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MATERNAL RELATEDNESS WITHIN DOUBLE BURIALS OF AN ANCIENT ALBANIAN TUMULUS

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MATERNAL RELATEDNESS WITHIN DOUBLE BURIALS OF AN ANCIENT ALBANIAN TUMULUS

By

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ABSTRACT

MATERNAL RELATEDNESS WITHIN DOUBLE BURIALS OF AN ANCIENT ALBANIAN TUMULUS

By

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A series of double burials from a tumulus near Kamenica, Albania were analyzed using mitochondrial DNA, with a goal of determining if individuals within a burial might be maternally related, and the extent of relatedness among burials. Bone samples, primarily segments of long bones, petrous portions and teeth, were collected, and DNA extracted. Segments of hypervariable regions 1 and 2 were sequenced and analyzed using a novel approach that looked for both sequence similarity and shared polymorphic sites. The data suggested seven of the eight double burials examined have the possibility of being maternally related. Because the double burials studied were distributed temporally, a patrilocal culture was indicated.

To my family and friends, for their unwavering support of all my pursuits

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Collaborative research efforts between scientists of different backgrounds can lead to the answering of questions unanswerable by either field alone. Changing world politics also offers new opportunities to investigate scientific questions. The Tumulus at Kamenica Archaeological Project, the excavation of the largest tumulus (burial mound) yet discovered in Albania, is an international, multidisciplinary investigation, taking advantage of collective expertise in the fields of archaeology, anthropology, and molecular biology. In particular, the synthesis of molecular biological techniques with archaeological and physical anthropological research has proven valuable in the study of the prehistoric Kamenica people.

Albania, the area of study, is located just north of Greece with its western border touching the Adriatic Sea. In 1991 Communism fell, leading to reform that included expansion of Albania's archaeological research agenda. Western scientists were invited to join this unique opportunity to unearth Albania's rich history, one that is much less understood than its surrounding countries. It is hoped that collaborative efforts among these scientists can create a better picture of the prehistoric villagers and shed light on who may be the ancestors of current Albanians.

The Tumulus at Kamenica

The site of Kamenica is a Late Bronze Age, Early Iron Age tumulus located in eastern Albania just outside the village of Kamenica in the southeastern corner of the fertile Korçë basin. The site was first identified in the early 1990s. Excavations began at the tumulus in 2000 as a rescue effort to preserve the burials after the site was badly

damaged by looters from 1997 to 1999. The Albanian Rescue Archaeology Unit developed a rescue and research project in collaboration with the Museum of Korçë and the Albanian Institute of Archaeology. Under the direction of Dr. Lorenc Bejko and Maria Grazia Amore, there were three seasons of excavation from 2000 – 2002, which unearthed approximately 400 human skeletons.

Bronze Age Tumuli of Southeastern Albania

Bejko (2000) published a comprehensive report examining mortuary customs from the southeastern portion of Albania during the Late Bronze Age. In an earlier publication he reported that cemeteries during this time quite often took the tumulus form (Bejko, 1994). The tumulus burial style was introduced to the Balkan region in the Early Bronze Age by pastoral peoples migrating from the Russian Steppes (Bejko, 2000). The author investigated 5 tumuli that ranged in size from approximately 2 – 6 m high with a 15 – 50 m diameter. The number of graves within a particular tumulus ranged from 18 to almost 300. Bejko found that tumuli commonly display a suite of archaeological features including a mound of soil and rocks, as well as a central grave placed within a large circle of rocks. This "Big Circle" is generally regarded as the first phase in construction of a tumulus. Because the center burial is thought to initiate the tumulus, it is hypothesized that this person was revered within the society. It is further hypothesized that other individuals from the same family were buried surrounding this individual, creating a family plot. According to Bejko (1994), single inhumations are the most common form of tumulus burial, although several other alternative burial treatments are observed, some of which include cremation and multiple individuals.

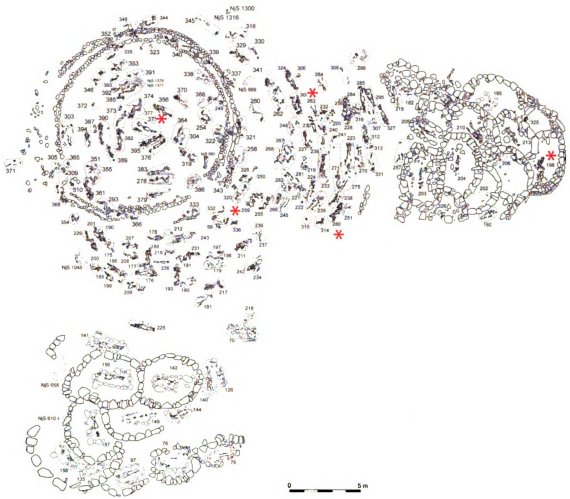
Description of the Tumulus at Kamenica

The Tumulus at Kamenica was utilized as a cemetery from approximately the 12th to 6th century B.C. Kamenica displays several of the classic tumulus features, yet it also is unusual in several ways. Figures 1 and 2 each illustrate a layer of the excavated portion of the tumulus. To begin, Kamenica exhibits a central individual surrounded by other burials encompassed within a large rock circle ("The Big Circle"), as shown in the upper left corner of Figure 1. Unlike the rock circle in most tumuli, this archaeological feature has a double ring of rocks, not a single ring.

The Tumulus is also unusual in its overall size, as well as its three-phase construction. Kamenica is the largest tumulus found in the region, spanning over 2000 m^2. In terms of the number of graves, Kamenica is the largest, with an estimated 800 total burials. A change in mortuary treatment is seen within the different phases of the tumulus. The Big Circle, considered the first phase, measured 13 m in diameter and contained the oldest set of burials. This portion of the tumulus dates to about 1200 to 1150 B.C., and included 35 soil graves. One adult burial was identified as the first in the tumulus, which skeletal analysis determined to be male. Phase 2 continued with soil burials outside of the double ring of rocks. This phase dates to about 1150 to 650 B.C., and had some 200 graves. Beyond the Big Circle and its outlying burials lie two areas termed Monumental Structures 1.1 (Figure 1 – upper right corner) and 3 (lower left corner), which comprise phase 3. Burials within the monumental structures were formed using rock walls, approximately 1 m high, and were filled with smaller rock, all presumably carried from some distance away. Overall, the main style of burial displayed was inhumation, with some cremations recovered. Although most inhumations were of

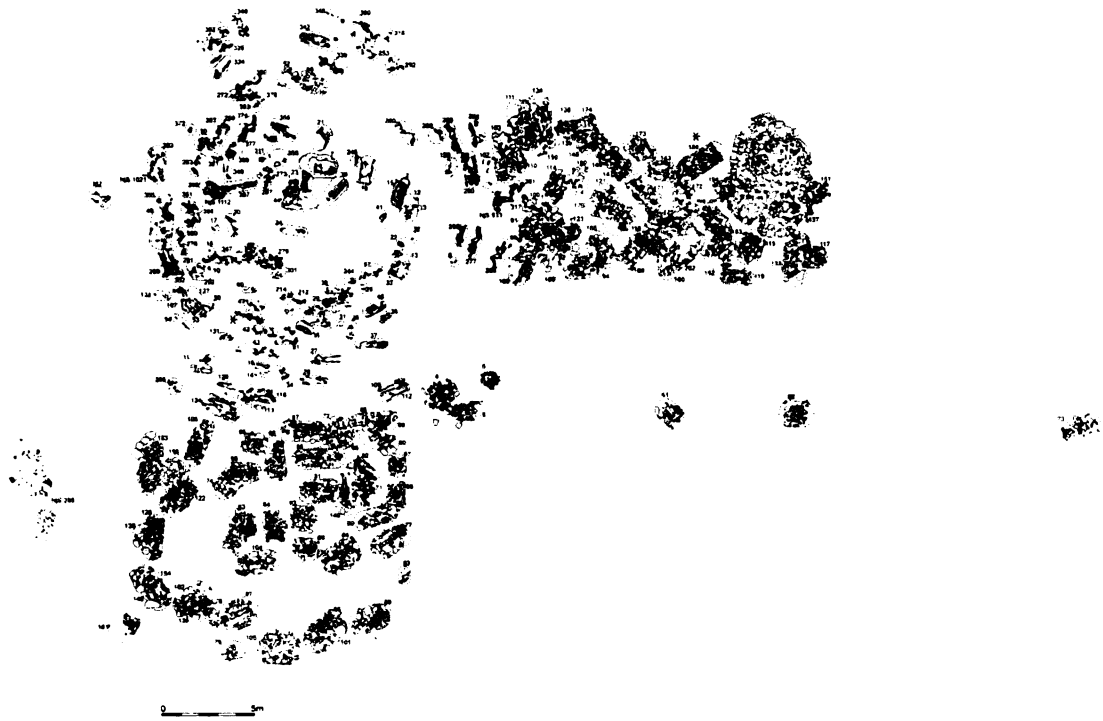
single individuals, there were numerous cases of "double burials" consisting of two individuals (Bejko and Amore, 2001).

Figure 1. Kamenica Tumulus: 12th – 6th Century B.C. Burials



The map depicts the layer of burials ranging from the 12th century B.C. within the Big Circle (upper left corner) to the 6th century B.C. within both Monumental Structures (upper right and lower left corners). Burials outside of the Big Circle are estimated to have been used between 11th and 8th century B.C. Double burials investigated during this research are marked with an asterisk.

Figure 2. Kamenica Tumulus: 11th – 6th Century B.C. Burials



The map depicts the upper layer of the tumulus. Burials above the Big Circle range from the 11th to 7th century B.C. while those above both Monumental Structures date between the 6th and 7th century B.C. Double burials investigated during this research are marked with an asterisk.

Genetic Analyses

Analysis of mitochondrial DNA (mtDNA) was used to investigate the genetic relatedness of the tumuli on a spatial and temporal level. Eight double burials were chosen from the tumulus to examine maternal relatedness within and among the burials, which would supply information regarding societal questions about the villagers that once lived in this area. Figure 1 depicts (with asterisks) five of the eight double burials, one within the Big Circle, three just outside of it, and one within Monumental Structure 1.1. Three double burials were chosen from those depicted in Figure 2, two above the

burials just outside of the Big Circle and one above Monumental Structure 1.1. Table 1 lists each burial's location, type, and estimated period of interment. Appendices 1 and 2 provide greater detail.

Burial Number	Style and Spatial Location	Century B.C.
46	Soil; out of BC	10
56	Soil; out of BC	9-8
184	Rocks, BAMS 1.1	6
198	Soil; MS 1.1	7
259	Soil	10
280	Soil	9-8
300	Soil	11
375	Soil, BC	12

Table 1. Double Burials

The table provides a list of sampled double burials with their burial composition and spatial location as well as the estimated period of interment. Abbreviations are BC = Big Circle, BAMS 1.1 = Burials Above Monumental Structure 1.1, MS 1.1 = Monumental Structure 1.1.

Ancient DNA

Several authors have addressed the extreme care that must be taken when working with ancient human DNA (Holland and Parsons, 1999; O'Rourke *et al.*, 2000). The procedures used to extract and amplify ancient DNA are the same used on present-day samples, therefore if human DNA were to contaminate the ancient sample, it is possible to generate results for the wrong nucleic acid. To avoid such issues, a number of precautions can be observed. Physical separation of the laboratory used for DNA isolation will help avoid contamination from the abundant DNA present after amplification. Reagent blanks and negative controls are used during all procedures to ensure reagents or DNAs have not become contaminated with foreign DNA. Lastly, experiments should be replicated, from the same bone analyzed multiple times, and/or by analyzing multiple bones from the same individual.

Many scientists have found ancient mtDNA isolation and analysis to be feasible. The first to successfully clone ancient mtDNA, from extinct *Equus quagga*, were Higuchi *et al.* (1984). Pääbo *et al.* (1985) followed by cloning nuclear DNA from 2400 year-old Egyptian mummy skin (Hagelberg and Clegg, 1991; O'Rourke *et al.*, 2000). In 1991 Hagelberg and Clegg were able to extract and amplify human tibia mtDNA from a site that carbon dated to 750 +/- 80 years before present. In 2002 a suspected Neandertal femur was found in France (Beauval *et al.*, 2005). The subsequent carbon dating placed its age at approximately 40,700 years before present while mtDNA analysis indicated the bone was indeed from a Neandertal.

DNA Preservation in Bone

There are several factors that can affect how well DNA is preserved in bone. During the initial phases of burial anaerobic bacteria, which have been shown to demineralize bone, break down soft tissues thus affecting the DNA within (Geigl, 2002). Soil conditions such as warm temperature, high moisture, and acidic pH are known to increase the rate of DNA degradation in bone (Parsons and Weedn, 1996; Smith *et al.*, 2003). High temperature and moisture content are also important for chemical processes that may adversely affect DNA, and increase microbial activity (Smith *et al.*, 2003). In agreement with Smith *et al.* (2003), Gotherstrom (2002) found that modern bones, which were pulverized and incubated with water, were less likely to yield DNA than those incubated under dry conditions. The damage incurred is very dependent upon the burial location and whether environmental conditions are such that these chemical and biological processes can occur (Smith *et al.*, 2003).

There are many types of degradative processes that can affect DNA. One of the most common is a hydrolytic process called deamination, which can alter pyrimidine nucleotides of the target molecule and result in the misincorporation of new nucleotides during PCR (Hofreiter *et al.*, 2001; Thomas *et al.*, 2003). It has been found that certain areas, or "hotspots", are more susceptible to damage than other areas of DNA (Thomas *et al.*, 2003). Tests performed on modern DNA suggest hotspots for degradation are generally located in non-coding regions and rapidly undergo mutation (Thomas *et al.*, 2003). Because the regions studied in the current study are non-coding, there is a possibility of difficulty. A problem may arise through mutation within the primer binding region causing amplification success to decline. Another issue is that only certain DNA molecules may have undergone mutation, resulting in differing sequences or haplotypes existing within a sample. DNA degradation can result in the acquirement of limited sequence information, making genetic-based conclusions difficult to reach. Much more study needs to be done with regard to the mechanics of ancient DNA degradation and until more is known, researchers must interpret data with caution.

Double Burials

Shown below are pictures of each double burial with descriptions of the location within the tumulus, the time to which the burial dates, the sex of the individuals within, and any archeological notes. Each individual within a burial has it's own number, designated by the Albanian archeologists as Nj.s.

Figure 3. Burial 46

A soil burial located outside of the Big Circle that dates to 10th century B.C. Individual one (Nj.s 233) is the skeleton of an adult male while individual two (Nj.s 234) is that of an adult female.

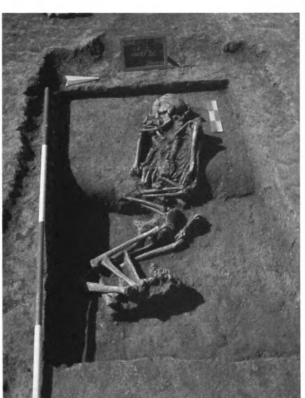


Figure 4. Burial 56

A soil burial located outside of the Big Circle that dates between the 8th and 9th century B.C. Individual one (Nj.s. 262) is the skeleton of a middle-aged adult, presumed male, while individual two (Nj.s. 263) is that of an adult female. The two skeletons appeared to have been placed holding one another, as their bones are intertwined.

Figure 5. Burial 184

A rock burial located above Monumental Structure 1.1 that is the youngest of the double burials dating back to 6th century B.C. Individual one (Nj.s. 710) is the skeleton of an adult male, while individual two (Nj.s. 711) is that of an adult female. The unusual body positioning, which cannot be easily viewed in the photograph, was head-to-toe.

Figure 6. Burial 198

A rock burial located within Monumental Structure 1.1 that dates to 7th century B.C. Individual one (Nj.s. 768) is the skeleton of an adult female, while individual two (Nj.s. 772) is that of a 3-5 year-old child. The adult female was buried with her hand cupping the child's head.

Figure 7. Burial 259

A soil burial located outside of the Big Circle that dates to 10th century B.C. Individual one (Nj.s. 1001) and individual two (Nj.s. 1002) are the skeletons of adult females.

Figure 8. Burial 280

A soil burial located outside of the Big Circle that dates between the 8th and 9th century B.C. Individual one (Nj.s. 1084) is the skeleton of an adult female, while individual two (Nj.s. 1085) is that of an adult male. The female skeleton was interred on top of the male skeleton.

Figure 9. Burial 300

A soil burial located outside of the Big Circle that dates to 11th century B.C. Indivdual one (Nj.s. 1164) is the skeleton of a 15 – 17 year-old male, while individual two (Nj.s. 1165) is that of a 14 – 16 year-old female.

Figure 10. Burial 375

A soil burial located within the Big Circle that is the oldest double burial dating to 12th century B.C. Both individual one (Nj.s. 1456) and individual two (Nj.s. 1457) are skeletons of adult males. One was an extended burial and the other a bundle burial, or a collection of an individual's bones.

MATERIALS AND METHODS

Sample Collection in Albania

Each skeleton had been collected and stored in a wooden box (with lid) and labeled with its burial number by an archeological team led by Dr. Lorenc Bejko and Professor Skender Aliu of the Albanian Institute of Archeology (Tiranë, Albania). Specific burials were chosen for sampling through collaborative effort by Dr. Bejko and Dr. Todd Fenton (Michigan State University, Dept. of Anthropology) based on estimation of the information mtDNA analysis could contribute to the Kamenica project. Skeletons were sampled first by bone type (preferably long bone, petrous portion of the temporal bone, or tooth) and then by integrity (see Appendix 3). Integrity of the bone samples was rated using a 0 through 5 numbering system based on their completeness. Teeth were rated in a similar fashion, but with some description adjustment made within the scale to represent their unique endurance. Rating systems were as follows:

Bones (excluding teeth)

0 – bone is complete; surface of bone has a sheen

1 – proximal/distal ends beginning to break; sheen may be present

2 – 3/4 to 2/3 bone present

3 – 1/2 to 1/4 bone present

4 – small pieces (1 – 4") of identifiable bone

5 – tiny (less than ½"), unidentifiable pieces of bone

Teeth

0 – no erosion visible

1 – little erosion

2 – moderate erosion

3 – great erosion, but crown still attached to root

4 – crown and root attached, but one or both is/are broken

5 – only crown or root present

Broken pieces of long bone were collected if available, or a cutting tool (either hacksaw or Dremel Rotary Tool) was used to cut wedges, generally from the mid-shaft region. Whole petrous portions and teeth (molars when available), which were almost always found loose, were chosen to further represent the skeleton. For labeling purposes, the first or the first few letters of the bone type were written on its surface using a Sharpie marker (Table 2). The bones were placed in plastic screw top containers labeled with burial number, skeleton number, and the tumulus area from which they were taken.

Table 2. Code used during sample collection

Code	Bone
A	Arm
C	Clavicle
Calc	Calcaneus
Car	Carpal
F	Femur
H	Humerus
M	Metatarsal
Man	Mandible
MC	Metacarpal
P	Petrous
PM	Pre - Molar
R	Radius
Rib	Rib
Scap	Scapula
S-P	Skull - Petrous
SO	Skull - Occipital
T	Tibia

Table 2 (cont'd).

U	Ulna
UM	Upper Molar
Phalanx	Phalanx
Fib	Fibula
V	Vertebrae

The code used to label bone samples upon collection.

DNA Isolation

Bone Preparation – Eight double burials were the focus of this research. Each of the 16 individuals was sampled separately, meaning only those bones associated with that skeleton were processed at any one time. All tools and containers associated with cleaning, sampling, weighing or storage were placed inside a UV illuminator and subjected to short wavelength UV irradiation for 6 minutes (on one or more sides) to destroy any surface DNA. Non-disposable items that could be thus treated were soaked in a 10% bleach solution for approximately 3 minutes. Gloves were worn at all times and were changed regularly to avoid contamination. Surface dirt was removed from bones by scrubbing with a test tube brush and 1x digestion buffer (20 mM Tris, 100 mM EDTA, 0.1% SDS) and rinsing with deionized water. Excess water was shaken off and the bones were moved to a separate drilling room. Drilling took place in an enclosed UV irradiated hood containing a clean sheet of bench paper that had previously undergone UV irradiation for a minimum of 5 minutes. The top layer of bone that had been exposed to soil was removed using the Dremel tool fitted with a sanding wheel. The sanding wheel was blotted with a 10% bleach saturated Chem-wipe to be reused on bones from the same individual.

Generating Bone Dust from Samples – Weigh boats measuring approximately two inches by two inches were labeled with the burial number and the type of bone being sampled. These were subjected to UV light on each side for 6 minutes. The UV hood was wiped down with a 10% bleach solution and equipped with a clean sheet of bench paper, which was then irradiated for a minimum of 5 minutes. A 1/16 inch drill bit fitted to the Dremel tool was used to drill into the bone; the dust was collected in the appropriate weigh boat and the mass was recorded. Five hundred μL of 1X digestion buffer was added to each weigh boat and the bone dust was scraped into a microcentrifuge tube using a metal spatula. To each tube 2 μL of proteinase K (final concentration of 0.4 mg/ml) were added, vortexed briefly and incubated overnight at 56°C. A reagent blank was created using 200 μL of digestion buffer and 1 μL of proteinase K.

DNA Extraction – Digestion reaction tubes were removed from the incubator and centrifuged at 14,000 rpm for approximately 5 minutes to pellet the bone material. The aqueous portion was transferred to a new tube and 500 μL of phenol were added. The tube was vortexed, centrifuged at high speed for 7 minutes, and the aqueous layer removed for a second phenol extraction. A chloroform extraction was performed in the same manner and the aqueous layer was then transferred to a new tube for storage.

Removal of Water Soluble Contaminants – Initially samples which exhibited any color after the phenol-chloroform extractions were further purified using Millipore Microcon Centrifugal Filter Devices-YM30 (adhering to the manufacturer's directions) washing three times with TE buffer (10 mM Tris, 1 mM EDTA). At a point later in the

research, a step became incorporated into the protocol where the aqueous layer was taken directly from the chloroform extraction and processed using a Microcon-YM30.

DNA Precipitation – Fifty μL of 3 M sodium acetate and 900 μL of cold 95% ethanol were added to the aqueous portion, vortexed for approximately 30 seconds and placed in a -20°C freezer for at least 30 minutes to precipitate the DNA. The sample was centrifuged at 5°C and 14,000 rpm for 15 minutes. All liquid was removed from the DNA pellet, which was then washed twice by adding 500 μL of cold 70% ethanol, centrifuging as above and removing all liquid. Pellets were vacuum dried and resuspended with TE according to the original bone dust mass (1 μL /mg).

DNA Amplification

MtDNA was amplified using either an Eppendorf Mastercycler or a Perkin Elmer GeneAmp 2400. Two amplification reactions were set up for each bone. The first reaction tube contained 1 unit HotMaster Taq DNA polymerase (Eppendorf), 0.2 mM each of dNTP (Promega), 2 μM of forward and reverse mtDNA primers (Genosys) (see Table 3), 10X HotMaster Taq buffer (Eppendorf), and 1 μL of sample DNA in a total volume of 20 μL. The second reaction tube received a 1:20 dilution of the DNA template from the first tube. PCR primer pairs forward 15989/reverse 16410, forward 16190/reverse 16410, forward 82/reverse 285, forward 82/reverse 484, and forward 155/reverse 484 (Table 3; http://www.afip.org/Departments/oafme/dna/) were used to amplify a segment of each hypervariable region under the following conditions: 2 minutes at 94°C, 38 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute, followed by 5 minutes at 72°C. A positive control was prepared for each set of

PCR reactions and a reagent blank was amplified alongside. In cases where the quantity of DNA was estimated (using agarose gels) at an insufficient level for sequencing, reamplifications were undertaken in which 1 μL of PCR product was used in place of the DNA template and the number of cycles was reduced to 20.

Table 3. MtDNA primers used for amplification and sequencing

Name	Sequence	Region	Product Size
F82	5'ATAGCATTGCGAGACGCTGG3'	HV2	203 bp
R285	5'GTTATGATGTCTGTGTGGAA3'	HV2	
F155	5'TATTTATCGCACCTACGTTC3'	HV2	329 bp
R484	5'TGAGATTAGTAGTATGGGAG3'	HV2	
F15989	5'CCCAAAGCTAAGATTCTAAT3'	HV1	421 bp
F16190	5'CCCCATGCTTACAAGCAAGT3'	HV1	220 bp
R16410	5'GAGGATGGTGGTCAAGGGGAC3'	HV1	

F=forward, R=reverse, the numbers refer to the position of the 5' base of the primer in the complete human mtDNA sequence (Anderson *et al.*, 1981). The first column lists primer names, followed by the sequence, the hypervariable region of mtDNA it targets, and the size of the amplicon resulting from the paired forward and reverse primers.

PCR Optimization – HV1 amplification did not exhibit problems (total lack of amplified DNA) during these experiments. HV2 amplification, however, was not as successful, requiring the testing of different PCR parameters. Using bone dust from a burial taken from the same tumulus, three variables were investigated: the addition of BSA to the PCR reaction, lowering the concentration of primer, and changes in the primer annealing temperature. The initial set of reactions included 10X BSA (1X = 10mg/mL) and an annealing temperature of 59°C. The second set of reactions contained 10X BSA and 1/5 of the original primer concentration. These were amplified at 60°, 61.5° and 62.5°C. It was determined that both a lower concentration of primer and a

primer annealing temperature of 60°C were optimal. The use of BSA and reduced primer concentration became standard in the protocol and were used throughout the research.

PCR Product Yield – Amplicon quantity was estimated via electrophoresis on a 3.0% agarose gel using 5 μL of PCR product. Products with a band the same size as the positive control were washed three times through a Microcon-YM30 using 300 μL of TE. When both the undiluted sample and the 1/20 dilution amplified, the PCR products were pooled.

Hypervariable Region Sequencing – Ten μL sequencing reactions were set up using 4 μL of Beckman CEQ DTCS Quick Start, 1 μL of primer, enough mtDNA to yield 25–50 fmol in the reaction, and if needed, water to reach 10 μL. The sequencing reaction consisted of: 30 cycles of 20 seconds at 96°C, 20 seconds at 50°C, 4 minutes at 60°C, followed by a hold at 4°C. Two μL of stop solution (0.8 μL of 3 M sodium acetate at pH 5.3, 0.8 μL of 0.5 M EDTA, and 0.4 μL of glycogen) were added and the reaction was vortexed briefly. Precipitation began with the addition of 30 μL cold 95% ethanol, after which the tube was vortexed vigorously for approximately 30 seconds, centrifuged at 14,000 rpm for approximately 7 minutes, and the liquid removed. Centrifuging at 14,000 rpm in between, the pellets were washed twice using 100 μL of cold 70% ethanol, the alcohol was removed, the samples were vacuum dried, and then resuspended in 40 μL of Beckman Coulter sample loading solution. The resuspended DNA was transferred to a 96 well plate, a drop of mineral oil added and the plate was loaded onto the Beckman Coulter CEQ 8000. Sequences were amplified using the LFR-1-60 program (capillary temperature 50°C, denature 120 seconds at 90°C, inject 15 seconds at 2.0kV, and separate 60 minutes at 4.2kV) with a separation time modification of 45 minutes.

DNA Sequence Analysis

MtDNA sequences were aligned to the Cambridge Reference Sequence (CRS; Anderson *et al.*, 1981) using BioEdit version 5.0.6. (Hall, 1997). The following nucleotide designations were used to label areas with more than one peak: Y designated C and T peaks, R (A and G), M (A and C), and W (A and T). All changes from the CRS were recorded and Mitomap (2005) was referenced to determine which polymorphisms had not been previously recorded. Bone types were analyzed to determine what percentage of them produced sequence. If all bones of a particular type gave at least one successful sequence, then that bone type was recorded as producing sequence 100% of the time. When bone condition allowed for resampling, it was performed at least once, yielding multiple sequences per individual. These sequences were compiled for each skeleton and those that were inconsistent with other sequences obtained from the skeleton were not included in further analysis. Sequences that possessed a change from the CRS in only the forward or reverse strand were also not included. Bone types that yielded sequence were then analyzed to investigate whether age of the burial or sex played a part in DNA preservation. Sex was also investigated to determine whether a correlation between it and visual preservation of the bone existed. Maternal relatedness was determined by comparing each skeleton's set of sequences for shared polymorphic sites, which were defined as at least one sequence from an individual exhibiting a change from the CRS (including Y, R, M, and W). Sequences from individuals within a double burial were aligned and both polymorphic sites and polymorphisms were recorded. Double

burials that contained more shared polymorphic sites or polymorphisms than unshared were recorded as possibly being maternally related.

RESULTS

Collection Phase

Overall, sections from 22 bone types, ranging in size from approximately 1/4 to 3 inches, were collected from 193 individuals. Table 4 lists the type and number of each bone collected and the tumulus sector in which the skeletons were found (Appendix 1 and 2 provide greater detail; technical note: images within this thesis are presented in color). The three most common types of bone collected were femora, petrous portions and teeth. Ten burials contained skeletons that had undergone cremation, which was made apparent through both discoloration and brittleness of the bone. Seven of these had reached very intense heat, producing bones that were white in color, while the remainder appeared black. These burials were distributed almost equally between the older sectors (Big Circle) and the more recent sectors (Monumental Structures 1.1 and 3).

Table 4. Bone Sample Collection from Kamenica Tumulus

Bone type	BC	MS 1.1	MS3	BAMS 1.1	BAMS 3	DB	Other	Total
Arm (unidentifiable)	3	0	0	0	0	0	0	**3**
Calcaneus	0	0	1	0	0	0	0	**1**
Carpal	0	0	0	0	0	0	1	**1**
Clavicle	1	2	2	1	0	3	1	**10**
Femur	50	10	13	14	19	25	29	**160**
Fibula	0	0	0	1	0	2	4	**7**
Humerus	31	4	6	6	16	11	18	**92**
Mandible	0	0	0	0	0	6	1	**7**
Maxilla	0	0	0	0	0	1	0	**1**
Metacarpal	0	0	0	0	0	3	2	**5**
Metatarsal	1	0	1	0	0	0	0	**2**
Pelvis	2	2	1	0	0	0	0	**5**
Petrous	38	5	11	11	15	18	29	**127**
Phalanx	1	0	0	0	2	2	3	**8**
Radius	5	1	0	0	2	8	1	**17**

Table 4 (cont'd).

Rib	0	1	0	1	0	0	1	**3**
Scapula	2	0	1	1	2	2	0	**8**
Skull (non-petrous)	9	7	4	5	5	2	8	**40**
Tibia	12	1	5	9	2	7	13	**49**
Tooth	41	8	14	10	14	28	31	**146**
Ulna	1	0	0	0	0	2	3	**6**
Vertebrae	0	0	1	0	0	0	1	**2**
Total	**197**	**41**	**60**	**59**	**77**	**120**	**146**	**700**

Bones types are distributed among the tumulus sectors from which they originated (labeled by archeologists) or if they belong to a double burial. BC = Big Circle; MS = Monumental Structure; BAMS = Burials above Monumental Structure; DB = Double Burial. The numbers represent bone quantity with the totals given per bone as well as per sector.

MtDNA Amplification and Sequencing Results

From the 193 skeletons that were sampled, eight females, seven males and one child, whose sex could not be determined, were the focus of this research. Among these 16 skeletons, DNA isolation was attempted from 62 different bones. These included 1 clavicle, 15 femora, 6 humeri, 11 petrous portions, 1 mandible, 1 metacarpal, 4 radii, 2 scapulae, 1 skull portion (non-petrous), 5 tibiae, 13 teeth and 2 ulnae (Table 5). Femur samples were divided equally between females and males, the mandible belonged to a male skeleton, the metacarpal to a female skeleton, the radii were all from males, while the ulnae were from female skeletons. Table 5 also provides the average integrity ratings of bones per sex with the majority exhibiting minimal disparity. Teeth, however, were on average in better condition within the female sex.

Table 5. Double Burial Bone Sex and Integrity

Bone	Female	Male	Average Integrity Rating		
			Female	Male	Combined
Clavicle	1	0	2.00	~	2.00
Femur	7	7	2.43	2.43	2.43
Humerus	4	2	2.50	3.00	2.75
Petrous	6	4	1.00	1.00	1.00
Mandible	0	1	~	4.00	4.00
Metacarpal	1	0	1.00	~	1.00
Radius	0	4	~	3.00	3.00
Scapula	2	0	4.00	~	4.00
Skull	1	0	4.00	~	4.00
Tibia	2	3	3.00	3.00	3.00
Tooth	7	5	2.29	1.80	2.04
Ulna	2	0	3.00	~	3.00

Bone types are distributed between female and male skeletons shown in the first three columns. The average integrity ratings for each bone type are given per sex in the last two columns.

The number of PCR reactions varied between the hypervariable regions as well as from bone to bone. Amplification was attempted on each bone a minimum of 2 times; results are displayed in Table 6. The femur from individual 234 (Burial 46), the petrous portion from individual 711 (Burial 184), the clavicle, humerus and tooth from individual 768 (Burial 198), and the femur, petrous portion and tooth from individual 772 (Burial 198) did not generate enough PCR product for sequencing, and therefore each reaction was reamplified to increase the yield. An attempt to amplify sequences of approximately 400, 300, and 200 bp was undertaken; the 200 bp amplicon yielded the most sequences.

Table 6. Attainable Sequences Within Double Burial Bones

Burial	Individual	Sex	Bone	Bone Rating	Result	
					HV1	HV2
46	233	Male	Femur	3	neg	neg
			Humerus	3	pos	pos
			Radius	3	pos	pos
			Tooth	1	pos	pos
	234	Female	Femur	2	pos	pos
			Humerus	2	pos	pos
			Skull	4	neg	neg
			Tooth	3	pos	pos
56	262	Male	Femur	1	pos	pos
			Petrous	1	neg	pos
			Radius	2	pos	pos
			Tooth	2	pos	pos
	263	Female	Femur	2	pos	pos
			Humerus	3	pos	pos
			Petrous	1	pos	pos
			Tooth	2	neg	neg
184	710	Male	Femur	3	pos	neg
			Mandible	4	pos	neg
			Tibia	3	pos	neg
			Tooth	1	pos	neg
	711	Female	Petrous	1	pos	neg
			Tibia	3	pos	pos
			Tooth	1	pos	pos
			Ulna	3	neg	neg
198	772	Female	Clavicle	2	pos	pos
			Femur	3	pos	pos
			Humerus	2	pos	pos
			Metacarpal	1	pos	pos
			Tooth	3	pos	pos
	768	? (Child)	Femur	3	pos	pos
			Petrous	1	pos	pos
			Tooth	1	pos	pos

Table 6 (cont'd).

259	1001	Female	Femur	2	pos	neg
			Humerus	3	pos	neg
			Petrous	1	neg	neg
			Tooth	3	pos	neg
	1002	Female	Femur	3	pos	neg
			Petrous	1	pos	neg
			Tibia	3	pos	neg
			Tooth	3	pos	neg
280	1084	Female	Femur	2	pos	pos
			Petrous	1	pos	neg
			Scapulae	4	pos	pos
			Ulna	3	pos	pos
	1085	Male	Femur	1	pos	pos
			Petrous	1	pos	pos
			Radius	3	pos	neg
			Tooth	2	neg	neg
300	1164	Male	Femur	3	neg	neg
			Petrous	1	pos	pos
			Tibia	3	neg	neg
			Tooth	3	pos	neg
	1165	Female	Femur	3	pos	neg
			Petrous	1	pos	neg
			Scapulae	4	pos	neg
			Tooth	1	neg	neg
375	1456	Male	Femur	3	pos	pos
			Petrous	1	pos	pos
			Radius	4	pos	pos
	1457	Male	Femur	3	pos	neg
			Humerus	3	pos	pos
			Tibia	3	pos	neg

Each double burial is listed in the first column followed by the individual skeleton reference numbers and sex. Each bone is listed along with its integrity rating (0 – 5) and whether a sequence was obtained, either positive or negative.

Appendix 3 displays hypervariable region data collected from the sequencing reactions for each double burial. All polymorphisms are listed across the top, starting with HV1, and following with HV2 when available. Column one lists the bones that produced sequence for each individual. Column two, labeled Bone Prep/PCR, indicates the bone prep (# 1 or # 2) followed by the particular PCR reaction, whether it was the 1st, 2nd etc., that had been set up for that particular burial. Those PCR reactions that indicate a second number (ex. Burial 198, individual 768, clavicle bone: 1.3.1) are reamplifications of that particular PCR reaction (3rd in this example). A positive result was given to any bone that yielded sequence at least one time (Table 6). Table 7 lists the percentage of bone yielding sequence based on its integrity rating. Sample sizes are given per bone rating with those rated 4 possessing the least number of bones. Bones rated 3 produced the greatest number of sequences in HV1, followed closely by a bone rating of 1, 2 and 4. For HV2, bones rated 2 produced the greatest number of sequences with those rated at 1 yielding 10% less. Bones rated 3 and 4 produced a lesser percentage with those at 4 producing the least number of sequences. When looking at a particular bone, reproducibility of sequences did occur between bone preps and between PCR reactions, but on multiple occasions two bones from the same individual did not concur between PCR reactions or preps (Appendix 3).

Table 7. Integrity Rating Compared With Sequence Production

Bone Integrity Rating	N	Percentage of Bone Yielding Sequence		
		HV1	HV2	Average
0	0	~	~	~
1	19	84	63	73
2	11	82	73	77
3	27	85	41	63
4	5	80	40	60
5	0	~	~	~

Integrity rating was compared to sequence production. The results from HV1 indicate there is not a powerful relationship between the visual appearance of a bone and the ability to obtain sequence. HV2 shows a slight relationship with bones rated at 4 producing the least number of sequences.

Displayed in Table 8 are sequencing results for HV1. A total of 52 bones (84%) produced DNA product that was successfully sequenced, while 10 (16%) did not. Of the 12 bone types, 6 produced sequences from all individuals, including clavicle, humerus, mandible, metacarpal, radius and scapula. The clavicle, mandible, metacarpel, radius and scapula, however, all possess sample sizes of 4 or less. The femur, petrous and tooth bones had sample sizes of 15, 11 and 13 respectively, and were successfully sequenced 87, 82, and 80% of the time. Seventy-eight percent of the teeth and 50% of the ulnae produced sequence, while the non-petrous skull portion resulted in none.

Table 8. HV1 Bone Type and Sex Analysis

Bone	Individual Number (Nj.s)		Positive Result (%)	Positive Females (%)	Positive Males (%)
	Positive Result	Negative Result			
Clavicle	768	~	100	100	~
Femur	234, 262, 263, 710, 768, 772, 1001, 1002, 1084, 1085, 1165, 1456, 1457	233, 1164	87	100	71
Humerus	233, 234, 263, 768, 1001, 1457	~	100	100	100
Petrous	263, 711, 772, 1002, 1084, 1085, 1164, 1165, 1456	262, 1001	82	83	75
Mandible	710	~	100	~	100
Metacarpel	768	~	100	100	~
Radius	233, 262, 1085, 1456	~	100	~	100
Scapula	1084, 1165	~	100	100	~
Skull	~	234	0	0	~
Tibia	710, 711, 1002, 1457	1164	80	100	67
Tooth	233, 234, 262, 710, 711, 768, 772, 1001, 1002, 1164	263, 1085, 1165	78	71	80
Ulna	1084	711	50	50	~

The 62 bones that were sampled are distributed based on whether they yielded interpretable sequence (positive) or not (negative). Under each of those headings are the particular skeleton (Nj.s) numbers. The combined percentage of each bone type that gave a positive result was determined. Those bones with both female and male constituents were analyzed separately to determine if there was a correlation between sex and the production of DNA sequence.

HV2 yielded far fewer sequences than HV1 (Table 9). A total of 33 bones (53%) produced DNA product that was successfully sequenced while 29 (47%) did not. Within the 10 bone types, the most productive bones were the clavicle and metacarpel at 100%, followed by 83% of the humeri and 78% of the radii. Both mandible and skull bones (excluding petrous) did not produce HV2 sequences. Comparing sequence production with integrity rating was difficult due to limited sample sizes (see paragraph above). However, looking at the data from Tables 5, 8 and 9 several relationships could be

formed. The metacarpel, which was well preserved, produced sequence 100% of the time, although the sample size was one. The clavicle, also well preserved, produced sequence 100% of the time and had a sample size of one. The teeth, with an average rating of 2.04, on the other hand, had an average sequence yield of 50%. HV2 sequencing of other bones also met with more limited success.

Table 9. HV2 Bone Type and Sex Analysis

Bone	Individual Number (Nj.s)		Positive Result (%)	Positive Females (%)	Positive Males (%)
	Positive Result	Negative Result			
Clavicle	768	~	100	100	~
Femur	234, 262, 263, 768, 772, 1084, 1085, 1456	233, 710, 1001, 1002, 1164, 1165, 1457	53	57	43
Humerus	233, 234, 263, 768, 1457	1001	83	75	100
Petrous	262, 263, 772, 1085, 1164, 1456	711, 1001, 1002, 1084, 1165	54	17	100
Mandible	~	710	0	~	0
Metacarpel	768	~	100	100	~
Radius	233, 262, 1456	1085	75	~	75
Scapulae	1085	1165	50	50	~
Skull	~	234	0	0	~
Tibia	711	710, 1002, 1164, 1457	20	50	0
Tooth	233, 234, 262, 711, 768, 772	263, 710, 1002, 1002, 1085, 1164, 1165	50	50	40
Ulna	1084	711	50	50	~

The 62 bones that were sampled are distributed based on whether they yielded interpretable sequence (positive) or not (negative). Under each of those headings are the particular skeleton (Nj.s) numbers. The combined percentage of each bone type that gave a positive result was determined. Those bones with both female and male constituents were analyzed separately to determine if there was a correlation between sex and the production of DNA sequence.

Combining results from HV1 and HV2, sexual disparity between sequence productions was investigated. Femur, humerus, petrous, tibia and tooth were collected from male and female skeletons. Femur and tibia both produced sequences more often

from female skeletons with 11 out of 14 and 3 out of 4 being positive. The bones that produced the greatest percentage of sequence for males were the humerus, petrous and tooth at 4/4, 7/8 and 6/10 respectively. The teeth produced similar results between the sexes.

Between the two hypervariable regions there may have been a change in amplification success when comparing the oldest skeletal material to those that were more recent (Table 10). This observation, however, must be taken in context with a sample size of 8 burials. The success of HV1 sequences did not appear to be greatly affected by the age of a skeleton. On the other hand, HV2 success was seen more often in younger burials. When comparing the two most extreme datasets in HV1, double burials 300 and 375 (oldest) and 184 and 198 (youngest), 94% of the bones produced sequences from the younger bones, while 79% were obtainable from the oldest set. HV2 differed as well with 62% of the younger burials and 36% of the oldest burials yielding sequence. There is, however, less of a disparity when comparing individual burials. Within HV1 the youngest burial, 184 from the 6th century BC, burial 280 from the 8–9th century BC and burial 259 from 10th century BC all had 88% of their bones produce sequence. Also, HV1 sequences were obtained from every bone in the oldest burial (375).

Table 10. Burial Age Compared With Sequence Production

Date (Century BC)	Burial	Obtainable Sequence (%)	
		HV1	HV2
6	184	88	25
7	198	100	100
9-8	56	75	88
9-8	280	88	63
10	46	75	75
10	259	88	0
11	300	63	13
12	375	100	67

A comparison of burial age versus obtainable sequence with estimated age of burial, double burial number, and the percentage of obtainable sequence from each hypervariable region; percentages were calculated by dividing the number of sequences produced in a burial by the total number possible.

Maternal Relatedness Within Double Burials

Potential maternal relatedness was assigned based on shared polymorphic sites or lack thereof within bone sequence. Given these criteria, individuals from seven of the eight (46, 56, 184, 259, 280, 300 and 375) double burials would be deemed maternally related (Appendix 4). The individuals from Burial 259 did not share polymorphisms in HV1, and HV2 mtDNA could not be amplified for analysis. Burial 198, containing the skeletons of an adult female (768) and a 3 – 5 year-old child (772), shared 2 polymorphisms, both in HV2. This burial, however, also contained a total of 5 polymorphisms that were not shared, 4 in the adult female and one in the child. Those found in the female's sequences were at positions 16270, 16292, 16311, and 16362. The child's sequences possessed a polymorphism at position 16318.

Previously Unrecorded CRS Polymorphisms

Polymorphisms that did not correspond with any currently in the Mitomap database (http://www.mitomap.org) were recorded (Table 11). A total of 5 polymorphisms fit into this category; three were transitions and two were transversions.

Table 11. Previously Unrecorded CRS Polymorphisms

Base	Mutation	Double Burial
162	C – T	198
167	C – T	56
175	A – C	198
181	A – G	198
16340	A – C	56

The CRS bases as defined by Anderson *et al.* (1981). The second column lists the CRS base followed by the polymorphism previously unrecorded in Mitomap (2005).

DISCUSSION

Collection Phase

Bone samples were collected from the Kamenica tumulus burials during the summer of 2003. The general rule used during sample collection was to examine what bones were available from a specific individual and to collect a minimum of four bone types, focusing on long bones, petrous portions and teeth, (particularly molars) more than others. The reasoning behind selecting these bones was based on their hardiness and their ability to survive for thousands of years. The petrous portion, being the hardest bone in the body, was investigated for its value to yield DNA, much as teeth have been (Gaytmenn and Sweet, 2003). The photos of each double burial displayed in the Introduction make the skeletons appear as if their bones were intact. Upon collection, however, the bones were found to be very fragile and most often fell apart.

As each bone sample was collected its condition was rated based on the integrity of intact material remaining after excavation. This rating system, inspired by Behrensmeyer's (1978) development of bone weathering stages (0 to 5) based on whole skeleton examination, was applied to single bones to determine if there was a correlation between bone appearance and the ability to obtain mtDNA. All bones from the double burials analyzed were rated 1 – 4; none were in pristine condition.

During the collection phase each petrous portion was rated 4 on the integrity scale because it is a fragment of the temporal bone. Upon later reflection, however, it was decided that the petrous portion should be treated as a unique bone type itself. After sampling a number of burials with petrous portions and discovering they were all very similar with respect to shape and integrity, the bone ratings were changed to 1. Petrous

portions appeared to remain quite stable, most likely due to their unique hardness, and if these could be associated with a skeleton, they were collected to investigate their value in forensic identification. Before this study there had been no documented DNA research performed on petrous portions. This may be because this portion of the skull is only accessible in broken crania, which was the case with the Kamenica tumulus material. The metacarpel bone was also rated 1. The clavicle bone was rated 2, with teeth, femora, and humeri possessing average integrity ratings of 2.04, 2.43, and 2.75 respectively. In general, long bones, petrous portions and teeth were in better shape than those bones with a flatter shape and less density, such as mandible and scapula. Interestingly, pelvic bones, which are also relatively flat and porous, often did not survive, making the sexing of skeletons problematic.

Bone ratings in Table 5 were compared with the bones that yielded mtDNA sequences most often (Tables 8 and 9). Upon comparison of the specific bone type's rating and sequence production, the most productive bones over both hypervariable regions were clavicle and metacarpel, followed by humeri and radii. The sample sizes of both clavicle and metacarpel were 1, meaning their inclusion in statistical comparisons was unreasonable. Those bones that possessed a larger sample size (femur, petrous portion and tooth) are the focus of this discussion.

When looking at HV1 the femur, petrous portion and tooth, all with ratings that fell in the upper half of the integrity scale, had average sequence production percentages ranging from the high 70's to high 80's. The same bone types in HV2, on the other hand, exhibited approximately a 30% drop in success rate. It is likely that the HV2 primer set is less robust than those used for HV1, leading to the decrease in sequence, however it is

also possible that the primer binding areas within HV2 were mutated to an extent that binding could not occur. Research by Clayton *et al.* (2004) suggested the position of a mutation within the binding site could affect primer binding. If the mutation is found close to the 5′ terminus of the primer binding sequence, it can destabilize primer annealing so that amplification either does not occur or is reduced in efficiency. The study also suggested that primer binding site mutations are quite rare. In bones of ancient origin, however, mutations are known to occur more frequently at sites that are particularly susceptible to mutation, termed "hotspots" (Thomas *et al.*, 2003). Alternate primer pairs that anneal to areas known to mutate at a lower frequency would be an option for amplifying these types of regions.

Bone integrity ratings were also compared with sequence production (Table 7) to determine whether a relationship between visual appearance of bone and the ability to obtain a DNA sequence existed. Looking at HV1 there was little indication of a relationship. Bones rated 1 through 4 had an average sequence production between 80% and 85%. The 6 bones that produced sequence 100% of the time in HV1 were rated anywhere from 1 to 4, further suggesting lack of a relationship. HV2, however, had a weak indication of a correlation between bone appearance and sequence production. Bones rated 1 and 2 produced sequence 63% and 73% of the time, while those rated 3 and 4 showed a drop in the sequence production with values of 41% and 40%. This information, however, must be taken in context with sample size. Bones rated 4 had the smallest sample size at 5, while those rated 3 had the largest at 27; bones rated 2 had a sample size of 11 and those rated 1, 19. Based on this information, a reduction in the ability to obtain HV2 sequence from a bone rated 2 and a bone rated 3 is given more

power than between those rated 3 and 4. When comparing bones rated 2 and 3 a drop in sequence production was seen, which may indicate bones that are more degraded are less likely to contain sequencable DNA. Those bones that worked 100% of the time were both rated 1 and 2, while those producing sequence less frequently were rated on average 2.75 and 3. The petrous portion however, possessed a rating of 1, and did not fall into the most productive category, with only 54% producing sequence. When analyzing HV2, in general, as sample size increased, average sequence production decreased, indicating those bones with smaller sample sizes may be skewing conclusions.

Generating Bone Dust from Samples

With the use of a drill, a precise portion of the bone can be sampled, which allows one to focus on a particular part of bone avoiding areas with greater degradation. Early in the sampling process a larger drill bit (1/8") was used. This was easily utilized on the larger bone samples, yielding a great amount of bone dust quickly, which, in some cases, also brought along dirt deposits. Drilling teeth was initially carried out using the same size drill bit, which was very difficult due to the tooth's small size. At that point, all bones were drilled using a 1/16" drill bit that was changed regularly. It was discovered that as a drill bit became dull, the bone was more likely to burn, which could only affect DNA adversely. This was an issue only with those bones that were very hard, such as teeth and most petrous portions. Use of the smaller bit also permitted more controlled access to pulp and dentin within the root body, which, in fresh teeth, have been shown to contain more DNA than other parts of the tooth (Gaytmenn and Sweet, 2003).

Sequence Analysis

MtDNA sequences from ancient bone samples are inherently difficult to obtain due to several factors. Chemical reactions and microorganisms within soil cannot only affect the bone structure, but also the DNA within. It is also believed that degradation of skeletal material has an adverse effect on the preservation of DNA (Gotherstrom, 2002) and unfavorable conditions such as high temperature, high moisture, and acidic pH have been shown to increase the rate at which this occurs (Parsons and Weedn, 1996; Smith *et al.*, 2003). Microorganisms favor areas of high moisture and warm temperatures and can produce enzymes such as endo- and exonucleases that digest DNA (Rogan and Salvo, 1990). Further, DNA often cannot be isolated from a bone sample without carrying along PCR inhibitors. The PCR inhibition that remained in some of the bone DNA after organic extraction indicated they were water-soluble substances, such as metal ions. The use of Microcon columns and the addition of BSA were necessary to respectively filter out or bind these substances, allowing the PCR reaction to go forward. Once these were incorporated into the analysis it was possible to greatly increase sequence production.

Because DNA in ancient samples tends to be highly degraded, what was isolated, amplified, and sequenced were short (~200 bp) fragments. Early on in the research, an attempt to amplify DNA pieces approximately 300 and 400 bp was made. The ~ 400 bp piece was attempted at the beginning of the research with few bones producing PCR product; those that did can be found in Appendix 3. Amplification of the ~ 300 bp piece was attempted with much the same result. Because amplification attempts of the ~ 200 bp often yielded PCR product, it became part of the standard protocol. In terms of analysis, the smaller the piece of DNA the less information there is available to work

with, which was an obvious disadvantage for the research, but a necessary tradeoff. Amplification of both hypervariable regions increased the number of bases available for investigation, but was not successful in all cases. For instance, HV2 could not be amplified from either individual in Burial 259. However, amplification and sequencing of HV1 was possible for seven of the eight bones taken from these two skeletons, showing that DNA was present. As mentioned above, it is possible that mutation(s) occurred within the HV2 primer binding sites, or that this region is particularly susceptible to degradation. Future research could employ multiple primer sets in an attempt to obtain more data from these samples.

An issue with sequence reproducibility within and between bones arose during analysis. Heteroplasmy, or the existence of more than one base at a particular mtDNA site (Holland and Parsons, 1999), is not uncommon and may help explain the results found in this study. Individuals 710 and 711 from Burial 184 displayed both C and T peaks at position 16290 in several bones. Looking specifically at individual 710, the mandible had both bases, while the tibia had a T at that position. When the DNA was amplified during a second PCR reaction, it displayed a C peak. Also, upon sequencing a different DNA prep of the mandible, it too had a C peak, as did the rest of the bones. Stochastic effects, resulting from the random sampling of DNA at very low copy number, may explain the appearance of one or two peaks within a sequence. It is likely in these experiments that very few mtDNA molecules were available for amplification. These few molecules may differ at a specific site due to heteroplasmy or mutation, and when amplifying very small quantities, one molecule may have been preferentially amplified. This can potentially result in differences among bone samples, among different regions of

the same bone, among DNA preparations from the same bone, or even among different PCR attempts from a single DNA preparation. Further, when heteroplasmy exists it becomes difficult to detect if the ratio of the two bases is greater than 5 to 1.

Perhaps for these reasons, more than 1 haplotype was detected in many of the individuals. Based on the few number of bases available for analysis, and the frequent occurrence of more than one haplotype from different bones of an individual (and in some instances, the same bone from an individual), it was necessary to use a novel approach during sequence analysis. Focusing on polymorphic sites, or those sites that showed regular sequence heterogeneity within an individual, the individuals within a double burial were analyzed for maternal relatedness. Greater confidence was given to those double burials that exhibited more polymorphic sites, either shared or unshared, representing maternally related or unrelated individuals respectively.

Maternal Relatedness Within Double Burials

Using both standard sequence alignments and the polymorphic site criteria, individuals in seven of the 8 double burials have the possibility of being maternally related, while Burial 198 was excluded. Six of the 8 double burials shared polymorphic sites. Burial 259 did not exhibit polymorphic sites, but the individuals shared the same haplotype. None of the eight double burials shared a common haplotype.

Once maternal relatedness was estimated, the meaning this relatedness (or lack thereof) had for the burials was considered. Double burials have been seen within other tumuli of this region, at a small number per tumulus (Bejko 2000). Why do these occur at all? An obvious possibility is that they represent family members, such as husband and

wife, siblings, or even mother and child, who died at the same time. It is also possible that they represent two unrelated individuals who happened to die at or about the same time and were thus buried together. Yet another possibility is that the individuals did not die at the same time, but were purposefully placed together. Finally, in some instance the bodies may have been accidentally buried at the same spot. Regardless of the origin, the assumption can be made that this practice was generally special in some way. Double burials were seen, albeit rarely (4%), throughout the ~800 years of tumulus use, seemingly indicating this type of mortuary treatment had a particular significance for the villagers.

Burial 300 held two teenagers; one was male and the other female. The DNA data indicated this pair might be maternally related, perhaps siblings. This seems reasonable, in that two young people died about the same time, perhaps by disease or other common cause, and as siblings they might naturally be buried together. However, it is possible this may not have been a true double burial. This was evident by the disturbance of one individual's bones upon interment of the second individual, indicating it was unlikely to have been a single burial event. Given this, it could be accidental that the first burial was disturbed, or it is possible that there was intent in placing the two individuals close together, as might be expected for a family burial site.

Burial 375 also may not have been a true double burial. This burial consisted of two adult males, one was extended within the grave and the other was a bundle burial. As with burial 300, the individuals within this grave appeared to have been placed in at separate times, indicated by the differing body treatments. The DNA haplotypes appeared to be similar indicating perhaps a sibling relationship. Speculation as to who

died first can be undertaken, but can reveal no true answer. It is known, however, the bundled male was well decomposed upon his interment into the double burial. In order to form a bundle of one's bones, most of the tissue that once held the body in anatomical position must be gone. This may indicate he was the first to die, was buried at a different location, and exhumed after the other male had died to take his place in the family burial plot.

The third questionable double burial was 184. This rock burial held an adult male and adult female in an uncommon head to toe orientation. Because their mtDNA sequences shared polymorphic sites, these too may have been maternally related, perhaps siblings. One possibility is the head to toe burial was intentional and has meaning, especially if this is a single interment. On the other hand, the skeletal orientation may have occurred inadvertently, due to the difficulty in depicting bone from rock, if the second individual was buried after decomposition had occurred.

The most compelling evidence for a true double burial is when skeletons are found with intertwining bones. The adult male and female from Burial 56 is a perfect example, with arm and leg bones encircling one another. The DNA data suggest these too may be maternally related. A sibling relationship is one possibility but this may be questionable due to the intimate nature of the body placement. It is plausible that the community was small and this male and female were married, most likely only distantly related. In a larger population it would be expected that differences between their haplotypes would be more evident, although in many cultures, including European royalty, marrying relatively closely related relatives is not uncommon. Further study of

burials within the same time period should lend more data to determine which was the case.

Burial 259 held 2 adult females that were placed in a similar fashion to Burial 56 indicating it too was likely a true double burial. The mtDNA data suggest these may be maternally related; possibly sisters.

Burial 46 consisted of an adult male and adult female. These individuals, as the mtDNA data suggest, appear to share a maternal relationship. As discussed with regard to Burial 56, a spousal relationship is a possibility; one that could be better understood if population size was known.

Burial 280 held an adult male and adult female. The female's body was placed on top of the male's body within the burial. This burial too, the archeologists felt, was a single interment. The mtDNA data indicated the individuals might be siblings or share some other form of maternal relationship.

Burial 198, which consisted of an adult female and 3 – 5 year old child, did not exhibit mitochondrial sequences that were similar, indicating this was not a mother-child pair as had been previously hypothesized. The skeletal positioning upon burial was quite interesting; the adult female's hand was placed cupping the child's head. A possibility is that the child was buried with a female from its paternal side or some other unrelated female who cared for this child.

The mtDNA sequences determined here can also provide information about village customs regarding marriage, specifically whether males or females relocated. In a matrilocal village it is custom for the male to enter his new bride's village and live as part of her family. In patrilocal villages, on the other hand, the female integrates into her new

husband's family. Because different maternal lines were seen within the tumulus over time, there is evidence to suggest the presence of a patrilocal village.

Previously Unrecorded CRS Polymorphisms

Five base changes within Burials 56 and 198 had not been previously recorded. Burial 56 had 1 transition and 1 transversion, while Burial 198 had 2 transitions and 1 transversion. Two of the transitions were C to T and the other was A to G. Both transversions were A to C changes.

Conclusion

The use of mtDNA to analyze maternal relationships can be useful when working with ancient skeletal material. The key with such an analysis is the ability to obtain as much genetic data as possible. Even though ancient DNA is generally very small in size, it is possible to gather the amount of information provided by one, larger amplicon by using primers that generate multiple smaller amplicons. The bones from Kamenica can be analyzed using this technique, but because the resulting sequences have not been shown to be entirely reproducible, care must be taken when conclusions are made. There are treatments, such as treatment with Uracil N-glycosylase, that can be used to help eliminate mutated bases, specifically the deamination of cytosine, which may help to reduce ambiguous sites (Hofreiter *et al.*, 2001; Thomas *et al.*, 2003). Strategies such as this might be applied to the Kamenica material, resulting in more defined haplotypes.

Analysis of eight of the double burials from the Tumulus at Kamenica has provided some insight into the villagers that once lived in the Korçë basin. Looking at

these eight double burials, it is estimated that 87.5% (7 out of 8) are consistent with maternal relatedness. Because the majority of double burials are believed to be single interment episodes, it seems likely the individuals died close in time with one another, suggesting death by disease or trauma. Regarding the possible genetic relationships of individuals within a grave; two hypotheses can be formed. The first is that the village was large and double burials were used to bury either immediate or extended family members, such as siblings or cousins. The second is that the village was small, and members often shared a maternal line, even if distantly related. The issue of village size may be resolved through analysis of additional burials within the same time period, leading to more answers about the ancient Kamenica villagers.

APPENCICES

APPENDIX I

Kamenica Tumulus: 12th – 6th Century B.C. Burials

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5m

APPENDIX II

Kamenica Tumulus: 11^{th} – 6^{th} Century B.C. Burials


5m
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APPENDIX III

Summary of MtDNA Sequences

Burial 46	CRS		16215	16223	16224	16234	16239	16242	16251	16256	16259	16260	16261	16262	16270	16271	16276	16278	16284
			A	C	T	C	C	C	C	C	C	C	C	C	C	T	G	C	A
233 Male; 234 Female	Bone Rating	Bone Prep/PCR																	
233 Humerus	3	2.1													T		G		
			T												T		G		
233 Radius	3	2.1																	
233 Tooth	1	2.1																	
														T					
234 Femur	2	2.1																	
			Y											Y					
234 Femur	2	2.1.1																	
			Y											Y					
234 Humerus	2	1.3																	
234 Humerus	2	1.2																	
234 Tooth	3	2.1																	

Burial 46	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
233 Male; 234 Female	Bone Rating	Bone Prep/PCR																	
233 Humerus	3	2.1	G	T							C								
			G	T							C								
233 Radius	3	2.1																	
233 Tooth	1	2.1																	
234 Femur	2	2.1																	
234 Femur	2	2.1.1																	
234 Humerus	2	1.3																	
234 Humerus	2	1.2																	
234 Tooth	3	2.1																	

Burial 46	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
233 Male; 234 Female	Bone Rating	Bone Prep/PCR													
233 Humerus	3	2.1	T												
233 Radius	3	2.1													
233 Tooth	1	2.1													
234 Femur	2	2.1													
234 Femur	2	2.1.1													
234 Humerus	2	1.3													
234 Humerus	2	1.2													
234 Tooth	3	2.1													

Burial 56	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
262 Male; 263 Female	Bone Rating	Bone Prep/PCR																	
262 Femur	1	1.4																	
262 Radius	2	1.4																	
262 Radius	2	2.1																	
262 Tooth	2	2.1																	
263 Femur	2	1.4																	
263 Humerus	3	1.4																	
263 Petrous	1	2.1																	
263 Petrous	1	2.2																	

Burial 56	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
262 Male; 263 Female	Bone Rating	Bone Prep/PCR																	
262 Femur	1	1.4																	
262 Radius	2	1.4																	
262 Radius	2	2.1																	
262 Tooth	2	2.1																	
263 Femur	2	1.4					Y												
							T												
263 Humerus	3	1.4																	
263 Petrous	1	2.1																C	G
263 Petrous	1	2.2																	

Burial 56	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
262 Male; 263 Female	Bone Rating	Bone Prep/PCR													
262 Femur	1	1.4													
262 Radius	2	1.4													
262 Radius	2	2.1													
262 Tooth	2	2.1													
263 Femur	2	1.4						C							
263 Humerus	3	1.4													
263 Petrous	1	2.1													
263 Petrous	1	2.2													

Burial 184	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
710 Male; 711 Female	Bone Rating	Bone Prep/PCR																	
710 Femur	3	2.2																	
710 Femur	3	1.3																	
710 Mandible	4	2.1																	
710 Mandible	4	2.2																	
					C														
710 Mandible	4	1.3																	
710 Tibia	3	2.2																	
				T															
710 Tooth	1	2.2																	
															T				
710 Tooth	1	1.3																	
711 Petrous	1	2.2.1																	
711 Petrous	1	1.3																	
711 Tibia	3	2.2																	
711 Tibia	3	1.3																	
711 Tooth	1	2.1																	
				Y															

Burial 184	CRS		16285 A	16286 C	16290 C	16291 C	16292 C	16294 C	16295 C	16296 C	16303 G	16304 T	16309 A	16311 T	16318 A	16319 G	16327 C	16340 A	16342 T
710 Male; 711 Female	Bone Rating	Bone Prep/PCR																	
710 Femur	3	2.2																	
																A			
710 Femur	3	1.3															T		
710 Mandible	4	2.1												C					
														C					
710 Mandible	4	2.2			Y														
					Y														
710 Mandible	4	1.3															Y		
710 Tibia	3	2.2			T											A			
					T											A			
710 Tooth	1	2.2					T												
							T												
710 Tooth	1	1.3												C					
711 Petrous	1	2.2.1																	
711 Petrous	1	1.3																	
711 Tibia	3	2.2																	
					Y														
711 Tibia	3	1.3												Y					
711 Tooth	1	2.1			Y											R			
					Y											R			

Burial 184	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
710 Male; 711 Female	Bone Rating	Bone Prep/PCR													
710 Femur	3	2.2													
710 Femur	3	1.3													
710 Mandible	4	2.1													
710 Mandible	4	2.2													
710 Mandible	4	1.3													
710 Tibia	3	2.2						C							
710 Tooth	1	2.2						C							
710 Tooth	1	1.3													
711 Petrous	1	2.2.1													
711 Petrous	1	1.3				C									
711 Tibia	3	2.2													
711 Tibia	3	1.3													
711 Tooth	1	2.1													

Burial 198	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
768 Female; 772 Child	Bone Rating	Bone Prep/PCR																	
768 Clavicle	2	1.3.1																	
768 Femur	3	1.5									T	T							
						T					T	T							
768 Femur	3	1.5							T		T	T							
						T			T		T	T							
768 Femur	3	2.1																	
768 Humerus	2	1.3.1																	
					C														
768 Humerus	2	1.5																	
768 Humerus	2	2.1																	
768 Metacarpal	1	1.5													T				
															T				
768 Metacarpal	1	2.1																	
768 Tooth	3	1.3.1											T						
													T		Y				
768 Tooth	3	1.5													T				
															T				

Burial 198	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
768 Female; 772 Child	Bone Rating	Bone Prep/PCR																	
772 Femur	3	1.3.1													T				
772 Femur	3	1.5																	
													M						
772 Femur	3	2.1																T	
				T			T											T	
772 Petrous	1	1.8.1		M											T				
				M															
772 Petrous	1	1.5																	
772 Petrous	1	2.1																	
772 Tooth	1	1.3.1								T									T
772 Tooth	1	1.5																	

Burial 198	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
768 Female; 772 Child	Bone Rating	Bone Prep/PCR																	
768 Clavicle	2	1.3.1																	
768 Femur	3	1.5				T		T	T	T		C	G						
						T		T	T	T		C	G						
768 Femur	3	1.5				T		T	T	T		C	G						
						T		T	T	T		C	G						
768 Femur	3	2.1												C					
768 Humerus	2	1.3.1												C					
														C					
768 Humerus	2	1.5																	
768 Humerus	2	2.1												C					
768 Metacarpal	1	1.5					T												
							T												
768 Metacarpal	1	2.1												C					
768 Tooth	3	1.3.1																	
768 Tooth	3	1.5					T												
							T												

Burial 198	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
768 Female;	Bone	Bone																	
772 Femur	3	1.3.1					T								T				
772 Femur	3	1.5						T		T		C							
								T		T		C							
772 Femur	3	2.1		T				T					G	C					
				T				T					G	C					
772 Petrous	1	1.8.1				Y	T								T				
772 Petrous	1	1.5													T				
															T				
772 Petrous	1	2.1																	
772 Tooth	1	1.3.1																	
772 Tooth	^{5}1	1.5													T				

Burial 198	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
768 Female; 772 Child	Bone Rating	Bone Prep/PCR													
768 Clavicle	2	1.3.1													
768 Femur	3	1.5		T			T		T	T	G	T	T	T	
768 Femur	3	1.5		T			T		T	T	G	T	T	T	
768 Femur	3	2.1													
768 Humerus	2	1.3.1													
768 Humerus	2	1.5			C										
768 Humerus	2	2.1						C							
768 Metacarpal	1	1.5						C							
768 Metacarpal	1	2.1						C							
768 Tooth	3	1.3.1													
768 Tooth	3	1.5						C							

Burial 198	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
768 Female; 772 Child	Bone Rating	Bone Prep/PCR													
772 Femur	3	1.3.1						C							
772 Femur	3	1.5													G
772 Femur	3	2.1													
772 Petrous	1	1.8.1													
772 Petrous	1	1.5													
772 Petrous	1	2.1													
772 Tooth	1	1.3.1													
772 Tooth	1	1.5													

Burial 259	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
1001 Female; 1002 Female	Bone Rating	Bone Prep/PCR																	
1001 Femur	2	1.1																	
1001 Humerus	3	1.1																Y	
				Y														Y	
1001 Humerus	3	2.1																T	
				T														T	
1001 Tooth	3	1.1																	
1002 Femur	3	1.1																	
1002 Femur	3	2.1																	
1002 Petrous	1	1.1																	
1002 Petrous	1	2.1																	
1002 Tibia	3	1.1																	
1002 Tibia	3	2.1																	
1002 Tooth	3	1.1																	
1002 Tooth	3	2.1																	

Burial 259	CRS		16285 A	16286 C	16290 C	16291 C	16292 C	16294 C	16295 C	16296 C	16303 G	16304 T	16309 A	16311 T	16318 A	16319 G	16327 C	16340 A	16342 T
1001 Female; 1002 Female	Bone Rating	Bone Prep/PCR																	
1001 Femur	2	1.1																	
1001 Humerus	3	1.1																	
1001 Humerus	3	2.1																	
1001 Tooth	3	1.1																	
1002 Femur	3	1.1																	
1002 Femur	3	2.1																	
1002 Petrous	1	1.1																	
1002 Petrous	1	2.1																	
1002 Tibia	3	1.1																	
1002 Tibia	3	2.1																	
1002 Tooth	3	1.1																	
1002 Tooth	3	2.1																	

Burial 259	CRS		16352	16354	16356	16357	16358	16362	16364	16366	16371	16375	16376	16378	16384
			C	C	T	T	C	T	C	C	A	C	C	C	G
1001 Female: 1002 Female	Bone Rating	Bone Prep/PCR													
1001 Femur	2	1.1													
1001 Humerus	3	1.1													
1001 Humerus	3	2.1													
1001 Tooth	3	1.1													
1002 Femur	3	1.1													
1002 Femur	3	2.1													
1002 Petrous	1	1.1													
1002 Petrous	1	2.1													
1002 Tibia	3	1.1													
1002 Tibia	3	2.1													
1002 Tooth	3	1.1													
1002 Tooth	3	2.1													

Burial 280	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
1084 Female; 1085 Male	Bone Rating	Bone Prep/PCR																	
1084 Femur	2	2.2																	
1084 Petrous	1	1.1																	
				Y															
1084 Scapulae	4	2.2																	
1084 Ulna	3	2.2																	
1084 Ulna	3	1.1																	
1085 Femur	1	2.2																	
				T															
1085 Petrous	1	2.2																	
1085 Petrous	1	1.1																	
1085 Radius	3	2.2																	
1085 Radius	3	1.1																	

Burial 280	CRS		16285 A	16286 C	16290 C	16291 C	16292 C	16294 C	16295 C	16296 C	16303 G	16304 T	16309 A	16311 T	16318 A	16319 G	16327 C	16340 A	16342 T
1084 Female; 1085 Male	Bone Rating	Bone Prep/PCR																	
1084 Femur	2	2.2																	
1084 Petrous	1	1.1																	
1084 Scapulae	4	2.2																	
1084 Ulna	3	2.2																	
1084 Ulna	3	1.1						Y											
								Y											
1085 Femur	1	2.2																	
1085 Petrous	1	2.2																	
1085 Petrous	1	1.1																	
1085 Radius	3	2.2			Y														
					Y														
1085 Radius	3	1.1			Y											R			
					Y											R			

Burial 280	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
1084 Female; 1085 Male	Bone Rating	Bone Prep/PCR													
1084 Femur	2	2.2													
1084 Petrous	1	1.1													
1084 Scapulae	4	2.2													
1084 Ulna	3	2.2													
1084 Ulna	3	1.1													
1085 Femur	1	2.2													
1085 Petrous	1	2.2													
1085 Petrous	1	1.1													
1085 Radius	3	2.2													
1085 Radius	3	1.1													

Burial 300	CRS		16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A
1164 Male; 1165 Female	Bone Rating	Bone Prep/PCR																	
1164 Petrous	1	1.1								Y					Y				
1164 Tooth	3	1.1								Y									
1165 Femur	3	1.1																	
1165 Femur	3	2.2		Y															
1165 Petrous	1	1.1								T					T				
1165 Petrous	1	2.2		T															
1165 Scapulae	4	1.1																	

Burial 300	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
1164 Male; 1165 Female	Bone Rating	Bone Prep/PCR																	
1164 Petrous	1	1.1												Y					
														Y					
1164 Tooth	3	1.1												C					
														C					
1165 Femur	3	1.1																	
1165 Femur	3	2.2																	
1165 Petrous	1	1.1																	
1165 Petrous	1	2.2																	
1165 Scapulae	4	1.1												Y					

Burial 300	CRS		16352 C	16354 C	16356 T	16357 T	16358 C	16362 T	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
1164 Male; 1165 Female	Bone Rating	Bone Prep/PCR													
1164 Petrous	1	1.1													
1164 Tooth	3	1.1													
1165 Femur	3	1.1													
1165 Femur	3	2.2													
1165 Petrous	1	1.1													
1165 Petrous	1	2.2													
1165 Scapulae	4	1.1													

Burial 375	CRS		16215	16223	16224	16234	16239	16242	16251	16256	16259	16260	16261	16262	16270	16271	16276	16278	16284
			A	C	T	C	C	C	C	C	C	C	C	C	C	T	T	C	A
1456 Male; 1457 Male	Bone Rating	Bone Prep/PCR																	
1456 Femur	3	2.1																	
1456 Petrous	1	2.1																	
1456 Petrous	1	1.1																	
								T											
1456 Radius	4	2.1																	
1456 Radius	4	1.1																	
										T					T				
1457 Femur	3	2.1																	G
																			G
1457 Femur	3	1.1																	
1457 Humerus	3	2.1																	
1457 Tibia	3	1.1																	
															Y				

Burial 375	CRS		16285	16286	16290	16291	16292	16294	16295	16296	16303	16304	16309	16311	16318	16319	16327	16340	16342
			A	C	C	C	C	C	C	C	G	T	A	T	A	G	C	A	T
1456 Male; 1457 Male	Bone Rating	Bone Prep/PCR																	
1456 Femur	3	2.1					Y												
							Y												
1456 Petrous	1	2.1																	
1456 Petrous	1	1.1																	
1456 Radius	4	2.1					T												
							Y												
1456 Radius	4	1.1																	
						T													
1457 Femur	3	2.1																	
1457 Femur	3	1.1																	
1457 Humerus	3	2.1					T												
							T												
1457 Tibia	3	1.1																	
							Y												

Burial 375	CRS		16352	16354	16356	16357	16358	16362	16364	16366	16371	16375	16376	16378	16384
			C	C	T	T	C	T	C	C	A	C	C	C	G
1456 Male; 1457 Male	Bone Rating	Bone Prep/PCR													
1456 Femur	3	2.1													
1456 Petrous	1	2.1													
1456 Petrous	1	1.1													
1456 Radius	4	2.1													
1456 Radius	4	1.1													
1457 Femur	3	2.1			C										
1457 Femur	3	1.1													
1457 Humerus	3	2.1													
1457 Tibia	3	1.1													

Burial 46	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
233 Male; 234 Female	Bone Rating	Bone Prep/ PCR																				
233 Humerus	3	1.1																		G		
233 Radius	3	1.1														G						
233 Radius	3	2.1																		G		
233 Radius	3	2.1.1																		G		
233 Tooth	1	2.1																		G		
				Y																		
233 Tooth	1	2.1.1																		G		
				Y																		
234 Femur	2	1.1																		G		
234 Femur	2	2.1.1																		G		
234 Humerus	2	1.2																		G	C	C
																					.C	C
234 Humerus	2	2.1																		G		
				C																		
234 Humerus	2	2.1.1																		G		
				C																		
234 Tooth	3	2.1																		G		
234 Tooth	3	1.1																		G		

Burial 56	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
262 Male; 263 Female	Bone Rating	Bone Prep/ PCR																				
262 Femur	1	1.4																		G		
262 Femur	1	1.1.1																		G	C	C
																					C	C
262 Petrous	1	1.4																		G		
262 Radius	2	2.2																				
262 Tooth	2	1.1.1															G			G	C	C
																	G			G	C	C
262 Tooth	2	2.2																		G		
263 Femur	2	1.4				Y												C	C	G		
						T																
263 Humerus	3	1.4																		G		
263 Petrous	1	1.4																	Y	G		
263 Petrous	1	2.2																				

Burial 184	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
710 Male; 711 Female	Bone Rating	Bone Prep/ PCR																				
711 Tibia	3	2.2.1									C									G		
			C								C											
711 Tooth	1	2.2.1																		G		

Burial 198	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
768 Female; 772 Child	Bone Rating	Bone Prep/ PCR																				
768 Clavicle	2	1.2										C			A							
768 Clavicle	2	2.7		C																		
768 Clavicle	2	2.7.1																		G		
				C																		
768 Femur	3	2.7																				
768 Femur	3	2.7.1																		G		
768 Femur	3	2.7.1																		G		
768 Humerus	2	1.1																		G	C	C
768 Humerus	2	2.7																		G		
768 Humerus	2	2.7.1																		G		
							C															
768 Metacarpal	1	1.2																		G		
768 Metacarpal	1	1.1																		G	C	C
					T						C									G	C	C
768 Metacarpal	1	1.5	C	C				G														
768 Tooth	3	2.7.1																		G		

Burial 198	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
768 Female; 772 Child	Bone Rating	Bone Prep/ PCR																				
772 Femur	3	2.7.1																				
772 Femur	3	2.7.1																		G		
772 Petrous	1	1.2		C																G		
				C																		
772 Petrous	1	1.5		C																G		
				C																		
772 Petrous	1	2.7																		G		
				C																		
772 Petrous	1	2.7.1		C																		
772 Petrous	1	2.7.1																		G		
772 Tooth	1	1.5		C																		
772 Tooth	1	2.7																		G		
772 Tooth	1	2.7.1		C																		

Burial 280	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
1084 Female; 1085 Male	Bone Rating	Bone Prep/ PCR																				
1084 Femur	2	2.1							G		C		G	C	A					G		
									G		C		G									
1084 Scapulae	4	2.1																				
1084 Ulna	3	2.1									C		G	C	A					G		
1085 Femur	1	2.1								T	C									G		
										T	C											
1085 Petrous	1	2.1							G		C		G	Y	R					G		
				Y					G													

Burial 300	CRS		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
1164 Male; 1165 Female	Bone Rating	Bone Prep/ PCR																				
1164 Petrous	1	1.1																				
																				G		
Burial 375	**CRS**		146 T	152 T	162 C	167 C	175 A	181 A	189 A	194 C	195 T	199 T	200 A	204 T	207 G	210 A	235 A	246 T	250 T	263 A	309.1 ~	315.1 ~
1456 Male; 1457 Male	Bone Rating	Bone Prep/ PCR																				
1456 Femur	3	2.1							G		C			C	A					G		
									G		C			C	A							
1456 Petrous	1	1.1																		G		
1456 Petrous	1	2.1																		G		
1456 Radius	4	2.1																		G		
1457 Humerus	3	2.1							G		C			C	A					G		
									G		C			C	A							

APPENDIX IV

Shared Polymorphic Sites

Burial Number	16215 A	16223 C	16224 T	16234 C	16239 C	16242 C	16251 C	16256 C	16259 C	16260 C	16261 C	16262 C	16270 C	16271 T	16276 T	16278 C	16284 A	16285 A	16286 C	16290 C
46												Y								
56																				
184		Y																		Y
198																				
259																				
280		Y																		
300								Y					Y							
375													Y							

Burial Number	16291 C	16292 C	16294 C	16295 C	16296 C	16303 G	16304 T	16309 A	16311 T	16318 A	16319 G	16327 C	16340 A	16342 T	16352 C	16354 C	16356 T	16357 T	16358 C	16362 T
46																				
56																				
184									Y		R									
198																				
259																				
280																				
300									Y											
375		Y																		

Burial Number	16364 C	16366 C	16371 A	16375 C	16376 C	16378 C	16384 G
46							
56							
184							
198							
259							
280							
300							
375							

Burial Number	146	152	162	167	175	181	189	194	195	199	200	204	207	210	235	246	250	263	309.1	315.1
	T	T	C	C	A	A	A	C	T	T	A	T	G	A	A	T	T	A	~	~
46																		G		
56																		G		
198		C																G		
280							G		C		G	Y	R					G		
375							G		C			C	A					G		

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