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Adriane Michelle Scola
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To the Graduate Council:

I am submitting herewith a thesis written by Adriane Michelle Scola entitled "Molecular Anthropology and the Punta Lobos Assemblage: DNA-Based Sex-Typing of Juveniles from Ancient Hair Samples." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts, with a major in Anthropology.

Lyle Konigsberg, Major Professor

We have read this thesis and recommend its acceptance:

Richard Jantz, Karla Matteson

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Thesis
2004
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Molecular Anthropology and the Punta Lobos Assemblage: DNA–Based Sex-Typing of Juveniles From Ancient Hair Samples

A Thesis
Presented for the
Master of Arts
Degree
The University of Tennessee, Knoxville

Adriane Michelle Scola
December 2004

ACKNOWLEGEMENTS

I would like to thank Dr. John Verano and Marla Toyne at Tulane University for the opportunity to work the Punta Lobos samples, my graduate committee members, Dr. Lyle Konigsberg and Dr. Richard Jantz, for their thoughtful critique and discussions throughout the course of this research, and especially Dr. Karla Matteson for her gracious donation of time, space, and knowledge- without which none of this would have been possible.

ABSTRACT

The recent proliferation of techniques suitable for DNA recovery in ancient samples has prompted anthropological researchers to explore molecular-based investigations of human remains from archaeological contexts. This study demonstrates the utility of ancient DNA analysis to strengthen a site-specific demographic profile from the Punta Lobos assemblage. DNA extraction via a silica-based extraction technique from juvenile hair bulb samples and further DNA amplification via high cycle-number PCR was undertaken to genetically type individual sex. Morphologically indeterminate subadult samples were typed as biological males when amplification was successful. The inclusion of preliminary amplification results from a mitochondrial DNA marker suggests that DNA concentration in the Punta Lobos hair samples is sufficient for an extended tri-system genetic analysis.

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Chapter 1

Introduction

Molecular genetic technology has given anthropologists novel analytical tools applicable to the study of human variation, evolution, and population structure. The advent of polymerase chain reaction (PCR) in particular has revolutionized and expanded the analytical repertoire in many biological fields, and opened the anthropological world to new interdisciplinary approaches (Kaestle and Horsburgh 2002). Beyond the use of classical genetic markers, ancient DNA research "allowed, for the first time, a *direct* incorporation of a temporal component in molecular analyses. Anthropologists were quick to adopt these new techniques for the production of previously unobtainable data, which they have applied to the traditional suite of anthropological research problems" (Kaestle and Horsburgh 2002:93).

Historical applications revolved around the successful extraction of endogenous DNA from ancient samples and the nature of DNA degradation over time (Handt et al. 1994; Handt et al. 1996; Lindahl 1993; Tuross 1994). The mid-nineties showed a proliferation of reliable extractions and sequence analyses which prompted researchers to explore questions regarding human variability, population structure, and human antiquity from archaeological contexts (Baker 2001; Doran 2002; Krings et al. 1997; Merriwether et al. 1994; Stone and Stoneking 1998). Recent studies have utilized ancient DNA analyses to provide a comprehensive picture of phenotypic, demographic, and genetic structure (Dudar

et al. 2003; Garcia-Bour et al. 2004; Gerstenberger et al. 1999; Keyser-Tracqui et al. 2003; Ricaut et al. 2004).

The following research aims to investigate the genetic component of a larger anthropological endeavor involving a pre-Contact skeletal assemblage from coastal Peru. Site conditions were such that large quantities of hair remained attached to many intact skeletons. These hair samples provide an opportunity for molecular studies that precludes destructive analysis of skeletal elements. The goal of this project is to successfully extract and amplify both nuclear and mitochondrial DNA from a small subset of this population in hopes of laying the groundwork for a future comprehensive genetic analysis.

The Archaeological Site

As part of an environmental impact study in 1997, Peruvian archaeologists uncovered human remains at the coastal Peruvian site of Punta Lobos, several miles from the modern-day fishing village of Huarmey. Further investigation yielded remains of roughly 200 individuals, with nearly 107 intact skeletons. In 2003, Dr. John Verano of Tulane University joined the excavation team headed by Hector Walde (Peru National Institute of Culture). Intrigued by early claims of mass ritual sacrifice, Verano and colleagues performed morphological assessments for sex and age and confirmed a population of mostly young men in their early 20's – 30's with some subadults and elderly men. Site preservation was excellent due to the dry, sandy, and relatively undisturbed environment such that textiles, hair, and some soft tissue remained. Each body

exhibited evidence of having been blindfolded, limbs bound from behind, and trauma marks across the cervical vertebrae and clavicle- an indication that slitting of the throat was the cause of death. Textile analysis revealed native manufacture consistent with the Chimu people, a pre-Incan society lasting from ca 1100-1476 AD.

The hair samples were collected by Dr. Verano during the 2003 field season at the Punta Lobos site and taken back to his lab at Tulane University. Samples from 74 individuals were prepared by J. Marla Toyne and sent to the Molecular Genetics lab at the University of Tennessee Medical Center, where all subsequent DNA analyses were carried out.

Andean Archaeology

The Andean Culture Area

The Andean cultural region of western South America encompasses the coastal lowlands, Andean highlands and the Amazonian forest margins extending southward from Columbia including Ecuador, Peru, western Argentina, Bolivia, and northern Chile (Burger 1995; Moseley 2001; Lanning 1967; Lumbreras 1974). In 1532, Francisco Pizarro and a small group of Spanish mercenaries encountered the vast Incan Kingdom of Tahuantinsuyu, or the "Land of the Four Corners." The wealthy metropolitan center of Cuzco was regarded as the imperial capital of the empire. At the time of Spanish conquest, the Incan empire was comprised of loosely consolidated regional polities and chiefdoms. Intergroup hostilities and political separatism increased dramatically after the

death of Wayna Copac, the last Pre-Hispanic ruler of Tahuantinsuyu. A small pox pandemic spread rapidly from Mexico prior to Pizarro's arrival; high mortality rates coupled with the social and political unrest created in its wake ravaged the continent leaving its citizens with no united defense against imperial enterprise. Pizarro's small contingency was able to claim control of Cuzco, imprison the Incan emperor Atawallpa, and bring the whole of the empire under the control of the Castilian Crown. Spanish chroniclers reproduced Incan rhetoric suggesting that before the rise of Tahuantinsuyu, the Peruvian landscape was dotted with "uncivilized" and scattered tribes, and little attention was paid to documenting the existence of pre-Incan cultures (Lumbreras 1974:179).

Environmental Setting

The Punta Lobos site is located in the Huarmey river valley of the Peruvian north-central coast. While the Pacific waters that border the Andean coastal shores are among the most productive marine environments on earth, the coastal region stretching inland towards the foothills of the Cordillera is characterized by harsh desert extremes. Moseley (2001; 1975) and Burger (1995) note that complex interactions between topographical features (namely the close proximity and steep vertical grade of the Andean mountain range, Pacific trade winds, the Corioles force, and four ocean currents) produce a stable arid environment. The Andean coastal desert maintains an average and uniform temperature of between 18 and 22 degrees C, and experiences only two minimally contrasting seasons of sun and fog with very little annual rainfall

(Moseley 1975:8). The juxtaposed, though highly stable, balance between the productive ocean and barren coast is thrown into flux during irregularly recurring periods when trade wind and ocean current patterns are disrupted. Known as El Niño, these events trigger mild to severe climatic effects that can ultimately devastate marine and terrestrial biomass. Andean civilizations, both past and present, have long endured these variable periods of pronounced environmental oscillations. These processes have contributed to the state of the archaeological record and should be considered when investigating Andean archaeological sites.

Foundations of Andean Archaeology

Moseley (1992) gives a brief introduction highlighting the history of Andean archaeology. He suggests that Andean archaeology takes root in the vast mining and looting enterprises quickly orchestrated by the Spanish after South American conquest. Monument quarrying revealed large stores of ancient ceramics, textiles, jewelry, and other artifacts that were subsequently housed in museums and private collections with no record of geographic or monument association. There were some individuals, however, that espoused a more "preservationist" attitude. As early as the 16th century, Piedro Cieza de León sought to document and safeguard Andean antiquities. During the late 18th century, Martínez de Compañón, the Bishop of Trujillo in the Moche Valley, "commissioned the mapping of ancient monuments, including Chan Chan and

the Huaca del Sol (Pyramid of the Sun), probably the largest mud brick mound ever erected in the continent" (Moseley 1992:17).

Alexander von Humbolt is credited with the first "systematic overview of the monuments of New World civilizations." (Moseley 1992:17) His 1814 publication *Vues de Cordillères et Monuments des Peuples Indigènes de l'Amérique*, legitimized antiquarian recording and description as a scholarly enterprise. In 1851, Mariano Eduardo de Rivero, in collaboration with J.J. Diego de Tschudi, published an archaeological description of the ruins of the central Andes. Solely a descriptive endeavor, it wasn't until E.G. Squier's *Peru: Incidents of Travel and Exploration in the Land of the Incas*, that the notion of time depth was brought to bear on Andean ruins. During his travels in the Lake Titicaca Basin in the 1870's, Squire recognized that different types of *chullpas* (masonry built towers) could be grouped as reflecting different construction periods. His insights influenced future researchers to classify Andean studies as aspects of Americanist vs. Hispanic history (Moseley 1992:18).

From 1876-77, Alphons Stubel, an influential German investigator, began a detailed mapping and descriptive study of the ancient ruins at Tiwanaku. Upon his return to Dresden, Germany, Stubel elicited aid from Max Uhle (a young museum worker pursuing studies on New World languages and Andean artifacts). Together, they produced *Die Ruinenstatten von Tihuanaco* in 1892. This seminal work "defined the art style of Tiwanaku, chronologically important because the Inca reported the metropolis to be in ruins when they first entered the region in the fourteenth century" (Moseley 1992:18). Uhle (whose work laid

the foundations for the "Berkeley school" of Andean studies) later set out to Peru and excavated the religious center of Pachacamac. After extensive excavations of the graves and tombs, Uhle documented distinctive artifact styles in relation to stratigraphic layers, thus providing evidence of pre-Incan Andean societies. The material remains from Uhle's excavations were analyzed by A.L. Kroeber and his students at the University of California at Berkley during the 1920' and 30's. Their findings echoed Uhle's interpretation that Tiwanaku and its associated style represented the center of an early empire that had conquered much of the Cordillera (and associated local styles) by force. During the 1950's, John H. Rowe and his students revised Kroeber's assessments of Uhle's materials and suggested that Andean evolutionary history reflected "periods of unity interspersed with periods of intermediate development" (Moseley 1992:18).

A second influential school, the "Peruvian school", was founded by native researcher Julio C. Tello. Tello served as director of the national archaeological museum in Lima. Internationally respected, he drew young students to Peru and created a thriving community for archaeological investigation. Tello's accomplishments include an interpretation of Chavin de Huantar (a richly decorated ceremonial platform) that led him to believe Chavin styles and iconography were rooted in early cultures of the Amazonian forests. Luis E. Valcarcel succeeded Tello as director of the national museum in 1930. His approach to Andean prehistory sought to integrate the archaeological ruins and materials within the context of native traditions and cultural continuity. "For decades he worked to actively promote ethnology, ethnohistory, and archaeology

to make the wholeness of the Andean past and present intelligible." (Moseley 1992:20).

Although many different chronologies have been developed, modified, and discarded by Andean researchers, John H. Rowe's periodization of Andean prehistory is still considered to be a valid chronological scheme because it is based on absolute time periods (Isbell 1997:18; Rowe 1970). The following discussion of the Late Intermediate Period is derived from Rowe's time scale.

The Late Intermediate Period and Chimor

The Late Intermediate Period of the Peruvian north coast is characterized by the rise and fall of the kingdom of Chimor. State formation occurred around 1000 AD and continued until 1470 AD when Incan conquest absorbed the coastal Chimu polities. Reaching from the Lambayeque to Rimac river valley (although there is some debate over the northern and southern boundaries), the 1000km long empire was governed from the large metropolis of Chan Chan in the Moche river valley. The state oversaw the expansion of pre-existing regional irrigation systems into large scale hydraulic networks that united smaller agricultural communities. Ethnohistorical and archaeological excavations suggest Chimor was the second-largest native Andean state (Lanning 1976; Lumbreras 1974; Moseley 2001).

Oral accounts revealed the formative lore of two coastal cities, each with its own dynastic legacy. One concerns the founding of Chan Chan by Taycanamu, while the other describes the formation of the Lambayeque region

by Naymlap, which was later absorbed by the larger Taycanamu dynasty (Lumbreras 1974; Moseley 2001).

According to legend, Naymlap arrived to the Lambayeque valley from the sea with a flotilla of balsa boats, accompanied by a large entourage including his wife, a greenstone idol, and 40 officials. Archaeological information suggests the region prospered after the demise of the Moche in Pampa Grande, roughly around 700-900 AD (Moseley 2001). The lord Naymlap had a senior son, Cium, who in turn had 12 sons that established 12 new settlements. The dynasty includes 12 rulers beginning with Naymlap and ending with Fempellec, who was tempted by a sorceress to move the stone idol and was punished with "30 days" of disastrous rains, floods, famine and disease. There is an unknown period of time between the demise of Fempellec and Chimor conquest from the south (Lumbreras 1974; Moseley 2001).

Although less embellished, the Taycanamu lore parallels Nayamlap's in that the founding ruler arrived from the sea by balsa boat. After settling in the Moche valley, Taycanamu's two sons expand the frontier to include the area between the Rio Santa and Rio Jequetepeque. The dynasty includes 5 to 7 pre-Incan rulers culminating in the great reign of Minchancaman, who was later captured by the Incas and brought to their capital (Lumbreras 1974; Moseley 2001).

Extensive archaeological excavations have been carried out at the Chimu imperial capital at Chan Chan (Lumbreras 1974, Moseley 2001). The city covers an area of a about six miles and is comprised of at least ten walled

compounds built successively as the urban population expanded towards the later part of the Late Intermediate Period. The compounds housed "streets, platforms, houses, reservoirs, platform mounds, and other public structures...they averaged about twenty acres in area, and the largest of them covered an area of some forty acres. Cemeteries, garden plots, and smaller residential units were distributed within and between the compounds" (Lanning 1967:153). Distinctive U-shaped buildings are a dominant architectural feature throughout Chan Chan and the smaller Chimu cities. Parsons and Hastings (1988) note that these buildings must have played a significant role "in controlling access to storerooms," and "together with their offertory caches of exotic artifacts and sacrificed humans and camelids, appear to manifest a distinctly state-level component of Chimu polity which can be traced widely in space, and whose distribution can be used to approximate the regional extent of Chimu imperial control" (Parsons and Hastings 1988:193).

Several smaller cities have been discovered in the Chimu domain which reflect the stylized architectural layout seen at Chan Chan. These include the more modern administrative centers of Farfan and Chiquitoy Viejo. Other communities within the region such as Pacatnamu, Jetepeque, Purgatorio, Chotuna, Apurlé, and Pampa Grande in the Lambeyeque drainage, suggest evidence of occupation stretching back to the Middle Horizon and even the Early Intermediate Period, and have little architectural resemblance to Chan Chan. The valleys of the north-central coast including Viru to Huarmey were likely home to more rural agricultural and fishing villages, of little concern to the governing body

and urban elite. Some researchers have suggested a distinct population decline for the southern river valleys, as opposed to general population stability in the larger communities to the north (Parsons and Hastings 1988).

Mortuary Practice and Sacrifice

In 1991, a symposium focused on Andean mortuary practices was held at Dumbarton Oaks in Washington DC. In *Tombs for the Living: Andean Mortuary Practices* (Dillehay 1995), researchers were invited to explore various biological, social, and economic aspects of Andean ritual sacrifice, burial patterns, and treatment of victims of war. John Verano contributed a paper entitled *Where Do They Rest? The Treatment of Human Offerings and Trophies in Ancient Peru*, wherein he investigates the nature of human remains found at the ceremonial center of Pacatnamu. Dated to the Late Intermediate Period, excavations at the front ceremonial entrance revealed three distinct strata of depositional events represented by 14 young male sacrifices. Each body exhibited extensive mutilation and the occurrence of insect remains suggested the bodies lay on the surface for some time. Biometric analyses were performed to compare morphologic features with contemporary Pacatnamu burial populations. Although these comparisons were not informative, isotopic ratios from bone collagen showed different ratios between the sacrificial and burial samples, suggesting the sacrificial victims relied on different dietary staples. This difference in diet history may reflect a difference in geographic origin, indicating the victims at the ceremonial entrance may have been displayed and mutilated war prisoners.

Verano describes the close association between the Moche and Chimu iconographic representations of prisoner sacrifice and notes the concordance between the pathologic signatures left on the skeletal remains and the iconographic and ethnohistoric description found in historic documentation.

The mass execution at Punta Lobos represents a significant historical event in pre-Hispanic coastal Peru. A thorough archaeological investigation similar to that described for the Pacatnamu site is integral to cultural and demographic inference. The genetic component, however, will offer insight into the relationships between individuals that can not be inferred by morphology alone.

Technical Aspects

DNA

DNA extracted from archaeological specimens is often highly fragmented due to accumulated chemical alterations over time (Brown 2001; O'Rourke et al. 2000). Structural damage from such processes as hydrolysis and oxidation severely hampers amplification efficiency, such that DNA fragments greater than 300 – 500 bp in length are rarely recovered (Handt et al. 1994; Lindahl 1993; O'Rourke et al. 2000; Poinar and Stankiewics 1999; Tuross 1994). DNA degradation, though highly influenced by macro-environmental processes, is also dependent on the archaeological micro-environment. Researchers have reported differential success rates when extracting from same-site individuals, and the relative state of soft or hard-tissue preservation does not necessarily

correlate to successful endogenous DNA amplification (O'Rourke et al. 2000; Vernesi et al. 1999).

When performing aDNA analysis on archaeological specimens, mtDNA has historically been the genetic system of choice due to its high cellular copy number. Many researchers have utilized mitochondrial haplogroup markers located on the mtDNA coding region, as well as analyzed sequence information from amplified fragments of the non-coding or hyper-variable region to investigate maternally transmitted haplotypes (Baker 2001; Carlyle 2000; Krings et al. 1997, Merriwether et al. 1994; O'Rourke et al. 2000; Stone and Stoneking 1998). Although mitochondrial DNA remains a useful marker to identify genetic relationships, heteroplasmy renders the molecules to a polyploid state, thus complicating subsequent sequence analysis (Baker 2001).

The Y-chromosome has become increasingly useful in light of recent exploration into the variety of Y-specific loci and techniques that allow for greater sensitivity (Bertranpetit 2000; Carvalho-Silva et al. 1999; Hammer and Zegura 2002; Jorde et al. 2000; Kayser et al. 2001; Kayser et al. 2000; Keyser-Tracqui et al. 2003; de Knijff et al. 2000; de Knijff et al. 1997; Schultes et al. 1999; Torroni et al. 1994). The Y chromosome exists as a single locus within the autosomal system; its haploid nature and non-recombining portion (NRY) allow for paternal lineage determination and provide an attractive complement to the maternally-inherited mtDNA system (Bianchi et al. 1998; Gerstenberger et al. 1999; de Knijff et al. 1997). Although the NRY of the Y chromosome exhibits the lowest level of polymorphism, it carries several classes of genetic markers including

microsatellites (STRs), minisatellites, single nucleotide polymorphisms (SNPs), and insertion/deletion polymorphisms (indels). Each marker class is defined by specific mutation rates and patterning, such that certain markers are better suited toward understanding genetic relationships between individuals, while others better reflect evolutionary trajectory and genetic differentiation between groups (Hammer and Zegura 2002).

Y STRs are useful markers for determining paternal relationships between closely related individuals, but offer little insight into population history. A suite of STRs are often used to construct Y chromosome haplotypes. Although haplotypes will give a measure of genetic diversity between male lineages, the level of genetic relatedness may be masked by the rapid rate of STR differentiation (Bosch et al. 1999; Hammer and Zegura 2002). Tandem repeats can fluctuate by one unit (either up or down) in a single generation, such that a father-son pair may have unique haplotypes- obscuring a true biological relationship. A second caution, when relying solely on haplotype analysis, concerns the patterning of haplotype differentiation by haplogroup background. "The recurrent nature of microsatellite mutation implies that haplotypes that are identical by state may not be identical by descent. In the same way that microsatellite haplotypes in each haplogroup will differentiate with time from the common ancestor from which they derive, haplotypes belonging to different haplogroups could occasionally converge with time as well" (Bosch et al., 1999:1633). It would be reasonable to assume, in light of the above statement, that identical haplotypes between individuals might not reflect common ancestry.

Single nucleotide polymorphisms (often biallelic in nature) are slower-mutating and have a lower occurrence of back and convergent mutation (Hammer and Zegura 2002; Kayser et al. 2000: de Knijff 2000). These markers represent more ancient differentiation events that dictate Y chromosome haplogroup characterization.

Recent studies of archaeological populations have fostered a three-way approach to investigating genetic relationships. The inclusion of autosomal, mtDNA, and Y chromosome markers offers 1) greater time depth when evaluating population settlement and migration patterns, 2) direct associations between closely related individuals, and 3) inference into paternal and maternal lineages allowing assessment of social inheritance patterns. For instance, in 2003, Keyser-Tracqui et al. published an aDNA study of a 2,000 year-old necropolis in the Egyin Gol Valley of Mongolia. The researchers performed a three-way analysis between autosomal, mtDNA, and Y chromosome genetic markers from skeletal elements to tease out genetic relationships between individuals. Excavations of the burial site yielded 99 individuals from 84 previously undisturbed graves. Skeletal preservation, as assessed by mineral/organic composition, was comparable to modern remains. DNA samples were extracted from bone fragments using a combination phenol/chloroform and silica-based method. Morphological sex determinations were corroborated by amelogenin typing of the sex chromosomes.

The authors deduced biological relationships between individuals using autosomal STR markers and constructed multi-allelic DNA profiles for 49

individuals. Comparisons between profiles were thought to reflect direct parentage if "a pair share an allele at each of the 9 loci tested; in this manner, a total of 9 pairs were identified as representing possible child-parent relationships" (Keyser-Tracqui et al., 2003:257). Y STR and mtDNA HV1 sequence analyses were used to verify the results of the autosomal markers. In most instances, these markers supported the close genetic relationships as defined by autosomal comparisons, but one discrepancy was noted between individuals that shared autosomal loci- neither shared the same Y haplotype or mtDNA sequence. Y STR analysis also revealed a spatial clustering consisting solely of males from a single patrilineage.

Other studies that have fostered a multi-system genetic approach include Gerstenberger et al.'s (2002) determination of residence patterns through maternal and paternal lineage determination for skeletal remains from 5^{th}-8^{th} century AD cemetery interments in Weingarten, Germany, Dudar et al.'s (2003) kinship system reconstruction of an Upper Canadian pioneer cemetery, and lastly Ricaut et al.'s (2004) investigation of two 2500 year old skeletal remains in the Sebystei Valley to deduce the genetic relationship between the two individuals and trace their genetic origins as reflections of Scytho-Siberian population movements.

Hair Studies

Researchers interested in ancient DNA recovery and analysis have employed various extraction methods to several types of preserved human tissues,

including bone, teeth, skin, and hair (Lin et al. 1995; Merriwether et al. 1994; Schultes et al. 1999; Vernesi et al 1999). Although archaeological assemblages tend to be dominated by osteologic remains, optimal climatic conditions, particularly those environments that promote a rapid rate of desiccation, may yield soft tissue specimens containing amplifiable endogenous DNA. An example of DNA extraction from well-preserved soft tissues includes the Doran et al. (2002) analysis of brain tissue from the Windover site peat bogs.

Several researchers have had success with DNA extraction from hair samples in both forensic and ancient contexts. Wilson et al. (1995) first provided an extraction protocol for mtDNA from human hair shafts. Baker (2001) and Baker et al. (2001) developed the silica/guanidine thiocyanate extraction method used in the present study to successfully generate mtDNA haplogroups and control region sequence data from forensic, historic, and ancient hair shaft samples. Lin et al. (1995) amplified nuclear DNA from 1300 year old mummies (hair, bone, and skin samples) from the Taklamakan desert to provide sex determinations.

Due to the unique and abundant preservation of hair, the Punta Lobos skeletal assemblage presents a rare opportunity for molecular studies that precludes destructive analysis of skeletal elements. In most instances, hair remained attached to patches of mummified scalp thus retaining the hair bulb or root- an ideal specimen for DNA extraction. During life, the bulb is an area of actively dividing epithelial cells (Baker 2001; Wilson et al. 1995) and, as opposed to the hair shaft, is a dense package of genetic information that does not contain

melanin. Since the pigment granules act as an inhibitor in the PCR reaction, hair shaft extractions must be diluted down until a balance between inhibitor content and DNA concentration allows for amplification (Wilson et al. 1995). Bulb extractions, however, provide higher DNA content and ultimately less enzymatic inhibitors.

The following study presents a preliminary molecular investigation for a subset of the Punta Lobos assemblage. Ancient DNA extraction was attempted for hair bulb samples from subadult individuals to determine 1) the genetic sex for each morphologically indeterminate subadult, and 2) the potential for a comprehensive genetic analysis in light of the relative state of nuclear and mitochondrial DNA concentration and degradation, as defined by PCR amplification efficiency.

Chapter 2

Materials and Methods

Several methods have been employed in the extraction of DNA from ancient samples including organic-based, silica-based, and combination techniques. Previous researchers in the University of Tennessee Medical Genetics laboratory have had much success with a silica/guanidine thiocyanate method as described in Boom et al. (1990) by Baker (2001). The following method for ancient DNA extraction from hair samples has been adapted from Baker (2001).

Contamination Precautions

Due to the highly degraded and fragmentary nature of ancient DNA samples, and the sensitive DNA binding capacity of silica, the preferential amplification of modern contaminating DNA is a serious concern. Several precautionary measures were employed to minimize the risks of modern contamination.

Disposable surgical masks, caps, gowns, sleeves, and latex gloves were worn at all times except during post-PCR analyses. Gloves were changed frequently during DNA extraction, isolation, and PCR preparation. All equipment, surfaces, and glassware were washed with 10% bleach, rinsed with 100% ethanol, and UV irradiated for 24 hours prior to use. Glass tissue homogenizers (Kontes Glass, Vineland, NJ) were soaked in 10% bleach for 2 hours, washed in

100% ethanol, rinsed with molecular biology grade water (hereafter referred to as MBG water), and UV irradiated for 24 hours. All solutions were made with MBG water, and all reagents were separated into single-use aliquots upon arrival. Only preloaded, aerosol-resistant, filtered pipette tips were used.

DNA extraction was carried out under a sterile, UV irradiated, positive pressure laminar flow hood. All PCR preparation took place in a separate room, inside a UV irradiated Clone Zone box (USA/Scientific Inc., Ocala, CA). Post PCR analyses were performed in two separate locations. Equipment in all areas was solely dedicated to a single purpose and equipment was never transferred between areas.

To insure that buffers and reagents were sterile prior to use, a series of blank extractions (the entire extraction protocol was carried out without the addition of any sample material) were analyzed. Sample material was added only after extraction blanks show no evidence of contamination. An extraction blank was included with all sample material extraction attempts, a PCR blank (all PCR reagents and with no DNA extract) was also included to monitor contamination during PCR preparation.

Extraction Buffer Preparation

Extraction buffer [10 M guanidine thiocyanate (GuSCN), .01 M Tris-HCl (pH 6.4), .2 M EDTA (pH 8.0), 1.3% Triton X-100] was prepared as described in Baker (2001). In a sterile, pretreated 50ml conical tube, eight grams of guanidine thiocyanate (GuSCN) was dissolved in 6.67ml of 0.1M Tris-hydrochloride (pH

6.4). Dissolution of the GuSCN was facilitated by swirling the tube periodically in a 60 degree C water bath. After the guanidine had completely dissolved, 1.47ml of 0.2M EDTA (pH 8.0) was added and inverted to mix. 1.67ul of Triton X-100 was then added to the tube. Lastly, 1.5 grams of silica (Sigma-Aldrich Co., St. Louis, MO) were added and vortexed thoroughly to bind any contaminating DNA. The buffer was then centrifuged at 2,000 x g for 15 minutes and the supernatant decanted into a pre-treated 50ml conical tube. The purified buffer was divided into single-use aliquots (~200ul) and stored at room temperature, in the dark, for no longer than three weeks.

Hair Selection

In instances where hair remained attached to pieces of scalp, three hairs were plucked with sterile tweezers away from any scalp material under a pre-treated laminar flow hood with positive pressure. Approximately 1cm of hair was cut with sterile surgical scissors from the root-end of each hair. In cases where no scalp remained, three loose hairs were selected and 1cm of each root-end were cut. Each hair was gently washed with 100% ethanol, followed by rinsing with MBG water. For each individual, 3cm of hair was placed in a .2ml tissue homogenizer (Kontes Glass, Vineland, NJ) containing 100ul of extraction buffer. After grinding the hair samples to facilitate cellular breakdown, the homogenate was transferred to a UV irradiated 1ml microcentrifuge tube. 100ul of extraction buffer was added to the homogenizer to rinse any remaining material from the

glass and the buffer was then pooled with the sample homogenate. The sample tube was left overnight (12-24hr) in a 60 degree C incubator with slight agitation.

DNA Isolation

A GENECLEAN kit (Bio 101, Vista, CA) was used for DNA isolation. Three volumes of sodium iodide (NaI) and 5ul of GLASSMILK® were added to the sample which was then placed in a 57 degree C incubator for 15 minutes, with slight agitation. The sample was centrifuged at 12,000 X g for 5 minutes to pellet the DNA-bound silica beads. The supernatant was discarded and the pellet resuspended in 500ul of New Wash® solution. Following two rounds of New Wash centrifugation (5 minutes) and resuspension, the pellet was dried at room temperature to evaporate any remaining ethanol and then eluted in 30ul of MBG water. After a final incubation for 10 minutes at 56 degree C, the sample was centrifuged for 5 minutes and the supernatant was added to a UV irradiated 1ml microcentrifuge tube and stored at -80 degree C until amplification.

Sex Typing of Subadults

To strengthen the demographic assessments of the Punta Lobos assemblage, sex determination from subadult hair samples was analyzed via the sex differentiated amelogenin locus. Archaeological interpretations are centered around an all-male assemblage composition, but morphological sex determination for pre-pubescent subadults is difficult to assess with any certainty (Faerman et al. 1995; Kaestle and Horsburgh 2002; Vernasi et al. 1999). The

amelogenin PCR markers isolate a small 106-112bp fragment of DNA on both the X and Y chromosomes, with a 6 nucleotide difference between the two (Brown 2001; Gerstenberger et al. 1999; Lassen et al. 1996; Vernesi et al. 1999). The resultant bands can be distinguished when resolved on an ethidium-bromide stained polyacrylamide gel. Males will show two bands of slight size difference while females will show a single band.

Successful amplification using the amelogenin markers will provide insight into the nature and extent of DNA degradation, as well as give a measure of autosomal DNA concentration. If the amelogenin markers repeatedly yield consistent results, we can assume that DNA content and relative state of degradation is sufficient for amplification of fragments greater than 100bp. Prior researchers have experienced an "allelic dropout" phenomenon associated with the 106/112bp amelogenin system, where the smaller X- specific sequence was preferentially amplified, yielding a false female sex identification (reported in Brown 2001 from Lassen et al. 1996). Multiple PCR runs off at least 2 different extractions should expose any anomalous results.

For the amelogenin markers (Table 2-1), 2ul of sample DNA was added to a PCR reaction mixture containing 10 mM AmpliTaq Gold enzyme buffer (PCR Buffer II, PE Applied Biosystems, Foster, CA), 1.7 mM MgCl2, 200 uM each dNTP, 10 pM each primer [Amelogenin F, R], and 2 units of AmpliTaq Gold (PE Applied Biosystems, Foster, CA), and brought to a 50ul total reaction volume with MBG water. Amplification was carried out in a Perkin Elmer Cetus programmable thermocycler. Cycling began with a 94 degree C denaturation/enzyme activation

Table 2-1. Sequence Data for Amelogenin F/R Primers

Primer	Size Range	Sequence
Amelogenin F	106/112	5' CCC TGG GCT CTG TAA AGA ATA GTG
Amelogenin R		5' ATC AGA GCT TAA ACT GGG AAG CTG

step for 10 minutes, followed by a 60 cycle repetition of 45 seconds at 94 degree C, 1 minute at 53 degree C, and 1 minute at 72 degree C, and ended with a final 72 degree C extension period for 7 minutes.

As a safeguard against modern contamination not detected in reaction blanks, amplification of a larger 320bp fragment found on chromosome 6 using in-house primers Hemo1 and Hemo2 (Table 2-2), was undertaken for ancient samples exhibiting amplification signal. 2ul of sample DNA was added to a PCR reaction mixture containing 10 mM AmpliTaq Gold enzyme buffer, 1.7 mM MgCl2, 200 uM each dNTP, 10 pM each primer [Hemo 1 and 2], and 2 units of AmpliTaq Gold, and brought to a 50ul total reaction volume with MBG water. Cycling began with a 94 degree C denaturation/enzyme activation step for 10 minutes, followed by a 60 cycle repetition of 1 minute at 94 degree C, 1 minute at 55 degree C, and 90 seconds at 72 degree C, culminating with a final 72 degree C extension period for 7 minutes.

Ancient samples yielding the strongest amplification signal with the amelogenin primers were selected for amplification with the mtDNA Haplogroup B markers (Table 2-3). 2ul of sample DNA was added to a PCR reaction mixture containing 10mM Amplitaq Gold enzyme buffer, 1.7mM MgCl2, 200uM each dNTP, 10pM each primer [HapB F and R], and 2 units Amplitaq Gold PCR, and brought to a total reaction volume of 50ul. PCR conditions are as follows: 10 minutes at 94 degrees C, 60 cycles of 1 minute 94 degree C, 1minute 52 degree C, and 1minute 72 degree C, followed by a final extension at 72 degree C for 7 minutes.

Table 2-2. Sequence Data for Hemo1/2 Primers

Primer	Size Range	Sequence
Hemo1	320	5' CCT AAA GAC GTA TTG CCC AAT
Hemo2		5' GAC TAG GGT GCC AGA CGG

Table 2-3. Sequence Data for Haplogroup B mtDNA (HapB F/R) Primers

Primer	Size Range	Sequence
HapB F	119	5' ACA GTT TCA TGC CCA TCG TC
HapB R		5' ATG CTA AGT TAG CTT TAC AG

PCR products (10ul) were electrophoresed on 10% TBE polyacrylamide slab gel, stained with ethidium bromide, then visualized on a UV transilluminator. All experiments contained two negative controls, as described previously, to assess possible contamination at different stages. Two positive controls were performed outside the dedicated aDNA clean-rooms and amplified on different days to avoid cross-contamination. The first contained female hair sample extract from the researcher (AMS) and the second contained concentrated anonymous male DNA from the Medical Genetics Laboratory. Each positive control was run on the same gel as ancient sample extracts for size comparisons.

Following successful resolution of amelogenin amplified product on a gel, extracts were amplified with 5' Fluorescein-labeled amelogenin primers (MWG Biotech, same sequence as Amelogenin F and R) for subsequent fragment analysis on the ABI Prism 310 Genetic Analyzer at the University of Tennessee Molecular Biology Sequencing Facility. Amplification of modern male DNA with 5' Rhodamine Red-labeled amelogenin primers (MWG Biotech, same sequence as Amelogenin F and R) was undertaken for a positive control. PCR fragments were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions. Raw data from the ABI Prism was analyzed using the associated Genotyper software program. Table 2-4 summarizes the settings and run specifications for fragment analysis on the ABI Prism 310.

Table 2-4. Settings for ABI Prism 310 Genetic Analyzer

Module	Inj. Sec.	Inj. kV	Run kV	Run C	Run Min.
GS STR POP 4 (1mL)	5	15.0	15.0	60	24

Chapter 3

Results and Discussion

Prior to amplification of extracted DNA from ancient materials, extracts from the female researcher's (AMS) hair bulbs were amplified with the amelogenin primers (MWG Biotech) to optimize amplification conditions. Once annealing and extension periods and temperatures repeatedly yielded strong amplification signal for modern hair samples, amplification with ancient samples was attempted. It should be noted that modern hair samples contained inhibitors that prevented successful amplification, such that serial dilutions of extracts were necessary to obtain amplification signal. Amplifications were most successful with dilutions of between 1:100 and 1:200.

Of the 74 individuals, hair samples from 14 juveniles and 2 morphologically determined males (Table A-1) underwent DNA extraction. Early amplification attempts were unsuccessful due to the presence of strong inhibitors. Serial dilutions of extracts coupled with an increase in magnesium chloride and AmpliTaq Gold concentrations failed to successfully amplify. Several extracts were spiked with modern concentrated human male DNA to confirm the presence of inhibitors, and in each attempt, the ancient DNA inhibitors either significantly decreased amplification efficiency or entirely prevented amplification of the modern purified DNA.

The addition of 2-5ug of BSA (Promega, Madison, WI) to each reaction significantly enhanced amplification efficiency when applied to serial dilutions of

ancient samples (Lin et al. 1995). The following dilutions proved most effective: 1:10, 1:25, 1:50, 1:75, and 1:100.

Each individual underwent 2 extraction attempts separated by at least several weeks. At least 2 amplification attempts with the amelogenin primers were performed on each sample extract. Of the 16 total individuals sampled, 4 juveniles and 1 adult male yielded consistent amplification signal for all extraction and amplification attempts. It is interesting (although preliminary) to note that individuals with no scalp present all failed to amplify (samples E2 and E47). Scalp material may have protected the hair root, thus depressing the rate of DNA degradation. Table 3-1 summarizes extraction and amplification attempts, conditions, and outcome of amelogenin amplification for all individuals.

For the amelogenin markers, all successful ancient sample amplifications produced the Y-specific band, but failed to amplify the smaller X-specific band when visualized on an ethidium bromide-stained polyacrylamide gel (Figure 3-1). Several researchers have experienced an allelic drop-out phenomenon when using the amelogenin system on ancient or degraded samples. Depending on which amelogenin system is used, most researchers have experienced the loss of the larger fragment (Faerman et al. 1995 for the 330 X/218 Y; Gotherstrom et al. 1997 for the 196 Y/136X; and Lassen et al. 1996 for the 112 Y/106 X). Vernesi et al. (1999), however, report the loss of the smaller X-specific (106bp) fragment for several individuals, as seen in the present study. While preferential amplification of the X allele can confound sex identifications (giving a false-

Table 3-1. Summary of Amplification Attempts, Reaction Conditions, and Resolution on Acrylamide Gel for Amelogenin Primers

Sample	# PCR Attempts/Success Amelogenin	Dilution	BSA Concentration	X/Y Bands on Gel
E2-1/2	3/0	~	~	~
E6-1/2	2/0	~	~	~
E36-1/2	4/2	1:10	30ug	Y
E11-1/2	5/5	1:25	40ug	Y
E10-1/2	3/3	1:50	20ug	Y
E47-1/2	2/0	~	~	~
E52-1/2	2/1	1:75	30ug	Y
E54-1/2	2/0	~	~	~
E68-1/2	2/0	~	~	~
E80-1/2	2/1	1:25	30ug	Y
E89-1/2	2/0	~	~	~
E93-1/2	2/0	~	~	~
ED3-1/2	2/0	~	~	~
E58-1-1/2	2/0	~	~	~
E58-2-1/2	2/0	~	~	~
E58caja82-1/2	2/0	~	~	~

For Each Sample, ½ Signifies 2 Independent Extractions, ~ = Unsuccessful Amplification

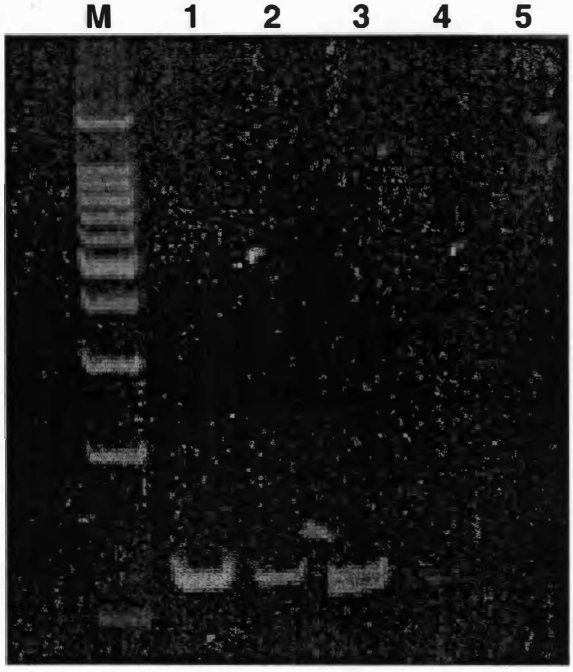

FIGURE 3-1. Amelogenin Amplification Product for Ancient Samples on Acrylamide Gel

M = 100bp markers, Lane 1 = Y-specific band (112bp) for adult male sample E11, Lane 2 = Y-specific band for juvenile male sample E10, Lane 3 = X + Y-specific bands (106/112bp) for concentrated modern male DNA, Lane 4 = extraction blank, Lane 5 = PCR blank

positive female identification), preferential amplification of the Y allele can only support a male identification.

The Hemo1 and Hemo2 primers were used to rule out the presence of modern DNA contamination. The larger 320bp nuclear marker failed to amplify in all ancient samples while producing strong amplification signals in both modern male and female DNA samples. These findings support the general tenet that an inverse relationship exists between fragment size and amplification efficiency for degraded DNA samples, such that fragments larger than 300bp are rarely recovered when analyzing endogenous ancient (or highly degraded) DNA (Lindahl 1993; O'Rourke et al. 2000; Tuross 1994; Whitaker et al. 1995).

Of the ancient samples that gave strong amplification of the Y-specific band on a gel with the Flourescein-labeled amelogenin primers (samples E11 and E10), fragment analysis with the ABI Prism 310 showed the presence of both X-specific and Y-specific peaks for each individual. Although the X-specific band was never present on an acrylamide gel, the 310 Genetic Analyzer can detect significantly lower concentrations of DNA and thus supports the evidence that the individuals typed are male. Table 3-2 contrasts the results from fragment analysis on the ABI Prism 310 and acrylamide gels.

Amplifications using the Haplogroup B mitochondrial markers were performed for 5 ancient samples that yielded amplification signal with the amelogenin primers (Table 3-3). Amplification was successful for each of the samples (E11, E10, E36, E54, and E80) after an equal amount (2-3 units) each of Amplitaq Gold and Taq enzyme (PE Applied Biosystems, Foster, CA) were

Table 3-2. Amplification with Flourescein-labeled Amelogenin Primers and Resolution of Fragments on ABI Prism Genetic Analyzer and Acrylamide Gel

Sample	Amelogenin FLR	X/Y Peaks ABI Prism	X/Y Bands Gel
AMS	+	X	X
CM	+	X/Y	X/Y
E11-2	+	X/Y	Y
E10-1	+	X/Y	Y

AMS = Extract from Female Researcher's Hair, CM = Concentrated DNA from Modern Male, + = Successful Amplification

Table 3-3. Amplification with mtDNA Haplogroup B Markers

Sample	mtHapB
AMS	+
CM	+
E11-2	+
E10-1	+
E36-1	+
E54-1	+
E80-1	+

AMS = Extract from Female Researcher's Hair, CM = Concentrated DNA from Modern Male, + = Band Visualized on Gel

added to each PCR reaction. Since inhibitors were likely the cause of amplification inefficiency for most extracts, Taq enzyme was added to both bind the inhibitors that prevent enzymatic activity and to "jumpstart" early rounds of amplification, thus increasing the number of target molecules for long range amplification. A dramatic increase in PCR product (as visualized on an acrylamide gel) was seen after the addition of Taq to the reaction mixture. Figure 3-2 shows the 117bp amplification product for several extracts. Amplification product was not limited to specific dilutions of sample extracts, as noted with amelogenin amplification, but rather was seen to work effectively in at least 2 to 5 serial dilutions for each extract. This technique should be applied to future PCR reactions of all samples.

Future Directions

The results of this study suggest that viable endogenous DNA persists in the hair bulbs of individuals from the Punta Lobos assemblage in sufficient quantities to warrant both nuclear and mitochondrial molecular investigation. Though preliminary, successful sex typing of 4 of the juveniles (and 1 adult) as biological males has strengthened the demographic assessments of the site. Successful amplification with the Haplogroup B marker for 5 individuals has given the foundational evidence necessary to proceed with an extended genetic investigation.

Future efforts should entail a comprehensive genetic analysis that includes Y-chromosome and mtDNA haplogroup and haplotype delineation for

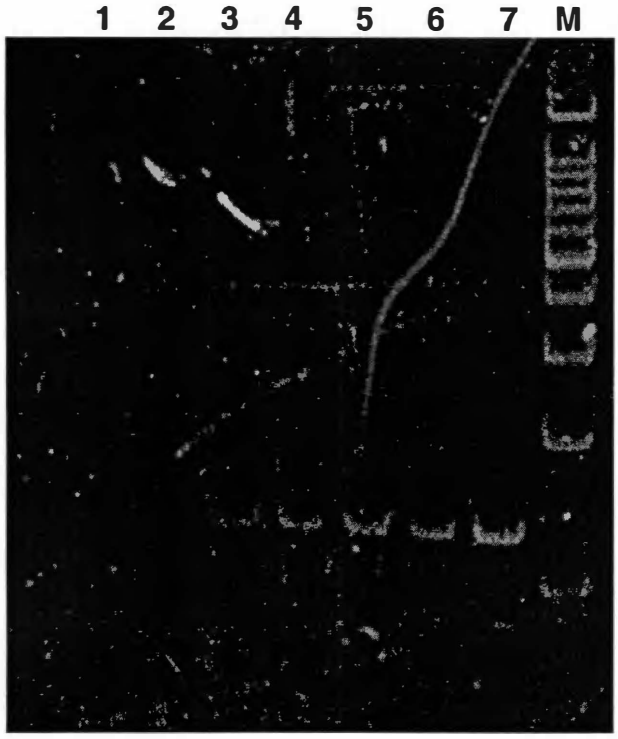

Figure 3-2. Haplogroup B mtDNA Amplification Product for Ancient Samples on Acrylamide Gel

Lane 1 = PCR blank, Lane 2 = Extraction blank, Lane 3 – 6 = Ancient samples (117bp product) E54, E36, E10, and E11 respectively, Lane 7 = Concentrated modern male DNA, and M = 100bp Markers

each of the 74 sampled individuals. The amplification efficiency of the Y-chromosome in tandem with the all-male defined assemblage suggests a Y-chromosome assay may be an ideal next step. Haplotype construction via Y-chromosome STR multiplexing will give a measure of paternally-defined genetic relationships, while biallelic SNP characterization will show more ancient differentiation events. If the Punta Lobos individuals represent a closely related population, then SNP-based haplogroups should be identical. The rapid mutation rates for STRs, however, should provide a basis for distinction between individual patrilineages (de Knijff 2000; de Knijff et al. 1997). The problem lies in discerning which specific STRs (and how many) will provide the greatest level of discrimination. Very few studies to date have characterized Y-chromosome haplotypes for pre-Contact South American remains (an exception being, Garcia-Bour 2004) Decisions concerning which loci are appropriate for analysis have to be based on rates of STR variability found in modern native South American populations (Bianchi et al. 1998; Bortolini et al. 2003; Carvalho-Silva et al. 2001; Mesa et al. 2000).

A publication by Tarazona-Santos et al. (2001) describes Y chromosome genetic structure across native South American populations as predicted by historical patterns of genetic drift and gene flow. "The following pattern of within- and among population variability emerges from the analysis of microsatellite data: 1) the Andean populations exhibit significantly higher levels of within-population variability than do the eastern populations of South America; 2) the spatial-autocorrelation analysis suggests a significant geographic structure of Y-

chromosome genetic variability in South America...; and 3) genetic distance analyses and the analysis of molecular variance suggest greater homogeneity between Andean populations than between non-Andean ones" (Tarazona-Santos et al., 2001:1485). Based on their findings of high within- population genetic diversity for western populations, the Peruvian region of South America should provide a rich source for Y chromosome haplotype variation.

Punta Lobos presents a unique opportunity for a thorough molecular population analysis. Unlike the most ancient burial contexts, the assemblage has no temporal incongruities. The mass execution of 200 individuals happened at a fixed point in time. There was no known site occupation and little opportunity for stratigraphic mixture. Large sample size and population contemporaneity allow for more sophisticated modes of statistical population genetic analyses. Haplogroups and haplotypes could provide insight into the degree of relatedness between individuals and determine the likelihood that they belonged to a single (or multiple) village(s). Through comparisons of genetic diversity in both maternal and paternal lineages, we may see the genetic patterns resulting from social residence structure (i.e. patrilocal vs. matrilocal inheritance) - a social construct with a genetic signature. Finally, a biospatial analysis may show clustering of related individuals to help reconstruct the time specific events of the execution.

Chapter 4

Conclusion

The bodies found on the beach at Punta Lobos weathered hundreds of years to tell a story. It is a story that requires painstaking efforts and widespread collaboration in its retelling. The results presented in this study offer a preliminary molecular investigation of this ancient skeletal population. Ancient DNA, like archaeological skeletal and cultural remains, has endured structural, environmental and chemical alterations over time, leaving only partial fragments of the antemortem whole for analysis. The piece-meal evaluation of small stretches of DNA can provide insight into intrapopulation relatedness and genetic history. And although plagued by similar archaeological pitfalls like sampling bias (and novel concerns such as modern contamination and preferential amplification bias), molecular analyses lend backbone and scientific integrity to archaeological interpretations. Anthropology is a discipline that requires the evaluation of many lines of evidence to make interpretations that must be modified in light of new finds, new techniques, and new theoretical applications. As such, the study of the past necessitates interdisciplinary collaboration.

The Punta Lobos site represents a complex archaeological assemblage from a poorly documented period of Andean political and cultural transition. A thorough genetic investigation, utilizing a tri-system genetic approach, will provide a valuable counterpart to the demographic, pathologic, morphologic, and

dietary site profile, and collectively enhance our understanding of these individuals' lives and deaths.

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Appendix

Table A1- Sex and Age Data for Extracted Individuals from Verano – Punta Lobos 2003 Collected Samples

Individual	Morphological Sex	Age Range
E2	3	12-15
E6	1	35-40
E36	3	9-11
E11	1	16-18
E10	3	10-12
E47	3	7-9
E52	3	9-10
E54	3	9-10
E68	3	10-13
E80	3	7-9
E89	3	14-17
E93	3	13-15
ED3	3	12-15
E58-1	3	8-10
E58-2	3	6-8
E58-caja82	3	7-9

For Morphological Sex, 1 = male, 2 = female, and 3 = unidentified

VITA

Adriane Michelle Scola was born in Providence, Rhode Island on April 22, 1978. She was raised in North Scituate, RI and attended North Scituate Elementary and Scituate Jr/Sr High School. After graduating in 1996, she briefly attended Skidmore College and transferred to Binghamton University, SUNY in 1998. She received her B.A. in anthropology and biology from Binghamton University in 2000. In 2002 she began a M.A. program in anthropology at the University of Tennessee, Knoxville and received her degree in 2004.

Adriane is currently pursuing her doctorate in biological anthropology at the University of Utah, Salt Lake City, UT.

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