using Mooder's et al. (2005) protocol (22) with modifications, on Eppendorf AG Thermocycler (Hamburg, Germany). Each 25 µl PCR reaction consisted of 1X PCR buffer (Invitrogen, NY, USA), 1.5 mM $MgCl_2$ (Invitrogen, NY, USA), 0.2 mM of each dNTP (Invitrogen, NY, USA), 0.4 mg/ml BSA (Roche), 1.0 µM of each primer (Invitrogen, NY, USA), and 1.25 U of Platinum *Taq* DNA Polymerase (Invitrogen, NY, USA). The thermocycler cycle started with a denaturing step at 95 degrees C for 2 minutes, and 40 cycles of 95 degrees C for 1 minute, annealing at 62 degrees C for 1 minute and extension at 72 degrees C for 1 minute. Two negative controls without DNA added were included in all PCR amplifications. The amelogenin PCR amplification product was visualized via the Gel Doc™ EZ System (Bio-Rad, ON, Canada) after gel-electrophoresis on 12% polyacrylamide gel and ethidium bromide (10 mg/ml) staining.

2.4. Y-Chromosome Single Nucleotide Polymorphisms (SNPs) analysis

2.4.1. Multiplex PCR amplification and SNaPshot reaction

Specific Y-chromosomal SNPs representative of East Asian and Siberian paternal lineages were selected (Figure 3.3). The 13 SNP markers were examined via two multiplex PCR amplification reactions using SNaPshot Multiplex kit (Life Technologies, NY, USA) via the ABI PRISM 3130xl Genetic Analyzer (Life Technologies, NY, USA) using POP-4® (life Technologies, NY, USA). The method was optimized for ancient DNA by adaptation of a published protocol (57). The PCR amplification reaction was performed using Eppendorf AG Thermocycler (Hamburg, Germany). Each 25µl PCR reaction consisted of 1X PCR buffer (Invitrogen, NY, USA), 4.0 mM $MgCl_2$ (Invitrogen, NY, USA), 400 µM of each dNTP (Invitrogen, NY, USA), 1.0 mg/ml BSA (Roche, BASEL, Switzerland), 2.0 U of Platinum *Taq* DNA Polymerase (Invitrogen, NY, USA), and the multiplex primer mixture (57) (Invitrogen, NY, USA). The PCR amplification reaction started with

a denaturing step at 95 degrees C for 2 minutes, followed by 37 cycles of denaturing on 95 degrees C for 30 seconds, annealing at 60 degrees C for 30 seconds, and extension at 65 degrees C for 30 seconds and finally a final extension step at 65 degrees C for 7 minutes. ExoSAP-IT PCR clean-up kit (GE Healthcare, Life Sciences, Quebec, Canada) was used for PCR clean-up to get rid of primers and unincorporated nucleotides. The SNaPshot results were analyzed on GeneMapper 4.0 software (Life Technologies, NY, USA) and Y-chromosomal haplogroups were assigned according to an updated human Y-chromosomal haplogroup tree (54).

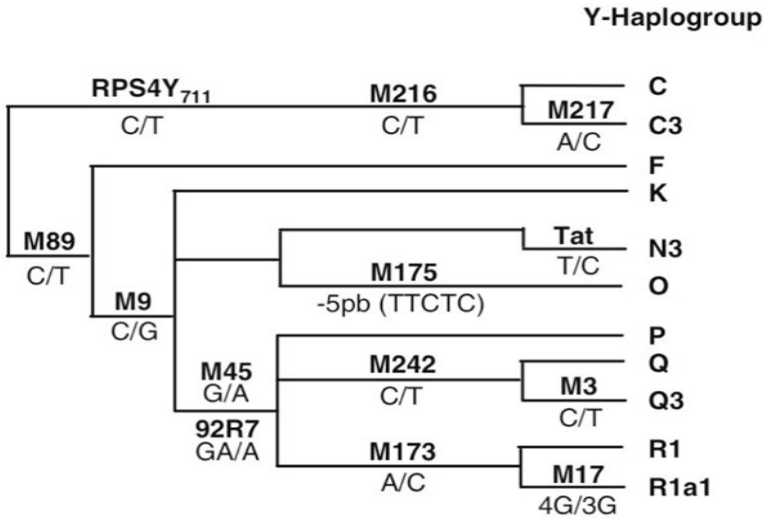

Figure 3.3. Phylogenetic tree for the tested 13 Y-chromosomal SNPs analyzed by this project. SNP names and sequence variations indicated above and under the lines, respectively. The haplogroups associated with the specific variations designated at the end of the line (57).

2.4.2. Y-chromosomal sub-haplogroups of Q-M242

Primers suitable for amplifying the SNP sites were designed using Primer3Plus software (68) (Table 2.2, Chapter 2). The amplicon size was less than 155 bp to match the criteria used with the SNaPshot multiplex PCR reaction. The PCR amplification reaction was identical to the amelogenin analysis except that the annealing temperature was 58 degrees C.

2.4. Evaluation of authenticity

Authenticity of all results obtained during the study was established through multiple analyses of the same sample following published guidelines (61). Two separate SNaPshot reactions, multiplex I (7-plex) and multiplex II (6-plex), were performed on each sample, and results rejected if there was discordance between SNaPshot I and SNaPshot II. Analysis of sub-haplogroups of Q-M242 was repeated twice for each sample. Because the study involved only East Asian and Siberian populations and their Y-chromosomal haplogroup distribution (54) any result demonstrating a SNP marker outside this haplogroup distribution of the population was excluded. Because the peak threshold on the electropherogram is 100 relative fluorescence units (RFUs), any peak less than this value was rejected. Fortuitously, only females were working with the samples in this study, minimizing the risk of contamination from male DNA.

2.5. Statistical analysis

To test the null hypothesis for the Y-chromosomal haplogroup distributions, i.e. that the EN cemeteries (Lokomotiv and Shamanka II) were continuous to LN-EBA cemeteries (Kurma XI and Ust'-Ida), Fisher's exact test was applied with a two-by-two contingency table. The table was extended to the size described by the number of populations and the number of haplogroups examined in the study. Fisher's exact test is analogous to two-way contingency chi-square test, but Fisher's exact test is always chosen when dealing with a small sample size as in this study's case as two-way contingency chi-square test requires that any given cell have a minimum frequency of five (69), which was not obtainable in our study for some haplogroups. Fisher's exact test was performed using the **SAS/STAT®** software.

3. Results

3.1. Analytical results

Amelogenin analysis for Lokomotiv samples by Mooder et al. (2005) identified 15 males and four females (22). Amelogenin analysis for Shamanka II samples by Thomson (2005) identified six males and one female (13), and another four Shamanka II males and two females were identified by amelogenin analysis as part of this study. For Ust'-Ida, 18 males and eight females were identified by Mooder et al. (2006) through amelogenin analysis. Finally for Kurma XI, six males and five females were identified by Moussa et al. (n/d) through amelogenin analysis (Table 3.2). Appendix 3.5 (Table) illustrates the discrepancies between morphological and molecular sex from all the four studied cemeteries.

Authentic Y-chromosomal results were obtained from a total of 36 males from the four cemeteries are represented in (Table 3.2). Only results meeting the authenticity criteria are included. The Lokomotiv cemetery showed a low success rate for Y-chromosomal analysis (7/17 males, 41%). However, higher success rates were obtained from the other cemeteries, Kurma XI (6/7 males, 86%), Ust'-Ida (14/16 males, 87%) and Shamanka II (9/9 males, 100%). Eight individuals from Lokomotiv and 2 from Ust'-Ida did not give results. Three individuals (two from Lokomotiv and one from Kurma XI) did not have Y-chromosomal haplogroups assigned (marked "not assigned" in Table 3.2), as some of the key SNPs (Figure 3.3) did not amplify either by SNaPshot I or II reactions. Three individuals from the LN-EBA (Ust'-Ida and Kurma XI) cemeteries show a previously undescribed polymorphism, L914SNP (T>G transversion) (Table 3.2).

Despite the low analytical success rate, Lokomotiv demonstrated the highest degree of heterogeneity in Y-chromosomal haplogroup distribution with four individuals belonging to haplogroup K-M9, two to haplogroup R1a1-M17 and one to haplogroup C3-M217. As mentioned earlier (1.3.1) the Lokomotiv graves are grouped in seven clusters (Appendix 3.1-Lokomotiv cemetery map) (23). Three males belonging to haplogroup K-M9 come from cluster 2 (LOK_1980.010.02, LOK_1980.016 and LOK_1980.022.02) and one comes from cluster 7 (LOK_1990.042). The two males belonging to haplogroup R1a1-M17 come from cluster 2 (LOK_1980.006 and LOK_1981.024.01), and the only male (LOK_1985.031.02) carrying C3-M217 Y-chromosomal haplogroup comes from cluster 4.

All individuals from the other EN cemetery, Shamanka II, belonged to one haplogroup, K-M9. There are two main grave clusters in Shamanka II (North West- NM and South East- SE), each of these two clusters has 'rows' of graves, and a row is, as cited by McKenzie, H (2005) "at least three closely associated parallel graves arranged in a more or less straight line" (88). Each of the analyzed Shamanka II males comes from a different row. SHA_2001.012 and SHA_2001.013.03 males come from 'row H'. SHA_2002.021.02, SHA_2002.021.03, SHA_2002.023.04, and SHA_2004.052.01 males come from 'row F'. SHA_2004.044.02 male comes from 'row K'. For SHA_2005.059.01 and SHA_2006.083.01 males both come from scattered graves that do not belong to a specific row (Personal communication with Dr. A.W. Weber and Dr. V. I. Bazaliiskii, 2012 (89))

The Y-chromosomal haplogroups from the LN-EBA cemetery Ust'-Ida, showed one individual with haplogroup K-M9, two individuals (Other, L914 SNP) and the remaining eleven individuals belonging to haplogroup Q-M242 and its sub-clade Q1a3-M346. In the other LN-EBA cemetery, Kurma XI, one individual carried the L914 SNP and all remaining individuals belonged to Q-M242/Q1a3-M346 (Table 3.2, Figure3.4). None of the analyzed individuals specifically from Ust'-Ida or Kurma XI belonged to sub-haplogroup Q1a2-M25.

Table 3.2. Amelogenin results, mtDNA and Y-chromosomal haplogroups from the male individuals belonging to the four studied cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI).

No	Cemetery & Master ID	Morphological Sex	Molecular Sex	Mitochondrial DNA Haplogroup	Y-chromosome Haplogroup
Lokomotiv (EN)					
1	LOK_1980.004	Female	XY [a]	D [a]	m.d.
2	LOK_1980.006	Male	XY [a]	F [a]	R1a1-M17
3	LOK_1980.010.02	Male	XY [a]	Other [a]	K*-M9
4	LOK_1980.010.03	Female	XY [a]	F [a]	Not Assigned
5	LOK_1980.012	Female	XY [a]	A [a]	m.d.
6	LOK_1981.013	Male	XY [a]	D [a]	Not Assigned
7	LOK_1980.014.03	Immature	XY [a]	U5a [a]	m.d.
8	LOK_1980.016	Male	XY [a]	D [a]	K*-M9
9	LOK_1980.017	Male	XY [a]	A [a]	m.d.
10	LOK_1980.022.02	Male	XY [a]	C [a]	K (2)
11	LOK_1981.024.01	Immature	XY [a]	F [a]	R1a1-M17
12	LOK_1984.027	Male	XY [a]	D [a]	m.d.
13	LOK_1985.031.02	Male	XY [a]	A [a]	C3-M217
14	LOK_1988.038.01	Female	XY [a]	F [a]	m.d.
15	LOK_1990.042	Male	XY [a]	G2a [a]	K-M9
16	LOK_1990.044.01	Male	XY [a]	F [a]	m.d.
17	LOK_1990.044.02	Male	XY [a]	F [a]	m.d.
Shamanka II (EN)					
1	SHA_2001.012	Undetermined	XY [b]	D *	K*-M9
2	SHA_2001.013.03	Probable Female	XY [b]	D [b]	K*-M9
3	SHA_2002.021.02	Male	XY [b]	G2a *	K*-M9
4	SHA_2002.021.03	Undetermined	XY [b]	A *	K*-M9
5	SHA_2002.023.04	Undetermined	XY [b]	D *	K*-M9
6	SHA_2004.052.01	Probable Male	XY	D	K-M9
7	SHA_2004.044.02	Undetermined	XY	C	K-M9
8	SHA_2005.059.01	Male	XY	C	K-M9
9	SHA_2006.083.01	Male	XY	C	K-M9

Table 3.2. (Continued…)

No	Cemetery & Master ID	Morphological Sex	Molecular Sex	Mitochondrial DNA Haplogroup	Y-chromosome Haplogroup
	Ust'-Ida (LN-EBA)				
1	UID_1987.005	Immature	XY [a]	Other [a]	Other* (L914 SNP)
2	UID_1987.009	Immature	XY [a]	A [a]	Q1a3*-M346
3	UID_1987.012	Male	XY [a]	D [a]	Q1a3-M346
4	UID_1988.016.01	Male	XY [a]	F [a]	Q1a3-M346
5	UID_1988.016.02	Male	XY [a]	Other [a]	Q1a3-M346
6	UID_1989.020.01	Male	XY [a]	C [a]	Q1a3-M346
7	UID_1989.026.01	Immature	XY [a]	A [a]	Other* (L914 SNP)
8	UID_1989.029	Male	XY [a]	A [a]	Q1a3-M346
9	UID_1989.030	Female	XY [a]	A [a]	m.d.
10	UID_1990.033.01	Male	XY [a]	Other [a]	Q1a3-M346
11	UID_1991.038	Male	XY [a]	C [a]	Q1a3*-M346
12	UID_1993.043	Male	XY [a]	G2a [a]	K*-M9
13	UID_1993.044.03	Immature	XY [a]	A [a]	Q1a3-M346
14	UID_1994.048	Male	XY [a]	C [a]	Q1a3*-M346
15	UID_1994.053.02	Immature	XY [a]	A [a]	m.d.
16	UID_1994.055.02	Male	XY [a]	G2a [a]	Q-M242
	Kurma XI (EBA)				
1	KUR_2002.001	Male	XY	D	Not Assigned
2	KUR_2002.007.02	Male	XY	D	Q1a3*-M346
3	KUR_2002.010	Probable Male	NA	Z	Q1a3*-M346
4	KUR_2002.015	Probable Male	XY	Z	Q1a3*-M346
5	KUR_2003.017	Probable Male	XY	D	Q-M242
6	KUR_2003.018	Probable Female	XY	D	Q-M242
7	KUR_2003.019	Probable Male	XY	F	Other* (L914 SNP)

- m.d.= missing data, no results were obtained from these samples.
- K*, R1a1*, Other*, Q1a3* results were obtained through a single-plex PCR amplification reaction.
- Not Assigned: the data obtained were not adequate enough to assign a Y-chromosomal haplogroup.
- [a] Molecular sexing and mtDNA haplogroup determined by Mooder (2004) (12).
- [b] Molecular sexing and for one sample mtDNA determined by Thomson (2005) (13).
- * re-analyzed samples from Shamanka II cemetery for more details (Chapter 4).

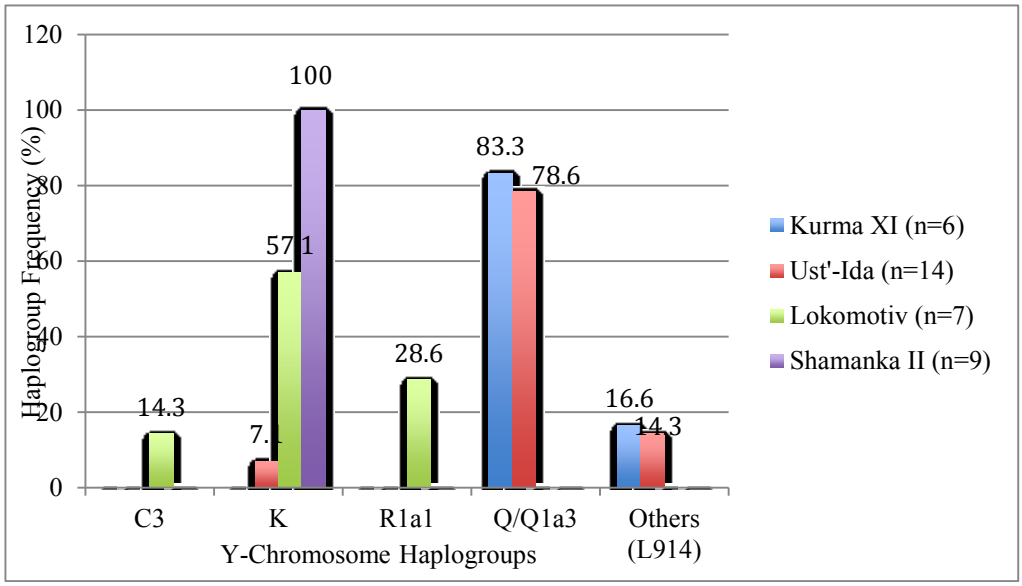

Figure 3.4. A column chart representing Y-chromosomal haplogroup frequency distribution in percentage for the four studied cemeteries (Lokomotiv, Shamanka II, Ust'-Ida, and Kurma XI).

Table 3.3. Explanation to fig 3.4 **(**Number of male individuals from each of the four studied cemeteries and the Y-chromosomal haplogroups they are assigned to).

Cemetery / Y-Chr. Haplogroups	C3	K	R1a1	Q/Q1a3	Others (L914)
Kurma XI (n=6)	0	0	0	5/6 (83.3%)	1/6 (16.6%)
Ust'-Ida (n=14)	0	1/14 (7.1%)	0	11/14 (78.6%)	2/14 (14.3%)
Lokomotiv (n=7)	1/7 (14.3%)	4/7 (57.1%)	2/7 (28.6%)	0	0
Shamanka II (n=9)	0	9/9 (100%)	0	0	0

3.2. Statistical results

Fisher's exact test results obtained from the two EN cemeteries, Lokomotiv and Shamanka II, showed a value (Fisher's exact test; p=0.0625) close to the statistical significance level 0.05. This might indicate a similar paternal background. However, the number of individuals analyzed may not be representative of the entire male population from the two cemeteries, and analyzing more male samples would be valuable.

The Fisher's exact test obtained from the two LN-EBA cemeteries, Ust'-Ida and Kurma XI, showed a strong statistical similarity (p=1.00), which is indicative of sharing the paternal origin between these two LN-EBA cemeteries.

Fisher's exact test obtained between Kurma XI vs. Lokomotiv and Kurma XI vs. Shamanka II showed significant statistical differences between them (p= 0.0023 and p=0.0002; respectively). Similarly, Fisher's exact test between Ust'-Ida and Lokomotiv (p= 0.0002), and Ust'-Ida and Shamanka II (p< 0.0001) also demonstrated a significant statistical difference.

4. Discussion

4.1. EN cemeteries populations' archaeological context

Individuals from the two EN cemeteries of Cis-Baikal showed a Y-chromosomal haplogroup distribution (Fisher's exact test; p=0.0625), which approached a statistical significance level p value of 0.05. The lack of Y-chromosomal haplogroup (only K-M9) variation in Shamanka II is striking. By contrast, there were three different Y-chromosomal haplogroups (K-M9, R1a1-M17, C3-M217) among the seven individuals represented at Lokomotiv, which indicates a high level of paternal heterogeneity at this cemetery. The statistics suggest a relatively close paternal biological relationship between the two EN communities. The two EN cemeteries are located on two different micro-regions in Cis-Baikal area, Lokomotiv on the Angara River Valley and Shamanka II on the South Baikal region. Stable isotope ratios, reflecting diet, from the two micro-regions show different signatures depending on the different types of fish and aquatic foods available in each area. Individuals from Lokomotiv and Shamanka II would be expected to show different stable isotope signatures because they are far apart from each other and located on two different fisheries, Lokomotiv on the riverine Angara and Shamanka II on lacustrine Lake Baikal. However, Lokomotiv and Shamanka II show similarity in their stable

isotope signatures ($\delta^{13}C$ and $\delta^{15}N$). These two large EN populations probably had similar adaptive strategies and access to both fisheries, the Angara and Baikal. Also, this can be an indication for an interaction between the populations of these two EN cemeteries (5).
As explained earlier (1.3.1), while there are common characteristics, such as use of red ochre as a mortuary ritual and common grave inclusions, between the two EN cemeteries (Lokomotiv and Shamanka II), there are differences in mortuary rituals and grave goods morphology (26). The differences between Lokomotiv and Shamanka II might also be relevant to Lokomotiv being heterogeneous in paternal Y-chromosomal haplogroup distributions. Further investigation of other male individuals from Lokomotiv and Shamanka II, to detect if the variation in haplogroup distribution would increase or decrease with increasing the sample size and if other Y-chromosomal haplogroups would be detected in Shamanka II, is necessary because the Y-chromosomal results from both EN cemeteries (Lokomotiv and Shamanka II) might be biased by the small sample size (Lokomotiv, n=7, and Shamanka II, n=9).
Lokomotiv show a high degree of variability in strontium isotope ratios within and between individuals proposing differences in mobility during the individual's life (20). Bone and teeth strontium isotope ratios from Lokomotiv (Fig. 3.5) showed a degree of inconsistency between individuals, suggesting differences in mobility between the individuals. Linking this variability to the Y-chromosomal haplogroup results at the individual level we observe that the only three males belonging to haplogroup K-M9 (LOK_1980.010, age 20-25 years, LOK_1980.016, age 45-55 years and LOK_1990.042, age 40-50 years) did not show a high degree of variability in $^{87}Sr/^{86}Sr$ between bones and teeth suggesting lower mobility during their lifespan. By contrast, the only male in the EN population belonging to haplogroup C3-M217 (LOK_1985.031.02, age 25-30 years) demonstrated a strikingly different $^{87}Sr/^{86}Sr$ ratio from the other three individuals at Lokomotiv from whom both strontium isotope and Y-haplogroup analysis is available. This suggested that he was born in one location, and although buried in the Lake Baikal area, spent his adulthood outside the region as his $^{87}Sr/^{86}Sr$ from femur is quite different to the other 16 individuals at Lokomotiv. Unfortunately, no further information could be gleaned about this individual. He was buried in a double grave in Cluster 4 (38) and therefore unlikely to be 'elite' because Mooder (2006) suggested that in the EN culture (Kitoi culture) 'elite' people were

buried in a single grave and mostly clustered in Cluster 2 of the Lokomotiv cemetery (38). The other individual (LOK_1985.031.01, age 35-50 years) buried with the C3-M217 male (LOK_1985.031.02), in a double grave, had an undetermined morphological sex (70).

For Shamanka II, no strontium isotope data from Shamanka II has been published yet. Regarding the Shamanka II individuals buried in different rows as was mentioned earlier in section (3.1) of this chapter, the assessment of Y-chromosome data and that all Shamanka II males beong to haplogroup K-M9 revealed no further patterning. However, this insight could change with investigating sub-haplogroups of K-M9 and with analyzing more Shamanka II males.

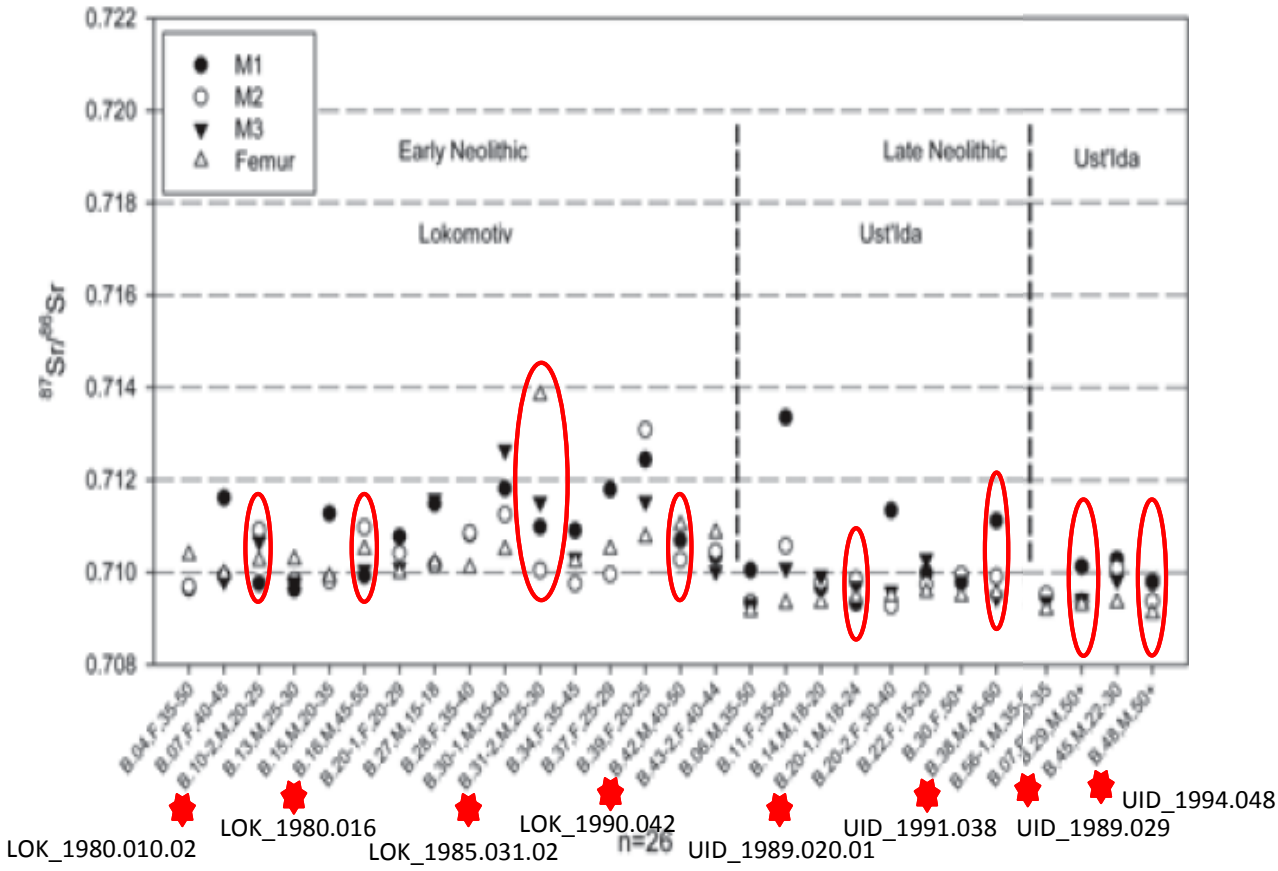

Figure 3.5. Strontium isotope ratios ($^{87}Sr/^{86}Sr$) measured in M1 (Molar 1), M2 (Molar 2), M3 (Molar 3) and Femur from Lokomotiv and Ust'-Ida individuals, the 'x' axis provides information about the burial numbers and sex and age of the individuals (B=burial, M=male, F=female, PM=probable male, and UND=undetermined sex). The 'y' axis represents the $^{87}Sr/^{86}Sr$ ratios. The individuals, with their Mastr IDs, circled and stared with red are the ones that Y-chromosomal results have been obtained from them (20).

Adapted with permission from [Identifying Hunter-Gatherer Mobility Patterns Using Strontium Isotopes. C.M. Haverkort, V.I. Bazaliiskii, N.A. Salvel'ev. University of Pennsylvania Museum. Copyright © 2010 Courtesy of the Penn Museum].

4.2. LN-EBA cemeteries populations' archaeological context

Individuals from the two LN-EBA cemeteries (Ust'-Ida and Kurma XI) showed no significant differences in their Y-chromosomal haplogroup distribution (Fisher's exact test; p= 1.00) with haplogroup Q-M242 being the predominant in both populations (n=11/14 individuals in Ust'-Ida, and n=5/6 individuals in Kurma XI). Ust'-Ida and Kurma XI belong to two different micro-regions in the Lake Baikal area (Figure 3.1). Y-chromosomal haplogroups from Ust'-Ida were obtained from 12 LN and two EBA individuals. All Kurma XI individuals from whom Y-chromosomal haplogroups were obtained belonged to the EBA period. There are many differences between the two cemeteries including mortuary rituals, grave structure and types as described previously (1.3.2).

Individuals of all ages, including subadults and adults, are buried at Ust'-Ida. However, at Kurma XI there were no individuals under the age of 15 or older than 50 years, which might hint that this is an 'exclusive' cemetery rather than a community burial ground (34). The two males (UID_1987.005 and UID_1989.026.01), who carry the Y-chromosomal L914 SNP in Ust'-Ida, are both juvenile (Table 3.1 and table 3.2). One male (UID_1987.005) was buried in a single grave, but the other male (UID_1989.026.01) was buried in a multiple grave. The only individual in Kurma XI carrying the Y-chromosomal L914 SNP (KUR_2003.019) was a young adult and was in one of the three sitting position graves at Kurma XI. There was no obvious relation between the two individuals at Ust'-Ida carrying the Y-chromosomal L914 SNP as both had different mtDNA haplogroups (Table 3.2). One was buried in a single grave (UID_1987.005) while the other was buried in a multiple grave (UID_1989.026.01). The Kurma XI individual (KUR_2003.019) carrying Y-chromosomal polymorphism L914 belonged to mtDNA haplogroup F (Table 3.2). However, unfortunately, no Y-chromosomal haplogroup data were obtained from the other two sitting burials in Kurma XI (i.e. samples did not amplify after several trials) to compare them genetically to each other.

The Strontium isotope ratios of individuals from Ust'-Ida show a low variability when compared to Lokomotiv individuals, which might suggest a lower mobility during lifetime (20). Unfortunately, no Strontium isotope data have been published for Kurma XI individuals yet.

Despite the many differences in burial practices and mtDNA haplogroup distribution between either LN Ust'-Ida or EBA Ust'-Ida and Kurma XI (Chapter 2), the Y-chromosomal haplogroup frequency and distribution is very similar, implying that both Ust'-Ida and Kurma XI had a similar paternal origin.

4.3. Correlation between maternal and paternal background in Cis-Baikal EN and LN-EBA populations

Comparing the Y-chromosomal haplogroup distribution findings to the mtDNA haplogroup distribution reflecting the paternal and maternal background might provide insight into the genetic relationship between the individuals in the various Cis-Baikal cemeteries. In Lokomotiv, the two individuals (LOK_1980.006 and LOK_1981.024.01) carrying R1a1-M17 Y-chromosomal haplogoup, both belong to mtDNA haplogroup F (22), and both are buried in cluster 2. These two males might be related but have insufficient evidence at present as there is a lack in other DNA sequence data, autosomal DNA analysis. The only individual at Lokomotiv (LOK_1985.031.02) with the Y-chromosomal haplogroup C3-M217, had the mtDNA haplogroup A, presented at a low frequency (13%, n=4/31) in Lokomotiv (22). This individual also showed different strontium isotope ratios, indicating a higher degree of mobility than other Lokomotiv individuals (20). Does his uncommon genetic background and unique strontium isotope ratio imply that he was from outside the Lokomotiv locality? All three individuals at Lokomotiv carrying the K-M9 Y-chromosomal haplogroup carried different mtDNA haplogroups (22) (Table 3.2), so no connection between maternal and paternal genetic background can be concluded. All males in Shamanka II cemetery belong to one Y-chromosomal haplogroup (K-M9), although there are five different mtDNA haplogroups represented (Table 3.2). The lack of variation in Y-chromosomal haplogroups in this population makes it difficult to draw conclusions about the genetic relationship between the individuals because they might be all related paternally, but further Y-chromosomal analysis would be required to analyze sub-haplogroups of K-M9.
In the Ust'-Ida burials, one major Y-chromosomal haplogroup, Q-M242/Q1a3-M346, is represented. The exceptions were two individuals (UID_1987.005 and UID_1989.026.01) with the novel Y-chromosomal SNP, L914 and one male (UID_1993.043) with the K-M9 haplogroup.

The two males in Ust'-Ida (UID_1987.005 and UID_1989.026.01) carrying the L914 SNP belong to different mtDNA haplogroups (Other and A, respectively) (Table 3.2). This finding indicates that there is no maternal relation between these two Ust'-Ida individuals carrying Y-chromosomal L914 SNP. Also, 83.3% (n=5/6) of males in Kurma XI belong to Y-chromosomal haplogroup Q-M242 or its sub-haplogroup Q1a3-M346. These males belong to either mtDNA haplogroup D (three individuals) or Z (two individuals), and the only male carrying Y-chromosomal polymorphism L914 has a different mtDNA haplogroup than the other males, F (Table 3.2). mtDNA haplogroup F is present in 25% (n=3/12) of individuals in the EBA Kurma XI cemetery sample (Chapter 2). The other two individuals carrying the mtDNA haplogroup F are females, and there are no other males in Kurma XI cemetery carrying Y-chromosomal polymorphism L914. Therefore, no relation can be drawn between mtDNA and Y-chromosomal haplogroups' distributions at the mean time.

4.4. Genetic context of the Cis-Baikal prehistoric population and the connection with the modern populations

The modern Cis-Baikal populations carry a heterogeneous distribution of Y-chromosomal haplogroups, each with a different origin and composition.

Haplogroup K-M9 is represented in both EN cemeteries (57.1%, n=4/7) in Lokomotiv and 100%, n=9/9 in Shamanka II), but with low frequency in the LN-EBA cemetery Ust'-Ida (7.1%, n=1/14) and absent in Kurma XI. It is believed that the origin of haplogroup K-M9 is from Southwest Asia (71). K-M9 is found with high frequency in south Siberian populations, for example, in the Tuvans and Mongolians and is also found in one central Asian population (Uigur) (72). K-M9 is an ancient haplogroup with an estimated age of 40,000-53,900 years. Haplogroup K with its designated M9 mutation is considered the ancestral haplogroup that defines each of L, M, NO, P, S and T haplogroups (54).

The second Y-chromosomal haplogroup found in high frequency in the LN-EBA populations is Q-M242 or its sub-haplogroup Q1a3-M346. Haplogroup Q-M242/Q1a3-M346 is found in 78.6% (n=11/14) of Ust'-Ida individuals and in 83.3% (n=5/6) of Kurma XI individuals. The approximate age of this haplogroup is 17,700 $\pm$ 4,800 years. Haplogroup Q-M242 is widely distributed across Siberia (58), and it is also widely distributed in Central to South Asia, West Eurasia and northern

East Asia, which might be indicative of Q-M242 expansion through northern Eurasia as a migratory route (73). Individuals carrying haplogroup Q-M242 might have migrated from Siberia via the Altai/Baikal area to the Americas (74-76), which suggests the presence of this haplogroup in the area just after the Last Glacial Maximum (59). A study done by Malyarchuk, B. et al. (2011) examining several northern East Asian sub-haplogroups of Q-M242, showed that Q1a3-M346 is more frequent than other sub-haplogroups (Q1a-MEH2, Q1a2-M25 and Q1a3a-M3) in Siberian populations. Q1a3-M346 is found is widely distributed in the Altaians, Todjins and Tuvinians, moderately distributed in Khakassians and Sojots, and rarely found in the Kalmyks population (59).

Haplogroup R1a1-M17 was identified in 28.6% (n=2/7) of males in the Lokomotiv population. This haplogroup is not only defined by the M17 marker, but also by M173 marker (53,54). R1a1 is widely distributed in Eurasia, including western Eurasia, southern Asia, central Asia and the Siberian population, particularly southern Siberia. It is believed that the R1a1 haplogroup is associated with the early migration of the Indo-Europeans eastward (77). The estimated age of R1a1-M17 is about 13,800 cal BP as estimated by the SNP evolution rate (78). It is also widely distributed in the southern and northern modern Altaian population (79). R1a1-M17 is found in the Tuvinian population (80), Khakassian population (81), and in the native Yakuts of the Sakha Republic (82). In prehistoric populations, the presence of the R1a1-M17 haplogroup was evident in the ancient Kurgan culture, a Late Bronze Age culture (5000 BC) found in southern Siberia. Twelve males analyzed from the ancient Kurgan people belong to R1a1-M17 haplogroup except for one belonging to C3 (83).

Haplogroup C3 is defined by the M217 SNP on the Y-chromosome. Only one C3 individual was found in the Cis-Baikal population. However, it is detected in several parts of Asia including the Central, South, Southeast and East of Asia, and also in Siberia and the Americas. There are many sub-haplogroups of C3 (84), but these were not investigated in this study. The haplogroup dated back in Siberia to 11,900 $\pm$ 4,800 years ago. A possible source of this haplogroup in Siberia is Mongolia and/or Lake Baikal (85).

The L914 SNP, a previously undescribed (T> G) transversion, was found in three LN-EBA individuals. In one of the Ust'-Ida individuals (UID_1989.026.01), L914 SNP was amplified in two

different samples, one bone (2009.190) and one tooth (2001.547). For the other Ust'-Ida individual, L914 SNP was amplified twice in two different occasions from the same bone sample (2009.177) (Table 3.1 and table 3.2). Reproducible results were also obtained from the Kurma XI individual defining L914 SNP (KUR_2003.019). These findings indicate that the L914 is a novel authentic SNP site on the LN-EBA individuals from Cis-Baikal prehistoric population. L914 SNP does not define a specific Y-chromosomal haplogroup and is not cited in the modern world population, which might indicate that this is a rare mutation site that is not defined yet.
No further statistical analysis (e.g. comparing the ancient populations genetically to the modern populations) was possible due to the small number of results obtained from each of the four cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI). Therefore, analyzing more samples from the cemeteries would allow for further investigations on the ancient populations of Cis-Baikal and comparing them genetically to the modern Siberian populations.

5. Conclusion

The EN and the LN-EBA populations at Lake Baikal might be paternally genetically distinct; however, this insight might change with analyzing more males from all the mentioned cemeteries as the number of males obtained in this study may not be representative of the entire population. The earlier conception of the biological discontinuity between the EN and the LN to EBA groups in the Cis-Baikal area suggested by Weber et al. (1995 and 2002) (1,86) might be supported now from the analysis of the paternal background of the area. The differences in the Y-chromosomal signatures of the EN and LN-EBA suggest displacement of males in the Cis-Baikal population during the EN period and replacement by a genetically different population during the LN and EBA. As shown in the results, there is little resemblance in the Y-chromosomal haplogroups shared between the EN and LN-EBA. There might be a similarity in Y-chromosomal haplogroup distribution between the two EN (Kitoi culture) cemeteries (Lokomotiv and Shamanka II), which might indicate a common paternal ancestor and possible social interaction, even if the new mtDNA data from Shamanka II showed that Lokomotiv and Shamanka II are maternally different from each other (Chapter 2). Sharing a common paternal ancestor between Lokomotiv and Shamanka II needs to be further investigated through analyzing more male samples from both cemeteries (Lokomotiv and

Shamanka II) as the results might be biased due to the small sample sizes from both cemeteries. The Y-chromosomal haplogroup distribution of the two LN-EBA cemeteries is similar, indicating a unified paternal origin for the area during the LN-EBA period. Interestingly; however, the mtDNA haplogroup distribution in EBA Kurma XI is different from LN Ust'-Ida and also from EBA Ust'-Ida (Chapter 2). Based on Y-chromosomal haplogroup analysis of both the EN and LN-EBA cemeteries, it is clear that the Y-chromosomal haplogroups of the prehistoric Cis-Baikal population are represented in the contemporary Siberian populations. The haplogroups found in the prehistoric study groups can mark migration events from the Lake Baikal area to the south towards Mongolia, and the Altai Republic as for haplogroup C3-M217 and R1a1-M17, respectively. Furthermore, the ancient hunter-gatherer groups that inhabited Lake Baikal played a major role in the formation of the Native American tribes evident in the existence of sub-haplogroup Q1a3 in the LN-EBA people. This is evident in the high frequency of sub-haplogroup Q1a3a-M3 in the Native American population (87). The sister sub-haplogroup of Q1a3a-M3 is sub-haplogroup Q1a3-M346 (59), which is identified in the LN-EBA populations. Analyzing more male samples would be beneficial to observe possibly other males carrying Y-chromosomal haplogroup C3-M217 and compare their strontium isotope ratios to examine if these males share similar mobility levels.

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Chapter 4

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Limitations in Working with Ancient DNA and How to Resolve Them

1. Introduction

There are several challenges in the retrieval and analysis of DNA from ancient specimens. Exogenous contamination is the most critical issue that compromises the authenticity of retrieved ancient DNA (aDNA) (1). Contamination can occur from the time of specimen excavation until the time of DNA extraction and analysis. Sources of contamination include human contact (excavators, examiners, laboratory personnel) and any reagents used during extraction or analysis (2). Cooper and Poinar introduced nine criteria for ancient DNA laboratories to follow in order to avoid contamination (3). Failure to follow these criteria may jeopardize the authenticity of results (4).

In addition, aDNA samples, either bone or teeth, may contain PCR inhibitors co-purified during extraction, which interfere with the PCR amplification process. PCR inhibitors make aDNA amplification extremely challenging and sometimes impossible. Thus, removing these inhibitors is essential to ensure successful PCR amplification (5-7). The common PCR inhibitors that are co-extracted with aDNA from hard tissues (bone and teeth) include humic acid (8), fulvic acid (9) and collagen type I (10). Extraction and amplification methods have been developed to significantly reduce these inhibitors (11,12).

When an organism dies, mutations and chemical changes accumulate in the DNA (13). DNA preservation depends on several variables such as environmental temperature and humidity (14). Dry weather and low temperature favor good DNA preservation presumably because they decrease the rate of destructive chemical processes (15), but may also depend on the location of the burial site and preservation conditions such as the depositional environment (16). Using gas chromatography/mass spectrometry, Hoss et al. (1996) measured the amount of damage in aDNA samples taken from cold regions and warm regions. They found that low temperature is an important factor for good long-term preservation, and the degree of DNA decay would decrease 10 to 25 folds for every 20 degrees C decrease in temperature (15). aDNA can be preserved from complete decay at an optimum temperature of 15 degrees C or less, and at optimum pH level 7.0 in dry conditions with very limited humidity (16).

Regardless of temperature and humidity, the burial environment still induces several types of DNA damage, including oxidation, alkylation and cross-linking, through natural agents such as microorganisms, ionizing and UV radiation (17).

The surrounding environment may cause DNA oxidation or hydrolytic damage. Over time, natural ionizing radiation degrades fully dehydrated DNA into shorter segments specifically by depurination and oxidative damage (1,13). DNA from ancient remains is also highly fragmented and cross-linked due to single and double stranded breaks (18). Invasion of decaying remains by putrefactive microorganisms and soil microorganisms accelerate the DNA decaying process in either soft or hard tissue (19).

Despite all the obstacles associated with aDNA analysis, the successful retrieval and analysis of aDNA is still possible. There are several examples documented in the literature indicating that aDNA can be successfully retrieved from ancient human samples using advanced sequencing technologies, for example, aDNA was obtained from 12,707-12,556 calendar years Before Present (BP) infant child remains from the Late Pleistocene Clovis burial site in western Montana, indicating that this individual belonged genetically to the ancestral population of many contemporary Native Americans (20). Moreover, DNA has been obtained from Neanderthals, the sister group of modern humans (21) who disappeared from the fossil record about 30,000 years ago (22), revealing evidence of their interbreeding with modern humans (21). aDNA was also retrieved from an archaic hominid found in the Denisova Cave 'Denisovans' in the Altai Mountains suggesting that Denisovans differ genetically from Neanderthals and present-day humans (23).

Reliable extraction methods have been developed to retrieve aDNA from human bones, teeth, and soft tissues (11,15,24). Increasingly sensitive PCR based technologies have facilitated the use of aDNA analysis in archaeological material and have helped reveal the genetic background of vanished populations. However, highly damaged aDNA causes an inverse relation between amplification efficiency and the size of the amplified product, which sets a limit on the length of the PCR amplification performed to only few hundred base pairs (25,26). A DNA sequence of more than a few hundred base pairs is most likely to be exogenous modern DNA (27).

In our aDNA laboratory, we follow strict criteria to avoid contamination with exogenous modern DNA and to ensure the authenticity of the results (3) by use of protocols designed to detect any contaminating DNA sequences. In preparation for this project, I have optimized several new aDNA extraction and PCR amplification techniques to address the technical challenges during sample analysis.

2. Contamination

2.1. Laboratory design

The ancient DNA laboratory was designed according to published guidelines for working with ancient DNA (3). It is located in the basement of the Medical Laboratory Sciences building. The laboratory at the University of Alberta consists of two separate rooms; the first room (the clean room) is dedicated to sample preparation, DNA extraction and preparation of PCR amplification. It is important to mention that a biological safety cabinet (Class II, Type A2, Thermo Scientific, USA) was installed in the ancient DNA laboratory in 2012. The biological safety cabinet decreases the chances of contamination by protecting the samples from being contaminated with the exterior airflow since the air flowing in the cabinet is sterile HEPA-filtered air. Prior to installing the new cabinet, two Dead Air Box workstations were used, one for DNA extraction and the other for PCR amplifications.

Prior to entering the clean room, researchers dressed in gowns, booties, sterile sleeves, gloves, masks and goggles. After entering the clean room another set of gloves was worn and sprayed with 30% (v/v) bleach (sodium hypochlorite).

The second room (post-PCR room) houses a thermo-cycler and gel electrophoresis equipment.

2.2. Summary of DNA extraction methods used previously

Former researchers in the aDNA laboratory used sample preparation and extraction methods adapted from published protocols to minimize the risk of contamination with extraneous DNA and to reduce the amount of PCR inhibitors in the extracted aDNA (28-34). The sample surface was decontaminated thoroughly. First, the outer surface of either bone or tooth sample was removed using a sterile scalpel. Then, the sample was immersed in 10% bleach, and finally the sample was UV irradiated for one hour. Boom's (1990) extraction method was the protocol used previously (29,33,34). This method involved incubating the sample with extraction buffer

(guanidium thiocyanate, 0.1 M Tris pH6.4, 0.5 M EDTA and Triton X-100) overnight in a 65 degree C water bath (Day 1). Then followed the silica binding step (Day 2) that involved incubating the sample supernatant from Day 1 with 500 µl extraction buffer and 40 µl silica on a rotator for two hours. The silica pellet was washed once with a wash buffer (guanidium thiocyanate and 0.1 M Tris pH6.4), then with 70% ethanol and finally with acetone. After leaving the silica pellet to dry, the DNA was eluted from the silica with 100 µl water through incubating the silica pellet plus water in a 56 degree C water bath for one hour (35). Then the resulting DNA was used for further PCR amplification reactions.

2.3. Contamination detection

The analysis of aDNA, particularly when analyzing human specimens, carries an exceptionally high risk of contamination that can occur from the time of field excavation to the time of DNA extraction and amplification. During the PCR amplification process, any modern exogenous contaminant DNA is preferably amplified over the degraded small amount of aDNA (3). Therefore, to detect any possible contamination several negative controls are applied during DNA extractions and PCR amplifications.

Throughout this project, great care was always taken to minimize the risk of contaminating the samples. Detection of contamination is facilitated by the use of negative controls. A negative control is a sample that is treated the exact same way as an ancient sample and undergoes all steps in either the DNA extraction or PCR amplification experiments, but no DNA is added to the negative control throughout the experiments. An amplification product appearing in the negative control where no DNA was added is taken as evidence of contamination.

2.4. Procedure for investigation of an incidence of contamination

In February 2009, contamination was determined in the Extraction Blank (EB) when a 176 bp amplicon was detected. This corresponds to the amplicon size of the mtDNA sequence after amplification with L16211 and H16346 primers (Chapter 2), when visualized on polyacrylamide gel. The EB is the negative control for the extraction procedure. It is subject to the entire extraction procedure but no bone or tooth powder (sample) is added. In this case, identifying the source of the contaminating mtDNA amplicon in the extraction procedure was crucial. Possible sources of contamination in the ancient DNA laboratory include any equipment,

pipettes, or reagents, either the extraction or the PCR amplification reagents. All work on the ancient samples was suspended during the contamination detection process to avoid further contamination of the ancient samples.

First, swabs were taken from different areas in the clean room including the sides and base of Dead Air Box workstations where extraction and PCR amplification preparation were performed, centrifuge, pipettes (10 µl, 100 µl and 1000 µl), tubes, mortars and pestles used to pulverize the samples. Prior to use, the cotton swabs (Fisher Scientific, NY, USA) were sterilized by autoclaving. The swabs were dipped in tubes containing 1000 µl extraction buffer and incubated in a 65 degree C water-bath overnight, following the DNA extraction protocol used for ancient samples (35) at that time. Then, the PCR amplification procedure, used for the aDNA-extracted samples, was applied to all swab extracts (30,31). After running the samples on a 12% polyacrylamide gel, there was evidence of widespread low-level contamination in the clean room as several swabs demonstrated the presence of the 176 bp mtDNA amplicons. For example, some pipettes showed contamination but not the mortars or pestles. However, this finding did not pinpoint the source of contamination. Therefore, a further investigation was undertaken to detect whether the source of contamination was the reagents used during the extraction protocol.

To test out the extraction reagents, two factors were taken into consideration. Firstly, the extraction procedure was performed in a biological safety cabinet in another laboratory to eliminate the risk of contamination from the equipment in the clean room. Secondly, because the ancient samples are very valuable and irreplaceable, another non-human DNA sample was used to investigate the possible source of contamination. With the help of Dr. Linda Chui (Department of Laboratory Medicine and Pathology, University of Alberta), bacterial DNA was extracted using the same Boom's (1990) silica-guanidinium thiocyanate extraction protocol used previously for aDNA samples (35). Two different strains of bacteria were used (*Corynebacterium diphtheriae* (Bacterial strain given by Maria Ackney) and *Escherichia coli* (Bacterial strain given by Dr. Linda Chui)), and the extractions were performed in Dr. Chui's laboratory (36) at the Provincial Laboratory for Public Health, University of Alberta Hospital. The extraction procedure was conducted using the same reagents used with the ancient samples.

The PCR amplification was carried out as described in Mooder et al. (2005, 2006) (30,31) but with the 16S universal primers (Invitrogen,NY, USA). The 16S universal primer is a primer designed to amplify the 16S ribosomal bacterial DNA gene, and it is widely used in bacterial phylogenetic studies (37), because this area is highly conserved in several species of bacteria (38). The PCR products were visualized by polyacrylamide gel electrophoresis. The extraction buffers were eliminated as a source of contamination because the two EBs showed no evidence of any DNA while bacterial DNA amplicons of the correct size were visible in the bacterial extractions.

The experiments suggested that the source of contamination was most likely the laboratory equipment and environment (i.e. the air in the clean room as the clean room does not have a separate ventilation system). Hence, the laboratory equipment and the Dead Air Box workstations for preparing the samples were all cleaned thoroughly (described later in section 2.4.1). Before continuing any further analysis on the ancient samples, new aliquots were prepared for the PCR amplification reagents including: $MgCl_2$, 10 X PCR buffer, dNTP and BSA. At this time, the Platinum *Taq* DNA polymerase and mtDNA primers (L16211, H16346) were not replaced with new aliquots to determine if they were the source of contamination.

To test whether the Platinum *Taq* DNA polymerase and/or the mtDNA primers (L16211, H16346) were contaminated, PCR amplification was performed, according to the protocol of Mooder et al. (2005) (30), in which the mtDNA primers (H16346, L16211) should not anneal with the bacterial DNA. No DNA was added to any of the PCR amplification reaction tubes in the clean room. The samples were transferred to the post-PCR room, where the bacterial DNA extract and modern human DNA positive control were added as a confirmatory step to demonstrate whether the PCR reagents were contaminated (bacterial DNA extracts) and that the PCR amplification reaction was performing properly (human DNA positive control). After running the PCR products on a polyacrylamide gel, two of the bacterial extracts showed evidence of contamination with the appearance of very faint bands with the size of 176 bp corresponding to the amplification of mtDNA region that the H16346 and L16211 primers were designed to amplify. There was no evidence of contamination in the negative controls including the EBs prepared during the bacterial extraction. The presence of amplicons corresponding to

the mtDNA region in the bacterial DNA extracts confirmed the presence of a random low level of DNA contamination in the laboratory.

mtDNA primers and the Platinum *Taq* DNA polymerase, reagents not replaced by fresh aliquots, were also considered as a possible source of contamination. The same PCR was repeated but replacing the mtDNA primers with amelogenin primers (Amel 1 and Amel 2) (39). After running the PCR products on a polyacrylamide gel, no amplification product was detected in either the bacterial DNA or the EBs, and the only amelogenin amplicon detected was from the female positive DNA control (at 106 bp).

From these experiments I was able to demonstrate that 1) there was DNA contamination at a low level on laboratory equipment, 2) that the extraction reagents showed no evidence of contamination 3) the mtDNA primer set was contaminated while the *Taq* polymerase was not. Tracking contamination was a time-consuming exercise that took about six months until ancient samples were analyzed with confidence again in the clean room. However, knowledge of a strategic technique in tracing back and identifying the source of contamination was gained to help avoiding future contamination problems. This was the most difficult issue dealt with through out this project, and one that could jeopardize the authenticity of the results.

2.5. Preventing contamination

Following this contamination issue, several new procedures were implemented in the ancient DNA laboratory and all experiments conducted in it.

2.5.1. Preventing contamination in the clean room and its equipment

a. The two sets of pipettes, (one for DNA extraction and one for PCR amplification), were replaced with new sets of pipettes (Eppendorf™ Research Pro Pipettes, Ontario, Canada)
b. Routine cleaning of the clean room was performed every two to three months, wiping the benches and the Dead Air Box workstations with concentrated (100% v/v) bleach (sodium hypochlorite), then washing them with HPLC grade water.
c. The clean room benches, the extraction and the PCR Dead Air Box workstations, the bone preparation hood and the centrifuge were all cleaned between each single usage with daily-prepared 10% (v/v) bleach (bleach destroys any contaminating DNA

from a variety of surfaces (40)). In October 2012, the extraction and PCR Dead Air Box workstations were replaced with a Class II (Type A2) biological safety cabinet (Thermo Scientific™, USA). The floor of the biological safety cabinet is made of stainless steel and bleach is not recommended for stainless steel. Therefore, two chemical resistance trays were used (Fisher Scientific, Ontario, Canada) (one for DNA extraction and one for PCR set-up) as bases to perform DNA extractions and PCR amplifications on them. The trays were washed with 100 % (v/v) bleach then rinsed with HPLC water and finally autoclaved at 121 degrees C for 30 minutes before starting to use them. Also, the trays were bleached regularly before taking them inside the biological safety cabinet and UV irradiated for four hours after each usage. The usage of the trays protected the stainless steel floor of the biological safety cabinet from being corroded. The floor and the sides of the biological safety cabinet were cleaned with 70% ethanol before and after usage.

d. The bone preparation hood was cleaned, from all sides, with concentrated bleach (100% v/v or 12% w/v bleach or sodium hypochlorite) between each sample preparation to avoid cross contamination between samples.
e. Sterile and filtered pipette tips were purchased instead of autoclaving non-sterile pipette tips.
f. Sterile disposable spatulas were used to collect bone or tooth powder after pulverization to avoid cross contamination between samples.
g. Supplies, not originally sterile, were sterilized by autoclaving for 30 minutes at 121 degrees C. These included tubes, gauze, tube racks and boxes, mortars and pestles.
h. The extraction and the PCR hoods were exposed to UV irradiation for four hours instead of one hour (41) to ensure maximum removal of any contaminating DNA. The same procedure was performed on the biological safety cabinet. UV irradiation destroys DNA and is effective in reducing DNA contamination from surfaces and reagents (41).

2.5.2. Preventing contamination from ancient samples

Slight modifications have been made to the cleaning process of bone and teeth samples, from the procedure previously described (29,33,34), to ensure complete decontamination of the sample surfaces from exogenous modern DNA or bacterial DNA.

a. The outer surface of the vertebral bone samples, after cutting the processes with a sterile saw, was scraped with a sterile scalpel. Then, the samples were immersed for 10 minutes in 16.7 % (v/v) bleach instead of 10% (v/v) bleach, which was shown to be insufficient to decontaminate bone or teeth samples (40). According to Kemp and Smith (2005) "…bone must be immersed in at least a 2% [w/v, which equals 16.7 % v/v bleach] sodium hypochlorite solution…to remove contamination from its surface" (40). The bleach was removed from the samples by immersing them in HPLC grade water for five minutes. Samples were then left to dry in the hood and exposed to UV irradiation for one hour on each side.

b. The outer surface of the teeth samples, after cutting the crown with a sterile saw, was removed with a sterile scalpel. Then, the root was immersed in 50% (v/v) bleach for 10 minutes, and rinsed with HPLC water. Kemp and Smith (2005) indicated that immersing bones or teeth samples in even 100 % v/v bleach would not destroy endogenous DNA in ancient samples because DNA binds to the hydroxyapatite that comprises the majority of the bones' and teeth's matrix (40). Therefore, immersing the teeth samples in 100% v/v bleach for 10 minutes was first tried, but no DNA was obtained from these samples. Thus, the concentration of bleach, for teeth samples, was reduced from 100% v/v to 50% v/v, which is still effective in decontaminating the teeth surface according to Kemp and Smith (2005) (40). The wet teeth samples were left to dry while being exposed to UV irradiation for one hour on each side.

2.5.3. Preventing contamination from extraction and PCR reagents

Extreme care was taken to avoid contamination in the extraction and the PCR amplification reagents while maintaining their integrity.

a. All glassware used to prepare reagents was washed with concentrated (100% v/v) bleach and detergent rinsed with Milli-Q water several times, and then autoclaved for 30 minutes at 121 degrees C.
b. Before adding the proteinase K to the extraction buffer (11), the buffer was filtered using a sterile PVDF filter (0.22 μM, Millex- GV, Sigma-Aldrich, Ontario, Canada) then autoclaved for 20 minutes at 121 degrees C.
c. The binding buffer was autoclaved for 20 minutes at 121 degrees C before adding the guanidium thiocyanate (GuSCN) because the GuSCN degrades when autoclaved (GuSCN, MSDS).
d. The washing buffer, before the addition of ethanol, and the silica suspension were autoclaved for 20 minutes at 121 degrees C.
e. The TE buffer used for DNA elution and the silica suspension were UV irradiated for 15 minutes before use.
f. The PCR reagents were aliquoted into small volumes and the PCR 10X buffer, dNTPs, $MgCl_2$ and BSA were UV irradiated for 10 minutes before preparing the PCR amplification mixture. Neither the primers nor the *Taq* DNA polymerase were UV irradiated, as the UV irradiation might cause the formation of pyrimidine dimers in the primers and deactivate the *Taq* DNA polymerase (41).

All protocols were followed by laboratory personnel working with the ancient samples to minimize the risk of contamination with exogenous modern DNA or cross contamination between samples.

2.5.4. PCR amplification protocol modifications

To reduce false positive results associated with excessive PCR cycles, the number of PCR cycles was reduced from 50 (31) to 40 for mtDNA PCR amplification reaction and from 60 (29) to 40 for amelogenin PCR amplification. Although, it is believed that 40 to 65 PCR cycles in aDNA research is a common procedure as some *Taq* Polymerase are less efficient in amplifying highly fragmented and degraded aDNA (*Taq* polymerases discussed below). Increased number of PCR amplification cycles might increase the risk of obtaining false positive results through the amplification of primer dimers or artifacts (12).

2.5.5. Discrepancies in DNA analysis

During this research, four cases of discordant results were identified while re-analyzing the mtDNA haplogroup distribution from the Shamanka II cemetery as part of a quality assurance exercise to ensure the reproducibility of the results previously obtained by other researchers in the laboratory.

Case 1: It is important to remember that BAP is a multidisciplinary project, and excavated samples have been assigned different identification numbers (IDs), Master ID and sample ID, over the course of several years of research by both Russian and Canadian scholars. The Master and sample IDs were developed to avoid any confusion between individual numbers and sample numbers, respectively. For example, vertebral bone sample 2002.235 and tooth sample 2002.257 both belong to the same individual with Master ID SHA_2002.021.02. The individual's Master ID indicates that this individual belongs to the Shamanka II cemetery (SHA), which was excavated in 2002 and the grave number is 21. This individual was the second individual in the grave, and was buried in a double or multiple grave. The previous researcher, who was working on the Shamanka II individuals (33), misidentified the sample IDs for one Shamanka II individual. This potentially created a discrepancy when assigning mtDNA and Y-chromosomal haplogroups to this individual. The former researcher analyzed the mtDNA from the bone sample with ID 2002. 238, and identified it as belonging to individual SHA_2002.021.02 (33) (Table 4.1); however, this sample actually belonged to individual SHA_2002.021.01. Each of these individuals, buried in the same grave, had a different mtDNA haplogroup; SHA_2002.021.02 carried a G2a mtDNA haplogroup, and SHA_2002.021.01 carried an F mtDNA haplogroup, but both of them had the same Y-chromosomal haplogroup, K (Chapter 3).

Cases 2, 3 and 4: Further discrepancy was identified during the analysis of three other Shamanka II individuals (Master IDs: SHA_2001.012, SHA_2002.021.03 and SHA_2002.023.04). Previous mtDNA analysis showed they belonged to mtDNA haplogroups U5a, F, and U5a, respectively (33). For two of these individuals (Cases 2 and 3), the analysis was repeated using different bone samples from the individual than the former researcher. For one individual (Case 4), the same bone sample, which the former researcher used, was re-extracted and analyzed (Table 4.1). The three individuals were found to belong to two different mtDNA haplogroups:

SHA_2001.012 belonged to mtDNA haplogroup D and SHA_2002.021.03 belonged to mtDNA haplogroup A, and SHA_2002.023.04 belonged to mtDNA haplogroup D (Table 4.1). The DNA extraction and PCR amplification were repeated twice to confirm the authenticity of the results, and the results were consistent with the first extraction.

Case 5: For one Shamanka II individual (Master ID: SHA_2001.013.03), a different bone sample, which the former researcher used, was extracted and analyzed, a concordant results with the former researcher was obtained from two separate DNA extractions and PCR amplifications, which confirms the authenticity of the result (Table 4.1)

Table 4.1. Re-analyzed cases from Shamanka II individuals.

Case	Shamanka II Master ID	Former Researcher's used ID & Sample Type	Moussa's used ID & Sample Type	Former researcher's mtDNA polymorphic sites and assigned haplogroup	Moussa's mtDNA polymorphic sites and assigned haplogroup
1	SHA_2002. 021.02	2002.235. (Bone)	2002.257. (Teeth)	16232, 16249, 16304, 16311 Haplogroup F; however, it was assigned an incorrect ID. The correct ID sample (2002.238. Bone) has SNPs at 16223, 16227, 16262, 16278 Haplogroup G2a	16223, 16227, 16278 Haplogroup G2a
2	SHA_2001. 012	2002.210. (Bone)	2009.060. (Bone)	16256, 16270 Haplogroup U5a	16223 Haplogroup D
3	SHA_2002. 021.03	2002.245. (Bone)	2009.070. (Bone)	16232, 16249, 16304, 16311 Haplogroup F	16223, 16290, 16319 Haplogroup A
4	SHA_2002. 023.04	2002.221. (Bone)	2002.221. (Bone)	16256, 16270 Haplogroup U5a	16223 Haplogroup D
5	SHA_2001. 013.03	2002.192. (Bone)	2009.061. (Bone)	16223 Haplogroup D	16223 Haplogroup D

However, two Ust'Ida individuals were also selected randomly to be re-analyzed for mtDNA, and no discrepancy was detected when the mtDNA haplogroups were compared with the previous results (Table 4.2) (29,31).

Table 4.2. Re-analyzed cases from Ust'-Ida.

Ust'Ida Master ID	Former Researcher's used ID & Sample Type	Moussa's used ID & Sample Type	Former researcher's mtDNA polymorphic sites and assigned haplogroup	Moussa's mtDNA polymorphic sites and assigned haplogroups
UID_1989.020.01	1995.150. (Bone)	2009.185. (Bone), 2001.418. (Teeth)	16223 and 16298 Haplogroup C	16223, 16298, 16327 Haplogroup C
UID_1989.026.01	1995.154. (Bone)	2009.190. (Bone), 2001.547. (Teeth)	16223, 16227, 16290, 16319 Haplogroup A	16223, 16227, 16290, 16311, 16319 Haplogroup A

No additional samples from these individuals were available for further analysis. Dr. Fiona Bamforth and I conducted an investigation of possible reasons for the discrepancy. The laboratory notebook from the research technician, who performed the first DNA extractions for some of the Shamanka II samples in 2005/2006, was the only source of information. After investigation, it was deduced that the ancient DNA laboratory demonstrated evidence of contamination during a six-month period in 2005/2006 when the samples were extracted. Measures to detect contamination were followed and all samples that showed any contamination were appropriately re-analyzed by the investigators.

There were several crucial points that were considered while investigating the Shamanka II discrepancy cases:

a. At the time the Shamanka II samples were extracted in 2005/2006, there were four people working in the aDNA laboratory on aDNA samples from the four burial sites. This was the only time that there were more than two people working in the laboratory. While protocols indicated that each individual prepared and used their own reagents, all equipment, for example, thermocycler, centrifuge, hoods, were shared. However, primers were shared on occasion for troubleshooting. This obviously increased the risk of contamination.
b. There was clear evidence from the laboratory notebook that contamination during extraction was identified and these extractions were rejected.
c. The Shamanka II samples were previously extracted by two different people, but not sure which samples were processed from these two extractions.
d. The former researcher's DNA sequencing results from Shamanka II were reviewed from the samples included as an appendix in her thesis and there was no evidence of heteroplasmy from the electropherogram, which might indicate contamination.
e. The previous researcher used 50 PCR cycles that might have produced false positive results.
f. Contamination was detected in the laboratory during my 2009 investigation and as a result, a more rigorous protocols for decontamination and detection of contaminating

DNA were instituted. Subsequently, all the analyses and extractions for this project were performed after the institution of these protocols.

g. I have mostly been the only person working in the aDNA laboratory apart from summer students or project students working under my supervision. This has reduced the risk of contamination.

In conclusion, the previous results demonstrate contamination was suspected but evidence of contamination could not be proved. This is an example of how difficult contamination is to detect despite using authenticity criteria previously established, and extractions that were performed on two separate occasions and analyzed at least twice. However, the introduction of even more rigorous protocols to prevent and detect contamination reduced these errors from recurring.

3. Overcoming the effects of PCR inhibitors

As previously mentioned, PCR inhibitors are co-extracted with DNA. Minimizing the amount of co-purified PCR inhibitors is a goal during DNA extraction and amplification to improve DNA recovery from ancient samples.

3.1. Reducing co-extraction of PCR inhibitors during DNA extraction

a. During extraction, the chaotropic salt GuSCN was used while binding with silica. According to Rohland and Hofreiter (2007), chaotropes (e.g. GuSCN) were found to be very efficient in reducing the co-purification of PCR inhibitors when compared to non-chaotropic salts such as sodium chloride (NaCl) that can co-purify PCR inhibitors (12). They have shown that combining GuSCN during the binding step with silica outperformed other methods with non-chaotropic salts (e.g. NaCl and KCl) (12).

b. The final elution step, while releasing the extracted DNA from the surface of the silica particles to the elution TE buffer (Chapter 3), was repeated twice to avoid transferring any silica particles that might interfere with any downstream PCR amplifications.

3.2. Reducing the effect of PCR inhibitors during PCR amplification

a. Bovine serum albumin (BSA) has been proven to enhance PCR amplification from ancient DNA extracts through overcoming the effect of PCR inhibitors (12). It is

believed that BSA increases the stability of the *Taq* DNA polymerase enzyme during the amplification reaction and reduces the loss of the reagents via adsorption to the tube walls (42). Therefore, the BSA concentration was increased in the mtDNA PCR amplification reaction from 0.015 mg/ml, as described previously (31), to 0.4 mg/ml per each 25µl mtDNA PCR reaction. BSA was also introduced, as a modification, to the Y-chromosomal multiplex PCR amplification reactions, in a final concentration of 1 mg/ml in the PCR reaction mix, as recommended for Y-chromosomal multiplex assays (12,43), but it was not used in the original Y-chromosomal multiplex PCR amplification published protocol by Bouakaze et al. (2007) (44).

These modifications improved PCR amplification efficiency through reducing the effect of PCR inhibitors. However, some apparently good quality bone or tooth powder generated from well-preserved samples could not be analyzed by PCR amplification, which might be due to the presence of PCR inhibitors, and this might explain the low success rate from Lokomotiv individuals for Y-chromosomal analysis. The success rate for mtDNA analysis from Lokomotiv samples was good (30), but this might be because of the high mtDNA copy number compared to the low copy number of the Y-chromosome in each cell.

Because of limited sample availability, each of the Kurma XI teeth was extracted twice using the same tooth powder, on two different occasions. For each individual, depending on the availability of bone or tooth samples from this individual, the powder, from either bone or tooth samples, is extracted twice at two different occasions to confirm the authenticity of the results. In some cases, where there is not enough bone or tooth powder for two separate extractions, the same bone or tooth powder used in the first extraction is used again for a second extraction (e.g. some Kurma XI samples).

About 25% of the Kurma XI (8 out of 30) tooth samples were successfully amplified for mtDNA from the same tooth powder only after the second extraction, and not from the first. A possible reason could be the elimination of PCR inhibitors during the first extraction, making the DNA available for PCR amplification after the second extraction. For these samples, the results were confirmed using other teeth samples from the same individual.

4. An attempt to analyze autosomal short tandem repeats (STRs)

Analyzing nuclear DNA markers in aDNA, other than Y-chromosomal DNA was attempted after the success in amplifying Y-chromosomal SNPs. The purpose of this trial was to examine the possibility of performing investigations on autosomal STRs. Autosomal STRs system is a well-established marker system that has been used by several archaeological, forensic and aDNA studies to determine genetic kinship between closely related individuals by biparental loci (45-48). After personal communication with Dr. Martin Somerville (Department of Medical Genetics, University of Alberta), a pilot study was conducted on five well-preserved Shamanka II bone samples, from which mtDNA results were previously successfully obtained, using the AmpFlSTR® Identifiler® kit for human identification (Applied Biosystems, NY, USA) (49). The kit is designed to amplify 15 STR loci plus the amelogenin polymorphism in one single reaction generating amplicon sizes ranging from 100 up to 450 bp. However, none of the samples amplified with any of the primer sets. Possibilities for the failure include a) the presence of PCR inhibitors, b) incompatibility of the kit with the highly degraded aDNA condition. However, more recently, a new kit designed for the analysis of highly degraded DNA has been introduced (AmpF*ℓ*STR® MiniFiler™ PCR Amplification Kit, Applied Biosystems, NY, USA). This kit amplifies eight STR loci plus the amelogenin marker of reduced size (miniSTRs), with amplicon sizes less than 200 bp. The miniSTR multiplexes were designed by moving the primer binding sites closer to the STR repeat unit (50,51). MiniSTRs are considered the best type of marker might be used to study highly degraded DNA as the aDNA (52,53).

5. Optimizing procedures for degraded DNA

aDNA is highly fragmented because of DNA damage, primarily by hydrolytic and oxidative processes after death (15) when all of the DNA repair mechanisms cease. Therefore, it is essential to select methods designed specifically for aDNA or modify methods to optimize aDNA analysis.

5.1. Reducing heat damage to DNA during extraction

a. A Dremel Rotary Tool Kit to drill inside the tooth root to extract the pulp was previously used for the Khuzhir-Nuge XIV teeth samples but was not successful in obtaining DNA (34). The heat created by the drilling mechanism might further

damage any remaining DNA. Therefore, instead of drilling inside the root, the whole tooth root was pulverized using a mortar and pestle after freezing the root in liquid nitrogen to avoid generating heat that might destroy aDNA.

b. Incubation at 65 degree C overnight in a water bath after adding the extraction solution to the bone or tooth powder is part of Boom's (1990) extraction method (35). The Rohland and Hofreiter (2007) aDNA extraction protocol, recommends incubation at 56 degree C, for only 1-3 hours, to improve the DNA yield through a better digestion of the bone powder in case of a coarse powder (11). However, the overnight incubation in 65 degree C step was completely omitted from the extraction protocol to avoid further degradation of the DNA by heat. Therefore, bone or tooth samples were completely pulverized until forming fine powder.

As a result of these modifications, analysis of two molar samples from Khuzhir-Nuge XIV was conducted. After optimization of aDNA extraction and analysis as described in section 5, an attempt to analyze mtDNA from two Khuzhir-Nuge XIV molar samples (1997.190 & 2003.639*)* from one individual (K14_1997.011), was successful. All previous attempts had been unsuccessful because of the poor preservation of the Khuzhir-Nuge XIV samples as a result of a cemetery flood (34). Both samples gave reproducible and concordant results showing that the individual belonged to mtDNA haplogroup D.

5.2. Primer design

PCR amplification primers were designed using Primer3Plus software (54) for Y-chromosomal Q-sub-haplogroup to produce a short amplicon size (between 81-155 bp) suitable for highly degraded DNA.

5.3. Selection of *Taq* polymerase

Different DNA polymerases were tested on a male positive control for the multiplex reaction, during optimization of the Y-chromosomal SNaPshot amplifications. Each of the *Taq* DNA polymerases has different properties, but all of them share hot-start enzyme activity, which is recommended to reduce any mispriming or primer-primer amplification (43), the tested *Taq* polymerases include:

a. Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, NY, USA)

Platinum® *Taq* DNA Polymerase High Fidelity contains a recombinant *Taq* DNA polymerase, and Platinum *Taq* antibody. It offers a higher amplification sensitivity over the regular Platinum® *Taq* DNA Polymerase.

b. HotStarTaq DNA Polymerase (QIAGEN, Toronto, Ontario)

   The HotStarTaq DNA Polymerase's specifications show that it has a chemically mediated hot start, and a unique PCR buffer that reduces nonspecific amplification products, primer dimers and background. It is also accompanied by the Q-Solution, which amplifies efficiently difficult templates (e.g. GC rich templates).

c. AmpliTaq Gold® DNA Polymerase (Applied Biosystems, NY, USA)

   AmpliTaq Gold® DNA Polymerase minimizes mispriming. Also, it has increased ability to counteract PCR inhibitors (55).

d. Platinum *Taq* DNA polymerase (Life Technologies, NY, USA)

   The Platinum *Taq* DNA polymerase is also a recombinant *Taq* DNA polymerase and contains Platinum *Taq* antibody. It is characterized by its high specificity and wide range of use in different PCR amplifications, and it produces high yield of the PCR product (12). The Platinum *Taq* DNA polymerase is particularly recommended for multiplex PCR reactions for its reduced mispriming activity (43).

The success of amplification for each *Taq* polymerase was evaluated through running PCR amplification experiments on modern positive control DNA using the mtDNA primers (H16346 and L16211) and also using the Y-chromosomal multiplex PCR amplification primers, and then the amplicon intensity was visualized under UV light after gel electrophoresis for presence of primer dimer products and the intensity of the band showing the PCR product. Platinum *Taq* DNA polymerase was the DNA polymerase of choice according to its best performance (Appendix 4.4).

6. Limitations in sample availability for aDNA analysis

The genetic component of the research in BAP had limited access to bones and teeth samples for each individual from all the cemeteries. The samples were pulverized and fully consumed during the DNA extraction process. Therefore, other investigators were unable to use them for other purposes. Vertebral bone samples were preferred as the sample size is greater and they

usually provide an adequate amount of bone powder for more than one DNA extraction process. However, the quantity of powder produced from pulverizing teeth samples was sometimes barely adequate for one extraction process. Fortunately, the DNA extraction protocol used in our laboratory (11) usually allowed the use of the same bone or tooth powder for a second extraction in case of sample scarcity. However, the DNA extract produced at the end of the extraction protocol was only 50 µl, and each PCR amplification reaction required between 4 µl (mtDNA PCR amplifications) and 7.5 µl (Y-chromosomal multiplex PCR amplifications). Each of these PCR amplifications was repeated at least twice to ensure the authenticity of the results. For these reasons, there were cases of insufficient sample for further analyses. Two of the Kurma XI individuals were assigned to Y-chromosomal haplogroup Q-M242, but no further PCR amplifications, to detect the sub-haplogroups of Q-M242, were possible because of sample depletion. Thus, all the optimization experiments were usually conducted on positive modern human DNA extracts in order not to deplete the limited ancient DNA samples.

7. Conclusion

In conclusion, analysis of DNA from historical samples is challenging in comparison to modern DNA. aDNA is highly fragmented and degraded because of environmentally induced mutations (1,13,56), it may contain PCR inhibitors and DNA quantity is minimal. Nonetheless, with the presence of specific and sensitive DNA extraction and PCR amplification protocols, the retrieval and analysis of aDNA is possible.

Avoidance of contamination with exogenous modern DNA is crucial and authenticity of the results must be established through multiple analyses of the same sample or different samples from the same individual. aDNA characteristics illustrate the uniqueness of this research and demonstrate the challenges in retrieval of aDNA from human remains.

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Chapter 5

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Conclusion and Future Directions

1. Summary of the project

The Lake Baikal region of Siberia has provided a rich environment for human existence for several thousand years. Canadian and Russian archaeologists have recovered a large number of well-preserved skeletal remains from discrete burial sites in the area, dating from 8000 to 4000 calender (cal.) years before present (cal BP) (1,2). The area was home to two temporally distinct populations, the Early Neolithic (EN, Kitoi) and the Late Neolithic-Early Bronze Age (LN-EBA, Serovo-Glazkovo). The EN group was separated from the LN-EBA group by about a 1000-year gap (hiatus) from which no human remains have been recovered. Several cemeteries have been excavated as part of an international multidisciplinary initiative, the Baikal Archaeology Project (BAP). These include one EN cemetery (Shamanka II) and two LN-EBA cemeteries (Kurma XI and Khuzhir-Nuge XIV). One EN cemetery (Lokomotiv) and one LN-EBA cemetery (Ust'-Ida) was excavated independently of BAP. BAP seeks to reconstruct the lifestyle of hunter-gatherers who lived around Lake Baikal through the application of research methods from archaeology, human osteology, bone chemistry, human genetics, and environmental studies (1). Archaeological data suggest cultural discontinuity between the EN and LN-EBA groups with differences in mortuary practices, diets and mobility patterns (1,3). The cemetery burials provide a unique opportunity to study prehistoric population genetics, in contrast to making inferences about prehistoric populations from extrapolation from modern DNA studies: 1) there are an unusually large number of well-preserved specimens from which DNA population specific polymorphisms can be examined to determine population origins, and genetic affiliations within and between cemetery samples established. 2) DNA analysis from human remains provides additional information for archaeologists to make inferences about social structure, family groupings and marriage patterns within each cemetery from studying graves and burial practices (e.g. single and multiple graves, presence or absence of ornaments and grave goods).

The significance of the project lies in the utility of DNA analysis as an archaeological tool in conjunction with traditional methods, such as stable isotope signatures and mortuary rituals, in order to make inferences about the prehistoric population structure.

This dissertation hypothesis focused on the examination of mtDNA from Shamanka II and Kurma XI cemeteries and examination of Y-chromosomal DNA from the four excavated

cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI) in the area that would identify genetic discontinuity and/or continuity through polymorphic distribitions between and within the EN and LN-EBA of prehistoric populations.

The aim of the project was to trace the maternal and the paternal lineages of the EN and LN-EBA populations by examination of mitochondrial DNA (mtDNA) and Y-chromosomal DNA (Y-DNA) polymorphisms, respectively. The degree of genetic continuity and/or discontinuity between and within the EN and LN-EBA cemeteries was established by DNA analysis and results *interpreted* within the archeological context of other BAP research methods (2,4-7). Major findings in this dissertation will be bolded in this chapter to highlight them.

Examination of aDNA from historical samples was challenging and strict authenticity criteria were implemented to avoid reporting any results showing contamination or discordant results (Appendix 5.1 has a summary of all aDNA results obtained from the project). The highly degraded and fragmented nature of aDNA (8-10) made it challenging to apply some analytical protocols e.g. the failure to obtain results from Shamanka II individuals after using *AmpFlSTR*® Identifiler® kit (Applied Biosystems, NY, USA). The difficulty in retrieval of aDNA may be due to the presence of PCR inhibitors, poor DNA preservation, or minimal DNA quantity. DNA amplification protocols were modified and optimized from published protocols (11-13) to increase the sensitivity and accuracy of analysis.

Avoidance of contamination with exogenous modern DNA was of crucial importance, and authenticity of the results was established through multiple analyses of the same sample. In the course of this project, a concrete working protocol was established that helped to reduce the possibility of any contamination either during sample preparation, DNA extraction or PCR amplification. This includes 1) assessing risk of random contamination from the surrounding laboratory environment, 2) increasing bleach concentration (used to eliminate exogenous contamination with modern DNA (14)) for washing bone samples prior to analysis.

As mentioned in the previous chapters of my dissertation, mtDNA and Y-chromosomal polymorphisms in archeological specimens give information on population origin, migration patterns, and the affinities between contemporary and prehistoric populations (15-17). Maternally inherited mtDNA has a high rate of mutation, leading to the accumulation of

population-specific polymorphisms, facilitating the study of population migration patterns through the female lineage (18,19). Loci on the Y-chromosome's NRY (Non-recombinant region) are haploid and inherited paternally and can be used to trace back the male lineage (20). Female and male migration patterns may differ (21).

2. Optimization of DNA extraction and analysis

During this project, several new aDNA extraction and PCR amplification techniques were optimized to address the technical challenges during sample analysis. This includes: 1) reducing the number of PCR cycles from 50 to 40 cycles, 2) testing several commercially available *Taq* polymerases to select the best amplification enzyme, 3) adjusting PCR reagent concentration (e.g. Mg^{2+} and BSA (Bovine Serum Albumin)) with the reaction conditions.

3. Conclusion of maternal and paternal background of the prehistoric population of Cis-Baikal

3.1. Maternal mtDNA conclusion

The maternally inherited mtDNA was analyzed previously from two EN cemeteries (Lokomotiv (22) and Shamanka II (23)) and one LN-EBA cemetery (Ust'-Ida (24)). The previous mtDNA analysis from the two EN cemeteries showed that Lokomotiv and Shamanka II individuals are statistically similar; therefore, the data from them (Lokomotiv and Shamanka II) were combined (25). The combined mtDNA analysis of 52 individuals from the two EN cemeteries shows higher frequencies of haplogroups D and F, and lower frequencies of haplogroups A and C (25). In contrast, analysis of 39 individuals from Ust'-Ida shows a higher frequency of haplogroups A and C and lower frequency of haplogroups D and F (25). This finding suggested a genetic discontinuity between the EN and LN-EBA populations of Cis-Baikal area in Siberia. It also indicated that the Cis-Baikal prehistoric population had gone through a significant change during the hiatus period, when no mortuary remains had been recovered (23-25). However, these findings alone were not sufficient to delineate the genetic background of the Siberian ancient population of Lake Baikal. There are two other cemeteries that are predominantly from the LN-EBA period, Kurma XI and Khuzhir-Nuge XIV. Khuzhir-Nuge XIV is the largest LN-EBA cemetery in the Little Sea micro-region of Lake Baikal. Comprehensive archaeological and osteological studies have been conducted on the cemetery's individuals giving a detailed

description on grave architecture, mortuary rituals, health and diet of individuals, morphological sex, and grave inclusions (26,27). Nevertheless, previous attempts to analyze DNA from individuals from Khuzhir-Nuge XIV were unsuccessful due to poor preservation of skeletal material (28). However, the modification and optimization of the extraction and the PCR amplification protocols during the course of this project facilitated the re-analysis of mtDNA from Khuzhir-Nuge XIV. **mtDNA from one Khuzhir-Nuge XIV individual (K14_1997.011) was retrieved from two different molar samples (1997.190 and 2003.639), extracted at two different occasions, assigning the individual to haplogroup D**. The success in extracting and analyzing mtDNA from a Khuzhir-Nuge XIV individual is very promising and may make further analysis on other individuals from this large cemetery possible.

Kurma XI is a smaller cemetery located also in the Little Sea micro-region of Lake Baikal. The 26 graves of Kurma XI date back to both EN and the EBA periods. Kurma XI consists of 6 EN graves and 20 EBA graves (29). Despite the small number of individuals available for analysis (17 individuals), fewer than the other three cemeteries, mtDNA results were retrieved from Kurma XI individuals (2 EN and 12 EBA individuals). The mtDNA haplogroup distribution from EBA Kurma XI shows a high frequency of haplogroup D (50%, n=6/12) and a low frequency of haplogroup A (8.3%, n=1/12), the new appearance of haplogroup Z (16.7%, n=2/12, not previously found in other Cis-Baikal populations), and the absolute absence of haplogroup C. Two EN individuals from Kurma XI, belonging to haplogroup F (Chapter 2), were actually found by radiocarbon dating to be from the Late Mesolithic period (30).

There were 21 individuals previously analyzed from the EN Shamanka II cemetery (23). From those 21 individuals, four individuals were re-analyzed (Chapter 4) and an additional six individuals were added to the Shamanka II population. This made mtDNA haplogroup distribution available from a total of 27 EN individuals from the Shamanka II cemetery. The 27 EN Shamanka II individuals show a high frequency of haplogroup D (40.7%, n=11/27) and a low frequency of haplogroup U5a (3.7%, n=1/27). A new statistical analysis was performed to estimate the genetic relationship between each of the four Cis-Baikal prehistoric populations taking into account the periods during which the cemeteries were used. The LN-EBA cemetery sample from Ust'-Ida was divided into two groups, one included the LN Ust'-Ida individuals

(n=29) and the other included the EBA Ust'-Ida individuals (n=10). All the new mtDNA results from both EBA Kurma XI and EN Shamanka II were compared statistically to the previously obtained results from EN Lokomotiv (31 individuals*)* with a high frequency of haplogroup F (48.4%, n=15/31) and a low frequency of haplogroup C (3.2%, n=1/31) (22) and LN Ust'-Ida (29 individuals) with high frequencies of haplogroups A (24%, n=7/29) and "Others" (27.6%, n=8/29) and a low frequency of haplogroup U5a (3.4%, n=1/29) and EBA Ust'-Ida (10 individuals) with a high frequency of haplogroup C (70%, n=7/10) and a low frequency of haplogroup A (30%, n=3/10) and no other haplogroups represented (24). The new statistical analysis showed that all of the cemeteries populations are significantly different from each other except for EBA Kurma XI and EN Lokomotiv. Also, the EBA Kurma XI and EN Shamanka II, both have significantly similar haplogroup distributions.

Surprisingly, the distribution of mtDNA haplogroups at EBA Kurma XI is more similar to Lokomotiv and to Shamanka II than to either LN Ust'-Ida or EBA Ust'-Ida. We expected EBA Kurma XI population to be maternally more similar to the EBA Ust'-Ida population than to the EN populations. These findings raise several questions regarding the maternal origin of the Cis-Baikal prehistoric populations who either lived during the EN period, LN period or EBA period. The concept of genetic discontinuity in the maternal background between the EN and the LN-EBA populations of Cis-Baikal is not a simple change or replacement in the origin of females during the hiatus period. The new data show that the Cis-Baikal region had a heterogeneous mtDNA haplogroup distribution during the EN, LN and EBA periods, but the two EN populations from Lokomotiv and Shamanka II share a common maternal ancestry with the EBA Kurma XI population. However, it has been suggested that Kurma XI is an 'exclusive' cemetery with some unique burial practices (31). Therefore, the EBA Kurma XI population might not be representative of the entire population that inhabited the Little Sea micro-region in this prehistoric time. Thus, mtDNA haplogroup analysis from Khuzhir-Nuge XIV cemetery would be very valuable and confirm one of two hypotheses. First hypothesis, if Khuzhir-Nuge XIV (mostly EBA) were similar to both the EBA Kurma XI and EN cemeteries' populations but different from Ust'-Ida, it would confirm the common maternal ancestry between EN and EBA (Kurma XI and Khuzhir-Nuge XIV) populations. Second hypothesis, if Khuzhir-Nuge XIV showed a different

haplogroup distribution to both EBA Kurma XI and EBA Ust'-Ida it would confirm the heterogeneity of the Cis-Baikal region with respect to maternal origins. All this emphasizes the importance of mtDNA analysis for individuals from Khuzhir-Nuge XIV.

Two individuals from the Kurma XI cemetery sample were found to belong to haplogroup Z. This is the first evidence of the presence of haplogroup Z in the Cis-Baikal prehistoric population. All individuals examined at all four cemeteries showed typical Asian haplogroups. Haplogroup Z is an Asian haplogroup (32) and is considered to be one of the dominant haplogroups in the northern East Asian populations (33). In modern populations, haplogroup Z is moderately distributed in northeast Asia (e.g. Itel'mens (6.3%, n=3/47) and Koryaks (5.8%, n=9/155)) (34), but is also present rarely in south Siberian populations for example, the Altai-Sayan population (35). Thus, the unique appearance of individuals belonging to mtDNA haplogroup Z from Kurma XI cemetery might indicate that haplogroup Z is an exclusive lineage to the Little Sea micro-region of Cis-Baikal.

3.2. Paternal Y-chromosomal DNA conclusion

Examination of the paternal lineage through Y-chromosomal polymorphisms is a novel approach to BAP and facilitates the assessment of the paternal DNA contribution within and between the EN and the LN-EBA groups, and complements the maternal data.

Y-chromosomal SNPs were analyzed using the SNaPshot multiplex technique, which was modified from a published protocol (36). The published method took into account the degraded state of aDNA and the multiplex primers were designed to amplify short segments of DNA (81 to 155 bps) (36).

Male individuals, for which molecular sexing was confirmed by amelogenin analysis, were analyzed from all the four cemeteries, Lokomotiv and Shamanka II (EN) and Ust'-Ida (LN-EBA) and Kurma XI (EBA). The lowest success rate for DNA retrieval was from Lokomotiv (41%, n=7/17) and the highest success rate was from Shamanka II (100%, n=9/9). Lokomotiv and Shamanka II showed some similarity in their haplogroup distribution (Fisher's exact test; p=0.0625, with the p value close to the statistical significance level of 0.05). This is likely due to the high frequency of haplogroup K (57.1%, n=4/7) in Lokomotiv and the dominance of haplogroup K (100%, n=9/9) in Shamanka II. Ust'-Ida and EBA Kurma XI also had a statistically

significant resemblance in their Y-chromosomal haplogroup distribution (p= 1.00). However, EBA Kurma XI was different from Lokomotiv (p= 0.0023) and Shamanka II (p=0.0002) in its Y-chromosomal haplogroup distribution.

Interestingly, Lokomotiv showed a greater degree of heterogeneity in its haplogroup distribution than all other cemeteries even with the low success rate of analysis. There were three Y-chromosomal haplogroups represented in Lokomotiv males, haplogroups K-M9, R1a1-M17 and C3-217, but only one haplogroup was represented in Shamanka II males, K-M9. For Lokomotiv specifically, a high degree of variable mobility was suggested from the variability of strontium isotope (Sr) ratios within and between Lokomotiv individuals (37).

The LN-EBA cemetery Ust'-Ida showed one individual with haplogroup K-M9, two individuals with an unidentified haplogroup (L914 SNP) and the remainder belonging to haplogroup Q-M242 and its sub-haplogroup Q1a3-M346. EBA Kurma XI showed one individual with the same unidentified haplogroup (L914 SNP) and the rest were Q-M242/Q1a3-M346. The Q-M242 sub-haplogroup Q1a3a-M3 (Q-M3), the most frequent haplogroup in the Native American population (38), is not found in the Kurma XI population. **Sub-haplogroup Q1a3-M346, a sister to Q1a3a-M3 (39), is identified in three of five males in EBA Kurma XI, which supports ancient links between the Siberian and the Native Americans (39).**

The L914 SNP, a (T> G) transversion, at ChrY position 4925671 (40), might be a rare mutation associated with a specific haplogroup not yet defined. This novel mutation was found in one Ust'-Ida individual (UID_1989.026.01) and amplified from two different samples, one bone (2009.190) and one tooth (2001.547). For the other Ust'-Ida individual (UID_1987.005), this SNP was amplified twice on two different occasions from the same bone sample (2009.177). Reproducible results were also obtained from the EBA Kurma XI individual defining L914 SNP (KUR_2003.019). These findings confirm the authenticity of the L914 SNP (Chapter 3). Further analysis of Y-chromosomal haplogroups for the individuals carrying the L914 SNP, would give us further insight on linkage of this SNP.

4. Interpretation of the genetics findings from mtDNA and Y-chromosomal DNA in the context of the BAP project

4.1. Cis-Baikal prehistoric populations' maternal and paternal genetic affinities

Previous mtDNA analysis from the Cis-Baikal prehistoric populations ~~on~~ from two EN cemeteries (Lokomotiv and Shamanka II) and one LN-EBA cemetery (Ust'Ida) proposed that the two populations are genetically distinct in their maternal origin (25). However, the new results from EBA Kurma XI and EN Shamanka II show that the Cis-Baikal has a heterogeneous mtDNA haplogroup distribution through the EN, LN and EBA periods. However, the new results suggest a common ancestry between the EN populations and EBA Kurma XI population because the mtDNA haplogroup distribution of Kurma XI is similar to the EN cemeteries, Lokomotiv and Shamanka II (Chapter 2). This finding raises many questions about the maternal background of the LN-EBA population of Lake Baikal. This heterogeneity in mtDNA haplogroup distribution in the Cis-Baikal region can be explained through the patrilocality post-marital residence (discussed below). During prehistoric time in the Lake Baikal area, it would appear that females were migrating from different surrounding areas to marry men who stayed in their birthplaces, as evidenced by the heterogeneous maternal background in Cis-Baikal. Previous mtDNA haplogroup distribution from Ust'-Ida suggested that the LN-EBA prehistoric population of Cis-Baikal has close affinity with the modern Siberian native groups (e.g. Buryats, Tuvinians, and Yakuts) and with the Iron Age cemetery (2300- 1800 cal BP), Egyin Gol, in Northern Mongolia (25). mtDNA halogroup distributions from LN Ust'-Ida still show close affinity with modern Siberian native groups (e.g. Buryats and Kets) and with the Egyin gol (Iron Age) prehistoric population. The EBA Kurma XI results combined with EBA Ust'-Ida results show a closer affinity with some east Siberian populations such as the Koryaks and Itel'men due to the novel appearance of haplogroup Z, which is more frequent in eastern Siberian populations (34,41). Also, the increased frequency of mtDNA haplogroup D, after pooling datasets from EBA Kurma XI and EBA Ust'-Ida, place the LN-EBA closer to the eastern Siberian population than previously proposed (25). The Lokomotiv and Shamanka II dataset combined shows that they have close affinity with a modern Siberian population, Todjins (Southern Siberian population), which was not proposed previously (25).

The novel Y-chromosome haplogroup data indicate that the EN and the LN-EBA populations at Lake Baikal are paternally genetically distinct and suggest a paternal discontinuity between the EN and LN-EBA populations of the Lake Baikal area. The differences in the Y-chromosomal signatures of the EN and LN-EBA suggest displacement of males in the Cis-Baikal population during the EN period and replacement by a genetically different population during the LN and EBA periods (Chapter 3).

The presence of Y-chromosomal haplogroup K-M9, with high frequency in the EN populations, shows that the EN prehistoric populations have a close paternal genetic affinity with south Siberian groups (e.g. Tuvinians and Mongolians). For the EN populations, the close affinity with south Siberian groups is also strengthened with the presence of Y-chromosomal haplogroup R1a1-M17, which is widely distributed particularly in southern Siberia (42).

The LN-EBA Cis-Baikal prehistoric populations are characterized by a high frequency of haplogroup Q-M242. Thus, the LN-EBA populations have a close affinity with most of the modern Siberian groups including, Sel'kup and Kets, because Q-M242 is widely distributed across Siberia (43). Individuals from the LN-EBA populations carry the Q1a3-M346 lineage, which is a sub-haplogroup from Q-M242. Those individuals affirm the close affinity of the LN-EBA populations to modern Siberian groups. A study of several northern East Asian groups by Malyarchuk, B. et al. (2011) examining specific sub-haplogroups of Q-M242, showed that Q1a3-M346 is more frequent than other sub-haplogroups, for example, Q1a*-MEH2 and Q1a2-M25 and even Q1a3a-M3 in the modern Siberian populations, including Altaians, Todjins and Tuvinians (39).

From mtDNA and Y-chromosomal haplogroup distributions, it appears that the prehistoric populations of the Cis-Baikal area have close maternal and paternal affinity with most of the contemporary Siberian populations surrounding the Lake Baikal area (e.g. Altaians, Todjins, Tuvinians, Buryats, Koryaks and Itel'men).

4.2. Cemetery genetics: implications for social organization of the EN and LN-EBA populations

Summing the mtDNA and the Y-chromosomal DNA haplogroup distributions from all four cemeteries lead us to a potential scenario for post-marital residence and marriage patterns during prehistoric times in Lake Baikal. Three of the cemeteries (Shamanka II, Ust'-Ida, and EBA Kurma XI) show low Y-chromosomal haplogroup diversity between males and high mtDNA haplogroup diversity, which suggests a patrilocal post-marital residence in ancient times. Patrilocality, when females move to their husbands' residence after marriage and men stay in their birthplace, is the norm in about 70% of the world's populations (44). According to Kelly, R.L. (1995 and 2013), patrilocality is the most common form of post-marital residence, at 65%, in hunter-gatherer societies (45,46). Patrilocal societies are usually characterized by a high level of diversity in mtDNA haplogroups and low level of diversity in Y-chromosomal haplogroups within the same group with the reverse situation between groups, and this heterogeneity in mtDNA haplogroups can also suggest an exogamy marriage pattern, when men are allowed to marry women from outside the group (47-50). The EN, LN and EBA populations have high diversity in mtDNA haplogroups, while EN Shamanka II, LN-EBA Ust'-Ida and EBA Kurma XI populations have low diversity in Y-chromosomal haplogroups. The only cemetery that showed heterogeneity in their Y-chromosomal haplogroup distribution was Lokomotiv, but the small sample size and the low success rate in Y-chromosomal DNA analysis from Lokomotiv is a limitation to suggest a specific post-marital residence or marriage pattern. Therefore, analysis of more Lokomotiv samples may elucidate the genetic signature from the prehistoric population of Lake Baikal by estimating the influence of social norms on their genetic diversity. Appendix 5.1 is a table summarizing all the mtDNA haplogroups and Y-chromosomal haplogroups from all four studied cemeteries.

4.3. Life histories: implication at the level of the individual

While the focus was on population genetics at the broad level, there is an interpretation at the individual level. For example, the Lokomotiv individual (LOK_1985.031.02, age 25-30 years, XY) is the only male in the EN population and in the entire Cis-Baikal examined individuals belonging to Y-chromosomal haplogroup C3-M217. This male was buried in a double grave with

another individual (Female, LOK_1985.031.01, age 20+ years) (51). This male (LOK_1985.031.02) carried mtDNA haplogroup A, which is found in moderate to high frequency in the Lokomotiv cemetery sample (25). This male (LOK_1985.031.02) demonstrated a strikingly different $^{87}Sr/^{86}Sr$ signature from the other three individuals at Lokomotiv from whom both strontium isotope (37) and Y-haplogroup analysis is available. This suggested that he was born in one location, and although buried in the Lake Baikal area, spent his adulthood outside the region as his $^{87}Sr/^{86}Sr$ from femur is quite different from the other 16 individuals at Lokomotiv.

5. Closing points

In conclusion, this research bridges Arts and Sciences and demonstrates the value of multidisciplinary research with contributions from researchers of different backgrounds to build a composite picture of the prehistoric Siberian hunter-gatherers. This study demonstrates the value of aDNA research as an archaeological tool contributing to predictions about the prehistoric populations' lifestyle. The uniqueness of the BAP was demonstrated by the unusually large number of well preserved skeletal remains together with evidence of social complexity of the studied populations observed in their mortuary practices. This research correlates mtDNA data with Y-chromosomal DNA results to establish evidence of female and male migration patterns and marriage patterns. The project contributes to our knowledge of the history of human habitation and movement patterns, specifically from Siberia eastwards to North America and connects the prehistoric and modern Siberian populations with the peopling of North America, as the Lake Baikal area human remains provide the oldest DNA data from Siberia. Trials to connect genetic affinities between prehistoric populations of Lake Baikal and contemporary groups of Siberia through applying new techniques used thus far with modern DNA. Overall, this study has the potential to make significant contributions to regional and global archaeology, and also contributes towards the development of better methods of working with highly degraded DNA.

6. Future directions

There is still work that could follow from this research:

I. Examine mtDNA polymorphisms from samples of individuals from Shamanka II (EN) and Khuzhir-Nuge XIV (LN-EBA) cemeteries.

mtDNA has been analyzed previously from 21 skeletal samples from Shamanka II (23), and six more Shamanka II individuals in the current study. This small number of samples does not represent the entire cemetery samples, as an additional 100 samples (bones and teeth) are available for analysis. Shamanka II is exceptional in its large number of well-preserved burials, the distinctive spatial organization of these graves in rows (chapter 3), and numerous grave inclusions (52,53).

After the modification and optimization of extraction and PCR amplification methods, it would be of a great benefit to examine mtDNA polymorphisms in Khuzhir-Nuge XIV individuals. As it was described earlier, the success in retrieval of DNA from teeth samples that was demonstrated in Kurma XI individuals can be applied to the Khuzhir-Nuge XIV samples as well.

II. Determine Y-chromosomal haplogroup distribution of more individuals from Lokomotiv as well as Shamanka II (EN) through the examination of Y-chromosomal SNPs.

Despite the low success rate in Y-chromosomal analysis from Lokomotiv, with the high level of heterogeneity in Y-chromosomal haplogroups distributions, indicates that it would be useful to analyze more male samples from Lokomotiv. Analyzing more samples would also assist in understanding post-marital residence and marriag patterns within the Lokomotiv prehistoric people. We might be able to detect an ancient case of matrilocality, where the woman stays in her birthplace and the man moves in to live with her and her family after marriage. In a matrilocality post-marital residence case, high Y-chromosomal haplogroup diversity would be detected (48).

III. Analyzing sub-haplogroups of Y-chromosomal haplogroup K specifically from Shamanka II individuals

As it was explained earlier, all of the Shamanka II males were assigned to Y-chromosomal haplogroup K-M9. Haplogroup K-M9 basically can define other Y-chromosomal haplogroups including, L, M, N, O, P, S and T (54). Therefore, individuals assigned to haplogroup K-M9, especially from Shamanka II, need further investigation to define the exact Y-chromosomal lineage they belong to. This can be achieved through designing a multiplex reaction for the Y-chromosomal lineages represented in the Siberian population and found under the main haplogroup K-M9.

IV. Examination of autosomal miniSTRs in multiple burials

Autosomal short tandem repeats (STRs) are a well-established marker system that can be used to determine genetic kinship between closely related individuals by biparental loci (15,16,55,56). Autosomal STRs could be genotyped in individuals from double and group burials to determine the relationship between those who have a biological kinship between each other. A new kit designed for the analysis of highly degraded DNA has been introduced (AmpFℓSTR® MiniFiler™ PCR Amplification Kit, Applied Biosystems, NY, USA). This kit amplifies eight STR loci plus the amelogenin marker of reduced size (miniSTRs), with amplicon sizes less than 200 bp. The miniSTR multiplexes were designed by moving the primer binding sites closer to the STR repeat unit (57,58). MiniSTRs are considered the best type of marker might be used to study highly degraded DNA as the aDNA (59,60).

V. Applying Next Generation Sequencing methodology to mtDNA analysis

Usually mtDNA typing techniques focus on the HV1 and HV2 regions to examine the mtDNA, which ignores the rest of the mtDNA genome and limits the resolution power to a very short segment of mtDNA (61). However, the technique of next generation sequencing was applied to a highly denatured and chemically damaged DNA sample with sequences less than 100 bp, and results were successfully obtained. Sequencing of the whole mtDNA genome using next-generation techniques has also been applied to archaeological samples (~ 2,500 years old) with successful results. This approach can provide the highest level of maternal discrimination (61). Applying all these methods and protocols to the BAP project may reveal more from these ancient populations and help to answer questions not only for the genetic research side of the project but also to all the other investigations involved in this multidisciplinary project.

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Appendices

Appendices for Chapter 2

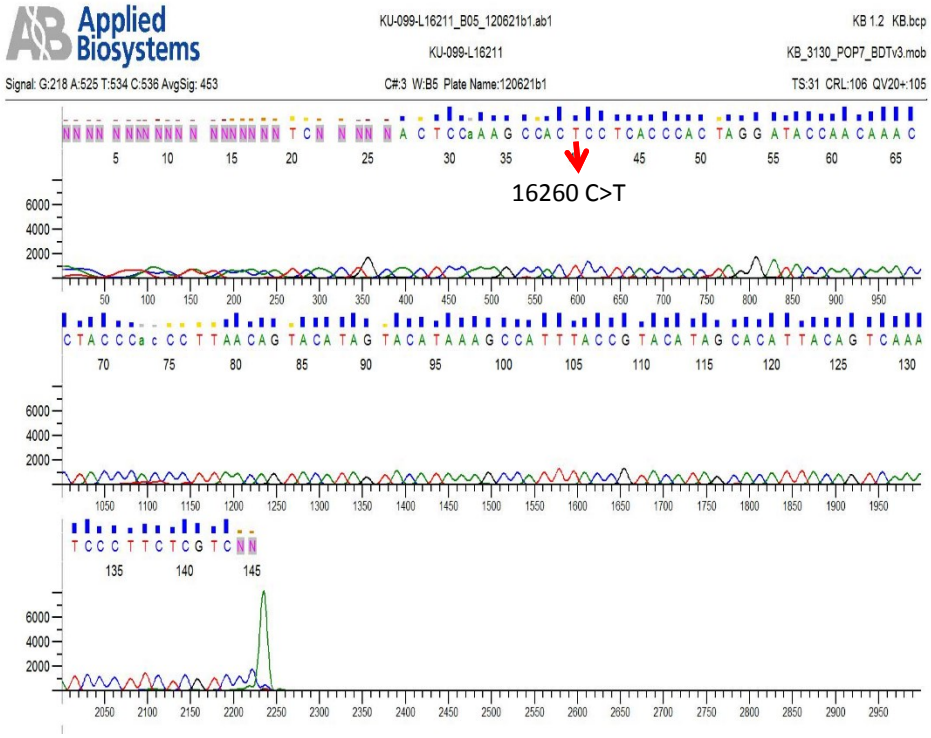
Appendix 2.1. Summary of the mtDNA haplogroups and their base substitutions, molecular sex assignment for all the EN Shamanka II individuals.

No	Master ID	Morphological sex	Molecular sex	Age (In years)	mtDNA haplogroups	HV1 variants +16000
1	SHA_2001.012	Undetermined	XY [(b)]	25-35	D *	223
2	SHA_2001.013.03	Probable female	XY [(b)]	18-19	D [(b)]	223
3	SHA_2002.021.02	Male	XY [(b)]	25-30	G2a *	223 227 278
4	SHA_2002.021.03	Undetermined	XY [(b)]	16-18	A *	223 290 319
5	SHA_2002.023.04	Undetermined	XY [(b)]	20+	D *	223
6	SHA_2004.052.01	Probable male	XY	20-24	D ^	223
7	SHA_2004.044.02	Undetermined	XY	20+	C ^	223 298 327
8	SHA_2005.059.01	Male	XY	35-39	C ^	223 298 327
9	SHA_2006.083.01	Male	XY	20-22	C ^	223 298 327
10	SHA_2002.023.05	Undetermined	XY [(b)]	20+	D [(b)]	N/A
11	SHA_2001.011.02	Male	NA [(b)]	30-40	D [(b)]	223 319
12	SHA_2001.011.01	Female	NA [(b)]	18-20	C [(b)]	223 298 327
13	SHA_2002.008	Male	NA [(b)]	35-40	A [(b)]	223 290 319
14	SHA_2001.014.01	Male	NA [(b)]	25-30	D [(b)]	N/A
15	SHA_2001.014.02	Female	NA [(b)]	20-25	F [(b)]	232 249 304 311
16	SHA_2001.019	Male	NA [(b)]	25-30	D [(b)]	223 319
17	SHA_2001.018	Male	NA [(b)]	25-29	C [(b)]	223 298 327
18	SHA_2001.016	Undetermined	XX [(b)]	20-25	U5a [(b)]	256 270
19	SHA_1999.007	Probable female	Undetermined [(b)]	20-30	D [(b)]	223
20	SHA_2001.015	Male	NA [(b)]	25-35	A [(b)]	223 290 319
21	SHA_2002.023.01	Probable male	Undetermined [(b)]	35-45	F [(b)]	232 249 304 311
22	SHA_2002.024.01	Male	NA [(b)]	25-35	D [(b)]	223 290 311 319
23	SHA_2002.022	Male	NA [(b)]	19-22	F [(b)]	232 249 304 311
24	SHA_2002.021.01	Male	NA [(b)]	25-30	G2a [(b)]	223 227 262 278
25	SHA_2002.024.02	Male	NA [(b)]	12-15	A [(b)]	223 290 319
26	SHA_2007.090	Male	XX	18-20	A ^	223 227 290
27	SHA_2007.096.02	Female	XX	30-35	D ^	223 319

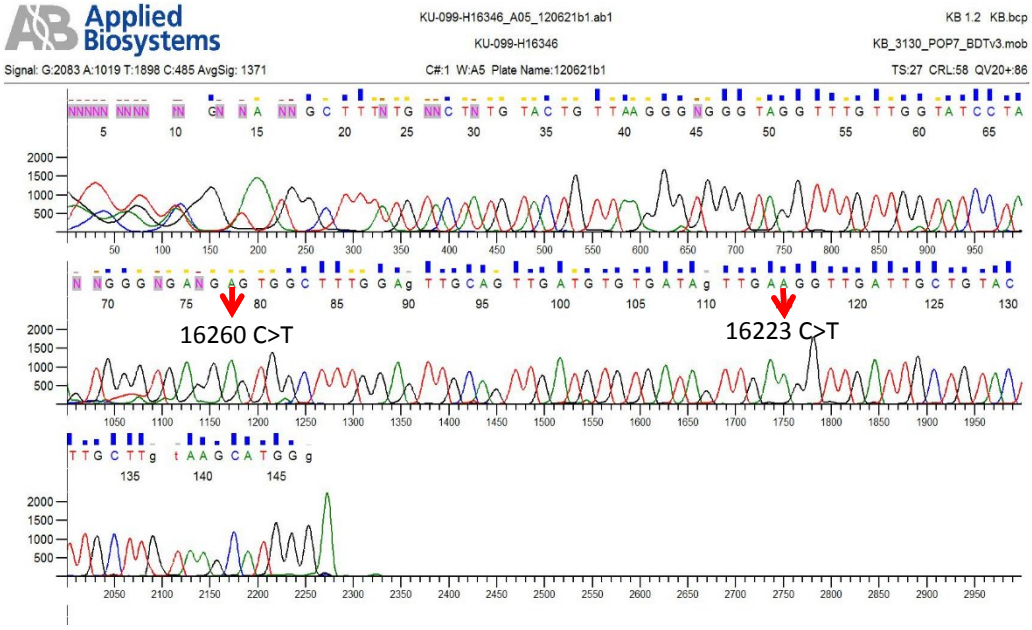
- [(b)] mtDNA and molecular sex from Shamanka II were determined by Thomson (2006) (Thomson,T. 2006)
- * re-analyzed mtDNA haplogroups from Shamanka II samples
- ^ newly analyzed Shamanka II samples

Appendix 2.2. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2002.010), tooth sample ID 2003.099. Two base substitutions at 16223(C>T) and 16260 (C>T), Haplogroup Z.

Forward:

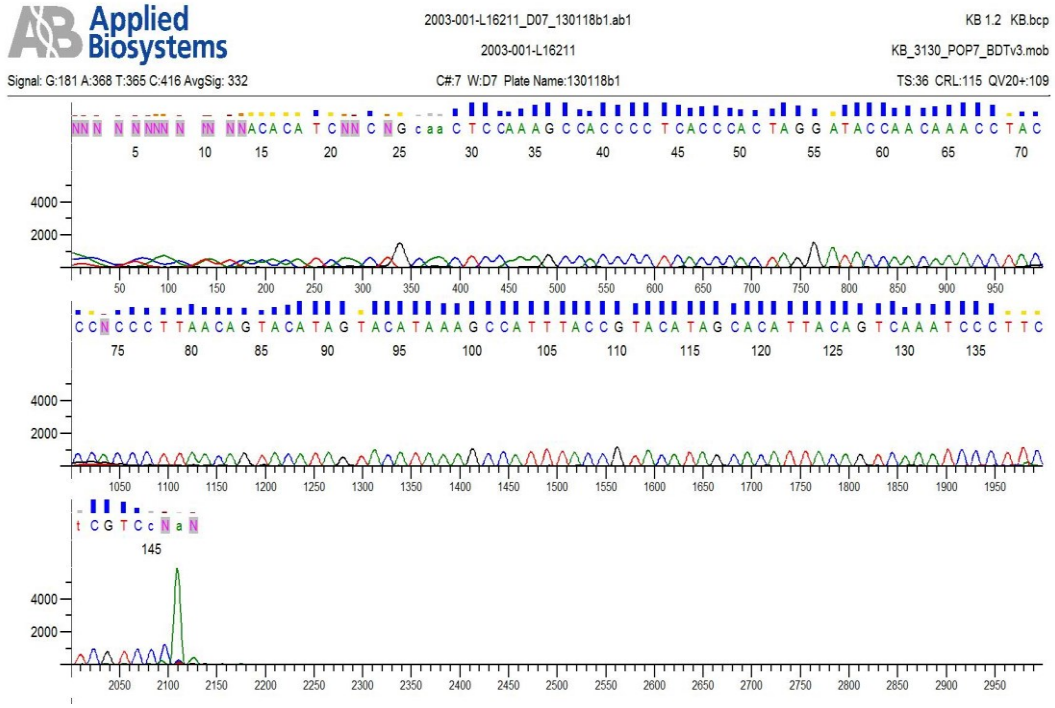


Reverse:

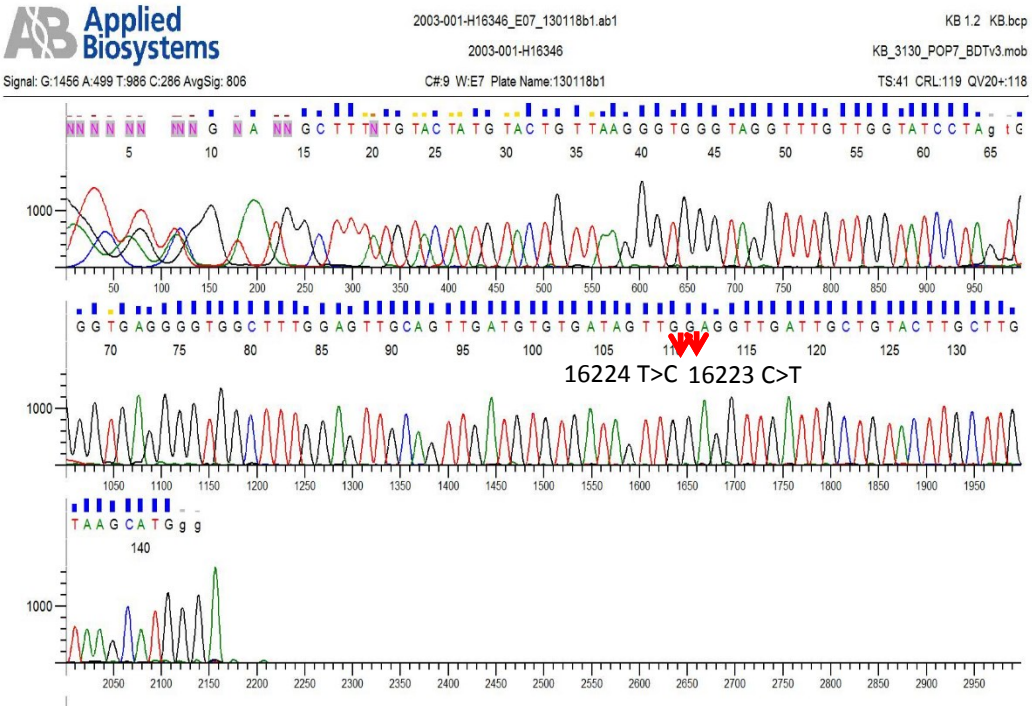


Appendix 2.3. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2003.018), tooth sample ID 2003.001. Two base substitutions at 16223(C>T) and 16224(T>C), Haplogroup D.

Forward:

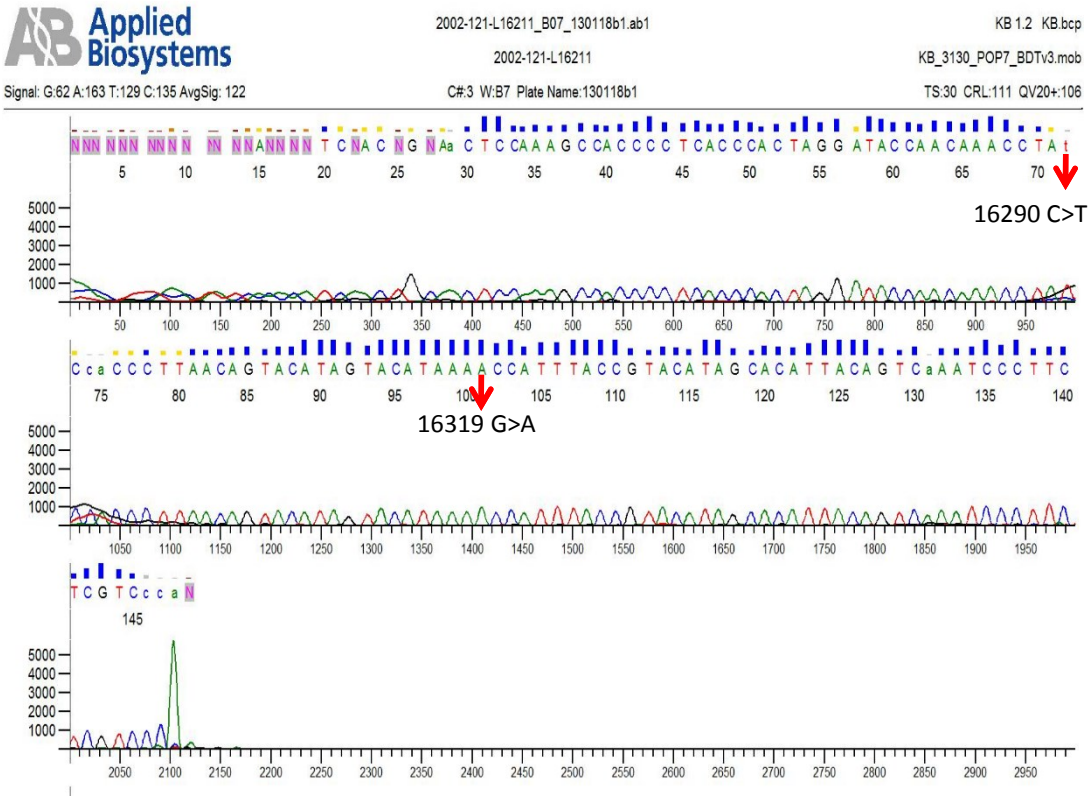


Reverse:

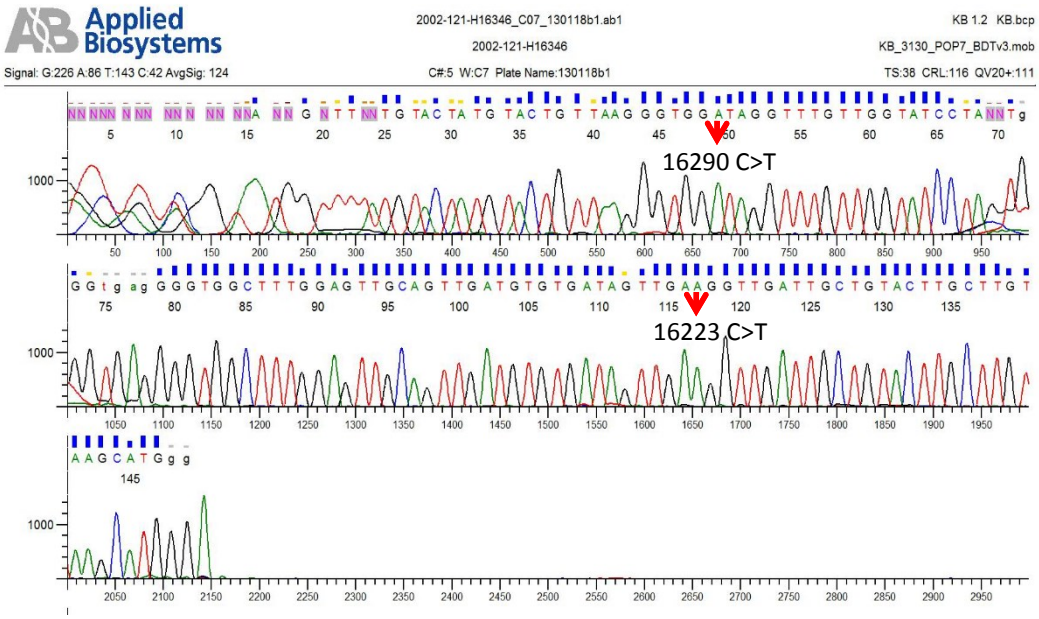


Appendix 2.4. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2002.013), tooth sample ID 2002.121. Three base substitutions at 16223(C>T), 16290(C>T), and 16319(G>A), Haplogroup A.

Forward:

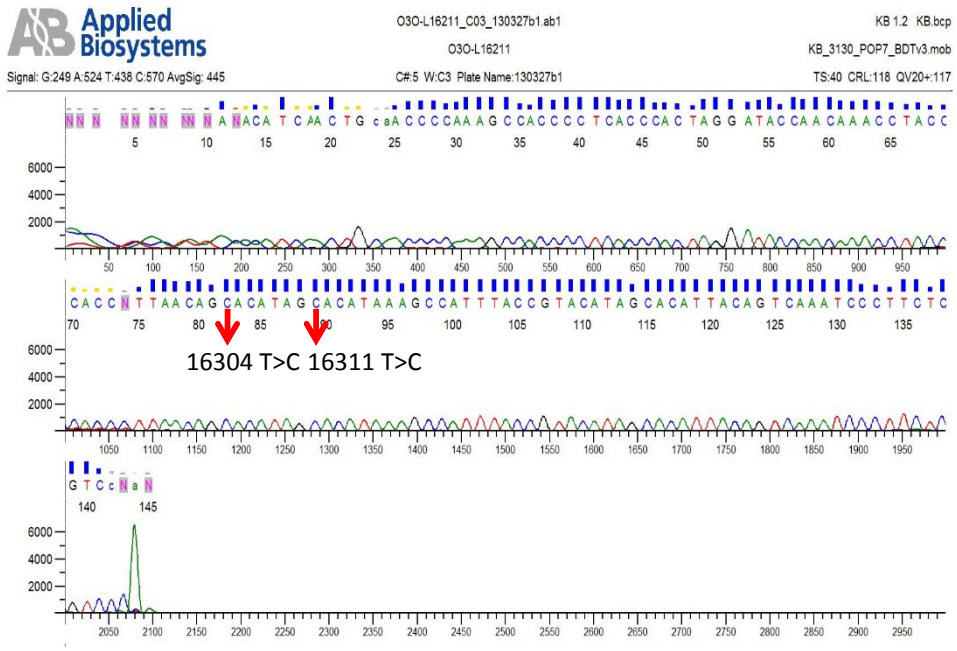


Reverse:

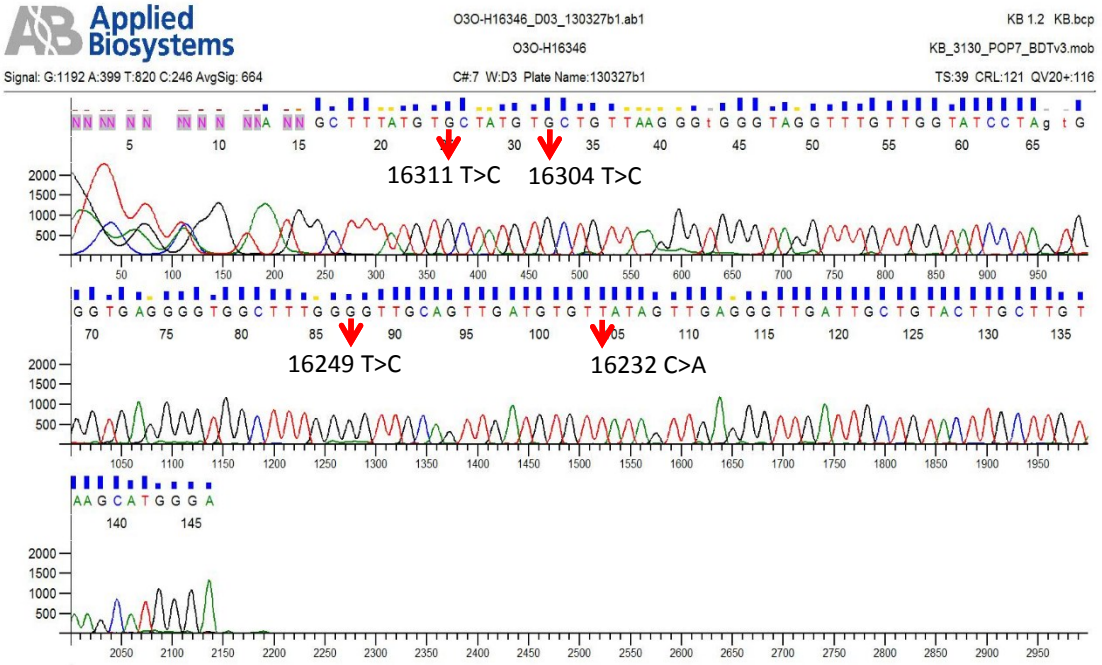


Appendix 2.5. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2003.024), tooth sample ID 2003.030. Four base substitutions at 16232(C>A), 16249 (T>C), 16304 (T>C), and 16311 (T>C), Haplogroup F.

Forward:

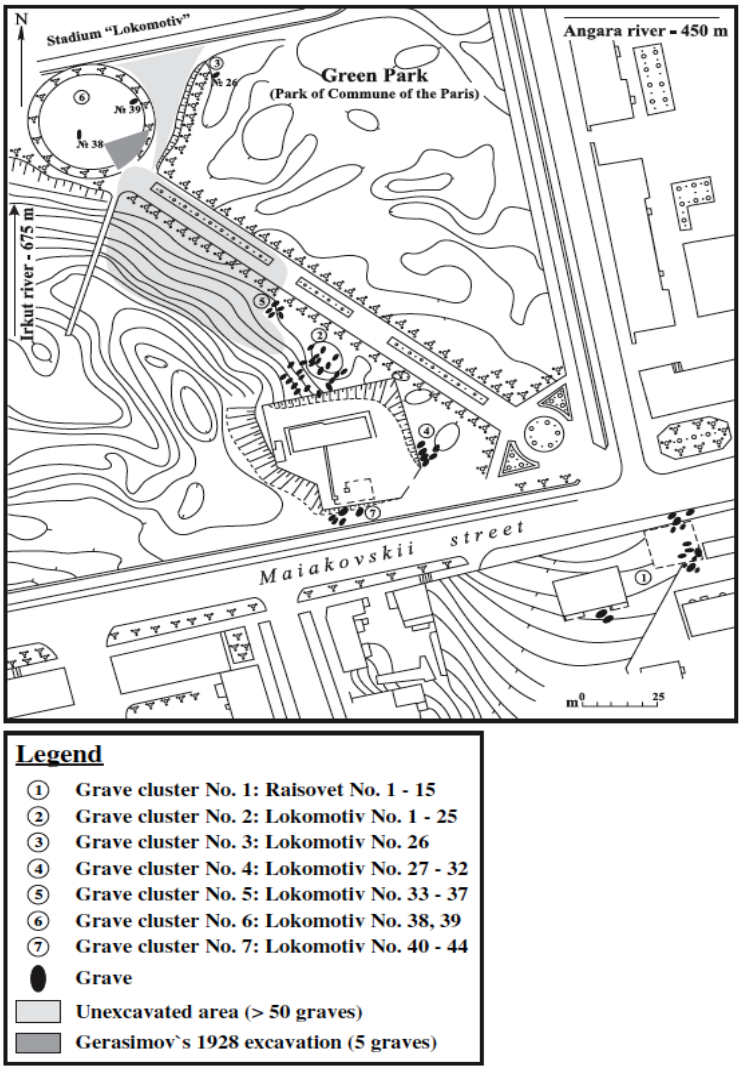


Reverse:

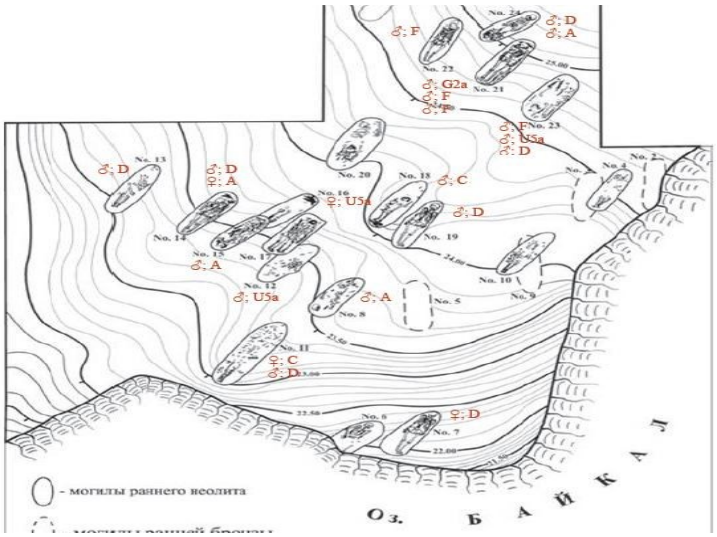


Appendices for Chapter 3

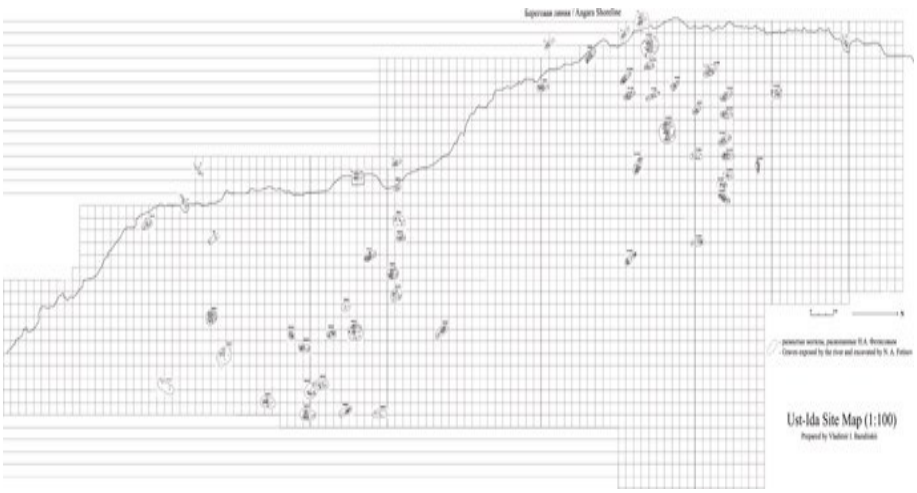
Appendix 3.1. Lokomotiv cemetery map (Mooder et al. 2005).



Appendix 3.2. Shamanka II cemetery map with mtDNA haplogroup and molecular sex assigned to the individuals (Thomson,T. 2005).



Appendix 3.3. Ust'-Ida cemetery map (Mooder,K.P. 2004).



Appendix 3.4. Reagent preparation

Extraction Buffer

The extraction buffer (0.45M EDTA (Sigma-Aldrich, Ontario, Canada), 0.25mg/ml proteinase K (Sigma-Aldrich, Ontario, Canada), pH 8.0) was filtered using a sterile PVDF filter (0.22 uM, Millex- GV). Each extraction required 10 ml of extraction buffer per ~ 0.5 g bone or tooth powder. The buffer was autoclaved for 20 minutes at 121degrees C and freshly prepared before each extraction.

Binding Buffer

Each sample required 40 ml of the binding buffer (5M Guanidium Thiocyanate (GuSCN, OmniPur, MA, USA), 0.025M NaCl (Fisher Scientific, NY, USA), 0.05M Tris (Invitrogen, NY, USA). The buffer was autoclaved for 20 minutes at 121 degrees C before adding the guanidium thiocyanate and freshly prepared before each extraction.

Washing Buffer

Each sample wash required 1 ml washing buffer (50% (v/v) ethanol, 0.125M NaCl, 0.01M Tris, 0.001M EDTA, pH 8.0). The washing buffer was autoclaved for 20 minutes at 121degrees C before adding the ethanol.

Silica Suspension

The silica suspension was prepared using 4.8 g silicon dioxide (~99% purity, Sigma-Aldrich, ON, Canada) suspended in Milli-Q water to a final volume of 40 ml and left to settle for 1 hour. After this, 39 ml of the supernatant was transferred to another container and left to settle for a further 4 hours. Finally, 35 ml of supernatant was discarded and 39.2 µl of 10N HCL (Fisher Scientific, NY, USA) was added to pellet. The silicon dioxide suspension was divided into small aliquots (80 ml), autoclaved for 20 minutes at 121 degrees C and stored in the dark. The reagent was stable for one month at room temperature. A 100 µl of silicon dioxide suspension was added to each sample.

Appendix 3.5. Discrepancies between morphological and molecular sex from four Cis-Baikal cemeteries (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI).

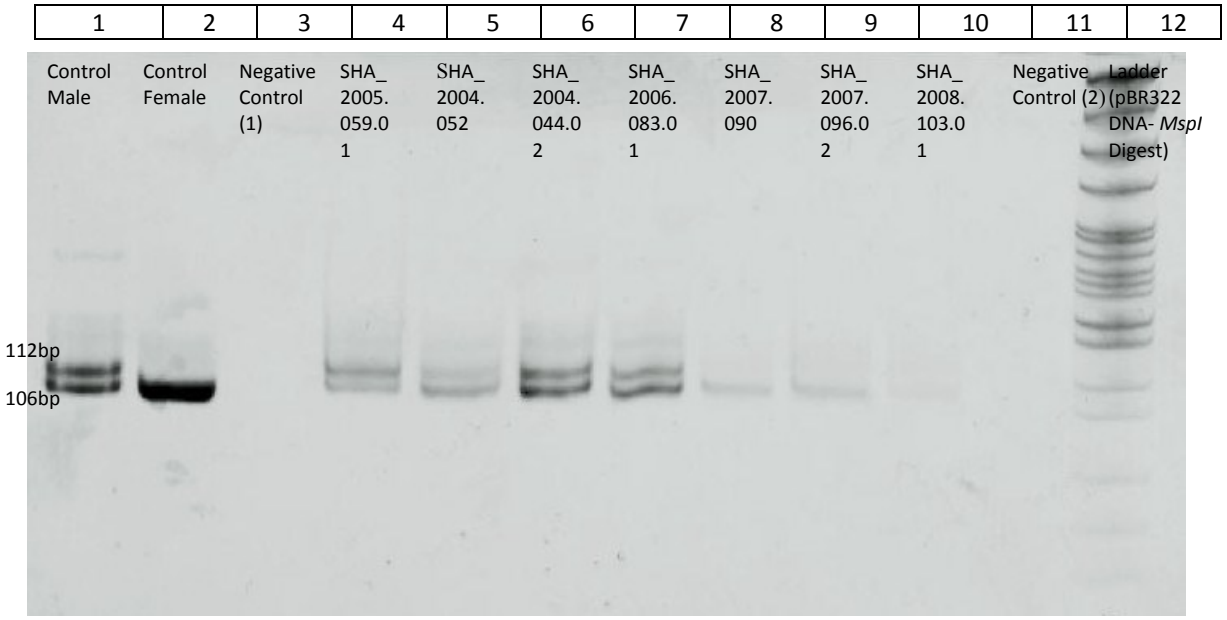
Master ID	Morphological Sex	Molecular Sex
LOK_1980.004	Female	XY [a]
LOK_1980.014.03	Immature	XY [a]
LOK_1981.024.01	Immature	XY [a]
LOK_1988.038.01	Ambiguous	XY [a]
SHA_2002.021.03	Undetermined	XY [b]
SHA_2002.023.05	Undetermined	XY [b]
SHA_2002.023.04	Undetermined	XY [b]
SHA_2001.012	Undetermined	XY [b]
SHA_2001.016	Undetermined	XX [b]
SHA_2001.013.03	Probable Female	XY [b]
UID_1987.009	Immature	XY [a]
UID_1989.030	Female	XY [a]
UID_1991.042	Female	XY [a]
UID_1989.026.04	Immature	XY [a]
UID_1989.026.05	Immature	XX [a]
UID_1989.030	Female	XY [a]
UID_1989.031	Immature	XY [a]
UID_1993.044.01	Immature	XX [a]
UID_1993.044.02	Immature	XX [a]
UID_1993.044.03	Immature	XY [a]
UID_1994.053.02	Immature	XY [a]
UID_1988.018	Immature	XX [a]
KUR_2003.018	Probable Female	XY
KUR_2002.007.01	Undetermined	XX

- [a] Molecular sexing determined by Mooder (2004) {{135 Mooder,K.P. 2004; }}.
- [b] Molecular sexing determined by Thomson (2005) {{134 Thomson,T. 2006; }}.

Appendix 3.6. Primers and PCR amplification conditions used for mtDNA amplification of the HV1 region and amelogenin (molecular sex) determination analyses.

Tested Region	Primer	Sequence	Length (bp)	Annealing Temperature	Primer Concentration	Reference
HVI	H16346	5'-GGGACGAGAAGGGATTTGAC-3'	176	56°C	1.0 µM	Mooder, K.P. 2004)
	L16211	5'-CCCATGCTTACAAGCAAGTA-3'	176	56°C		
Amelogenin	Amel1	5'-CCCTGGGCTCTGTAAAGAATAGTG-3'	106/112	62°C	1.0 µM	(Mannucci, A. 1994)
	Amel2	5'-ATCAGAGCTTAAACTGGGAAGCTG-3'	106/112	62°C		

Appendix 3.7. Polyacrylamide gel stained with ethidium bromide illustrating an example of PCR amplification of amelogenin. Lanes 1 and 2 show the male and female positive controls, respectively, lanes 3 and 11 show the PCR negative controls, lanes 4, 5, 6 and 7 show males of Shamanka II, lanes 8, 9 and 10 show females of Shamanka II, and lane 12 shows the ladder.



Appendix 3.8. Primers used for the Y-chromosome two multiplex PCR reactions to amplify 13 regions containing Y-chromosomal SNPs (Bouakaze,C. 2007).

Marker	PCR primer sequence (5'→3')		Amplicon size (bp)	Primer Concentration (µM)
	Forward	Reverse		
Multiplex I				
M173	TTTTCTTACAATTCAAGGGCATTTAG	CTGAAAACAAAACACTGGCTTATCA	81	0.3
M175	AGTACCCAAATCAACTCAACTCCAGTG	CTGATACCTTTGTTTCTGTTCATTCTTGA	100	0.2
M217	GATTCTTTAACTTGTGAAGGAGAATGAA	CGTAAGCATTTGATAAAGCTGCTGTG	115	0.3
M45	GGTGTGGACTTTACGAACCAACCTT	TATCTCCTGGCCTGGACCTCAGAAG	119	0.4
$RPS4Y_{711}$	TGGTGGGATGTTGTTTTTCTCTCCT	CAACAGTAAGTCGAATGCCCTTTCC	123	0.45*
M17	ATTGGGGAATACCTGGTCATAACA	AATAGTTTGGCCACTTAACAAACCC	124	0.4
M216	TCCTCAACCAGTTTTTATGAAGCTAGA	GCAAAAGATAATTGTTCCAGGGTAAGC	145	0.2
Multiplex II				
M9	TCAGGACCCTGAAATACAGAACTGC	TTGAACGTTTGAACATGTCTAAATTAAAG	93	0.3
92R7	TTTAAATCCCTCCTATTTGTGCTAACCA	CACTTCTTTTCAGAAAAATGCATGAAC	107	0.35
M3	AATGTGGCCAAGTTTTATCTGCTG	GGCATCTTTCATTTTAGGTACCAGCTC	111	0.15
TAT	GACTCTGAGTGTAGACTTGTGA	GAAGGTGCCGTAAAAGTGTGAA	112	0.2
M89	TGGATTCAGCTCTCTTCCTAAGGTTAT	CTGCTCAGGTACACACAGAGTATCA	135	0.2
M242	GCATAGAAAGTTTGTGCAAAAAGGTGAC	GGGCTTTCAGCATAATACCTTACCTAGAA	155	0.25

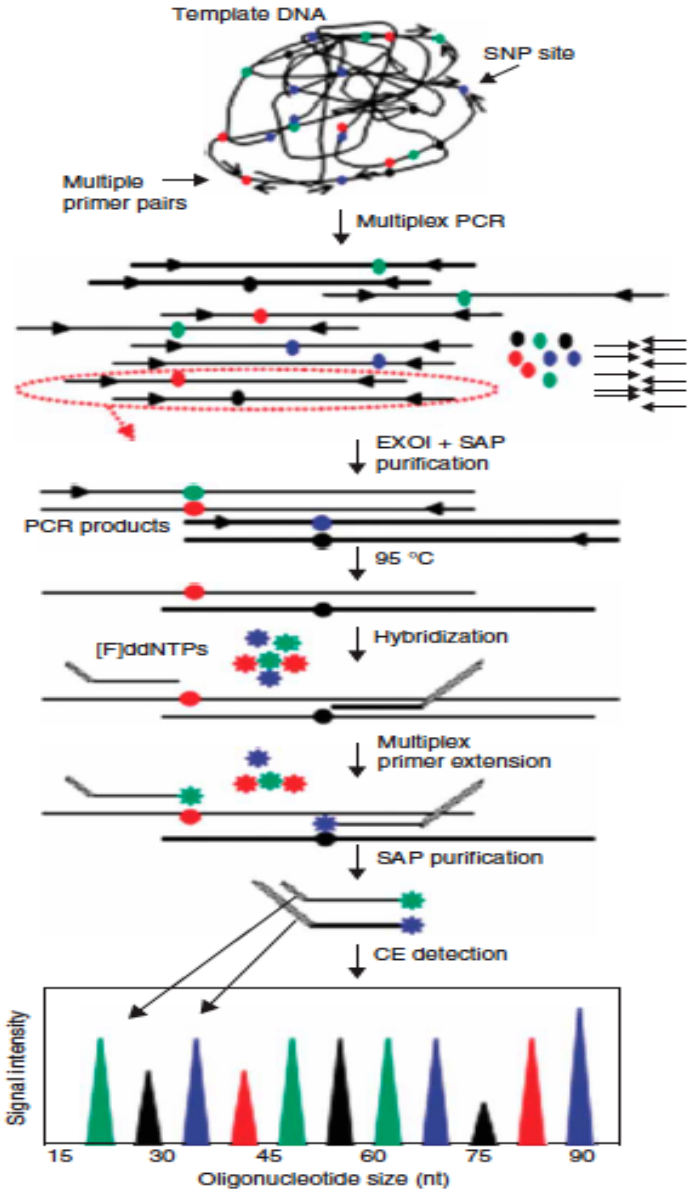
*The primer concentration was modified from the published concentrations.

Appendix 3.9. Primers used for the Y-chromosome two SNaPshot reactions (Bouakaze,C. 2007)

Marker	Poly (dC)	Neutral sequence (5'→3')	Target specific sequence (5'→3')	(*)	Primer size (nt)	Primer Concentration (µM)
SNaPshot I						
M175	2	AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	CACATGCCTTCTCACTTCTC	F	62	0.2
M217	-	-	TTATGTATTTTTCCTTCTGAAGAGTT	R	26	0.1
M45	-	CGTCGTGAAAGTCTGACAA	CTCAGAAGGAGCTTTTTGC	R	38	0.7*
RPS4Y$_{711}$	-	ACTAGGTGCCACGTCGTGAAAGTCTGACAA	GGCAATAAACCTTGGATTTC	F	50	0.5
M17	-	TGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	CCAAAATTCACTTAAAAAAACCC	R	60	0.18
M216	21	AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	TGCTAGTTATGTATACCTGTTGAAT	R	86	0.4*
SNaPshot II						
M9	12	AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	CATGTCTAAATTAAAGAAAAATAAAGAG	R	80	0.27
92R7	-	TAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	CATGAACACAAAAGACGTAGAAG	R	56	0.47*
M3	-	-	GGTACCAGCTCTTCCTAATT	R	20	0.05*
TAT	2	AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAA	GCTCTGAAATATTAAATTAAAACAAC	R	68	0.13
M89	-	GCCACGTCGTGAAAGTCTGACAA	AACTCAGGCAAAGTGAGAGAT	R	44	0.2
M242	-	GAAAGTCTGACAA	AAAAGGTGACCAAGGTGCT	F	32	0.2

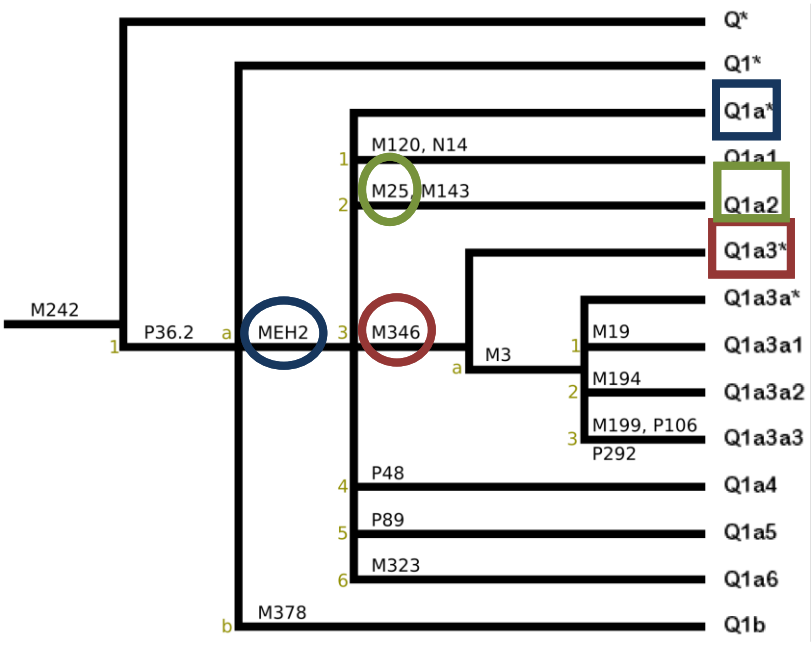
*The primer concentration was modified from the published concentrations.

Appendix 3.10. A flow chart summarizing the multiplex reaction and the SNaPshot steps, the colors represent the four DNA bases dyed with defined fluorescent dyes (Sanchez,J.J. 2006).



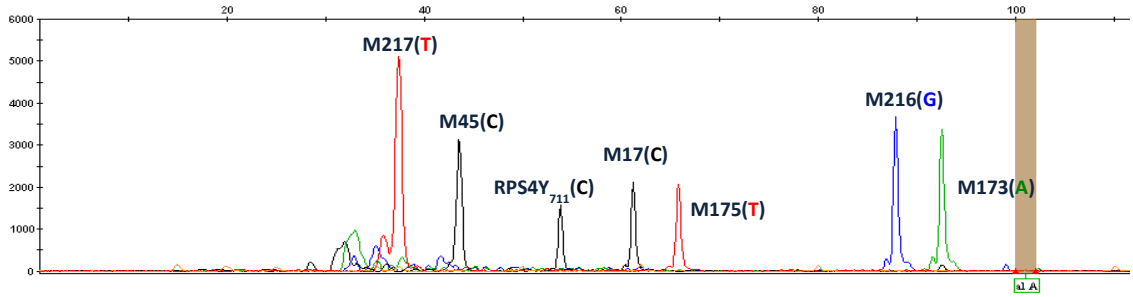
Adapted with permission from [Developing multiplexed SNP assays with special reference to degraded DNA templates. Juan J Sanchez, Phillip Endicott. Nature Protocols. 1(3). Copyright ©2006 Nature Publishing Group] (License number 3537380517257)

Appendix 3.11. Phylogenetic tree representing sub-haplogroups of the major haplogroup Q, which is identified by M242 SNP site of the Y-chromosome (Karafet,T.M. 2008). We are interested in three sub-haplogroups (Q1a*, Q1a2 and Q1a3* squared) that are defined by three Y-chromosomal SNPs (MEH2, M25 and M346 circled, respectively).

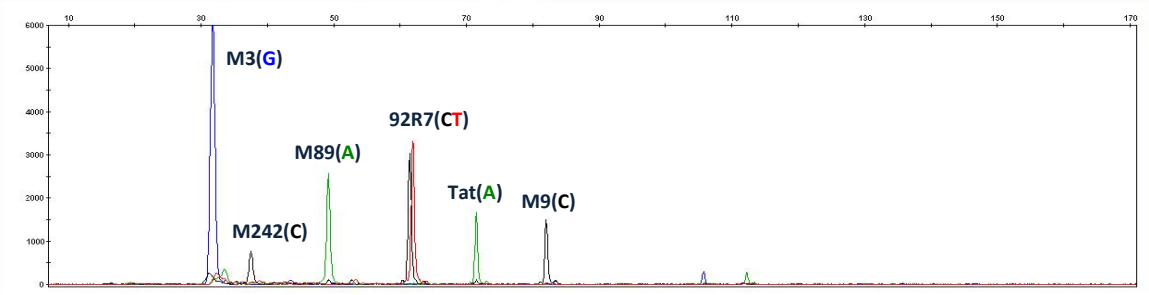


Adapted with permission from [New binary polymorphisms reshape and increase resolution of the human Y chromosomal haplogroup tree. Karafet,T.M.; Mendez,F.L.; Meilerman,M.B.; Underhill,P.A.; Zegura,S.L.; Hammer,M.F. Genome Research. 18(5). Copyright © 2008 Cold Spring Harbor Laboratory Press]. Genome Research is an open access journal, which permits non-commercial use, distribution, and reproduction in other forums, provided the original authors and source are credited.

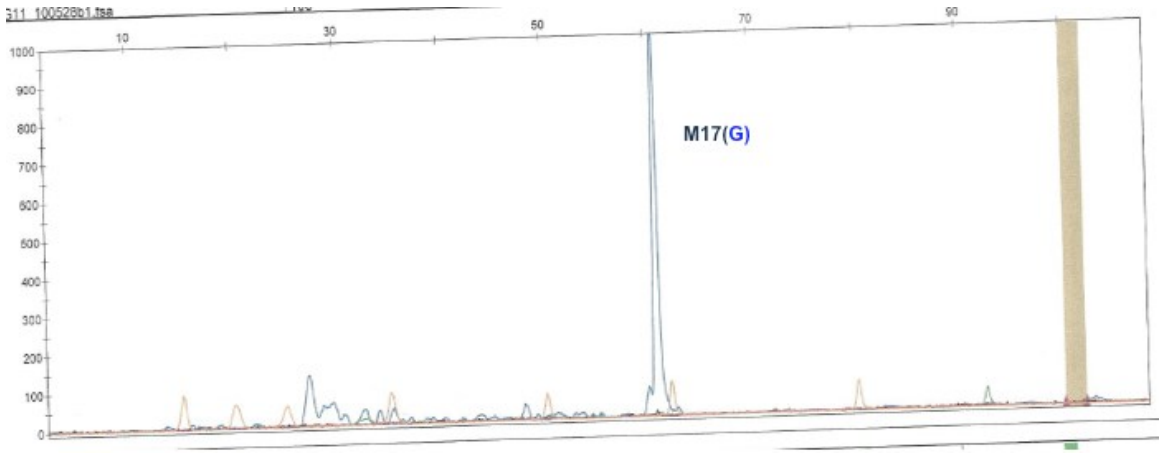
Appendix 3.12. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Lokomotiv individual (LOK_1990.042) representing Haplogroup K. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



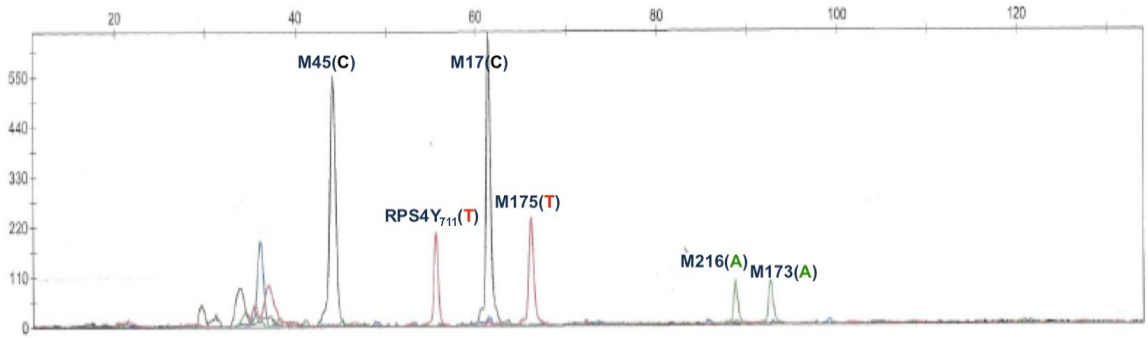
Appendix 3.13. (continued) Y-DNA sequence electropherogram obtained from SNaPshot II analysis of Lokomotiv individual (LOK_1990.042) representing Haplogroup K. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



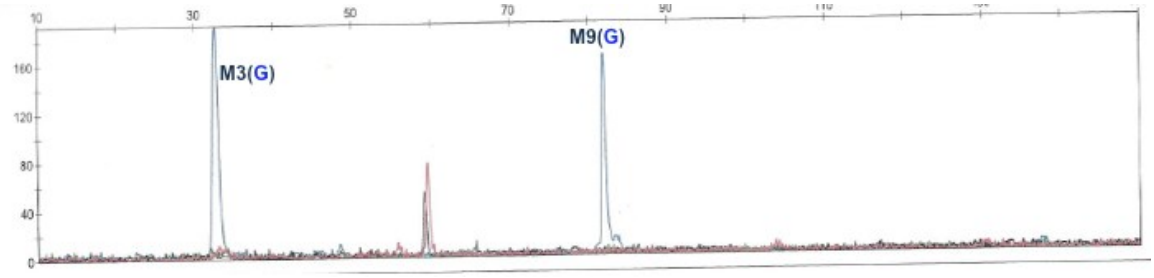
Appendix 3.14. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Lokomotiv individual (LOK_1980.006) representing Haplogroup R1a1. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



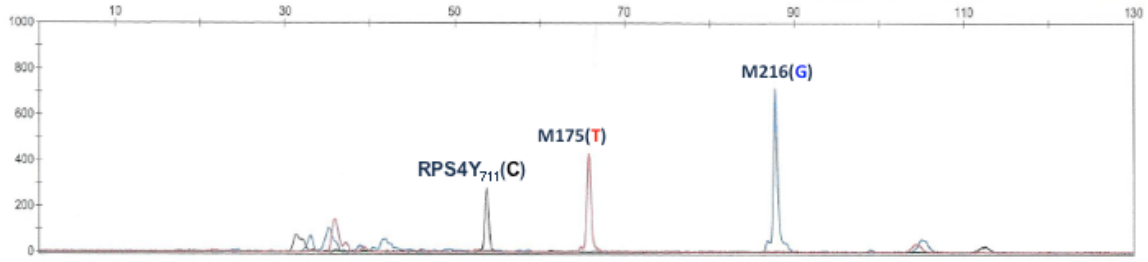
Appendix 3.15. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Lokomotiv individual (LOK_1985.031.02) representing Haplogroup C3. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



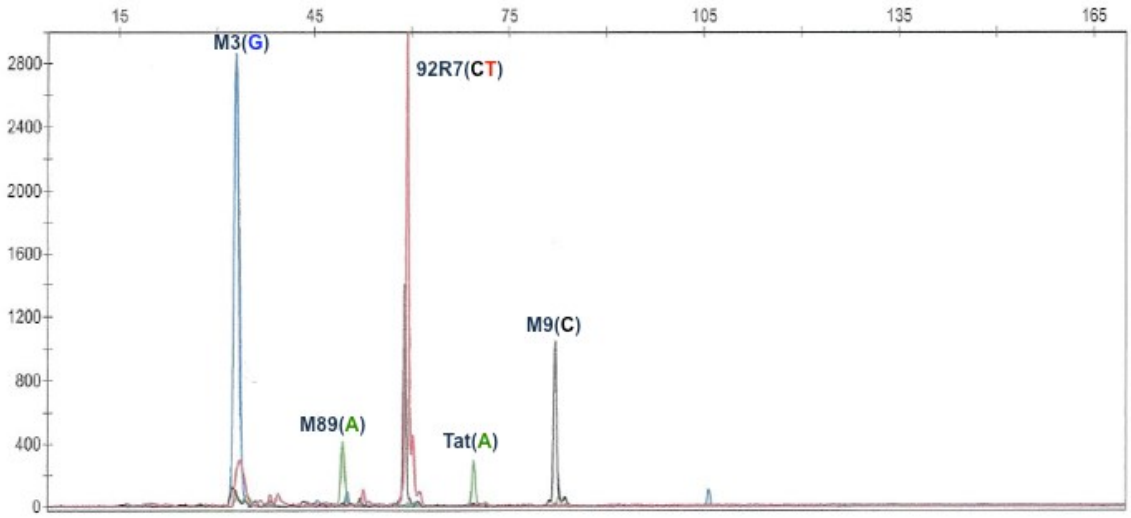
Appendix 3.16. (continued) Y-DNA sequence electropherogram obtained from SNaPshot II analysis of Lokomotiv individual (LOK_1985.031.02) representing Haplogroup C3. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



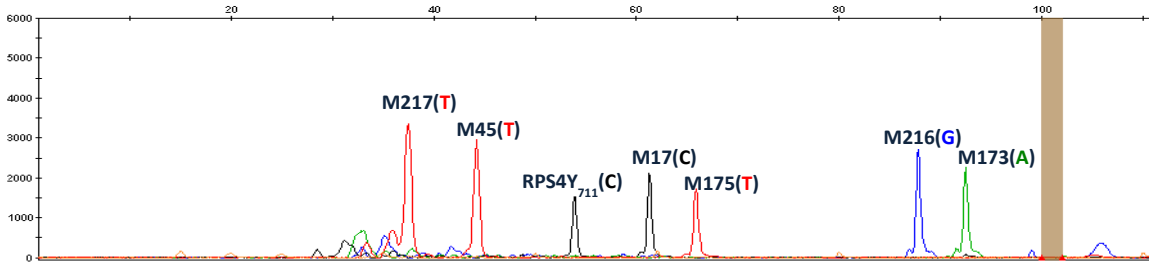
Appendix 3.17. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Lokomotiv individual (LOK_1980.010.03) representing no haplogroup assigned due to some missing SNPs. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



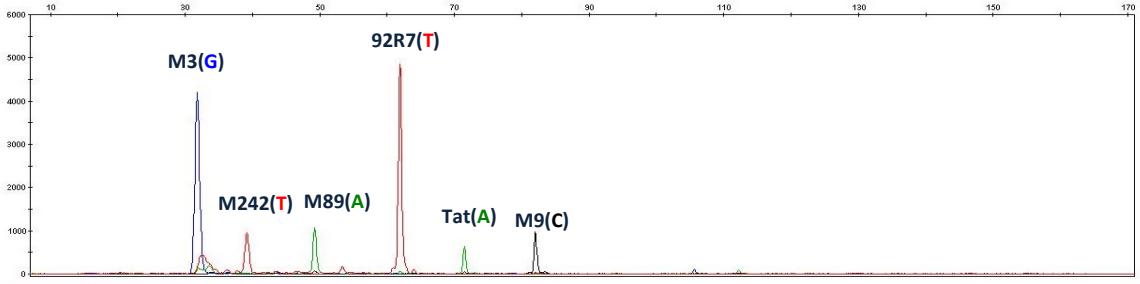
Appendix 3.18. (continued) Y-DNA sequence electropherogram obtained from SNaPshot II analysis of Lokomotiv individual (LOK_1980.010.03) representing no haplogroup assigned due to some missing SNPs. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



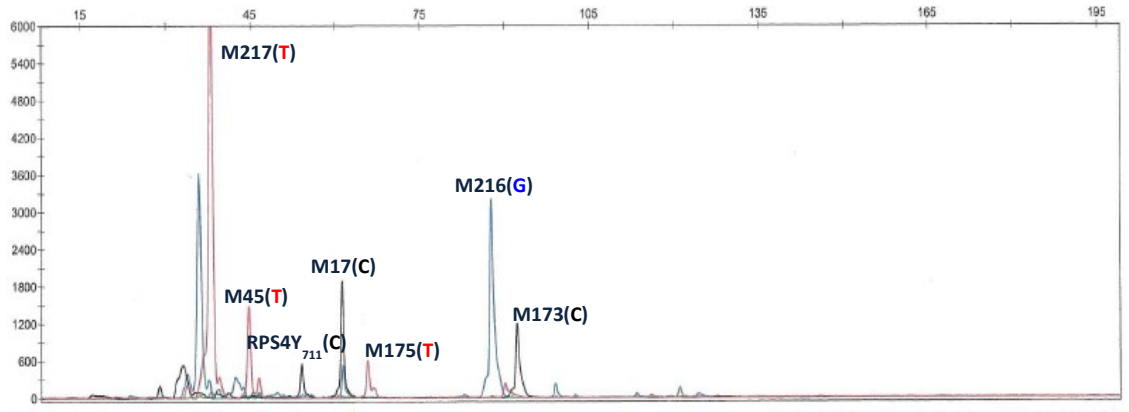
Appendix 3.19. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Ust'Ida individual (UID_1989.029) representing haplogroup Q. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



Appendix 3.20. (continued) Y-DNA sequence electropherogram obtained from SNaPshot II analysis of Ust'Ida individual (UID_1989.029) representing haplogroup Q. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.

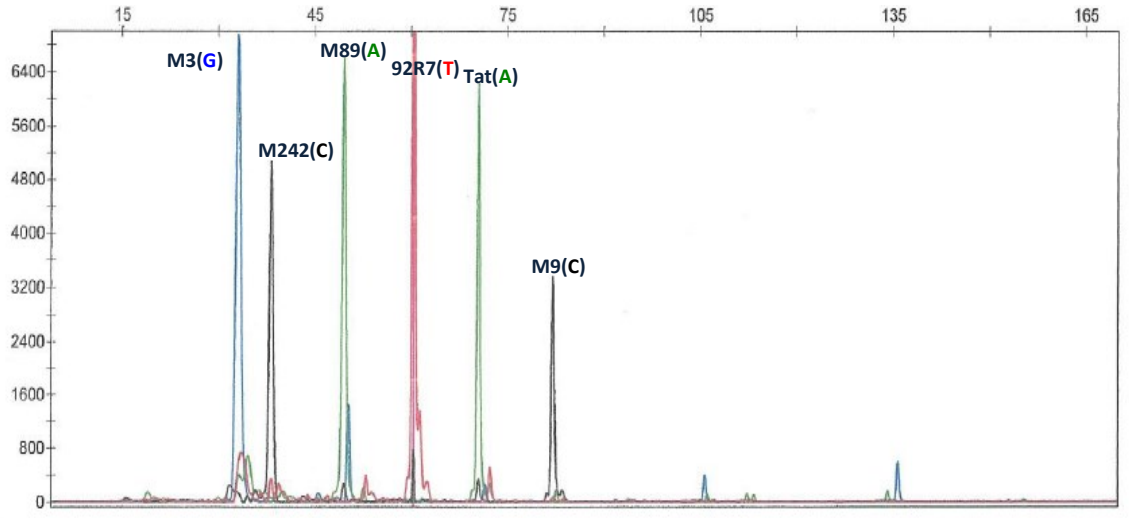


Appendix 3.21. Y-DNA sequence electropherogram obtained from SNaPshot I analysis of Male control. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.

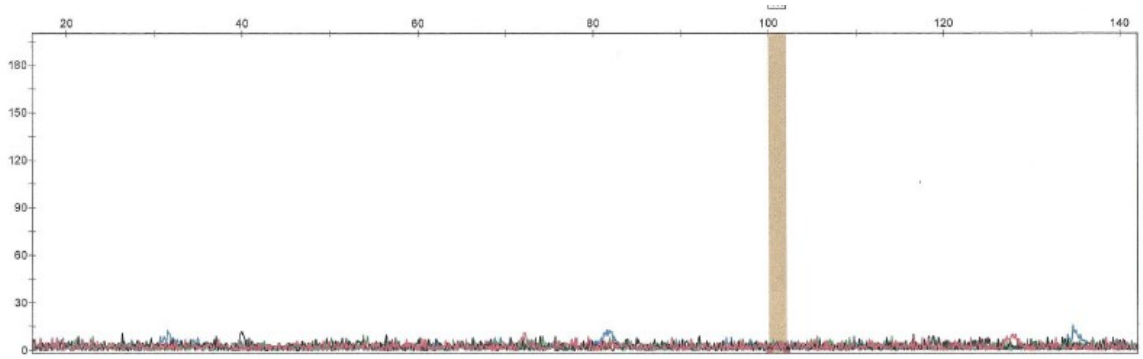


(Haplogroup R1)

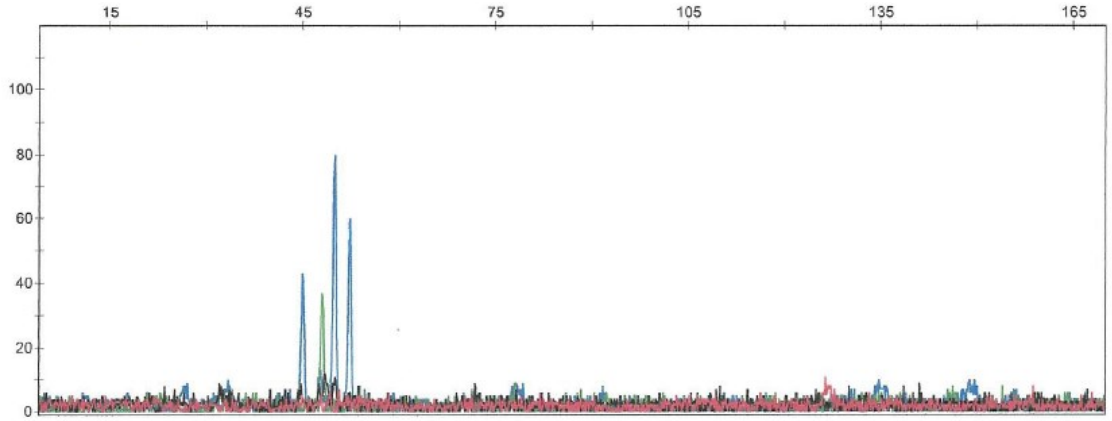
Appendix 3.22. (continued) Y-DNA sequence electropherogram obtained from SNaPshot II analysis of Male control. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



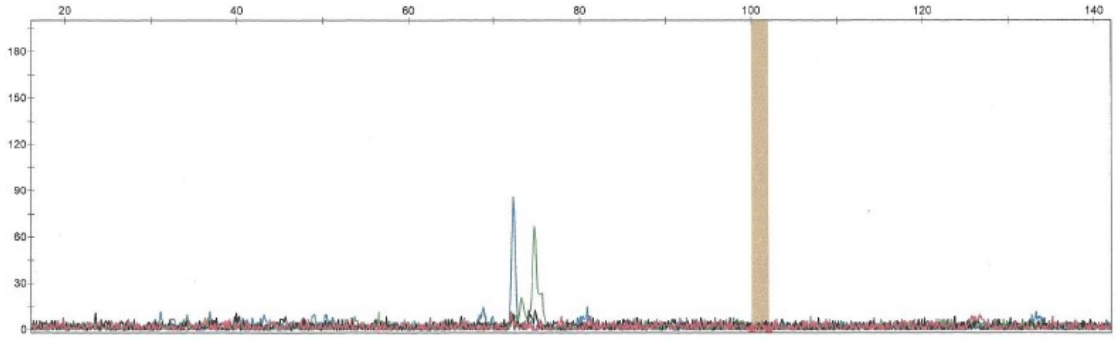
Appendix 3.23. Negative Control sequence electropherogram obtained from SNaPshot I. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



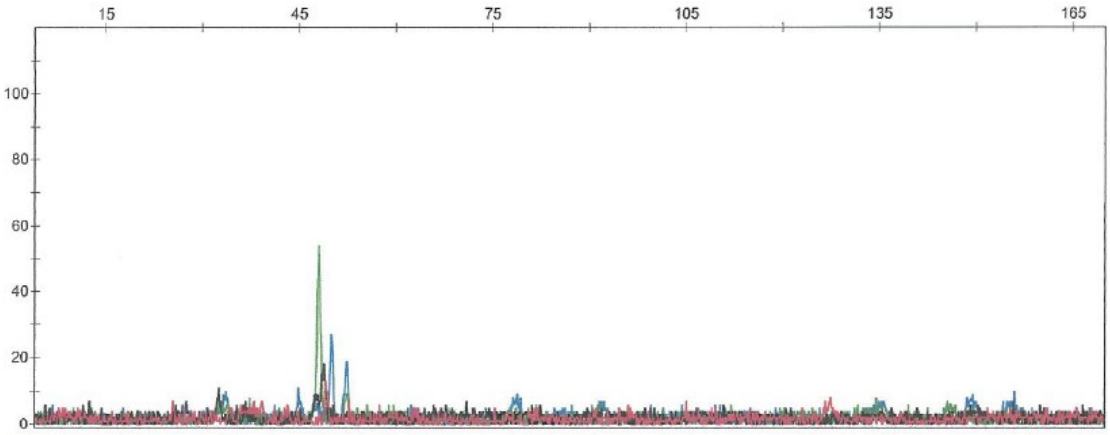
Appendix 3.24. (continued) Negative Control sequence electropherogram obtained from SNaPshot II. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.



Appendix 3.25. Female Control sequence electropherogram obtained from SNaPshot I. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.

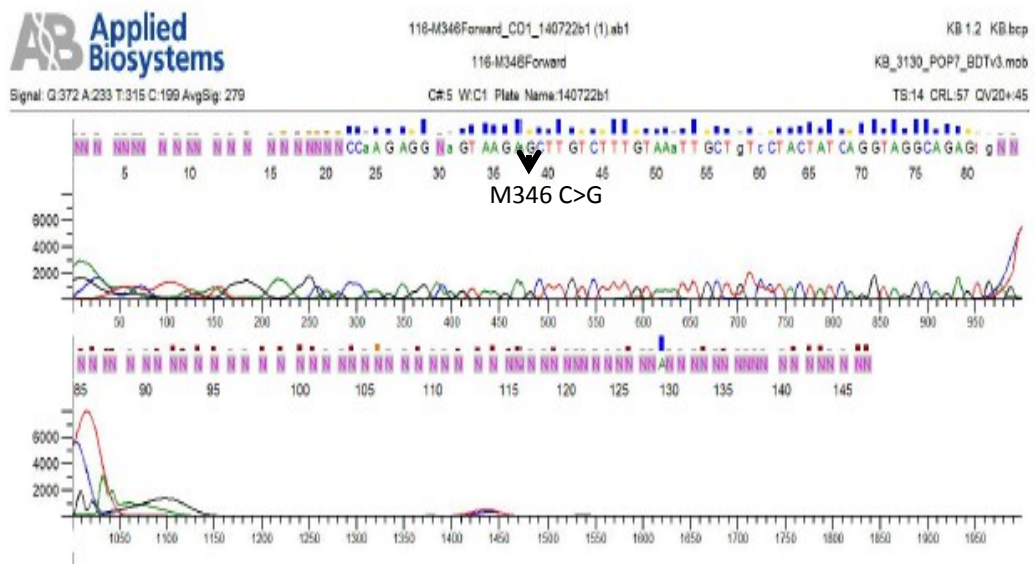


Appendix 3.26. (continued) Female Control sequence electropherogram obtained from SNaPshot II. These plots were obtained using GeneMapper 4.0 software. Each peak labeled with the SNP marker and the base substitution designating it.

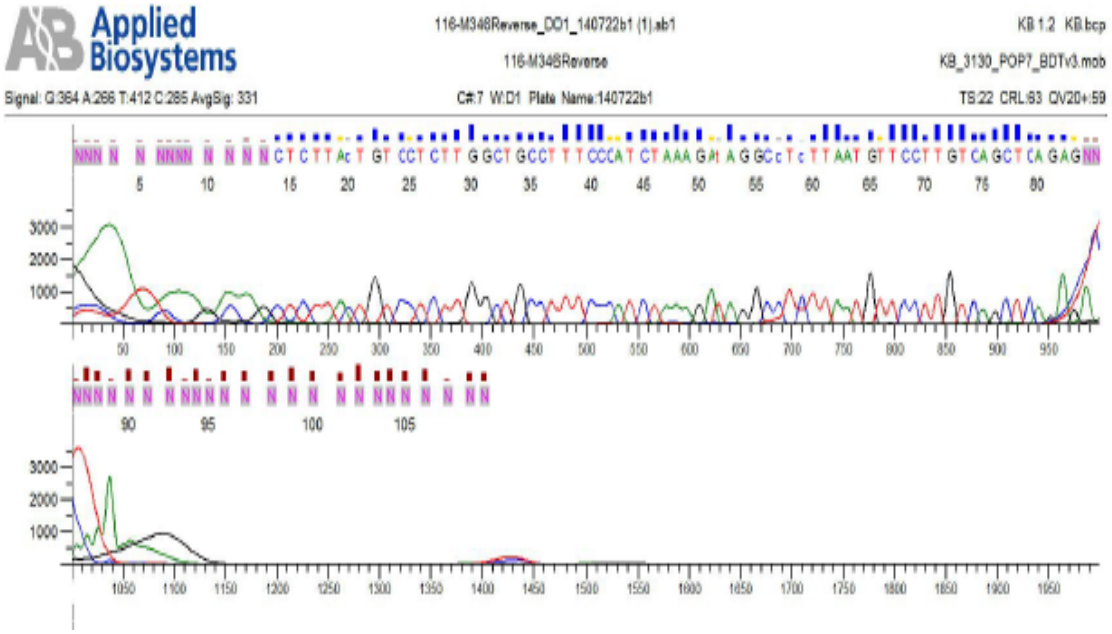


Appendix 3.27. Q1a3-M346 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2002.007.02) with M346 (C>G).

Forward:

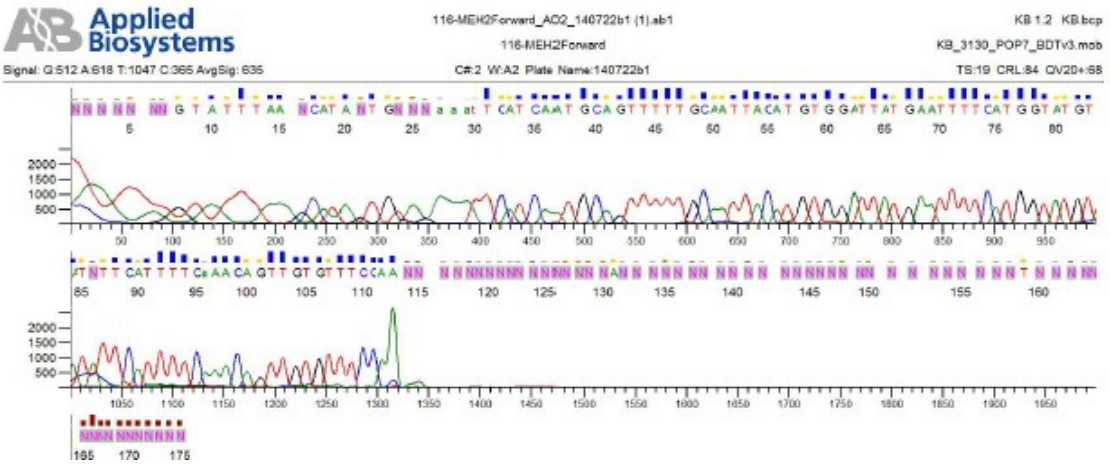


Reverse:

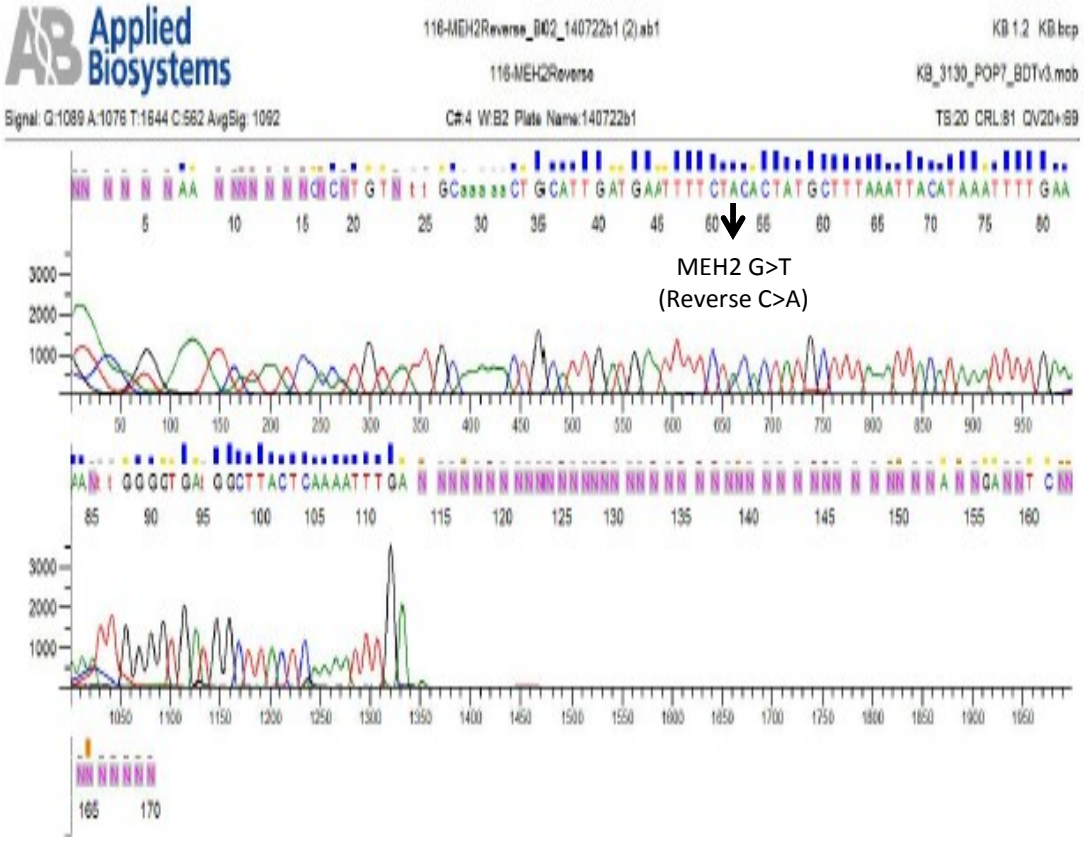


Appendix 3.28. Q1a*-MEH2 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Kurma XI individual (KUR_2002.007.02) with MEH2 (G>T).

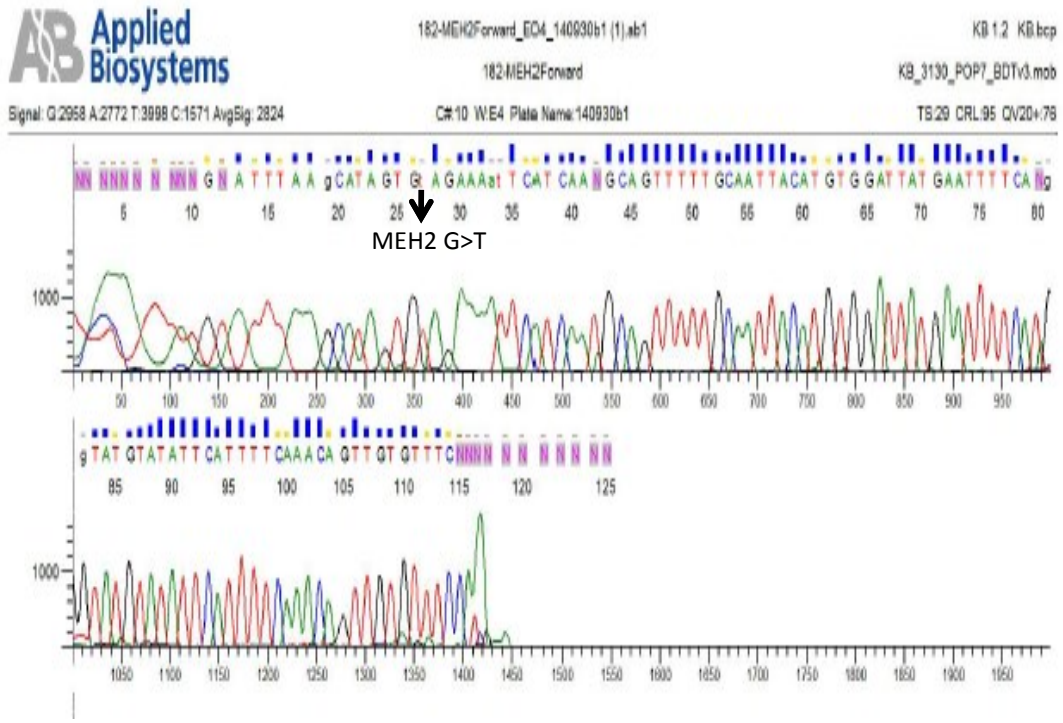
Forward:



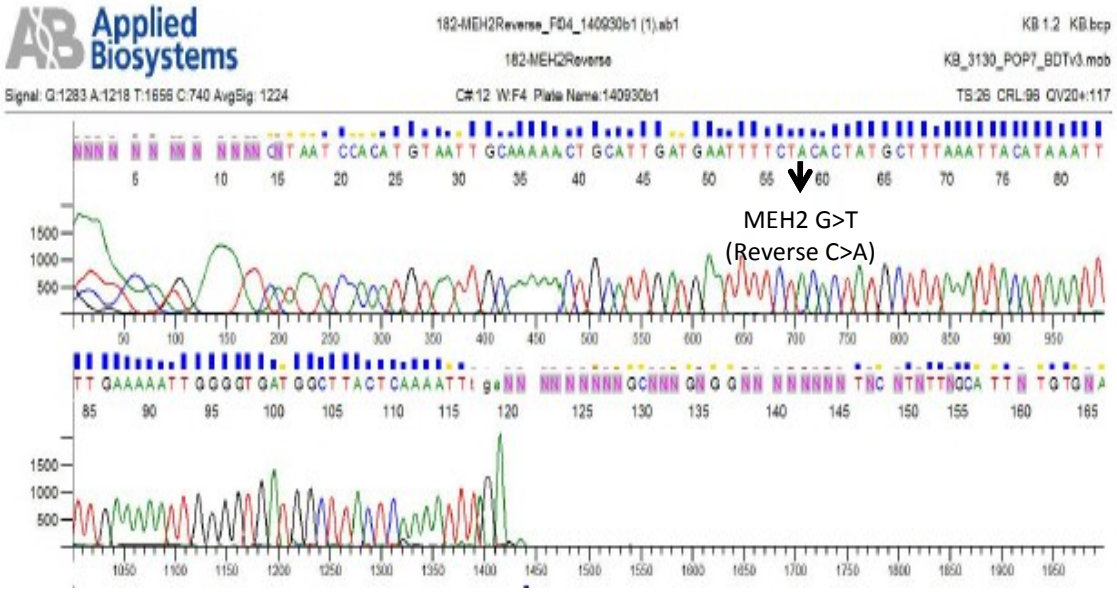
Reverse:



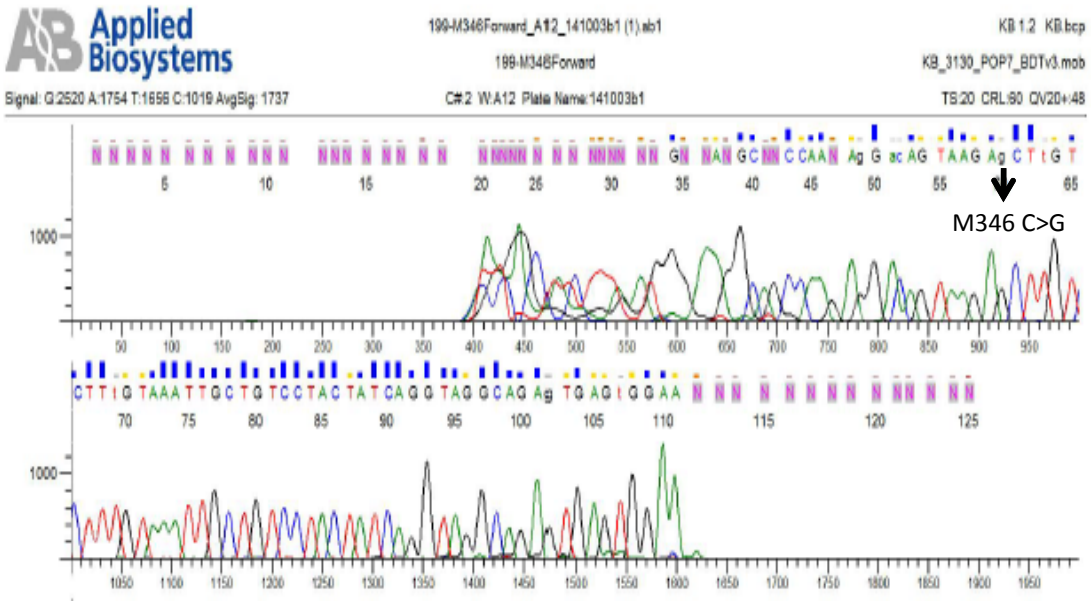
Appendix 3.29. Q1a*-MEH2 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Ust'-Ida individual (UID_1988.016.01) with MEH2 (G>T).
Forward:



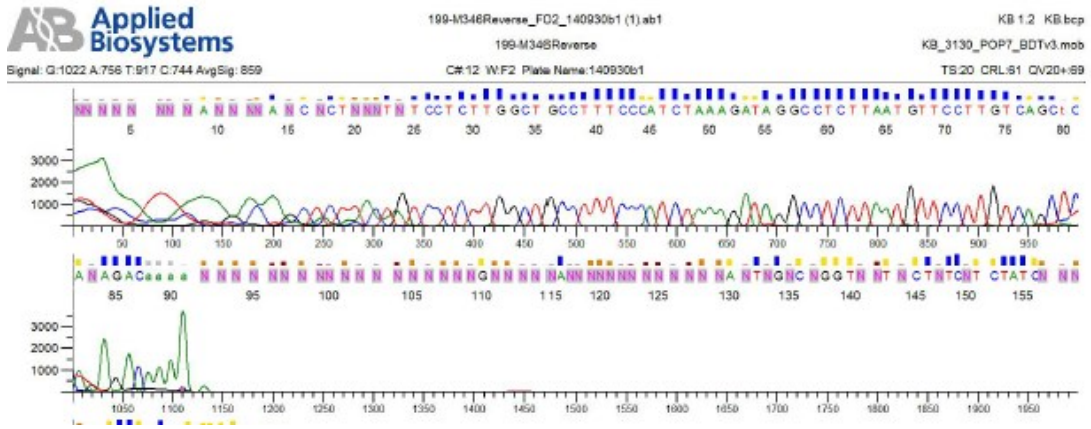
Reverse



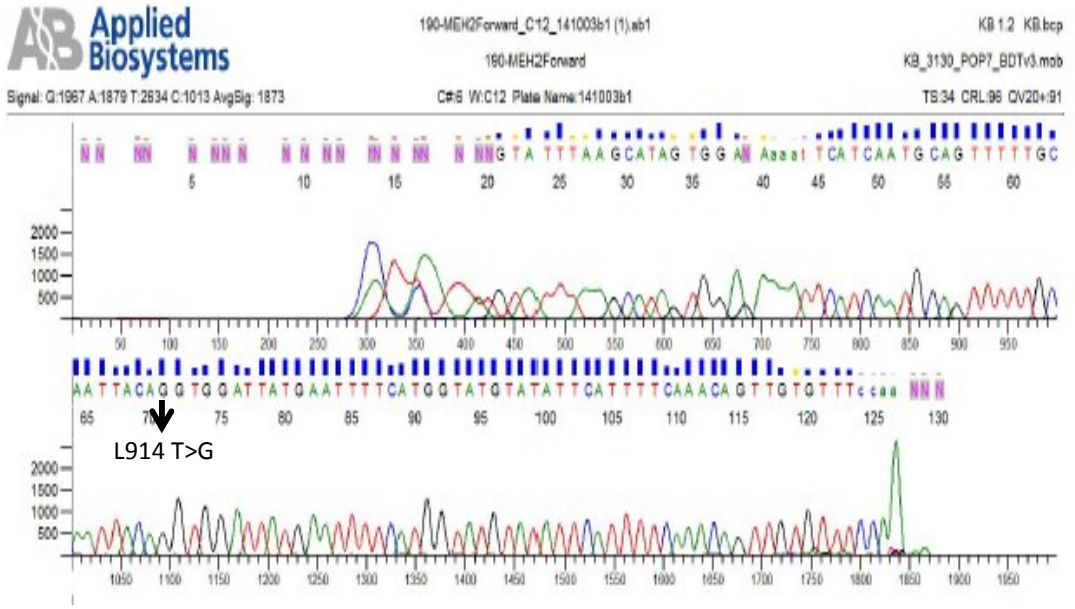
Appendix 3.30. Q1a3-M346 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Ust'-Ida individual (UID_1991.038) with M346 (C>G).
Forward:



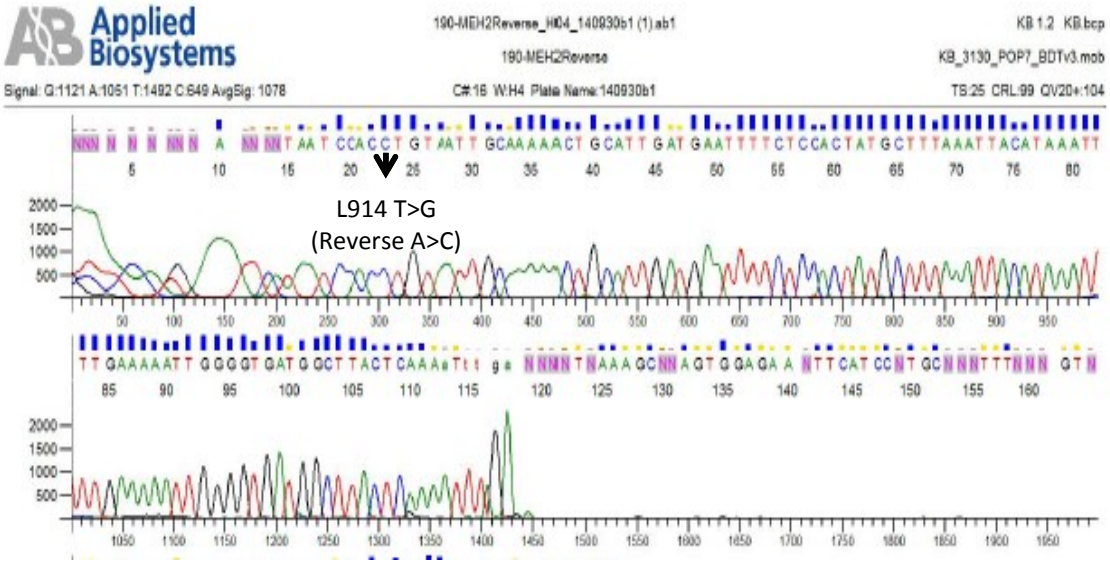
Reverse:



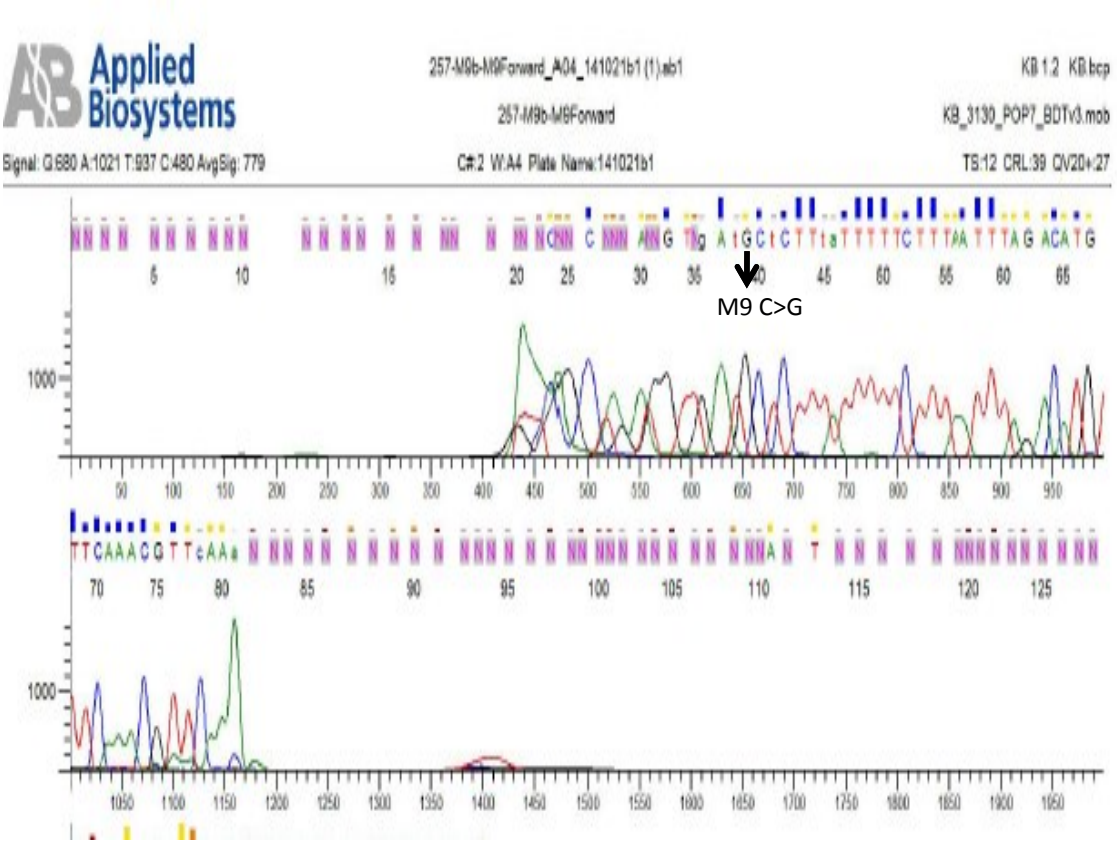
Appendix 3.31. Q1a*-MEH2 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Ust'-Ida individual (UID_1989.026.01) with MEH2 primer representing L914 SNP (T>G) site.
Forward:



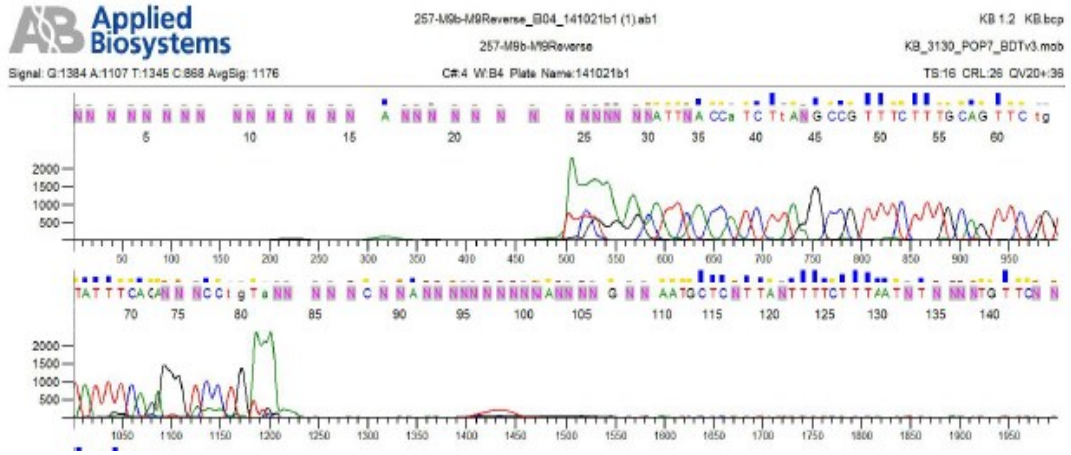
Reverse:



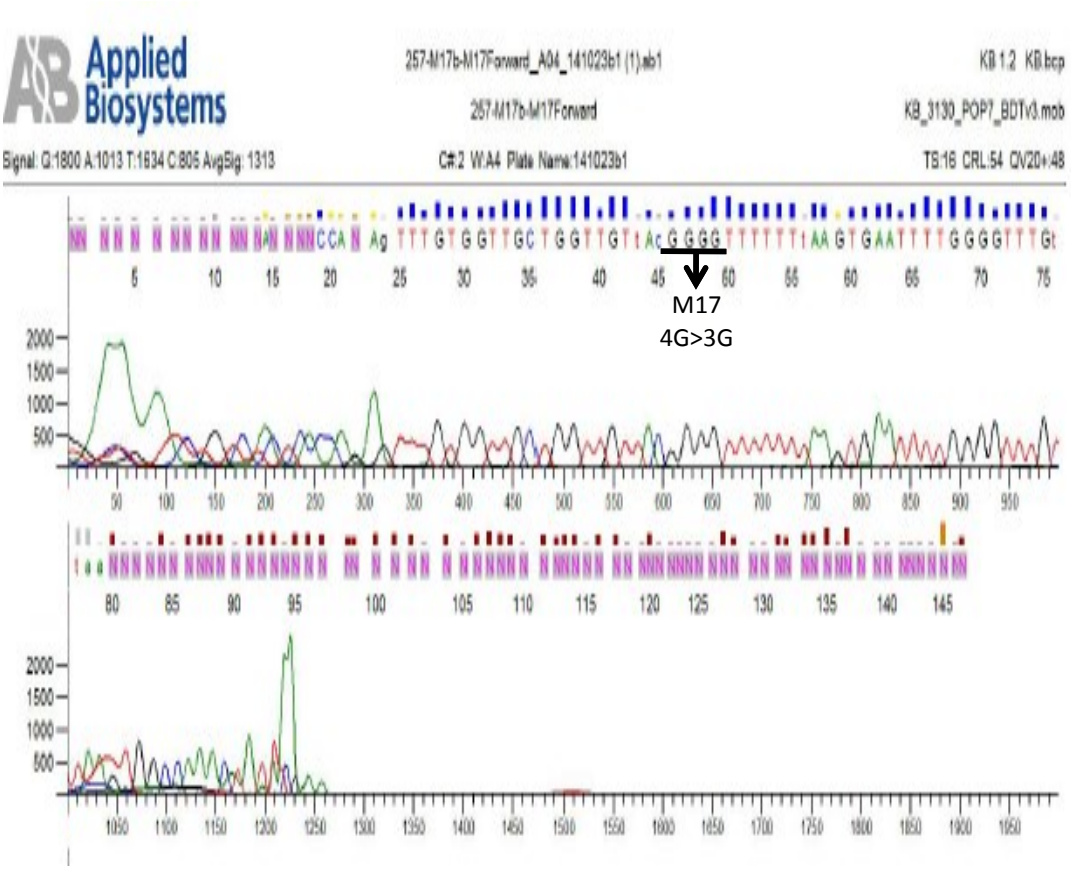
Appendix 3.32. K-M9 sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2002.021.02) with M9 (C>G).
Forward:



Reverse:



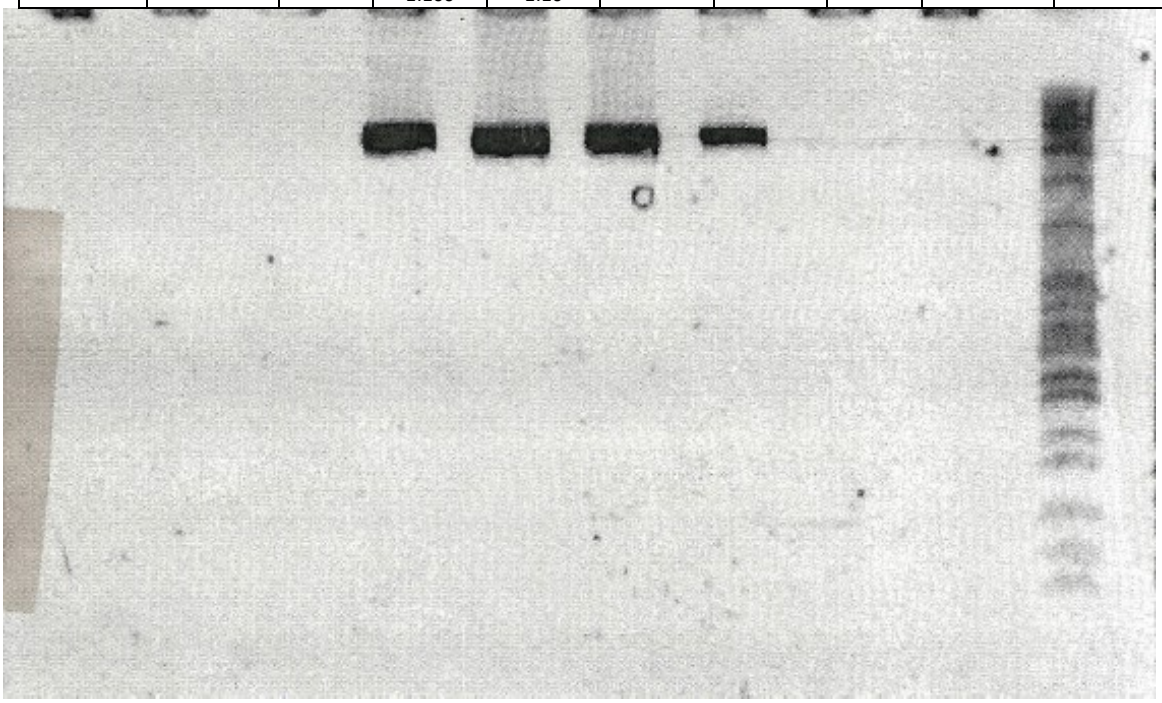
Appendix 3.33. R1a1-M17 sequence electropherogram obtained from the forward (top) sequences of Shamanka II individual (SHA_2002.021.02) with M17 (4G>3G).
Forward:



Appendices for Chapter 4

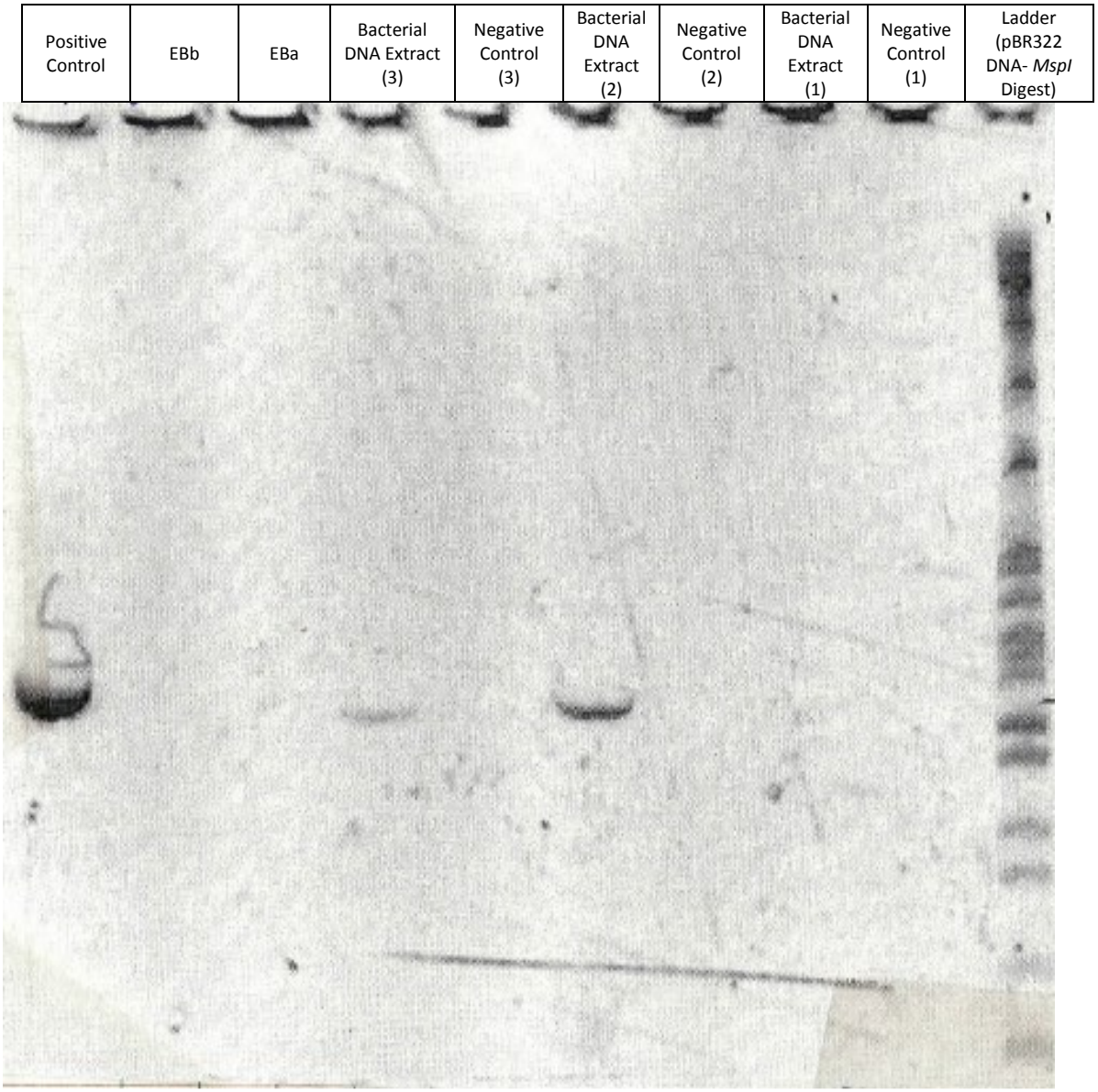
Appendix 4.1. Polyacrylamide gel electrophoresis picture illustrating the Bacterial PCR amplification reaction with 16S universal primers to test if the source of contamination was the extracting reagents

Negative control (2)	Master Mix	EBb	Bacterial Extract dilution 1:100	Bacterial Extract dilution 1:10	Bacterial Extract (2)	Bacterial Extract (1)	EBa	Negative Control (1)	Ladder (pBR322 DNA- *MspI* Digest)



Only the bacterial DNA gave amplification results with the 16S primers indicating that the source of contamination is not the extracting reagents. The amplicon size is at ~500 bp.

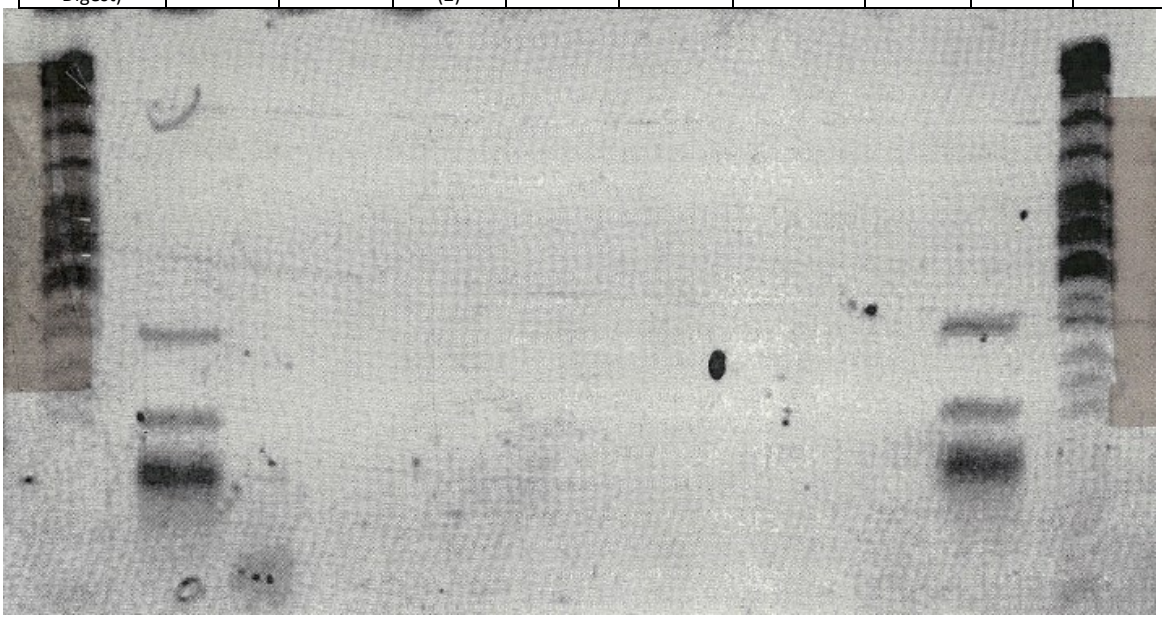
Appendix 4.2. Polyacrylamide gel electrophoresis picture illustrating the evidence of contamination from mtDNA primers (H16346, L16211) through using bacterial DNA extract for PCR amplification.



Bacterial extract (2) and Bacterial extract (3) show a faint band at 176 bp, which is the mtDNA amplification product when conducting PCR with H16346 and L16211.
The negative controls and the EBa and EBb are not shoing any evidence of contamination. Also, bacterial extract 1 is not showing any evidence of contamination indicating that the contamination exists but with very low quantity.

Appendix 4.3. Polyacrylamide gel electrophoresis picture illustrating PCR amplification of bacterial DNA extract using the Amelogenin primers (Amel 1 and Amel 2).

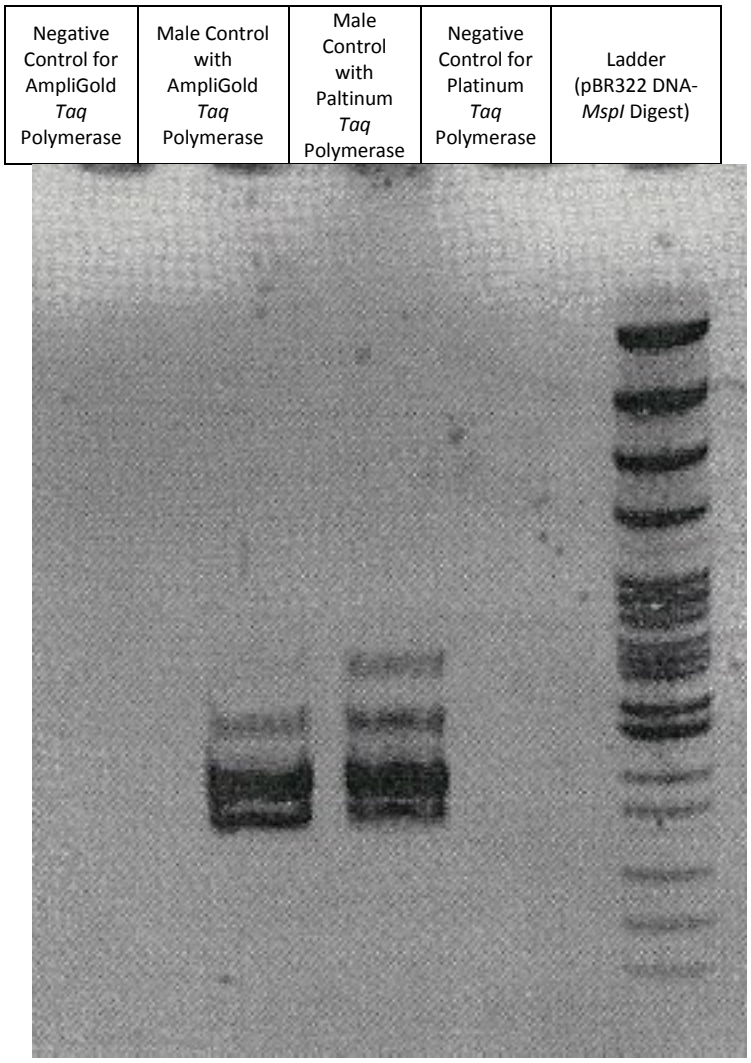
Ladder (pBR322 DNA- *MspI* Digest)	Positive Control (2)	Negative Control (2)	Bacterial DNA Extract (2)	Master Mix (2)	Master Mix (1)	Bacterial DNA Extract (1)	Negative Control (1)	Positive Control (1)	Ladder (pBR322 DNA- *MspI* Digest)



Master Mix is the PCR mix only without adding bacterial DNA extract, which is also considered a negative control. The excess use of negative control is to detect the slightest amount of contamination in any of the negative control.

The two bacterial DNA extracts did not show any amplification product indicating that there is no contamination detected through the PCR amplification using the Amel 1 and Amel 2 primers. The positive controls were for a female, so there is a big band with amplicon size 106 bp. There is a strong evidence of unspecific priming that could be due to the annealing temperature (60 degree C) that was later increased to 62 degree C.

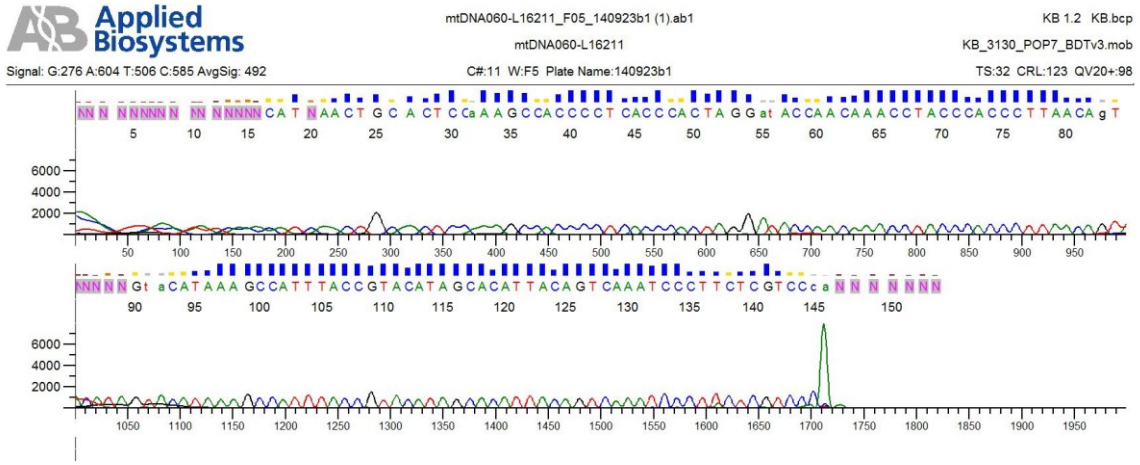
Appendix 4.4. Polyacrylamide gel electrophoresis picture illustrating Y-chromosomal multiplex PCR amplification reaction of positive male control using two different types of *Taq* DNA polymerase (AmpliGold *Taq* DNA Polymerase, and Platinum *Taq* DNA Polymerase).



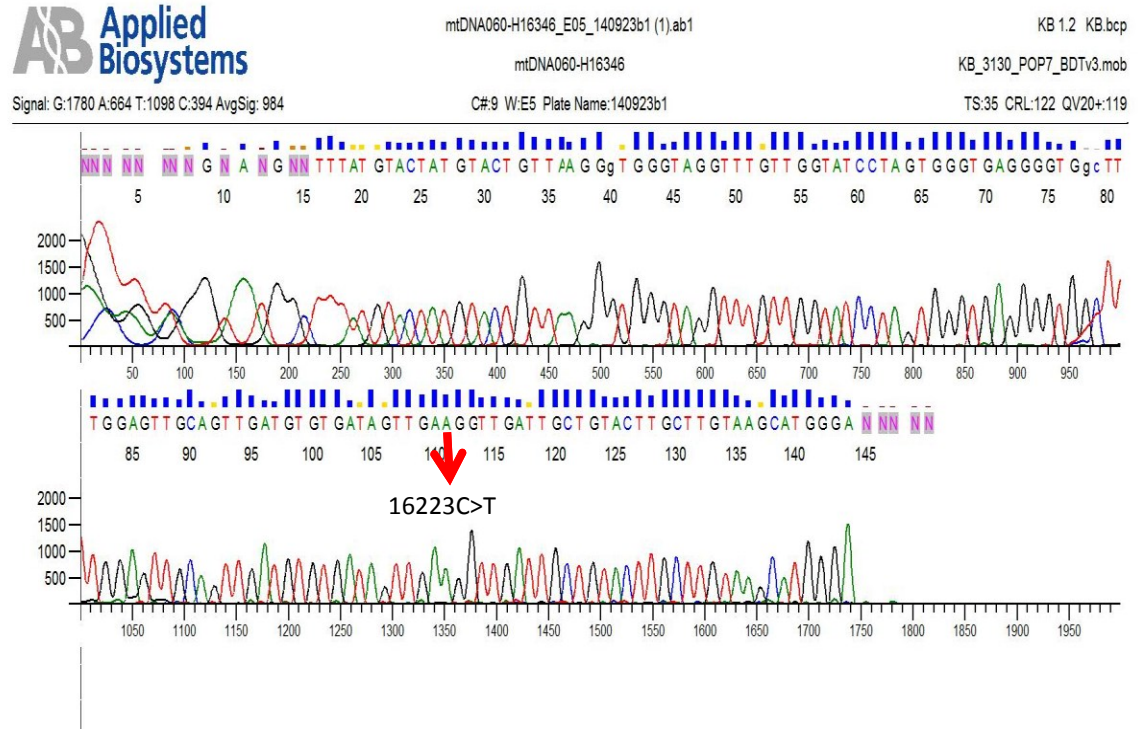
From the Y-chromosomal multiplex amplification results, it is obvious that Platinum *Taq* DNA polymerase is better than the AmpliGold *Taq* DNA polymerase, evident in the presence of all the expected bands from the multiplex reaction and the intensity and clarity of the bands.

Appendix 4.5. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2001.012), bone sample ID 2009.060. One base substitution in the reverse sequence at 16223 (C>T), Haplogroup D.

Forward:

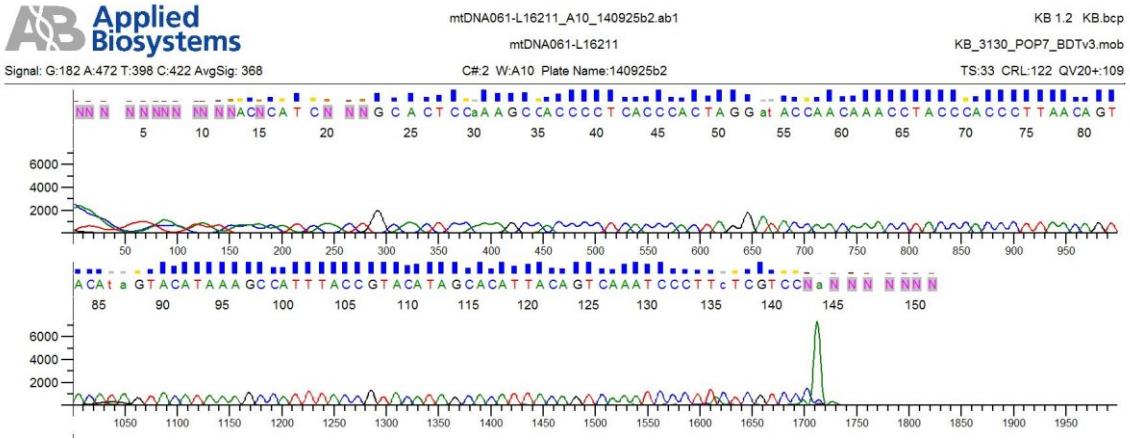


Reverse:

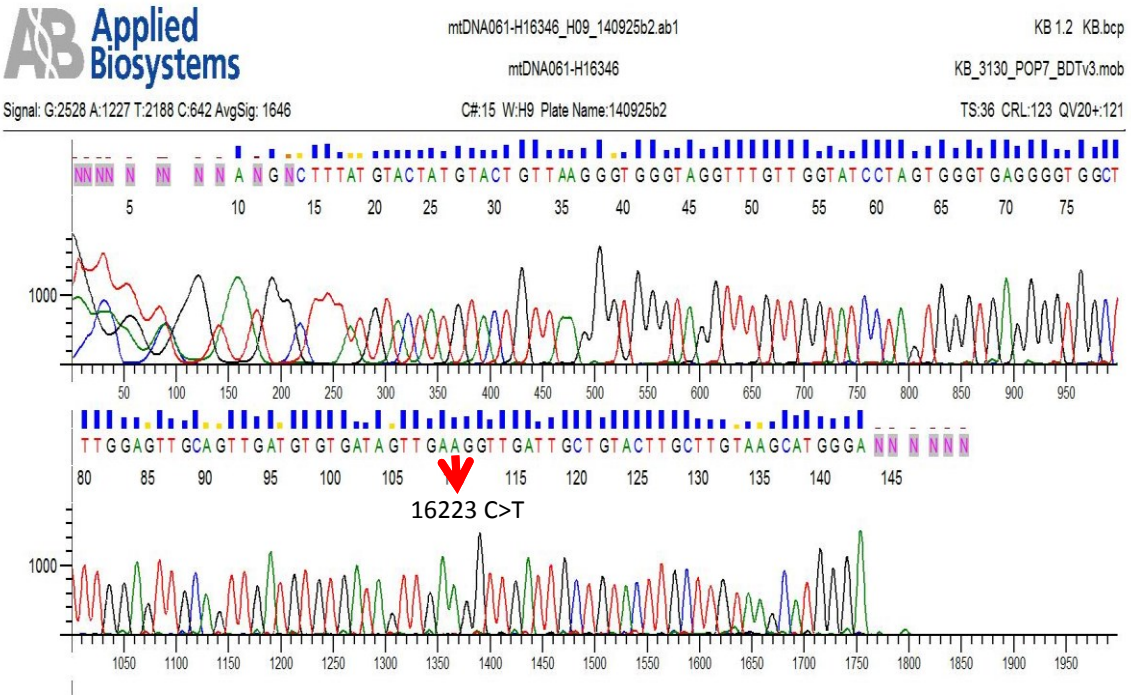


Appendix 4.6. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2001.013.03), bone sample ID 2009.061. One base substitution in the reverse sequence at 16223 (C>T), Haplogroup D.

Forward:

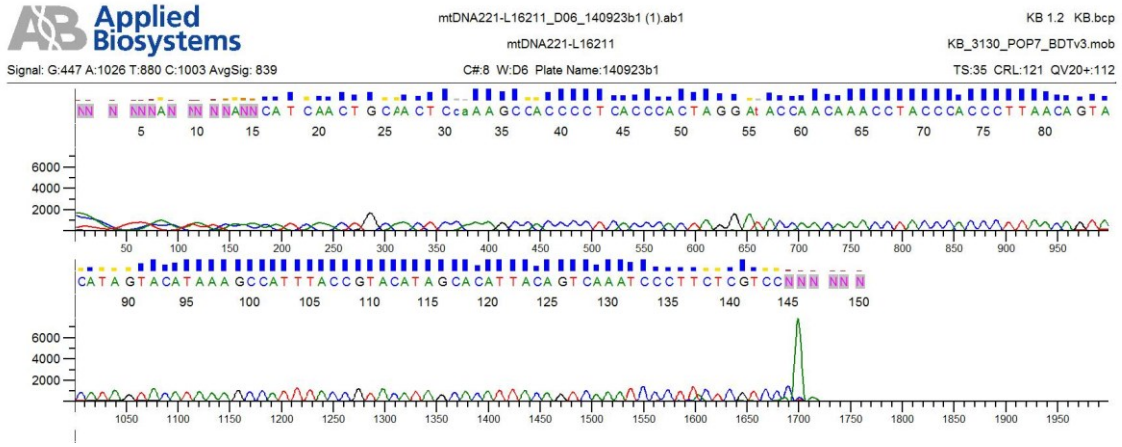


Reverse:

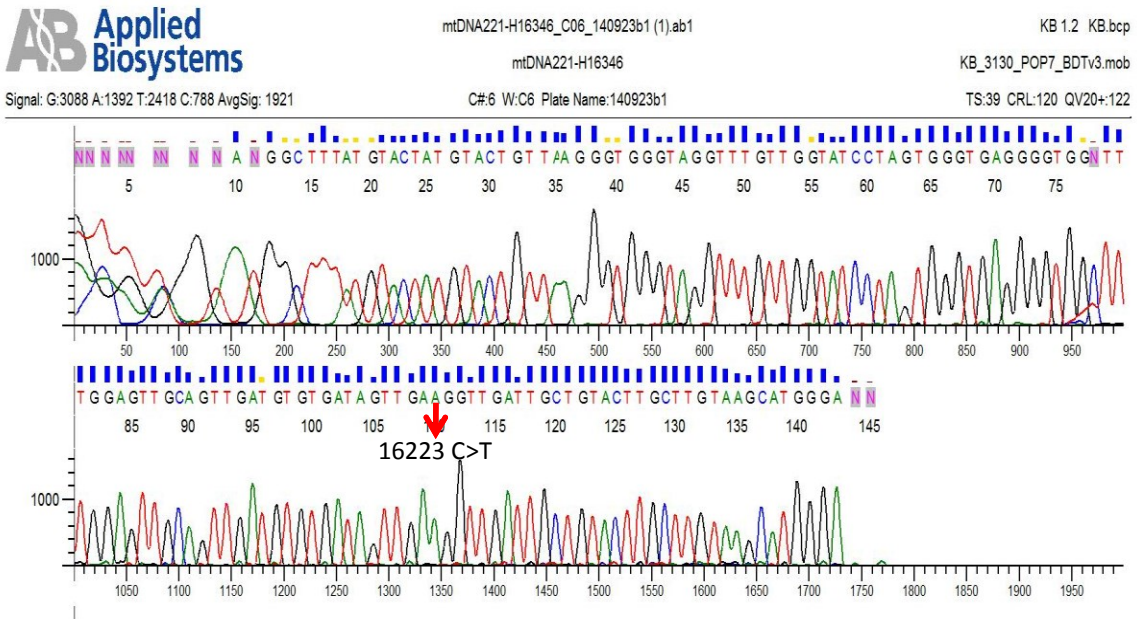


Appendix 4.7. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2002.023.04), bone sample ID 2002.221. One base substitution in the reverse sequence at 16223 (C>T), Haplogroup D.

Forward:

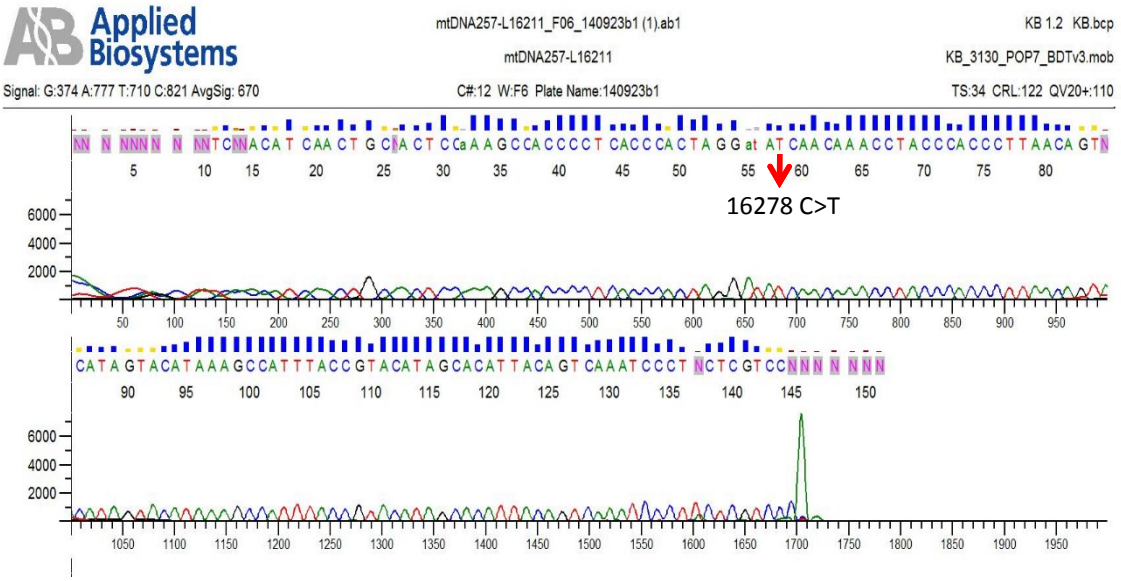


Reverse:

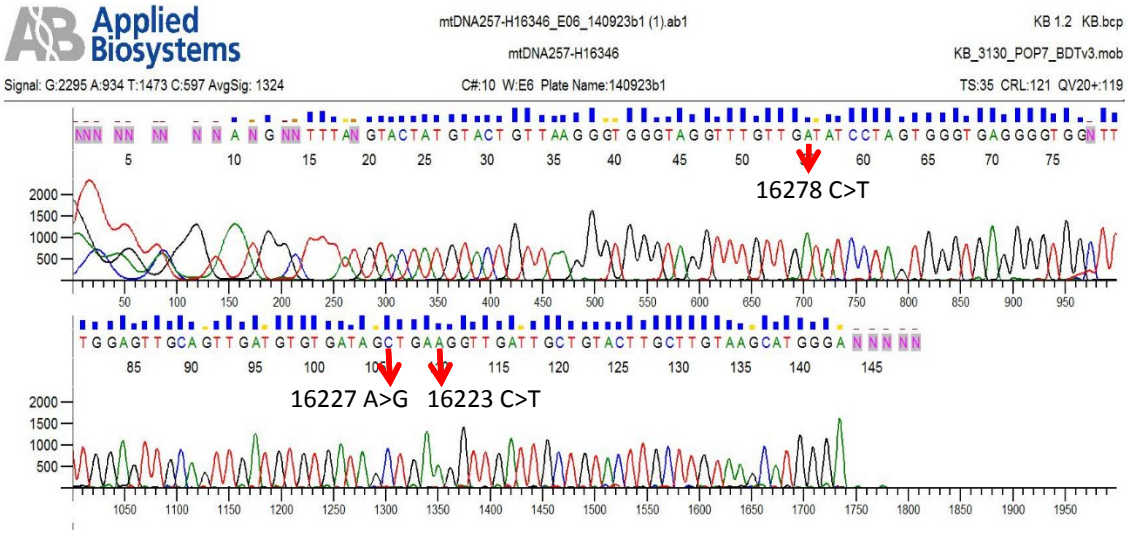


Appendix 4.8. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2002.021.02), tooth sample ID 2002.257. Three base substitutions at 16223 (C>T), 16227 (A>G) and 16278 (C>T), Haplogroup G2a.

Forward:

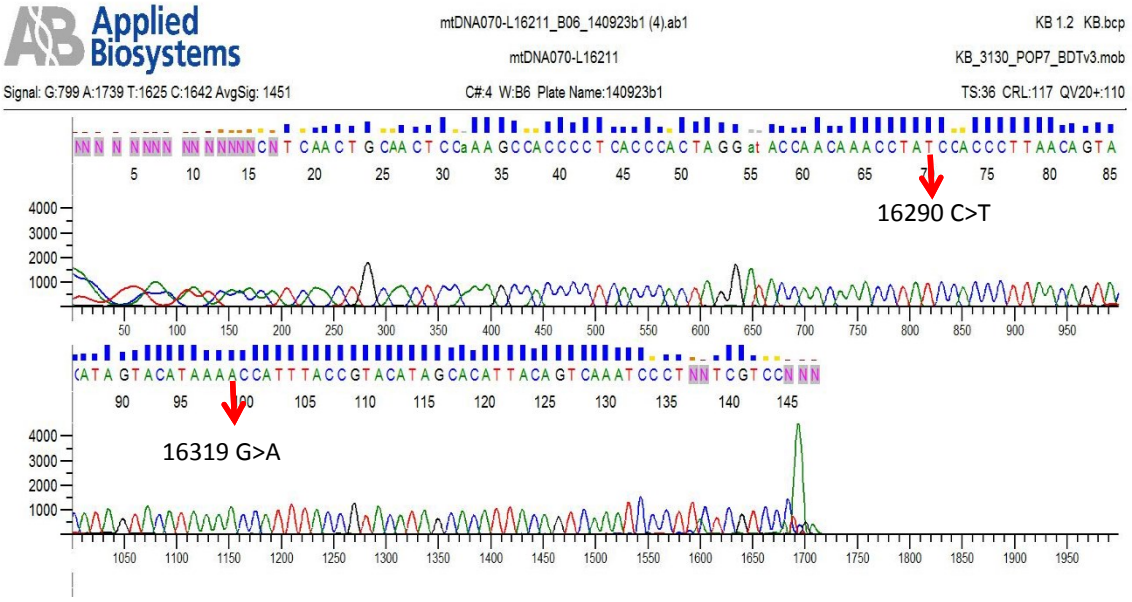


Reverse:

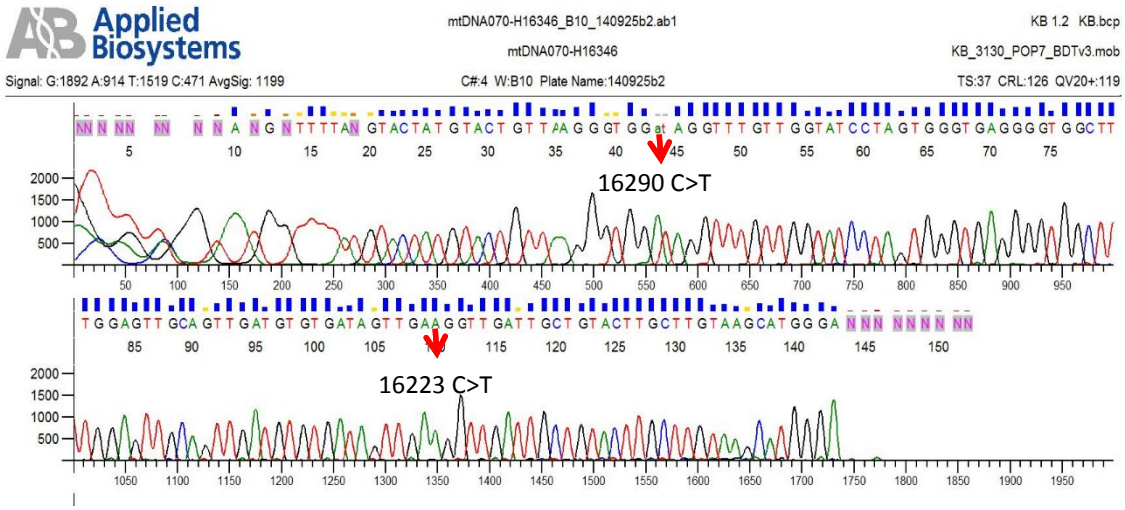


Appendix 4.9. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Shamanka II individual (SHA_2002.021.03), bone sample ID 2009.070. Three base substitutions at 16223 (C>T), 16290 (C>T) and 16319(G>A), Haplogroup A.

Forward:

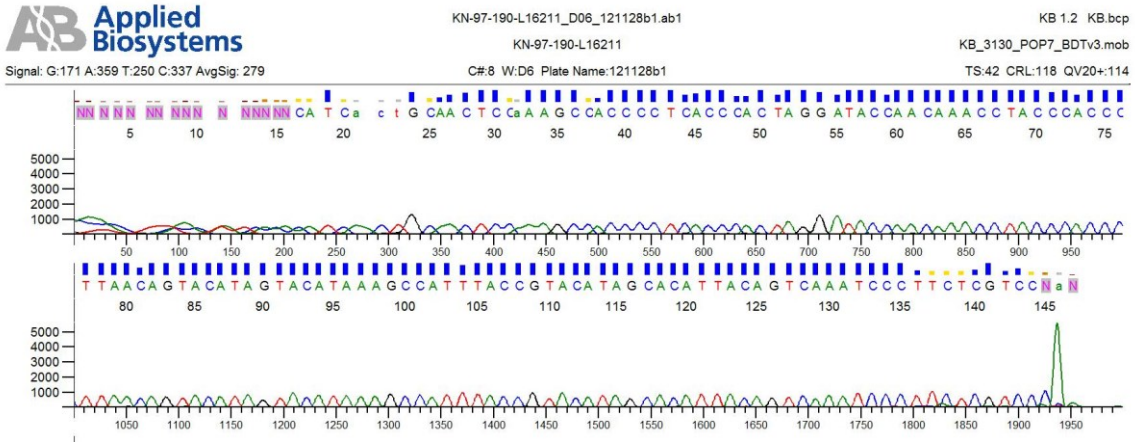


Reverse:

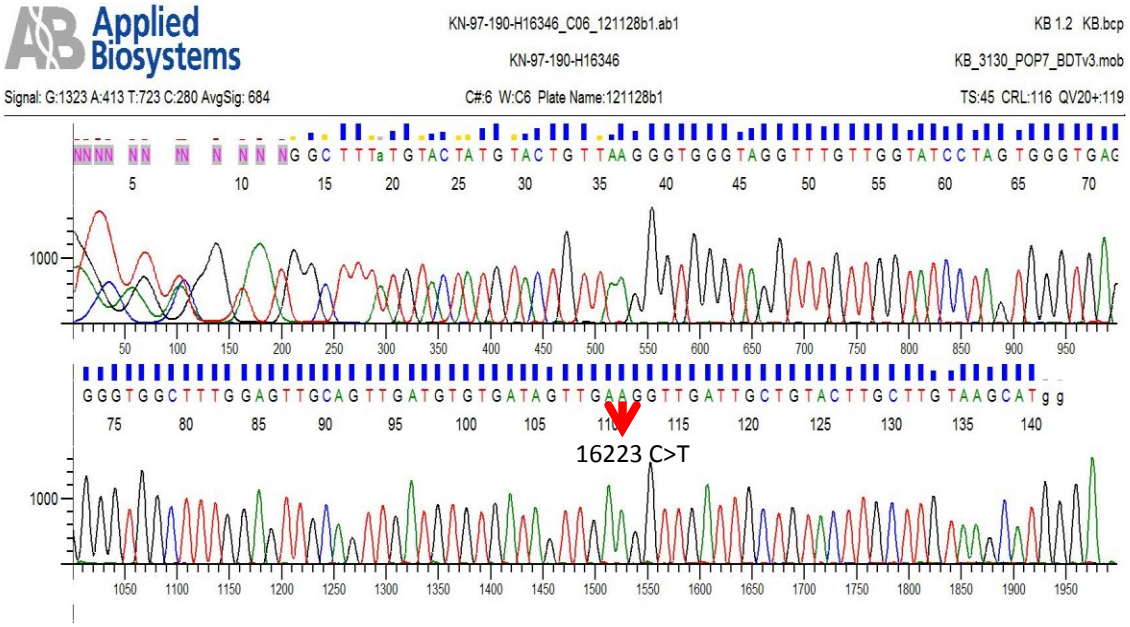


Appendix 4.10. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Khuzhir-Nuge XIV individual (K14_1997.011), tooth sample ID 1997.190. One base substitution in the reverse sequence at 16223 (C>T), Haplogroup D.

Forward:

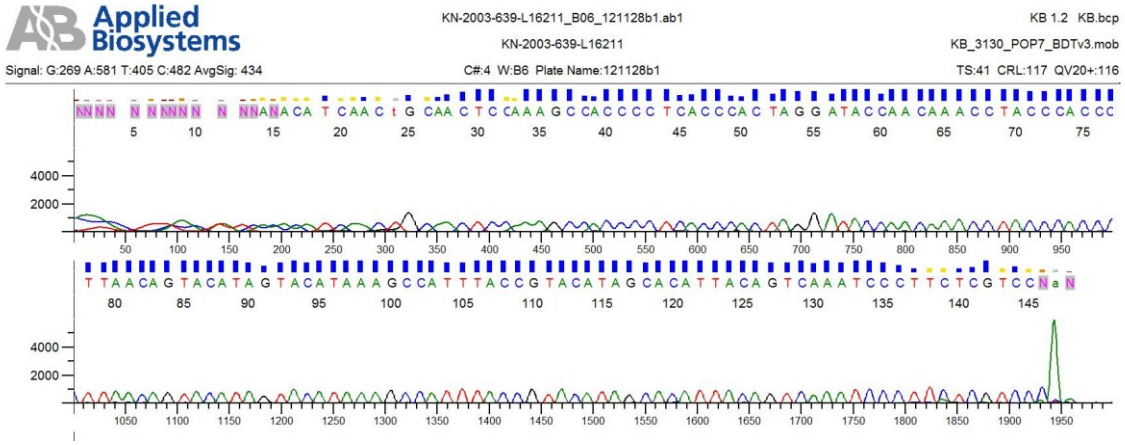


Reverse:

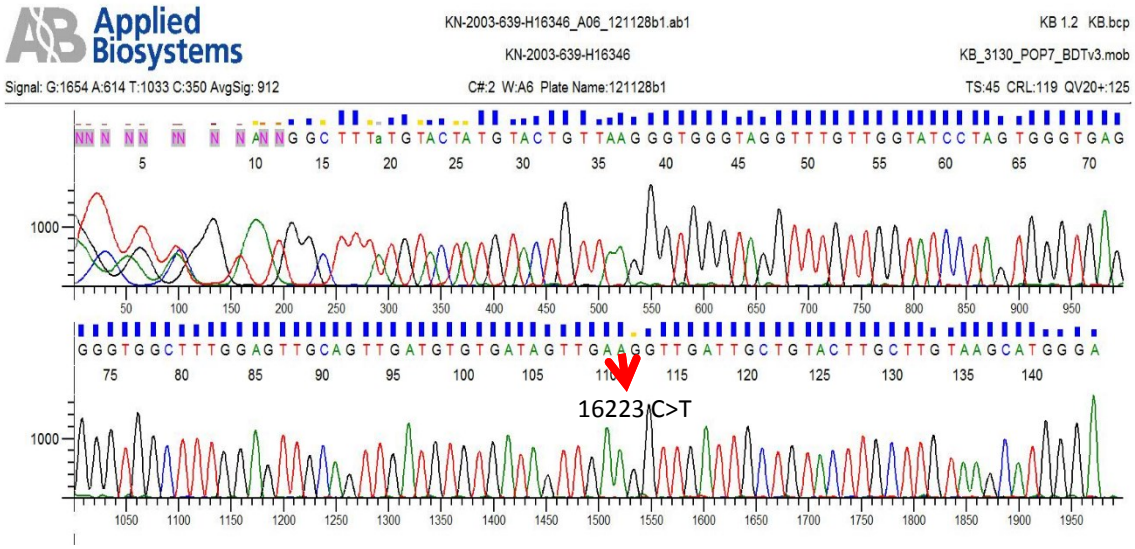


Appendix 4.11. MtDNA sequence electropherogram obtained from the forward (top) and reverse (botton) sequences of Khuzhir-Nuge XIV individual (K14_1997.011), tooth sample ID 2003.639. One base substitution in the reverse sequence at 16223 (C>T), Haplogroup D.

Forward:

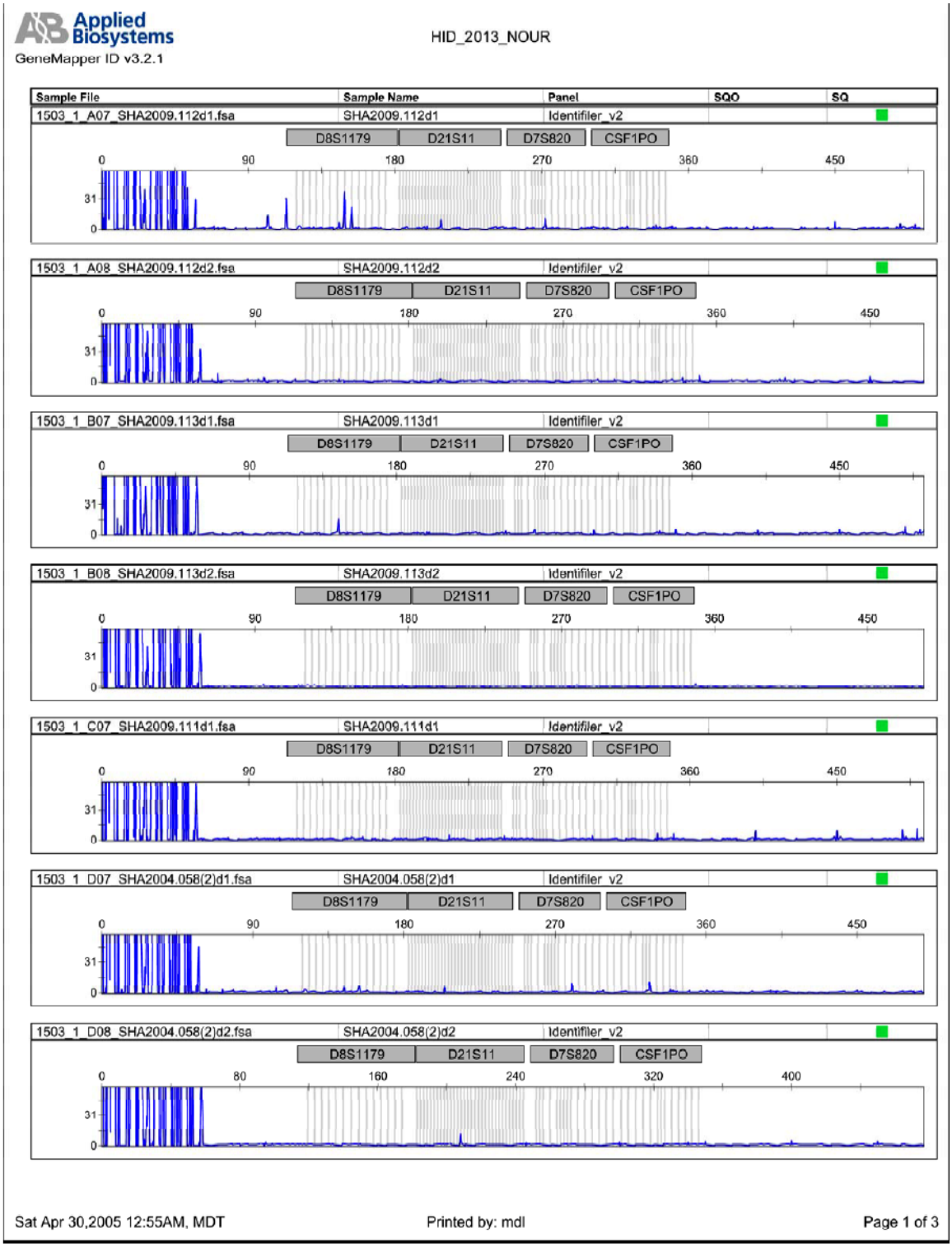


Reverse:



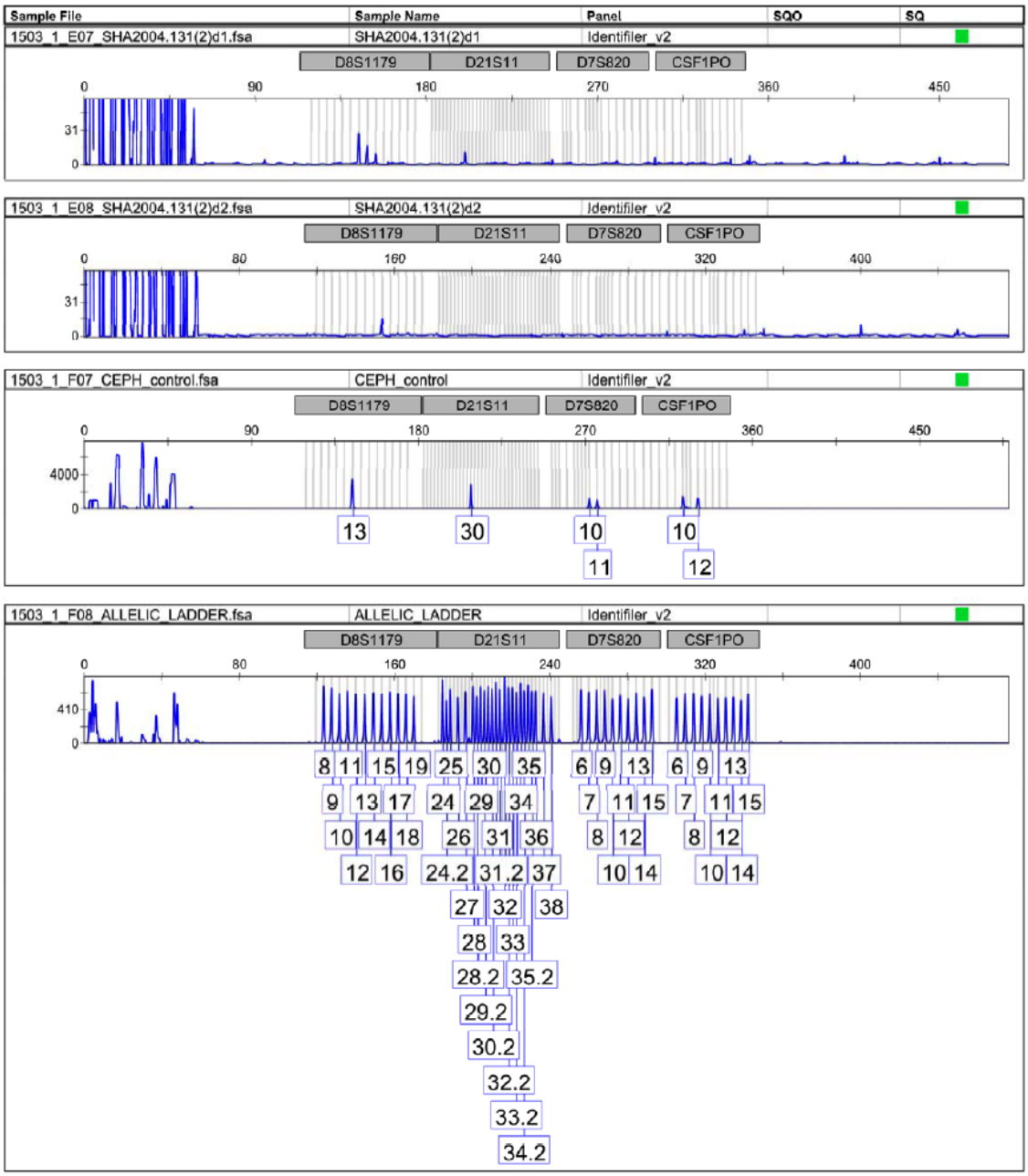
Appendix 4.12. Shamanka II samples and the trial to amplify autosomal STRs using AmpFlSTR® Identifiler® (Applied Biosystems, USA) kit. The trial was unsuccessful and the evidence is attached below represented with the four dyes 6FAM, NED, PET and VIC.

6FAM



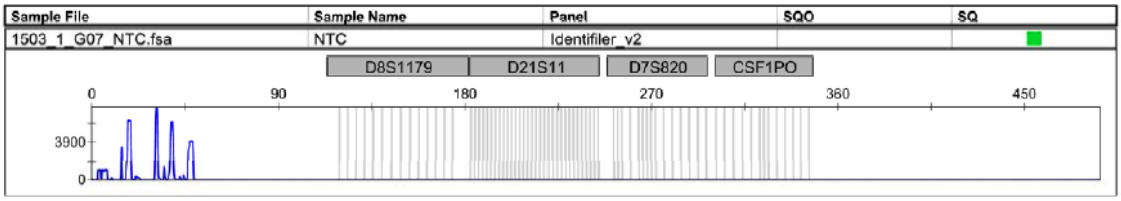
Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR

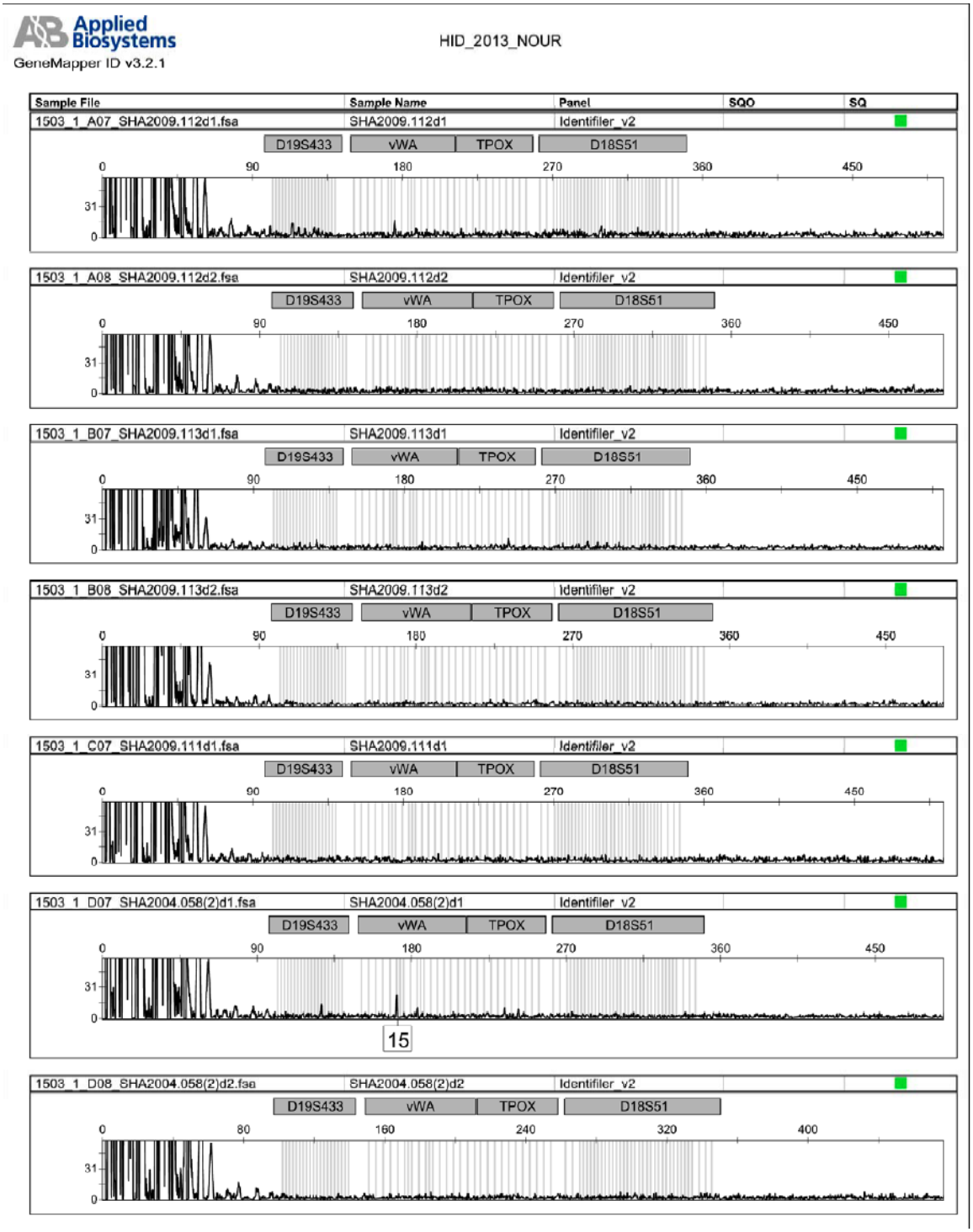


Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR



NED


Applied Biosystems
GeneMapper ID v3.2.1
HID_2013_NOUR
Sample File
Sample Name
Panel
SQO
SQ
1503_1_A07_SHA2009.112d1.fsa
SHA2009.112d1
Identifiler_v2
D19S433
vWA
TPOX
D18S51
1503_1_A08_SHA2009.112d2.fsa
SHA2009.112d2
1503_1_B07_SHA2009.113d1.fsa
SHA2009.113d1
1503_1_B08_SHA2009.113d2.fsa
SHA2009.113d2
1503_1_C07_SHA2009.111d1.fsa
SHA2009.111d1
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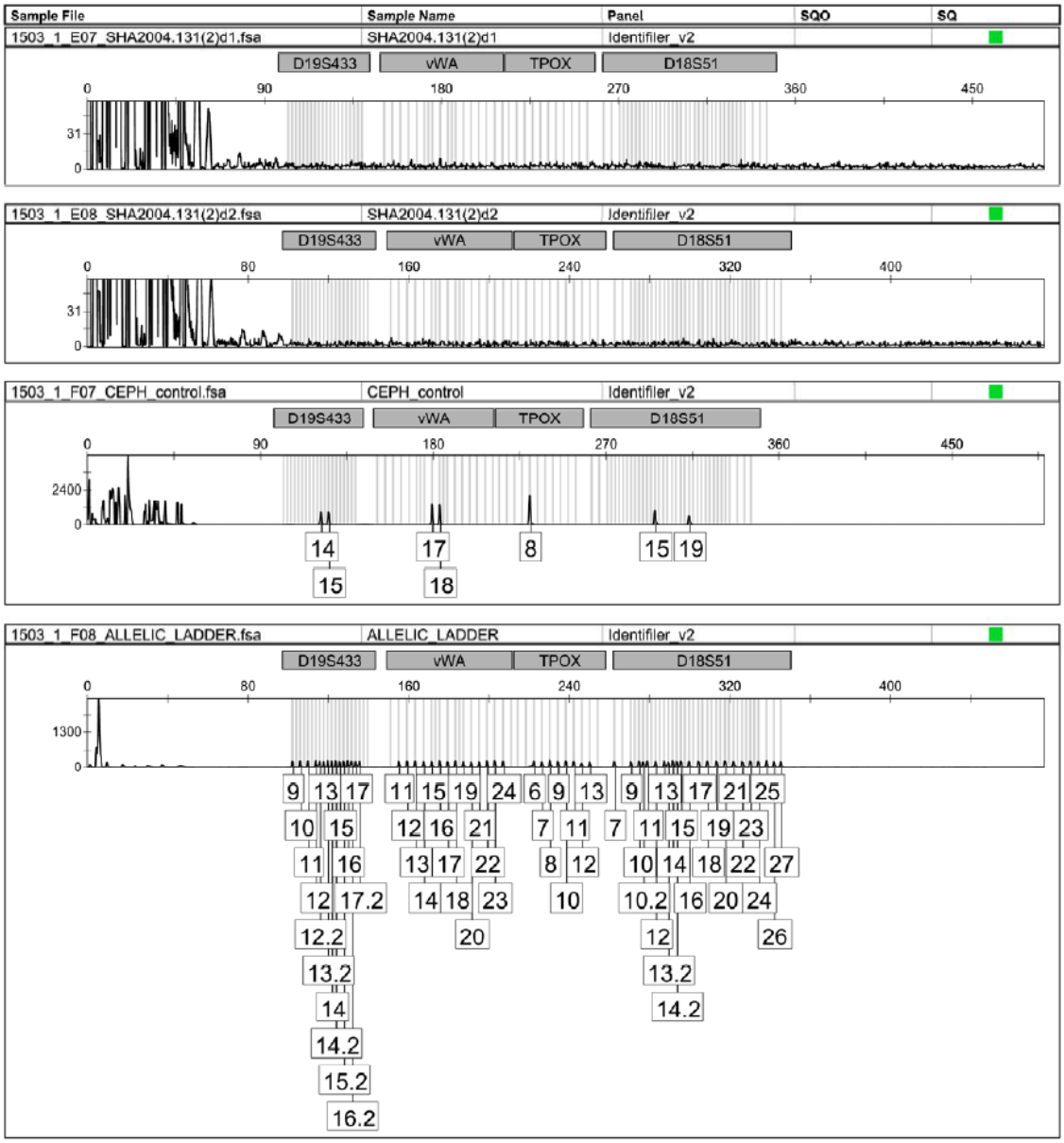
SHA2004.058(2)d1
15
1503_1_D08_SHA2004.058(2)d2.fsa
SHA2004.058(2)d2

Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR



Applied Biosystems
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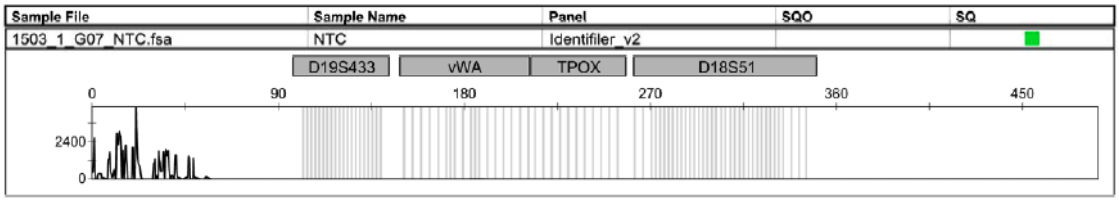
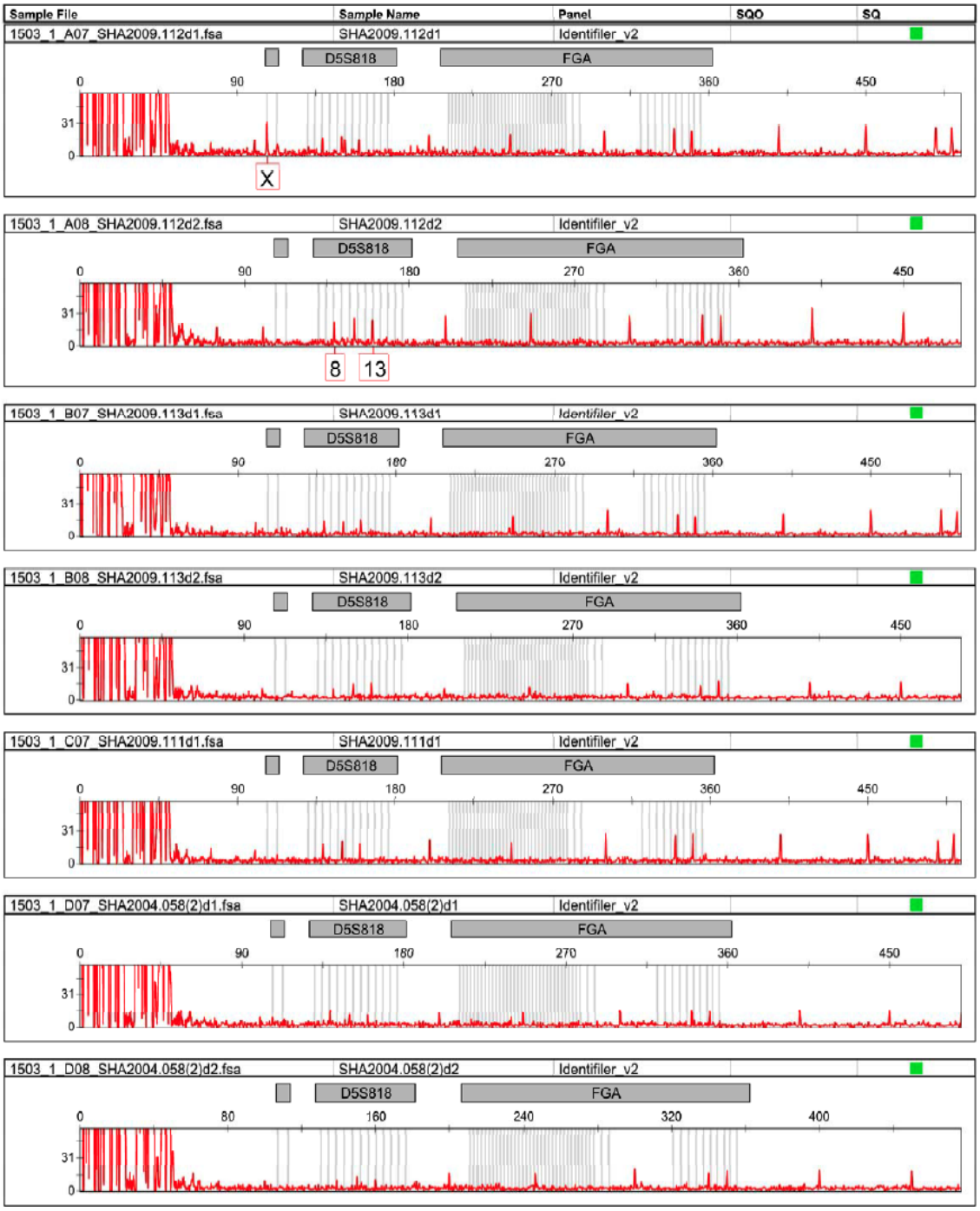


PET

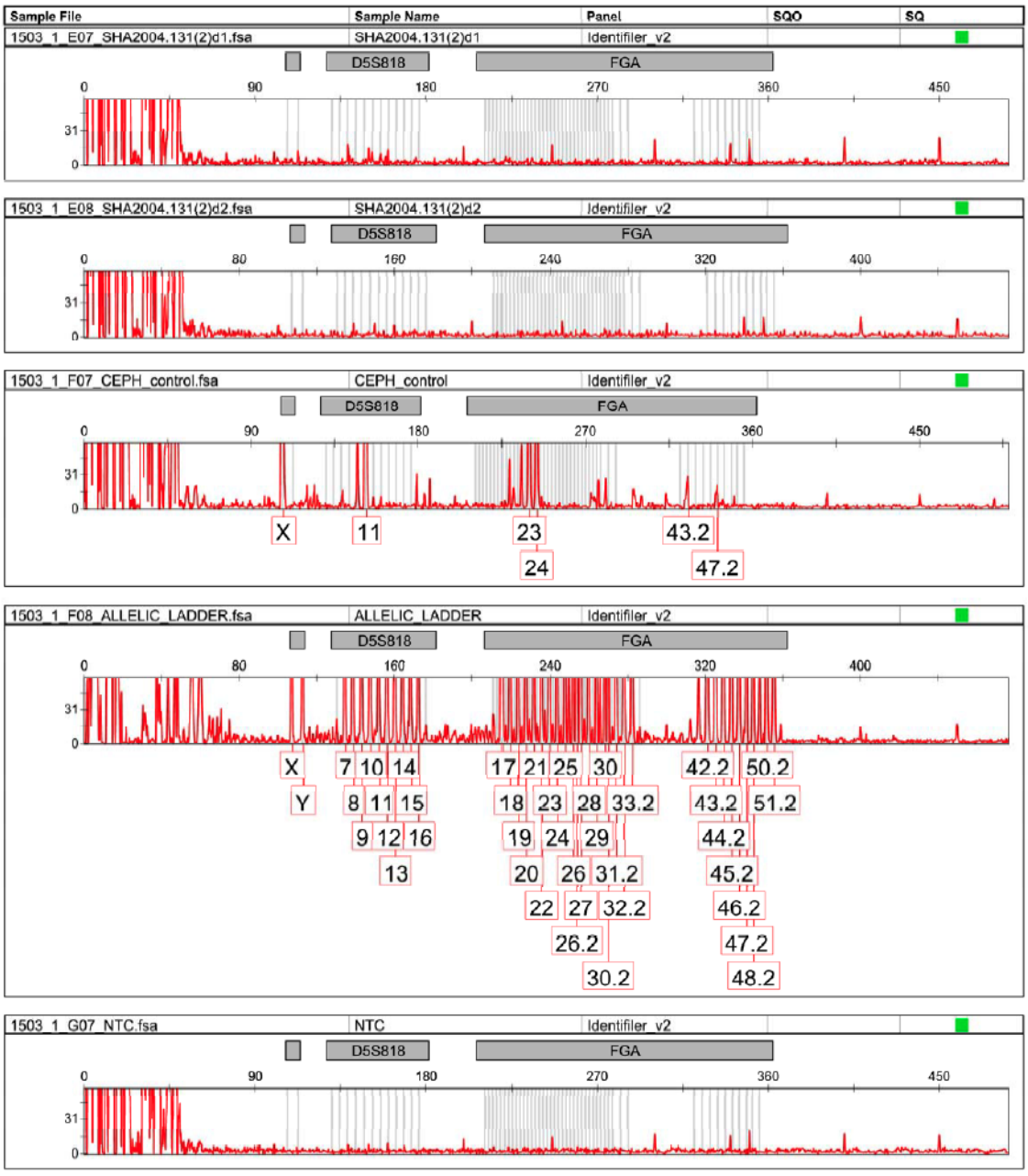
Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR



Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR

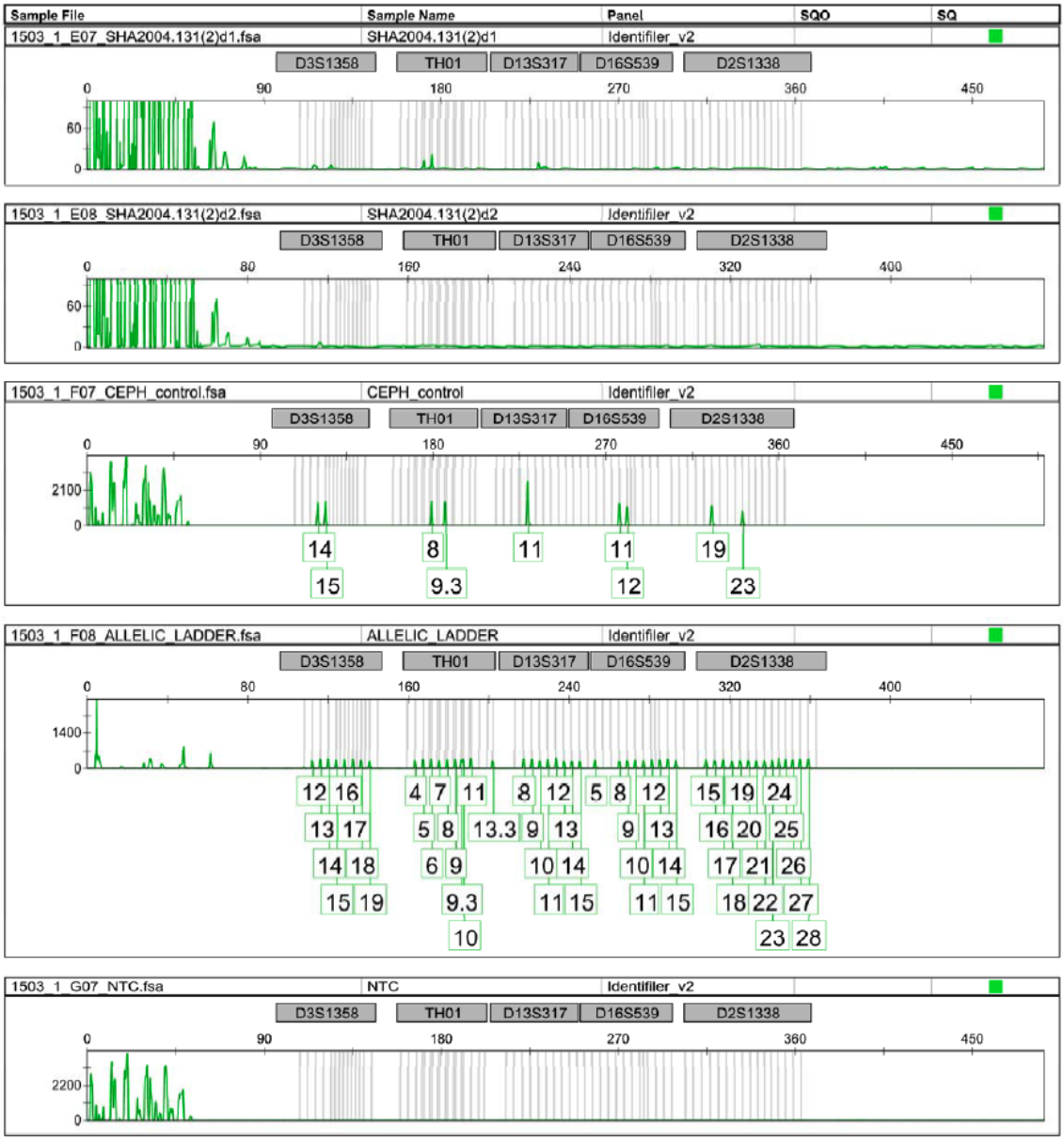


VIC


Applied Biosystems
GeneMapper ID v3.2.1
HID_2013_NOUR
Sample File
Sample Name
Panel
SQO
SQ
1503_1_A07_SHA2009.112d1.fsa
SHA2009.112d1
Identifiler_v2
D3S1358
TH01
D13S317
D16S539
D2S1338
1503_1_A08_SHA2009.112d2.fsa
SHA2009.112d2
1503_1_B07_SHA2009.113d1.fsa
SHA2009.113d1
1503_1_B08_SHA2009.113d2.fsa
SHA2009.113d2
1503_1_C07_SHA2009.111d1.fsa
SHA2009.111d1
1503_1_D07_SHA2004.058(2)d1.fsa
SHA2004.058(2)d1
1503_1_D08_SHA2004.058(2)d2.fsa
SHA2004.058(2)d2

Applied Biosystems
GeneMapper ID v3.2.1

HID_2013_NOUR



Appendices for Chapter 5

Appendix 5.1. Summary of the genetic work conducted on all the cemeteries from Lake Baikal (Lokomotiv, Shamanka II, Ust'-Ida and Kurma XI and one individual from Khuzhir-Nuge XIV) including amelogenin results, mtDNA and Y-chromosomal haplogroups.

Cemetery site	Period	Morphological sex (Weber et al. 2011)	Molecular sex	mtDNA haplogroups	Y-Chr haplogroups
LOK_1980.004	EN	Female	XY [(a)]	D [(a)]	3 attempts to extract DNA failed
LOK_1980.006	EN	Male	XY [(a)]	F [(a)]	R1a1
LOK_1980.010.02	EN	Male	XY [(a)]	Other [(a)]	K*
LOK_1980.010.03	EN	Female	XY [(a)]	F [(a)]	Not Assigned
LOK_1980.012	EN	Female	XY [(a)]	A [(a)]	2 attempts to extract DNA failed
LOK_1981.013	EN	Male	XY [(a)]	D [(a)]	Not Assigned
LOK_1980.014.03	EN	Undetermined	XY [(a)]	U5a [(a)]	2 attempts to extract DNA failed
LOK_1980.016	EN	Male	XY [(a)]	D [(a)]	K*
LOK_1980.017	EN	Male	XY [(a)]	A [(a)]	2 attempts to extract DNA failed
LOK_1980.022.02	EN	Male	XY [(a)]	C [(a)]	K
LOK_1981.024.01	EN	Undetermined	XY [(a)]	F [(a)]	R1a1
LOK_1984.027	EN	Male	XY [(a)]	D [(a)]	4 attempts to extract DNA failed
LOK_1985.031.02	EN	Male	XY [(a)]	A [(a)]	C3
LOK_1988.038.01	EN	Female	XY [(a)]	F [(a)]	2 attempts to extract DNA failed
LOK_1990.042	EN	Male	XY [(a)]	G2a [(a)]	K
LOK_1990.044.01	EN	Male	XY [(a)]	F [(a)]	2 attempts to extract DNA failed
LOK_1990.044.02	EN	Male	XY [(a)]	F [(a)]	2 attempts to extract DNA failed
LOK_1980.014.05	EN	NA	XY [(a)]	F [(a)]	Didn't have any samples from this individual
LOK_1980.002.01	EN	Female	m.d. [(a)]	F [(a)]	
LOK_1980.002.03	EN	NA	m.d. [(a)]	F [(a)]	
LOK_1980.015	EN	Male	m.d. [(a)]	D [(a)]	
LOK_1980.018	EN	Female	m.d. [(a)]	F [(a)]	
LOK_1981.024.02	EN	Male	m.d. [(a)]	F [(a)]	
LOK_1984.028	EN	Female	m.d. [(a)]	U5a [(a)]	
LOK_1984.029	EN	Probable Female	m.d. [(a)]	F [(a)]	
LOK_1985.030.01	EN	NA	m.d. [(a)]	F [(a)]	
LOK_1985.036	EN	Female	XX [(a)]	F [(a)]	
LOK_1986.037	EN	Female	XX [(a)]	F [(a)]	
LOK_1988.038.02	EN	Female	m.d. [(a)]	A [(a)]	
LOK_1988.039	EN	Female	XX [(a)]	D [(a)]	

Cemetery site	Period	Morphological sex (Weber et al. 2011)	Molecular sex	mtDNA haplogroups	Y-Chr haplogroups
SHA_2001.012	EN	Undetermined	XY [b]	D	K*
SHA_2001.013.03	EN	Probable Female	XY [b]	D [b]	K*
SHA_2002.021.02	EN	Male	XY [b]	G2a	K*
SHA_2002.021.03	EN	Undetermined	XY [b]	A	K*
SHA_2002.023.04	EN	Undetermined	XY [b]	D	K*
SHA_2004.052.01	EN	Probable Male	XY	D	K
SHA_2004.044.02	EN	Undetermined	XY	C	K
SHA_2005.059.01	EN	Male	XY	C	K
SHA_2006.083.01	EN	Male	XY	C	K
SHA_2002.023.05	EN	Undetermined	XY [b]	D [b]	Didn't have any samples from this individual
SHA_2001.011.02	EN	Male	m.d. [b]	D [b]	
SHA_2001.011.01	EN	Female	m.d. [b]	C [b]	
SHA_2002.008	EN	NA	m.d. [b]	A [b]	
SHA_2001.014.01	EN	Male	m.d. [b]	D [b]	
SHA_2001.014.02	EN	Female	m.d. [b]	F [b]	
SHA_2001.019	EN	Male	m.d. [b]	D [b]	
SHA_2001.018	EN	Male	m.d. [b]	C [b]	
SHA_2001.016	EN	Undetermined	XX [b]	U5a [b]	
SHA_1999.007	EN	Probable Female	Undetermined [b]	D [b]	
SHA_2001.015	EN	Male	m.d. [b]	A [b]	
SHA_2002.023.01	EN	Probable Male	Undetermined [b]	F [b]	
SHA_2002.024.01	EN	Male	m.d. [b]	D [b]	
SHA_2002.022	EN	Male	m.d. [b]	F [b]	
SHA_2002.021.01	EN	Male	m.d. [b]	G2a [b]	
SHA_2002.024.02	EN	Undetermined	m.d. [b]	A [b]	
SHA_2007.090	EN	Male	XX	A	
SHA_2007.096.02	EN	Female	XX	D	
SHA_2008.103.01	EBA	NA	XX	G2a	
UID_1987.005	LN	Undetermined	XY [a]	Other [a]	Other* (L914 SNP)
UID_1987.009	LN	Undetermined	XY [a]	A [a]	Q1a3*
UID_1987.012	LN	Male	XY [a]	D [a]	Q1a3
UID_1988.016.01	LN	Male	XY [a]	F [a]	Q1a3
UID_1988.016.02	LN	Male	XY [a]	Other [a]	Q1a3
UID_1989.020.01	LN	Male	XY [a]	C [a]	Q1a3
UID_1989.026.01	LN	Undetermined	XY [a]	A [a]	Other* (L914 SNP)
UID_1989.029	EBA	Male	XY [a]	A [a]	Q1a3

Cemetery site	Period	Morphological sex (Weber et al. 2011)	Molecular sex	mtDNA haplogroups	Y-Chr haplogroups
UID_1990.033.01	LN	Male	XY [(a)]	Other [(a)]	Q1a3
UID_1991.038	LN	Male	XY [(a)]	C [(a)]	Q1a3*
UID_1993.043	LN	Male	XY [(a)]	G2a [(a)]	K*
UID_1993.044.03	LN	Undetermined	XY [(a)]	A [(a)]	Q1a3
UID_1994.048	EBA	Male	XY [(a)]	C [(a)]	Q1a3*
UID_1994.053.02	LN	Undetermined	XY [(a)]	A [(a)]	2 attempts to extract DNA failed
UID_1994.055.02	LN	Male	XY [(a)]	G2a [(a)]	Q
UID_1994.055.01	LN	Undetermined	XY [(a)]	C [(a)]	Didn't have any samples from this individual
UID_1987.011	LN	Female	m.d. [(a)]	G2a [(a)]	
UID_1988.014	LN	Male	m.d. [(a)]	F [(a)]	
UID_1988.018	LN	Undetermined	XX [(a)]	U5a [(a)]	
UID_1989.019	EBA	Male	m.d. [(a)]	C [(a)]	
UID_1989.020.02	LN	Female	m.d. [(a)]	Other [(a)]	
UID_1989.022	LN	Female	XX [(a)]	Other [(a)]	
UID_1989.024	EBA	NA	XX [(a)]	C [(a)]	
UID_1989.026.04	LN	Undetermined	XY [(a)]	A [(a)]	Didn't have any samples from this individual
UID_1989.026.05	LN	Undetermined	XX [(a)]	F [(a)]	
UID_1989.030	LN	Female	XY [(a)]	A [(a)]	3 attempts to extract DNA failed
UID_1989.031	LN	Undetermined	XY [(a)]	Other [(a)]	Didn't have any samples from this individual
UID_1990.033.02	LN	Undetermined	m.d. [(a)]	Other [(a)]	
UID_1991.036.01	LN	Undetermined	XX [(a)]	D [(a)]	
UID_1991.040.01	EBA	Female	XX [(a)]	C [(a)]	
UID_1991.041	LN	NA	m.d. [(a)]	G2a [(a)]	
UID_1991.042	EBA	Female	XY [(a)]	A [(a)]	Didn't have any samples from this individual
UID_1993.044.01	LN	Undetermined	XX [(a)]	A [(a)]	
UID_1993.044.02	EBA	Undetermined	XX [(a)]	A [(a)]	
UID_1993.045	EBA	Male	m.d. [(a)]	C [(a)]	
UID_1994.047	EBA	Male	m.d. [(a)]	C [(a)]	
UID_1994.051	EBA	Male	m.d. [(a)]	C [(a)]	
UID_1994.052	LN	Female	m.d. [(a)]	Other [(a)]	
UID_1994.054	LN	Male	m.d. [(a)]	C [(a)]	
KUR_2002.001	EBA	Male	XY	D	2 attempts to analyze the Y-chr failed

Cemetery site	Period	Morphological sex (Weber et al. 2011)	Molecular sex	mtDNA haplogroups	Y-Chr haplogroups
KUR_2002.007.01	EBA	Undetermined	XX	D	
KUR_2002.010	EBA	Probable Male	m.d.	Z	Q1a3
KUR_2002.013	EBA	Male	m.d.	A	
KUR_2002.014	EBA	Female	XX	F	
KUR_2002.015	EBA	Probable Male	XY	Z	Q1a3
KUR_2002.016	EBA	Probable Female	XX	F	
KUR_2003.017	EBA	Probable Male	XY	D	Q
KUR_2003.018	EBA	Probable Female	XY	D	Q
KUR_2003.019	EBA	Probable Male	XY	F	2 attempts to analyze the Y-chr failed
KUR_2003.021	EN	m.d.	m.d.	m.d.	3 attempts to extract DNA failed
KUR_2003.022	EN	Probable Female	XX	F	
KUR_2003.024	EN	Probable Female	XX	F	
KUR_2003.026	EBA	Probable Male	m.d.	D	
KUR_2003.027	EN	m.d.	m.d.	Inconsistent results	3 attempts gave inconsistent results
K14_1997.011	EBA	Male	m.d.	D	

- m.d.: missing data, no obtainable results
- (a) samples analyzed by Mooder (2004) (Mooder,K.P. 2004).
- (b) samples analyzed by Thomson (2006) (Thomson,T. 2006).
- All Y-chromosomal work is part of this study.

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