MOLECULAR IDENTIFICATION AND ANALYSIS OF TREPONEMATOSIS (SYPHILIS, BEJEL, YAWS, OR PINTA) IN ANCIENT MUMMIFIED REMAINS FROM NORTHERN CHILE AND SOUTHERN PERU

A

DISSERTATION

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

August 2008

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By

Michelle Kaye

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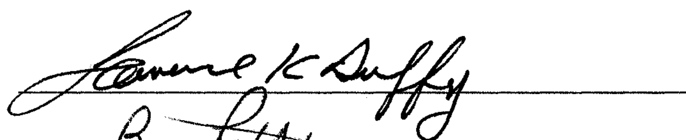

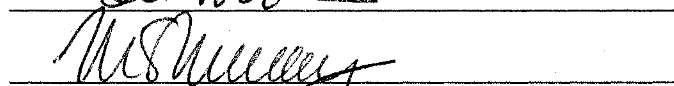

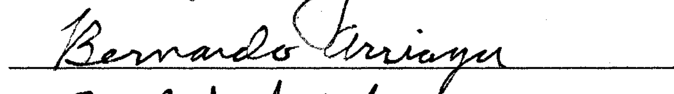

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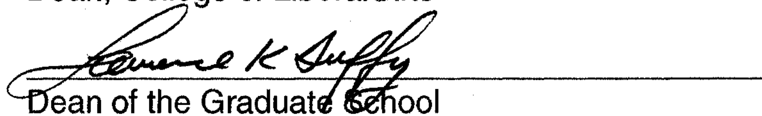

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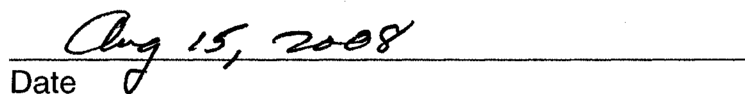

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Abstract

The sequencing of *Treponema pallidum* subsp. *pallidum,* the bacterium that causes syphilis, and the identification of a family of 12 genes with sequence similarity that allows scientists to distinguish between treponemal subtypes has opened up a new line of inquiry for biological anthropologists. This research contributes genetic evidence of pre-contact treponematosis in the Americas; by combining osteological and molecular evidence with data on environment and cultural practices, it also furthers our knowledge of human-pathogen interaction.

This research assessed the presence of treponematosis, a bacterial spirochete, in the DNA of skeletal and mummified human remains from northern Chilean cemeteries dating from 5000 BC to AD 1100. The objectives were to: (1) determine whether treponemal DNA could be successfully recovered, amplified, and identified by subspecies from ancient bone and tissue, (2) compare any ancient sequences generated to the modern strains present in the National Center for Biotechnology Information (NCBI) GenBank database, (3) test the null hypothesis that treponematosis was not present in the New World before European contact, and (4) explore which cultural factors may have contributed to the spread of treponematosis in these groups.

This research established a foundation for future treponemal studies through the development of primers and protocols for the analysis of ancient treponemes. The results of this study suggest that the inhabitants of this region suffered from a systemic bacterial infection, likely a chronic form of non-venereal

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treponematosis: yaws or bejel. Potential treponemal DNA was recovered from bone in an individual dated 202 cal BC – cal AD 3 from the Azapa valley. An investigation of Chinchorro artificial mummification suggests that their mortuary practice likely did not result in a higher frequency of treponematosis, as compared to later and inland groups. Rather, status and socioeconomic factors may have played a role in differential infection rates between those mummified in complex styles and those in natural or less complex styles. Further analysis of human remains with suspected treponemal lesions is necessary to reconstruct the history of treponematosis, improve our understanding of their pathogenesis, and guide scientists in developing preventative measures.

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ACKNOWLEDGMENTS

Many hands were involved in the development and completion of this dissertation. I am indebted to my committee members (Dr. Joel Irish, Dr. Lawrence Duffy, Dr. Ben Potter, Dr. Maribeth Murray, and Dr. Bernardo Arriaza) for their patience and guidance in helping me fine-tune this dissertation. Special thanks go to Dr. Joel Irish, professor of Biological Anthropology at University of Alaska Fairbanks for his long-suffering role as my esteemed advisor. Without his satirical asides and witticisms neither this dissertation, nor my current (and debatable) remaining sanity would have been possible. I am also grateful to Dr. Bernardo Arriaza, Leticia Latorre Orrego, Raúl Rocha, María Flores, Anita Flores, and the employees of the Museo Arqueólogico San Miguel de Azapa for sharing with me their love and respect for these very special mummies and skeletons.

Huge thanks also go to Dr. Frank Cipriano, Director of the Conservation Genetics Laboratory at San Francisco State University who mentored me in molecular techniques and allowed me to indulge my curiosity in all things ancient and forensic. Stephen and Renée Fratpietro of the Paleo-DNA Laboratory, and Dr. Carney Matheson at Lakehead University deserve thanks for their help in the laboratory and for sharing with me their knowledge of ancient DNA analysis. Special thanks also go to the Morales and McKinstry families, for their hospitality; to my husband Brian Fitzgerald and my family for their support and unrelenting

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patience; and to the students working in the Conservation Genetics Laboratory who accepted me as one of their own. The Anthropology Department at UAF, Dr. Daniel Odess, and the Geist Fund graciously provided funding for this project. Dr. Charmie Godornes and Dr. Sheila Lukehart kindly donated their time and laboratory skills to test the new primers. Karen Kosiek, secretary to the Anthropology Department at UAF deserves many thanks – without her kind words, sage advice, and dedication to the students and faculty no advancement would have been possible. I am also grateful for the support I received from my friends Holly Stelton, Jenny Newton, Bonita Dainowski, Dr. Steven Symes, Dr. Greg Hampikian, Dr. Richard Harrington, Dr. Brett Riddle, Larry Okumoto, Roland Wessling, Jefferson Ballew, and Mike Weiss who provided encouragement, advice, laughter, libations, and most importantly escape. I got to do something that not many people get to do – and for that I am grateful.

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Chapter I.

Introduction

The study of treponemes has changed drastically since the late 1990's; the sequencing of *Treponema pallidum* subsp. *pallidum* (*T.p. pallidum*), the agent that causes syphilis (Fraser et al., 1998), and subsequent discoveries that allow scientists to distinguish genetically among the treponemal subspecies that affect skeletal elements has opened up a whole new line of inquiry to biological anthropologists. This research builds upon these past discoveries (Blanco et al., 1997; Norris et al.,1998; Pennisi, 1998; Pillay et al., 1998; Weinstock et al.,1998; Bosch, 1999; Kolman et al., 1999; Radolf et al., 1999; Centurion-Lara et al., 2000a,b, 2006; Stamm and Bergen, 2000; Zink et al., 2002; LaFond et al., 2003, 2006; Bouwman and Brown, 2005; Gray et al., 2006; Radolf and Lukehart, 2006).

Only 10 years ago, the debate over treponematosis revolved around two questions: 1) what is the evidence for the development and disbursement of treponemal infections, and 2) do the treponemes represent four discrete infections or just one that has changed into unique subspecies over time due to environmental or demographic variations (Holcomb, 1934, 1935, 1941; Hudson, 1958a,b, 1965; Harrison, 1959; Cockburn, 1961; Hackett, 1963,1967; Allison et al., 1982, 1984; Baker and Armelagos, 1988; Stirland, 1991:39-47; Engelkenz et al., 1991; Ortner, 1992a,b; Rothschild and Rothschild, 1995; Trembly, 1996;

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Aufderheide and Rodriguez-Martin,1998:154,166; Saunders et al., 2000; Morton and Rashid, 2001; Sefton, 2001)?

Scientists can now answer some of these questions. While the origins and spread of the treponemes still represent a valid area of inquiry, new insights into their genetics, protein membrane, oxygen metabolism, transport systems, and antigenicity have provided scientists with a greater understanding of the biology of these pathogenic bacteria (Centurion-Lara et al., 2006; Norris and Weinstock, 2006:19-39; Radolf and Lukehart, 2006). In particular, these advances elucidated the genome for venereal syphilis, and identified a family of 12 *Treponema pallidum* repeat (*tpr*) genes that vary among the treponemal subspecies (Fraser et al., 1998; Weinstock et al., 1998; Kolman et al., 1999; Cameron et al., 2000; Centurion-Lara et al., 2000a,b; Stamm and Bergen, 2000; LaFond et al., 2003, 2006; Bouwman and Brown, 2005; Centurion-Lara et al., 2006; Gray et al., 2006). Scientists now recognize that there are genetic differences among the treponemal subspecies. Data suggest that the genus *Treponema* evolved from a common ancestor in bacterial evolution, possibly hundreds of millions of years ago; the *T. pallidum* species, while closely related may have diverged tens of thousands of years ago (Norris and Weinstock, 2006:34; Radolf and Lukehart, 2006). A recent phylogenetic study has spurred debate by suggesting that *T. pallidum* first arose in the Old World (specifically in Africa) in a nonvenereal form of treponematosis called yaws (*T.p. pertenue*-OW) (Harper et al., 2008). The Old World generally refers to those areas known to

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Europeans, Asians, and Africans before the 15th century voyages of Christopher Columbus. Included in the Old World are Europe, Asia, Africa, and their surrounding islands. This Old World strain of yaws spread with early humans to the Middle East and Eastern Europe where it changed into a nonvenereal form of treponematosis called bejel (*T.p. endemicum*); early migrants carried bejel to the Americas, where it changed into a New World yaws strain (Harper et al., 2008). The New World generally refers to the Americas and Australia. This New World strain (*T.p. pertenue*-NW Americas) was then reintroduced to the Old World via European explorers, "becoming the progenitor" of the venereal form, syphilis (*T.p. pallidum*) (Harper et ai., 2008; Mulligan et al., 2008). The New World yaws (*T.p. pertenue*-NW) from South America possessed characteristics similar to the *pallidum* subspecies while remaining nonvenereal (Harper et al., 2008). Harper and colleagues (2008) point out that there were no bejel strains for them to draw from in South America, and they had only two indigenous yaws strains from the same locale; therefore, they say, it is possible that an unknown strain more closely related to subspecies *pallidum* (syphilis) once existed in South America (Harper et al., 2008). Other molecular researchers question the conclusions that the phylogenetic study draws about the evolution of the treponemal subspecies; they are not convinced that they are "discrete agents" (Mulligan et al., 2008). Another consideration is the scarcity of osseous and epidemiological evidence for the non-endemic treponemal diseases in European antiquity.

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Biological anthropologists have an important contribution to make to treponemal research. First, they can study the skeletal and mummified remains of ancient people for osteological and molecular evidence of treponematosis. Secondly, they can make inferences about the behavior of people with respect to infectious disease. Modifications in behavior and culture affect the dynamics of disease - how and whether it proliferates, and when it re-emerges (Grassly et al., 2005:417; Ferguson, 2007:733). Analyses that encompass the biology of the pathogen and the behavioral response of the affected population inform our understanding of human-pathogen interactions (Ferguson, 2007:733). Through participant observation or inferred societal changes, anthropologists can learn how societies influence the transmission and biology of pathogens (Ferguson, 2007:733).

With few exceptions (Rogan and Salvo, 1989, 1990a,b, 1994; Rogan, 1991; Rogan and Lentz, 1994; Kolman et al., 1999, Bosch, 1999; Bouwman and Brown, 2005) previous anthropological research on treponematosis has focused on ethnographic, archaeological, and osteological data (Gudger, 1910; Hrdlička, 1914; Moodie, 1929; Hackett, 1957; McArthur, 1968; Cockburn, 1971; Brothwell, 1976; El-Najjar, 1979; Beyon and Siegal, 1981; Lowenthal, 1982; Allison et al., 1982; Allison, 1984; Elting and Starna, 1984; Powell, 1988a,b; Pringle, 1998; Hutchinson, 1993; Yakinci et al., 1995; Arriaza, 1995a,b; Rothschild and Rothschild, 1995; Mansilla and Pijoan, 1995; Trembly, 1996; Standen and Arriaza, 2000a,b; El Molto et al., 2000a,b; Buckley and Tayles, 2003a,b; Mays et

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al., 2003; Mitchell, 2003). Molecular treponemal research on archaeological samples has only been attempted by a handful of researchers (Bouwman and Brown, 2005; Barnes and Thomas, 2006; von Hunnius et al., 2007), and by only two recently in South America (Rogan and Lentz, 1994; Kolman et al., 1999).

The current research assesses the presence of treponematosis, a spirochetal infection, in the DNA of skeletal and mummified human remains from northern Chilean cemeteries. While samples were collected from several cultures, a focus of this research was the Chinchorro culture -- a pre-ceramic, pre-metallurgical fishing and gathering group from the Atacama coast of northern Chile, dating from approximately 7020 ± 255 BC to 1110 ± 380 BC (Arriaza, 1995a:39-43). The Chinchorro, who lived in and around the modern city of Arica, shared an elaborate mortuary ritual that involved natural and artificial mummification (Arriaza, 1995a; Sutter and Mertz, 2004). Their remains exhibit high frequencies of chronic periostitis in the tibiae in contrast to data available from post-Chinchorro populations; this condition may be indicative of treponemal infection. This relatively high rate of infection is unusual in a small nonagricultural population that did not travel or trade widely. Three main forms of treponematosis that affect skeletal elements are addressed herein: venereal syphilis, bejel, and yaws.

Molecular techniques are used to recover and analyze ancient mitochondrial DNA and treponemal bacteria from these mummies and skeletons. The results provide further skeletal and molecular evidence of the presence of a

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treponemal or treponemal-like infection in the Americas before European contact (Bogdan and Weaver, 1992; Milner, 1992; Ortner, 1992a,b; Powell, 1988a,b, 1990, 1991, 1992; Saunders et al., 2000). Special attention is given to factors such as regional ecology, climate, population density, and settlement patterns that could influence the epidemiological model for the early Andean cultures studied.

According to Arriaza (1995a, 2000), the high frequency of bone pathologies in the Chinchorro population may be a consequence of handling decomposed cadavers, and a result of their artificial mummification practice. That is, their practice of saving the skin, eviscerating some organs, filling the body cavities, stitching incisions, and painting the deceased may have enabled the spread of infectious bacteria from the dead to the living via surface cuts on the latter.

The current research contributes to the discussion of the origin and evolution of treponematosis (Hudson, 1958a:34:22-24; 1965; Cockburn, 1961:221-8; Hackett, 1963:7, 1967:152-69; Stirland, 1991:39-47; Rothschild and Rothschild: 1995:1406). Recovery of a molecular sequence suggesting the presence of treponematosis in prehistoric South America may encourage further molecular analysis of this pathogen in ancient peoples. Ultimately, this research may help scientists obtain a better understanding of changes to this pathogen through the comparison of past and present treponemal sequences.

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Rogan and Salvo (1990a,b) recovered nuclear and mitochondrial DNA in nine mummies from the northern Chilean coast. Rogan and Lentz (1994) recovered DNA sequences resembling modern syphilis (*T.p. pallidum*) from two Chinchorro mummies. Kolman and colleagues (1999) detected syphilis in a 200-year-old skeletal sample from Easter Island.

This research builds upon these previous studies. A specific goal was to determine whether treponemal DNA could be recovered from ancient bone, (contra Bouwman and Brown 2005) and to assess the attribution of the Chinchorro bone pathologies to a potential form of treponematosis (Standen et al., 1984; Allison et al., 1984; Standen and Arriaza, 2000b). Molecular anthropology, as applied to the study of disease can identify bone pathology; it can be used to trace the co-evolution of humans and pathogens and is a useful diagnostic tool for past and present disease. As a bacterial infection, treponematosis spreads through surface cuts, bodily fluids, and vaginal or anal intercourse; it can increase an individual's susceptibility to other diseases like HIV/AIDS, and is subject to increasing antibiotic resistance. While this research contributes to the understanding of treponematosis history in South America, it may also have some potential to help scientists glean information about its: 1) genetic composition, 2) effects on human populations, and 3) prevention.

Research Objectives

This study addresses four objectives. The first involves the assessment and use of a unique molecular experiment to recover treponemal DNA from

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ancient Andean archaeological samples. The second combines osteological and molecular analyses to provide a more definitive picture of the pathogen that causes the osseous lesions in these populations. The third generates new radiocarbon dates for these ancient samples to estimate their age and establish that the disease affecting them was not transmitted through European contact. The last objective is to consider the mortuary ritual and if it caused increased transmission of the bacterium from the deceased to the living who prepared the dead. The four objectives are reiterated below; the null hypothesis is stated, followed by an explanation of how it will be evaluated.

(a.) Treponemal DNA cannot be successfully recovered and amplified from ancient bone, tissue, and/or organs using molecular techniques. Research by Bouwman and Brown (2005) suggested that treponemal DNA could not be recovered from ancient and historical bone. Here efforts were made to extract treponemal DNA from ancient bone, amplify, clone, sequence, and replicate any positive results. In addition, the viability of tissues and their ability to retain spirochetes was examined. If treponemal DNA could be recovered from soft tissue, then mummified tissues are a potentially viable source for treponemal spirochetes.

(b.) The pathologies present on the Chilean mummies do not indicate a probable treponemal infection. If DNA extracted from these mummies matches or closely resembles treponemal sequences in the National Center for

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Biotechnology Information (NCBI) GenBank Database, then a (or a probable) treponemal infection likely existed, and the null hypothesis can be rejected.

(c.) Treponematosis was not present in New World before European contact. DNA was extracted from tissue and/or bone to determine if viable treponemal spirochetes could be recovered, amplified, cloned, sequenced, and replicated from Chilean mummies and skeletons. Samples were radiocarbon dated, as funds permitted, to determine age. If treponemal sequences were recovered from pre-Contact samples then treponematosis was present in this region before European contact.

(d.)The elaborate mortuary ritual practiced by the Chinchorro was not the likely cause of a higher frequency of treponemal infection in that group compared with inland populations that did not practice artificial mummification. Statistics on potential treponemal infections in the Chinchorro and in later regional valley populations were compared. Standen and Arriaza (2000b) previously collected these data. If after careful examination of environmental, geographic, osteological, and social-cultural data, no other probable contributing factors could be found to explain the higher rate of infection in the Chinchorro, then their burial ritual likely contributed to the frequency of infection. Burial practices, including mummification styles and techniques, are considered alongside other factors to determine the likelihood that mummification contributed to the spread of the disease. Given that the Chinchorro population is

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ancient, had no written records, and was not the subject of ethnographic reports, any conclusions reached are tentative.

Potential Contributions

This research contributes genetic evidence indicating the presence of a potential treponemal infection in the Americas prior to European contact. It presents two sets of primers that target diagnostic regions that allow scientists to distinguish among syphilis, yaws, and bejel. Positive molecular results test the validity of, and perhaps advance the osteological analysis and estimation of treponemal subtypes. This research contributes bioarchaeological information on how mortuary practices might have affected the rate of disease transmission within a population.

All treponemal diseases are present today and represent public health concerns. In the United States, the rate of primary and secondary syphilis increased between 2001 and 2006, primarily in the men having sex with men (MSM) population. According to the Center for Disease Control (CDC), the estimated proportion of primary and secondary syphilis cases attributable to MSM increased from 4% in 2000 to 62% in 2004 (CDC, 2006a,b; Heffelfinger et al., 2007). Among all men in the United States the rate increased from 3.0 cases per 100,000 in 2001 to 5.7 cases per 100,000 in 2006; in women in the United States it increased from 0.8 cases per 100,000 in 2004 to 0.9 cases per 100,000 in 2005, and 1.0 cases per 100,000 population in 2006 (Erbelding, Johns Hopkins, 2003; CDC, 2006a,b). Venereal syphilis disrupts the epithelial surfaces

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of the body and as a result raises the chance of HIV transmission (Kolman et al., 1999;Erbelding, Johns Hopkins, 2003). This increase in disease incidence highlights the need for a better understanding of the treponemes (Kolman et al., 1999). In addition to syphilis, pinta may still be present in rural pockets of Central and South America; yaws is present in South America (Guyana), central Africa (Democratic Republic of Congo and Republic of Congo), Papua New Guinea, and Southeast Asia (Indonesia, East Timor); and bejel remains, at a low level, in the Middle East (Turkey, United Arab Emirates), Asia, and Australia (Antal et al., 2002:90; Harper et al., 2008). Moreover, the World Health Organization reports that after years of reduced global levels of disease, yaws is making a comeback, primarily in central Africa, due to a lack of attention by local and international health providers (WHO, 2007).

The study of ancient DNA is multidisciplinary. This research integrates clinical, biological, and anthropological methodologies to investigate the genetics and epidemiology of a potential treponemal infection. The analysis of additional human remains with suspected treponemal lesions is necessary to reconstruct the co-evolutionary history of humans and treponemes, improve our understanding of treponemal pathogenesis, and guide researchers in creating and implementing preventative measures (Kolman et al., 1999:2063).

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Chapter II.

The Mummies of Northern Chile and Southern Peru

This research reports on the analysis of treponemal DNA from mummified bone and tissue samples of 15 individuals from 11 Arica and Azapa valley archaeological excavations/sites in northern Chile. Samples were taken from individuals in multiple sites due to curatorial constraint. These groups lived between the modern borders of Chile and Peru. The sites represent several cultures: Chinchorro, Faldas del Morro, Alto Ramírez, Tiwanaku, Cabuza, Maitas (Maytas)-Chiribaya, San Miguel, and Desarrollo Regional.

Cultural affiliations were determined by previous researchers through a combination of radiocarbon dating, burial practices, and ceramic and textile traditions (Bird, 1943, 1946; Mostny, 1964; Dauelsberg,1969, 1974, 1982, 1985; Focacci, 1969; Núñez, 1976; Berenguer, 1978; Santoro, 1980a,b; Muñoz, 1981, 1983, 1989, 1993; Muñoz and Chacama, 1982, 1993; Mujica et al., 1983; Allison et al., 1984; Allison 1985a,b; Mujica, 1985; Muñoz and Focacci,1985; Rivera and Rothammer, 1986; Berenguer and Dauelsberg, 1989; Focacci and Chacón, 1989; Schiappacasse et al., 1989; Rivera, 1991; Guillen, 1992; Kolata, 1993; Arriaza, 1995a; Goldstein,1995; Sutter, 2000. 2006; Sutter and Mertz, 2004). When the archaeologists listed the associated culture on their original data recording forms, the individuals had not been radiocarbon dated; therefore, there

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may be some discrepancies between the associated culture and the chronology in which that culture has been placed.

Using the chronology described by Sutter (2006) for the prehistoric peopling of the Azapa valley, the earliest date to the Archaic Period (circa 8000 - 1000 BC); others date to the Formative Period (circa 1000 BC - AD 500), the Middle Intermediate Period or Middle Horizon (circa AD 500 - AD 1100) and, the most recent, to the Desarrollo Regional Period (circa AD 1100 - AD 1470) (Arriaza, 1995a:125-132; Muñoz, 2004:218-220; Sutter, 2006:63-66).

Four sites represent probable Chinchorro culture (Morro-1, Morro- 1/6, Yungay-372, and PLM-8). The remaining seven (AZ-70, AZ-71, AZ-115, AZ-140, AZ-141, PLM-3, and PLM-6) belong to a variety of cultures that followed the Chinchorro. While the coastal cultures of PLM-3 and PLM-6 shared the Chinchorro's maritime focus, the valley cultures had a mixed economy, which included maritime resources, animal husbandry, horticulture and, in the later periods, agriculture. The later inland cultures differed from the Chinchorro in that they were subject to changes in culture, technology, and genetic continuity through migration from the north and east. While these later cultures developed pottery, metallurgy, and woven textiles, they did not practice Chinchorro-style artificial mummification. Two sites, Morro-1 (Standen, 1991) and Morro-1/6 (Foccaci and Chacón, 1989) are located within 50 m of each other, and as noted are a single cemetery; however, for reasons unknown, they were given separate designations.

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The morro, for which sites Morro-1 and Morro-1/6 are named, is a headland near the coastal southwest edge of Arica. From the top of it, one can peer to the west over the Pacific Ocean, east toward the Azapa valley, north to the modern city of Arica and beaches Chinchorro and Las Manchas, and south toward beaches El Launcho, La Lisera, Brava, and Corazones. The Yungay-372 site, named for the street where archaeologists excavated human remains, is approximately 200 m northeast of the El Morro site (Morro-1/6), which lies at the northeast base of the morro. The sites of PLM-8, PLM-3, and PLM-6 are located 2 km south of the Morro sites on El Launcho Beach in Arica; however only PLM-8 is part of the Chinchorro culture, the others are considered to be part of the Desarrollo Regional culture (Alvarez, 1969). The remaining non-Chinchorro sites (AZ-70, AZ-71, AZ-115, AZ-140, and AZ-141) are located approximately 12 km east of Arica in and around the modern town of San Miguel de Azapa. The different site designations represent excavation seasons and neighboring cemeteries (Sutter and Mertz, 2004:132).

Because several cultures are included within the research sample, this chapter includes a brief cultural history, which describes some of the main cultural changes. The Chinchorro and their practice of artificial mummification are a focus of this research, and are discussed in detail. This chapter details their mummification types to understand better, how treponemal infections may have been spread through the mortuary practice. It also briefly addresses the practice of natural mummification, which was the norm for the non-Chinchorro

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groups. None of the cultures that followed the Chinchorro practiced the Chinchorro's unique style of artificial mummification; they instead allowed the bodies to desiccate naturally via the arid environment.

The research sample is summarized in Table 1 using chronological periods for the Azapa valley (Sutter, 2006). Within this table the individual's burial is listed in the exact terms used by the archaeologist(s) and museum staff; some individuals have only tomb listings, some have tomb and túmulo listings. Túmulos are ceremonial mounds that are found marking later Azapa valley burials; they are thought to be a result of the diffusion of culture and ideas from northern populations migrating from the altiplano and circum-lacustrine area surrounding Lake Titicaca to the valley in the Formative and later periods (Rocha, pers. comm., 2008). Túmulos are thought to have been both markers of ancestral land (burial markers) and monuments to the landscape and hidden hydrologic resources (Rocha, pers. comm., 2008). They were constructed of varying layers of sand and vegetal fibers, and they received much veneration from local populations who often deposited offerings on them (Rocha, pers. comm., 2008). It is unknown why some tombs are given numeric and alphanumeric designations and túmulo are given alphanumeric designations. It is possible that numeric and alphanumeric designations may designate tombs with unlabeled túmulo; however, as noted, all information comes directly from the archaeological summary card placed with each mummy/skeleton. Without further information, it is impossible to know if this is the case. The radiocarbon

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Table 1 Research samples

Count	Designation	Cemetery/ Site	Burial No.	Mummy Type	Sex	Estimated Age	Cultural Association/ Coast or Valley	Chronological Period (Sutter 2006)
1.	CHI-1-06	Morro-1/6	Tomb 7	Natural	M	40+ yrs.	Chinchorro/Faldas del Morro/Coastal	Archaic
2.	CHI-2-06	AZ-140	Tomb 23	Natural	M	35-40 yrs.	Maitas (Maytas)-Chiribaya, San Miguel/Valley	est. Middle Intermediate Period-Desarrollo Regional
3.	CHI-3-06	AZ-115	Tomb 16B	Natural	M	35-40 yrs	Alto Ramirez/Valley	est. Formative-Middle Intermediate Period
4.	CHI-4-06	AZ-70	Tomb C2, Túmulo 7	Natural	F	40-45 yrs.	Alto Ramirez/Valley	Formative
5.	CHI-5-06	PLM-6	Tomb 28	+Unknown	M?	< 35 yrs.	Desarrollo Regional/coastal	est. Desarrollo Regional
6.	CHI-6-06	Morro-1	CH. 22	+Unknown	M	Adult	Chinchorro/ Coastal	Archaic Period
7.	CHI-7-06	PLM-8	Tomb 3	Red/Blk	F	35-40 yrs.	Chinchorro/ Coastal	est. Formative Period
8.	CHI-8-06	Morro-1	Tomb 27, C5	Natural	M	25-30 yrs.	Chinchorro /Coastal	est. Archaic Period
9.	CHI-9-06	AZ-71	Tomb 601	Natural	M	40-45 yrs.	Cabuza, Tiwanaku/Valley	Formative Period
10.	CHI-10-06*; CHI-11-06*	Morro-1	Tomb 28, C9	Mud	F	20-25 yrs.	Chinchorro /Coastal	Archaic Period
11.	CHI-12-06	AZ-141	Tomb. 53	Natural	M?	~ 20 yrs.	Cabuza/Valley	est. Middle Intermediate Period
12.	CHI-13-06	AZ-71	Tomb 169	Natural	M?	13-15 yrs.	Cabuza, Tiwanaku/Valley	est. Middle Intermediate Period-Desarrollo Regional
13.	CHI-14-06	PLM-3	Tomb 142	Natural	M	~50 yrs.	Desarrollo Regional/Coastal	est. Middle Intermediate Period
14.	CHI-15-06	AZ-140	Tomb 140	Natural	F	18-20 yrs.	Maitas (Maytas), San Miguel/Valley	est. Middle Intermediate Period
15.	CHI-16-06	Yungay-372	str. 1, cuerpo 1B	Natural	F	Young Adult	UNK♦/Coastal	est. Archaic Period

* CHI000010-06 and CHI000011-06 is the same individual
♦ The culture for this individual is not known, although it is thought to be Chinchorro
est. = estimated chronological period (radiocarbon dates unavailable)
*CHI-10-06 and CHI-11-06 are the same individual
? = sex is estimated

dating information for the dated sites is summarized in Table 2. Estimated dates for the nine undated sites are summarized in Table 3. Figure 1 presents these dates graphically. Radiocarbon results generated for this dissertation also appear in Chapter 5. The radiocarbon reports appear in Appendix B.

Table 2 Summary of radiocarbon dated samples

Site	Associated Culture	Sample ID	Lab Number	Conventional Radiocarbon years BP	Calibrated Age (2 sigma)	δ13C/12C	Material	Reference
Morro-1/6, T.7	Chinchorro/ Faldas del Morro	CHI-1-06	LLNL CAMS 134138	3955 ± 30 BP	2475-2215 cal BC	-15.0‰	Human bone (R, acromion pr. scapula)	This dissertation
AZ-70, T. C2, Túmulo 7	Alto Ramirez	CHI-4-06	Beta -231777 AMS	2380 ± 40 BP	720-390 cal BC	-18.3‰	Human trunk muscle/skin tissue	This dissertation
Morro-1, CH 22	Chinchorro	CHI-6-06	Beta -231779 AMS	3830 ± 40 BP	2460-2140 cal BC	-17.4‰	Human trunk muscle/skin tissue	This dissertation
Az-71, T. 601	Cabuza/ Tiwanaku	CHI-9-06	LLNL CAMS 133582	2140 ± 30 BP	202 cal BC-cal AD 3	-13.0‰	Human bone -- Corpus sterni	This dissertation
Morro-1, T28/C9	Chinchorro	CHI-10-06/ CHI-11-06	Lab no. I-13651	3670 ± 100 BP	2281-1698 cal BC	unreported	Human muscle/lung tissue	Allison et al. (1984:163-165)
PLM-3, T.142	Desarrollo Regional	CHI-14-06	Beta- 231778 AMS	1130 ± 40 BP	Cal AD 780-1000	-10.1‰	Human muscle/skin from pelvic region	This dissertation

Table 3 Summary of samples with estimated ages

Site	Associated Culture	Sample ID	Period	Approximate Chronological Age (Sutter, 2006)
AZ-140, T. 23	Maitas (Maytas), San Miguel	CHI-2-06	est. Middle Intermediate Period	ca. AD 500 - AD 1100
AZ-115, T. 16B	Alto Ramirez	CHI-3-06	est. Formative	ca. 1000 BC - AD 500
PLM-6, T. 28	Desarrollo Regional	CHI-5-06	est. Desarrollo Regional	ca. AD 1100 - AD 1470
PLM-8, T. 3	Chinchorro	CHI-7-06	est. Formative Period	ca. 1000 BC - AD 500
Morro-1, T. 27/C5	Chinchorro	CHI-8-06	est. Archaic Period	ca. 8000 BC - 1000 BC
AZ-141, T. 53	Cabuza	CHI-12-06	est. Middle Intermediate Period	ca. AD 500 - AD 1100
AZ-71, T. 169	Cabuza	CHI-13-06	est. Middle Intermediate Period	ca. AD 500 - AD 1100
AZ-140, T. 140	Maitas (Maytas), San Miguel	CHI-15-06	est. Middle Intermediate Period	ca. AD 500 - AD 1100
Yungay 372, Str. 1, Cuerpo 1B	Unknown	CHI-16-06	Unknown	Unknown

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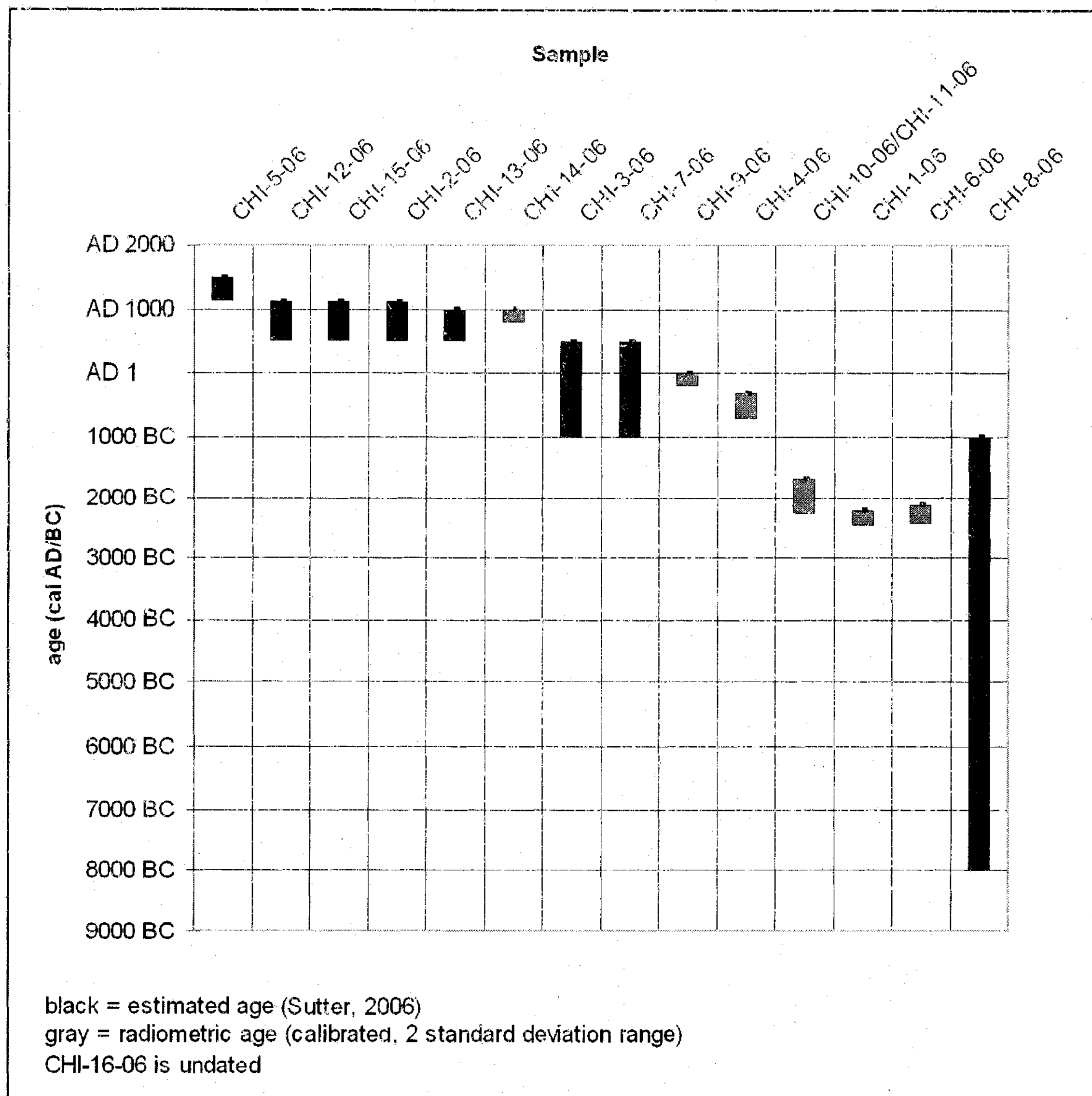

Figure 1 Sample dates

Table 4 includes data on the number of treponemal cases per site within the research sample (Arriaza, pers. comm., 2005). The sites are displayed in estimated chronological order (some sites are not dated). For PLM-6 (sample CHI-5-06) and Yungay (sample CHI-16-06) there are no data.

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Table 4 Research sample: treponematosis cases per site

Site	Chronological Period (Sutter 2006)	Cases of Treponematosis	Total Population	Percent Affected Per Site
Morro-1	est. Archaic Period	17	96	17.7%
Morro-1/6	Archaic	10	69	14.4%
Yungay-372	est. Archaic	--	--	—
Azapa-70	Formative	1	54	18.5%
Azapa-115	est. Formative	1	37	2.70%
Playa Miller-8	est. Formative Period	1	9	11.1%
Playa Miller-3	est. Middle Intermediate Period	1	2	50%
Azapa-71	est. Middle Intermediate Period	6	158	3.79%
Azapa-140	est. Middle Intermediate Period	6	151	3.97%
Azapa-141	est. Middle Intermediate Period	1	53	1.88%
Playa Miller-6	Desarrollo Regional	—	—	—

est. = estimated chronological period (radiocarbon dates unavailable)

Regional Environment and Geography

Definition of the Area:

The individuals studied here lived on the coast and in the valleys of what is now southern Peru and northern Chile between the modern cities of Ilo, Peru and Antofagasta, Chile in the Atacama Desert (Figures 2 and 3). This area covers about 900 km ^{2} and is located at latitude 17.24 S and longitude 70.71 W (Arriaza, 1995a:32). To the west is the Pacific Ocean, to the north is modern-day Peru, and to the east are the *altiplano* (high plains), the Andes, and modern Bolivia and Argentina. In southern Peru and northern Chile, the average coastal temperature ranges from a minimum of 15 - 22 °C (Arriaza, 1995a:31). The Atacama Desert is arid, with an average rainfall of 0.8 mm.

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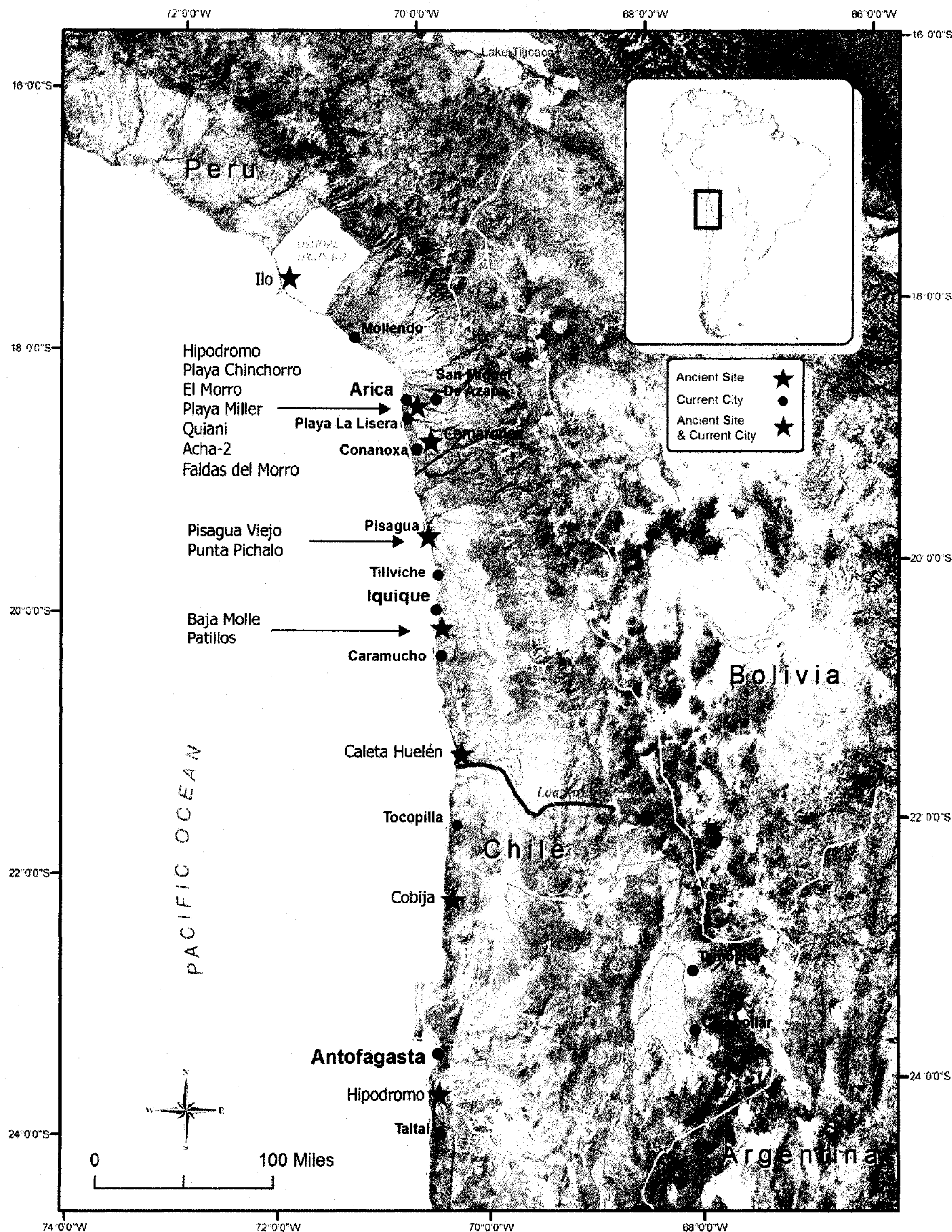

Figure 2 Prehistoric Andean sites along the Pacific coast of Peru and Chile (Map by Jesse Cohen)

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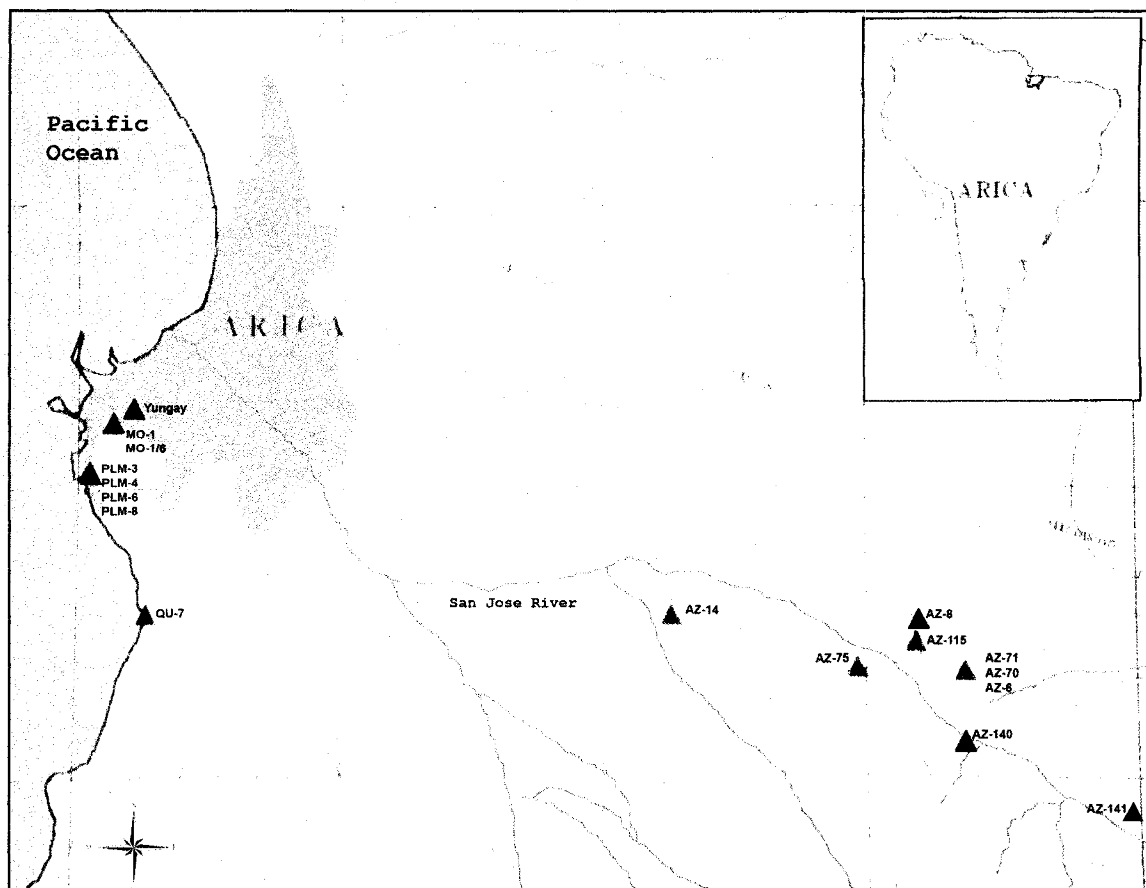

Figure 3 Archaeological sites discussed in dissertation (Map adopted and modified with permission from Standen and Arriaza, 2000b)

Arica and other coastal cities of the Atacama Desert are in the *pampa*, or dry desert coastal region. This area receives moisture from the Pacific Ocean and has small streams, springs, and seeps that provide a fertile ecosystem (Arriaza et al., 2001:32). Southern Peru and northern Chile comprise a seismically and volcanically active area that has experienced numerous earthquakes along the Peru-Chile Trench, located 160 km off the coast. The trench is a result of the subduction of the Nazca Plate under the South American Plate. The trench and the western portion of the Andean central plateau form the

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"Bolivian Orocline" - a curved mountain belt that defines the northern slope of southern Peru, northern Chile, and Bolivia.

Geography:

The area is part of the South-Central Andes; it begins in the drainage of the Tambo River in Peru, continues south to the drainage of the Loa River in Chile and includes the desert beyond Taltal in Antofagasta and the border of Copiapó (Guillen, 1992:89). To the east, this region includes the Titicaca Basin, which incorporates parts of modern day southern Peru, northern Chile, eastern Bolivia, and Argentina (Guillen, 1992:89). The people discussed here centered their communities in the Arica-Camarones area. The best evidence of their mortuary ritual is present from Arica to Cobija, along the coast (Arriaza, 1995a). Arica is modern Chile's northernmost city lying 18 km south of the modern (2008) Peruvian border; the surrounding region is a focus of this dissertation.

The South-Central Andes encompass many mesoclimates (Figure 4), which are defined by altitude, the Andean chain, and the Antarctic current (Llagostera, 1989; Núñez, 1989; Wise, 1991; Guillen, 1992). Summaries for five grouped ecological areas identified and discussed by Guillen (1992:89-96) within the South-Central Andes are summarized below.

The Circum-Lacustrine Area:

This area includes the Titicaca Basin and plateaus stretching 3,850 to 5,200 meters above sea level (masl). It is a wet, low-temperature area with grasslands, shrub forests, and "umbelliferous plants" (plants with flowery, leafy

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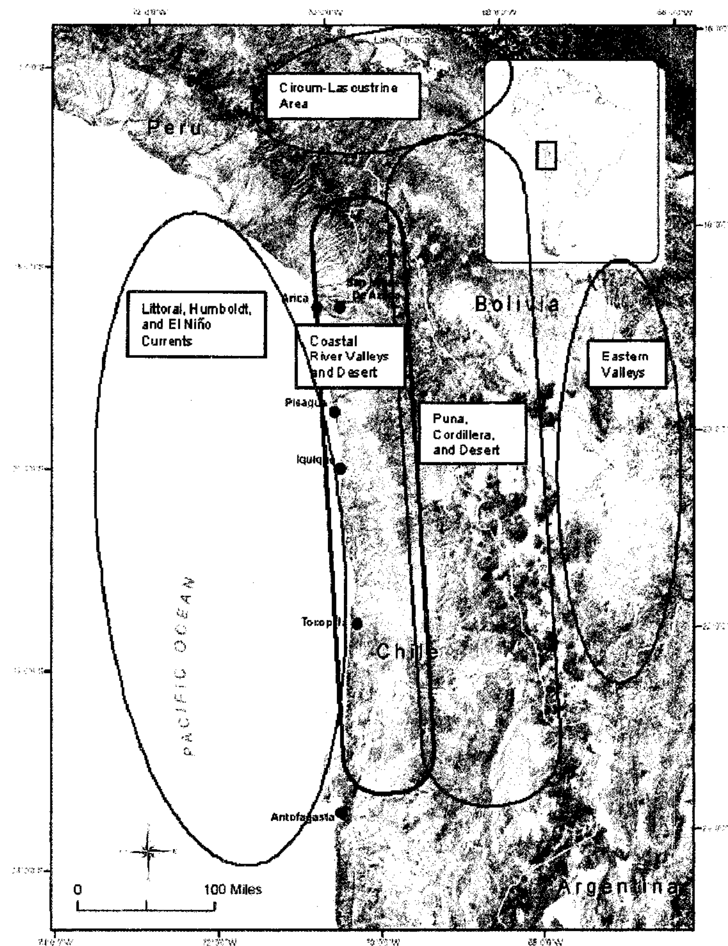

Figure 4 Approximation of ecological areas in the South-Central Andes

heads) (Guillen, 1992:90). Fauna include rodents such as chinchilla (*Chinchilla laniger*), vizcacha (*Lagidum visaccacia* and *Lagadum Peruanum*), and guinea pig (*Cavia porcellus*), and camelids such as vicuña (*Vicugna vicugna*), guanaco (*Lama guanicoe*), alpaca (*Vicugna pacos*), and llama (*Lama glama*). There is also a wide variety of birds. Gulls include the Gray Gull (*Larus modestus*), Band-tailed Gull (*Larus belcheri*), Kelp Gull *(Larus dominicanus), a*nd Swallow-tailed Gull (*Creagrus furcatus*). Other birds include the Torrent Duck (*Merganetta*

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armata), Steamer Duck *(Tachyeres patachonicus)*, White Tufted Grebe (*Podiceps rolland*), Pied-billed Grebe (*Podilymbus podiceps*), Great Grebe (*Podiceps major*), Plumbeous Rail Coot (*Rallus sanguinolentus*) Spot-Flanked Gallinule coot (*Porphyriops melanops*), Red-Gartered Coot *(Fulica armillata)*, White-winged Coot (*Fulica leucoptera*), Red-fronted Coot *(Fulica rufifrons*), Andean Ibis (Theristicus branickii), Black-faced Ibis (*Theristicus melanopis*), and White-Faced Ibis *(Plegadis chihi*) (Guillen, 1992:90-91). There are also many types of fish, including two species of kilifish (Orestias *cuvieri and* Orestias *chungarensis*) and catfish (Trichomycterus *areolatus)*.

The Puna, Cordillera, and the Desert:

The *puna* includes the dry altiplano of Chile, a plateau 4,000 masl with mountains and volcanoes ascending to 6,000 masl (Guillen, 1992:90). Average precipitation is about 300 mm (Núñez, 1989). Several small rivers and oases exist, including San Pedro de Atacama, Calama, and Tarapacá (Guillen, 1992:90-91). The riverine *cordillera* begins at 1,500 masl and extends to 3,000 masl (Guillen, 1992:90-91). It receives heavy rainfall.

Farther south is the Atacama Desert, a salt flat that encompasses parts of Chile, Bolivia, and Argentina. Flora includes shrubs, short columnar cacti, herbs, and tubers (Guillen, 1992:90-91). Fauna include camelids (vicuña, guanaco, alpaca, and llama) Andean Deer (*Hippocamelus bisulcus*), rodents (chinchilla, vizcacha, and guinea pig), and numerous birds (Puna Flamingo (*Phoenicoparrus*

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jamesi), Puna Rhea (*Rhea pennata tarapacensis*) and Puna Ibis *(Plegadis ridgwayi*)) (Guillen, 1992:91).

Eastern Valleys:

This is an area in Bolivia named the *Yungas* or *Montaña*, which includes valleys east of the Andes (Osborne, 1964; Guillen, 1992:91-92). It is humid and subtropical with hills and gorges. Precipitation is 700 - 800 mm per year; the mean annual temperature is 16 - 18 °C (Guillen, 1992:91). Flora and fauna are plentiful, and the soil is fertile (Guillen, 1992:91-92). Trees include Cedar (*Austrocedrus chilensis*), Big-Leafed or American Mahogany (*Swietenia macrophylla*), Common Walnut (*Juglans regia*), Kapok Ceiba (*Ceiba pentandra*), Pará Rubber (*Hevea brasiliensis*), Guayacán Soap Brush *(Guaiacum coulteri*), Cincona or Quinine Tree (*Cinchona officinalis*), Palm (Astrocaryum chonta) and trees of the *Polyiepis* and *Juglans* genera. Other plants include zarzaparilla (*Ribes cucullatum*), boldo *(Peumus boldus*), copal (Copaifera officinalis), and vilca *(Anadenanthera colubrina)*. Many crops grow here, including sweet potato (*Ipomoea batatas)*, manioc *(Manihot esculenta*), peanuts (*Arachis hypogaea*), tobacco *(Nicotiana tabacum*), and fruits including, Cherimoya (*Annona cherimola*), Guava *(Psidium littorale*), Pacay *(Inga feuilleei*), and Coca *(Erythroxylon coca*).

The Coast:

This area encompasses *lomas* (small foothills), river valleys, coastal desert, and the littoral (shoreline) (Guillen, 1992:91-92). From latitude of 3°- 30°

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south it is a desert (Guillen, 1992:91-92). The lomas rise 200 - 800 masl and receive cold air and fog from June to November (Guillen, 1992:92). Plants growing here include lichens such as *Menegazzia chrysogaster*, various species of *Bryophyta* mosses, and grasses such as *Spartina densiflora*. Succulents with roots include species of *Begonia* and *Hymenocallis*, tubers include species of Solanium and *Physalis*, and there are xerophytic bushes and cacti including species of *Copiapoa* (Engel, 1973). Fauna in the area include camelids (vicuña and guanaco), deer (*Blastocerus dischotomus)*, and small carnivores "Culpeo" Fox (Pseudalopex culpaeus), rodents (vizcacha), and lizards including many from the species *Liolaemus* (Guillen, 1992:92). Wildlife is abundant along the coast (and its valleys), as evidenced by accumulations of guano from seabirds, including species of shearwaters (*Colonectris and Puffinus*), Wilson's Petrels (*Oceanites oceanicus)*, gulls, and pelicans (*Pelacanus thagus)*. There are also bats, including Atacama Myotis (*Myotis atacamensis*) and Chilean Myotis (*M. chiloensis*). There are numerous fish in the waters including Spanish Sardines (*Sardinella aurita and Sardinops sagax*), anchovies (*Engraulis ringens),* mackerel (*Scomber japonicus Peruanus*), and Jurel or Yellow mackerel (*Trachurus murphyi*). Sea mammals, including South American Sea Lions (*Otaria flavescens),* are also present (Arriaza, 1995a:31) in the Pacific Ocean.

Coastal River Valleys and Desert:

Rivers originating in the Andes flow east to west across the desert. The Tambo, Lluta, Camarones, and Loa Rivers flow to the Pacific. The Osmore,

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Sama, Calina, Azapa, Codpa, and Camiña Rivers flow seasonally never reaching the ocean (Guillen, 1992:93). The nearby Camarones delta harbors many swamp-based plants, and has even more fresh water ones (Guillen, 1992:92-93). The valleys are more humid than the coast. Land animals include rodents, fox, deer, camelids, birds, and additional small mammals. Shrimp are found in the rivers, including South American Freshwater Shrimp (*Cryphiops caementarius*) (Guillen, 1992:93).

Surveys conducted by the staff of the Museo Arqueológico San Miguel de Azapa (MASMA), Universidad de Tarapacá in 1996 and 1997 identified 18 species of plants in this region. Most are found at river mouths and include species such as Gramma Salada (*Distichkis spicta*), Brea or Sorona (*Thesssaria absinthiodes*), and species of Junquillo (*Scrypus*) (Arriaza et al., 2001:32). Various grasses, tortoro reeds, and bushes, including *Pluchea chingoyo* and *Bacharis petiolata*, predominate.

Littoral, Humboldt, and El Niño Currents:

In the Pacific Ocean, the Humboldt Antarctic current forms an upwelling zone off the littoral, about 50 km wide that runs the coast from Valparaíso, Chile, to Chimbote, Peru (Guillen, 1992:94; Núñez, 1994; Llagostera, 1979). Many fish inhabit these cooler waters, which are less saline and oxygenated and have higher levels of phosphate compared to surface water (Guillen, 1992:92). Phytoplankton is present in the upper levels; in the lower levels are fish, mollusks, crustaceans, echinoderms, tunicates, cephalopods. Mollusks and fish

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remains exist in large numbers in the midden debris. Following the current are sea birds and sea mammals (Guillen, 1992:94).

The Humboldt Current from the Pacific Ocean contributes to the climate by bringing moisture from the ocean to the Andes (Arriaza, 1995a:31). The Humboldt Current changes in temperature as it meets warm water moving south toward Ecuador. El Niño conditions affect it. Moisture from El Niño currents can cause rain, mudslides, and flooding in the desert, as well as droughts in the highlands. When the Humboldt Current moves south, the warm water can cause plankton to die and decompose, the result is a gas that kills some marine life. Water temperature also rises 6 to 9 degrees, adding moisture and causing some fish to swim closer to shore or farther south (Guillen, 1992:94-95).

Cultural History of the Region

The South-Central Andes encompass a vast geographical and ecological region (Guillen, 1992:98, Núñez, 1994). There are a number of regional archaeological chronologies that vary and are difficult to compare (Uhle, 1919; Bird, 1946; Munizaga, 1957; Núñez, 1965; Dauelsberg, 1969; Wiley, 1971; Hyslop, 1976; Núñez 1976; Núñez, 1978; Mujica, 1985; Rivera, 1991; Llagostera, 1992; Guillen, 1992:98; Arriaza, 1993, 1995a), they are based on tool technology, ceramics, site type, stratigraphy, mummification technique and style, and ^{14}C dates.

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Defining Chinchorro Culture:

The Chinchorro are the founding population of the Arica region and archaeologists have outlined several different archaeological entities with multiple names, including: Chinchorroid, Chinchorro complex, Chinchorro culture, Chinchorro tradition, Arica Aborigines, Camarones Complex, Quiani Complex, Early Quiani, Late Quiani, Quiani I, and Quiani II (Bird, 1946; Alvarez, 1969; Bittmann and Munizaga, 1977; Núñez, 1969; Rivera, 1975; Núñez 1976; Olmos and Sanhueza, 1984; Llagostera, 1989) (Figure 5). According to Arriaza (1995b:14), "the study of the Chinchorro is often muddled by semantics. Several terms are found in the literature that in some references is only vaguely defined." These terms include phases, periods, horizons, cultures, and traditions. Mummies from this region have been studied since 1917, during this time the field of archaeology has undergone numerous theoretical changes, and as such, there have been multiple grouping methods developed and used by multiple authors (Uhle, 1917; Wiley and Phillips, 1958; Arriaza, 1995a) (Table 5). Quiani is the most problematic of the cultural sequences because it describes three different entities: (1) pre-ceramic sites Quiani 1 and 2 (Bird 1946), (2) Quiani or Quiani Complex -- terms used by Bird to describe pre-ceramic cultural sites excavated by Mostny (1964), and (3) a later horticultural stage, Quiani 7 described by Dauelsberg (Arriaza, 1995a). Quiani 1 and 2 (Bird, 1943, 1946), Quiani 9 (Muñoz and Chacama, 1982), and the Quiani and Camarones Complex (Llagostera, 1989, 1992) all comprise "Chinchorro culture" (Arriaza, 1995a:18).

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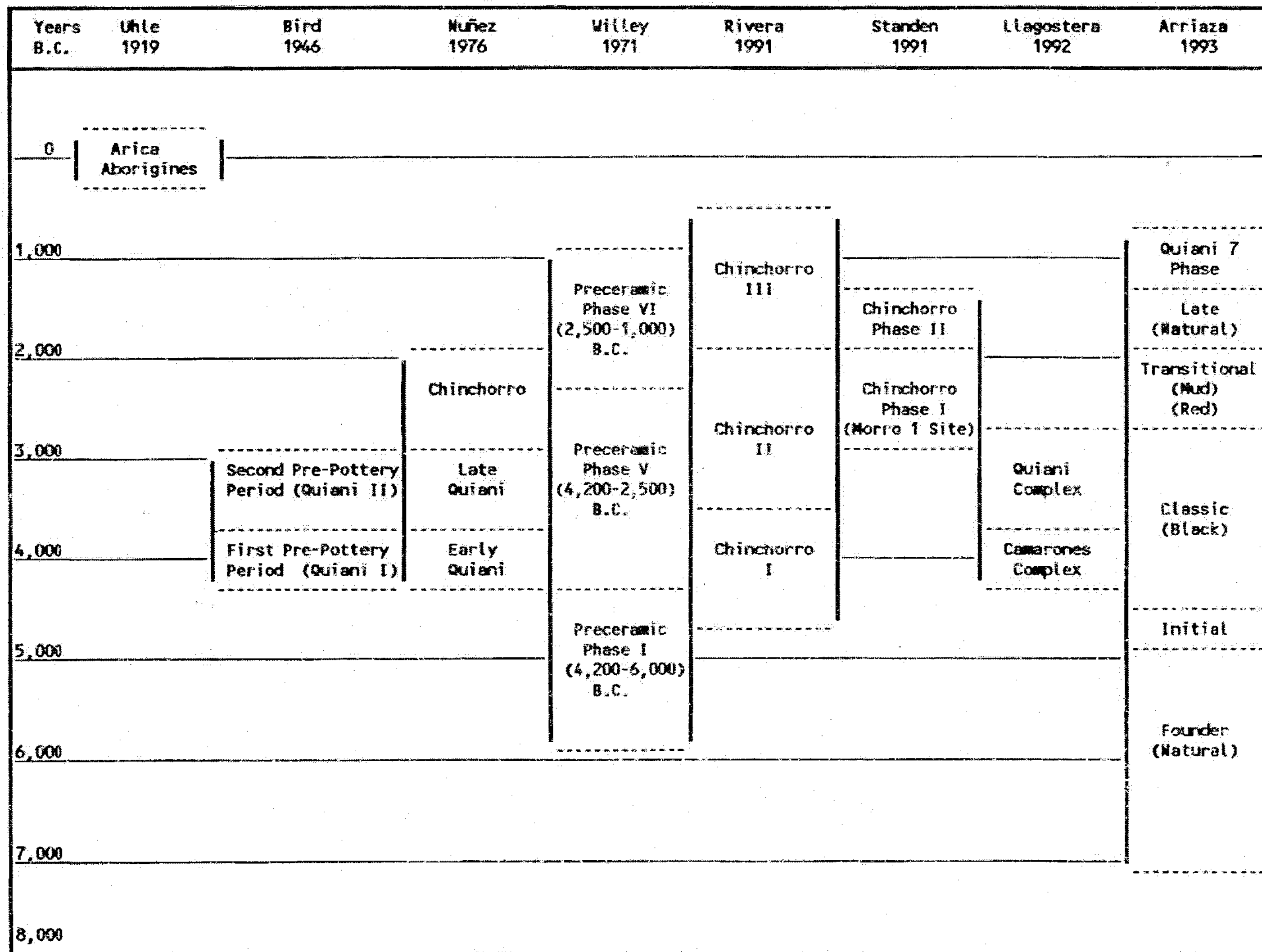

Figure 5 Various Chinchorro chronologies (Graph courtesy of B. T. Arriaza, 1995a:19)

However, Quiani 7, dated circa 1500 to 1300 BC, occurs completely in the Formative Period (2547 ± 171 BC and 528 ± 91 BC), though some consider it a late transitional Chinchorro site (Dauelsberg, 1974; Arriaza, 1995a:17-20, 39).

Table 5 Cultural names for Chinchorro mummies (Table courtesy of B.T. Arriaza, 1995a:11)

Cultural Name	Reference
Arica Aborigines	Uhle (1917, 1919)
First and Second Pre-Pottery Periods	Bird (1946:587-589
Patillo Complex	Schaedel (1957:25)
Chinchorro	Dauelsberg (1963:201) (In: True and Núñez, 1971:66)
Chinchorro Complex	Núñez (1965:46)
Chinchorro Tradition	Rivera (1975:28)
Chinchorro Cultural Complex	Bittmann and Munizaga (1976:64)
Chinchorro Culture	Alvarez (1961) (In Bittmann and Munizaga, 1977:120)
Camarones Complex	Llagostera (1992:92-93)
Quiani Complex	Llagostera (1992:92-93)

These cultural sequences incorporate adaptations related to subsistence, technology, and mortuary practices. The earliest sequences emphasized ethnological names and a comparison of burials in northern Chile (Uhle, 1919; Tartaglia, 1980). According to Uhle, the most characteristic feature of this culture was their care and veneration of the dead, and their distinct artificial mummification practice that was evident in most burials (Bittmann and Munizaga, 1976:61). Culture is determined by “a complex of regularly associated traits” (Childe, 1929; Renfrew and Bahn, 2000:163). Here the mortuary process defines the “culture.” Uhle referred to this culture as “Uhle’s aboriginals” it was

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later renamed the "Chinchorro complex." A "complex" is a chronological subdivision of artifact types. The Chinchorro mummified their dead in various techniques. Uhle (1917, 1922) define three classes: (1) simple treatment (wrapped in a mat): (2) complex treatment that consisted of three phases (a) removal of viscera, (b) desiccation of cavities with fire and filling of the cavities with packing materials, and (c) treatment of the surface to help the corpse maintain a life-like form), and (3) mummies covered with a layer of clay.

Uhle (1917, 1922) also noted differences in burial techniques as well as the degree of evisceration and preservation. These differences were attributed to one of three possibilities. First, it may have involved a practice of revisiting the bones, such as that done in Native North American ossuary burials. In these instances, the deceased was placed in a location, usually above ground, for a culturally prescribed period; after that, the body or parts of it were placed into a secondary burial (Uhle, 1917, 1922; Bittmann and Munizaga, 1976:77). This practice could explain the loss of soft tissue, skeletal elements, and occasional substitution of skeletal elements from one individual to another (Bittmann and Munizaga, 1976:77). Second, there may have been different practices of preparing the dead. Third, the treatment of corpses may have been "more successful" in some cases than in others; therefore, the differences in preparation may simply be accidental (Bittmann and Munizaga, 1976:67-76). A lack of knowledge in regards to the mummification techniques is one of the limits of this form of classification. It is not possible to know the origins of this practice,

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what role mummification played in the cultural system, or whether it relates to other cultural changes that occurred in the Americas around 5000 BP (3812 ± 108 BC) (Bittmann and Munizaga, 1976:86-87; Rivera, 1991). There is also no way of knowing how unifying the mummification practice was and whether it was developed locally or because of diffusion (Bittmann and Munizaga, 1976:86-87; Rivera, 1991).

A second chronology is based on stratigraphic profiles and cemeteries in Arica (Bird, 1946; Tartaglia, 1980). Later chronologies rely on ^{14}C dates for northern Chile, including the cities of Tarapacá, Antofagasta, and San Pedro de Atacama (Bittmann and Munizaga, 1977; Bittmann, 1984; Rivera, 1991). Within these chronologies, "Chinchorro culture" is differentiated from "Chinchorro tradition" (Arriaza, 1995a:14-20; Arriaza, 1995b:35, Rivera, 1975, Rivera, 1991). In archaeology, a "tradition" is "a socially transmitted form or unit (or a series of systemically related form units) which persists in time" (Thompson, 1956:38; Lyman and O'Brien, 2004:381). Here site type and artifacts define "the tradition" (Uhle, 1919; Bird, 1946; Munizaga, 1957; Tartaglia, 1980). A "horizon" links a number of phases in neighboring areas, phases that share cultural patterns but exist for shorter periods than traditions (Fagan, 1978:379; Pool, 2007). Horizon also can refer to an archaeological sequence (Wiley and Phillips, 1958). To be clear, the focus of this discussion is "Chinchorro culture," as defined by Arriaza (1995a:14-16), which considers the Chinchorro pre-ceramic, pre-agricultural, and without cotton or woven cloth.

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Chronology of the South-Central Andes:

The cultural history of the South-Central Andes includes five main periods (Mujica et al., 1983; Guillen, 1992:22,100-103; Sutter, 2006). These periods are not identical to those defined by Sutter (2006). Summarized in Table 6 are the two chronologies. The original dates in BP are displayed alongside the calibrated dates to maintain the integrity of the dating system. Dates reported in BP were calibrated using Stuiver and Reimer (1993) Method A with a 40-year Southern Hemisphere correction or the Cologne Radiocarbon Calibration & Paleoclimate Research Package calibration curve CalPal2007_HULU. When the standard deviation was not reported in the original articles ± 100 was arbitrariiy entered when using the CalPal 2007_HULU calibration program because the program would not work without entering a standard deviation; the dates should be considered approximate.

Table 6 Chronologies for the South-Central Andes and for the Azapa valley

Period	Date (South-Central Andes)*	Date (Azapa valley) (Sutter, 2006)
Archaic (Pre-Ceramic Period)	11,000 BP -- 4000 BP (~10,973 ± 117 BC - ~ 2547 ± 171 BC)	8000 BC -- 1000 BC
Formative	4000 BP -- 1500 BP (~2547 ± 171 BC - ~AD 500)	1000 BC -- AD 500
Middle Intermediate Period (Middle Horizon, Middle Period)	AD 500 -- 1000	AD 500 -- AD 1100
Late Intermediate (Desarrollo Regional)**	AD 1000 -- AD 1450	AD 1100 -- AD 1470
Late Period	AD 1450 -- AD 1532	Not Defined

*Dates were calibrated using CalPal 2007. Periods discussed by Guillen(1992)
**The tern Desarrollo Regional is used only in reference to the Azapa valley

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Santoro (1989) and Santoro and colleagues (2005) have also subdivided the Archaic Period into three phases. These appear below in Table 7.

Table 7 Santoro's (1989) subdivisions of the Archaic Period

Period	Date
Early Archaic Period	11,000 BP – 8000 BP (~10973 ± 117 BC - ~6903 ± 145 BC)
Middle Archaic Period	8000 BP to 6000 BP(~6903 ± 145 BC to ~4911 ± 125 BC)
Late Archaic Period	6000 BP to 4000 BP (~4911 ± 125 BC to ~2547 ± 171 BC)

For clarity, the first five main periods from the chronology of the South-Central Andes, with the addition of Santoro's subdivision of the Archaic Period or Pre-ceramic Period, are used in this chapter to discuss Andean regional chronology (Mujica et al.,1983; Santoro, 1989; Guillen, 1992:22,100-103; Santoro et al., 2005; Sutter, 2006;). However, because other region specific chronologies exist for northern Chile and Peru there may be some discrepancy in which chronological periods certain sites are placed in subsequent chapters; this may also be the case for definitions used by other authors discussing the same sites or their associated cultures (Stanish et al., 1989; Guillen, 1992:98; Sutter, 2006). Table 8 presents a summary of some Northern Chilean cultures within the chronology for the South-Central Andes (Mujica et al., 1983; Santoro, 1989; Guillen, 1992:22,100-103; Santoro et al., 2005:330; Sutter, 2006). The defining cultures have been listed for each period; however, there is continuance of some into multiple periods.

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Table 8 Northern Chilean cultures within the chronology of the South-Central Andes (Early Archaic Period – European contact)

Dates	Period	Northern Chilean Culture
11,000 BP to 8000 BP (10,973 ± 117 BC to 6903 ± 145 BC),	Early Archaic Period	Chinchorro, Acha
8000 BP to 6000 BP (6903 ± 145 BC to 4911 ± 125 BC).	Middle Archaic Period	Chinchorro
6000 BP to 4000 BP (4911 ± 125 BC to 2547 ± 171 BC).	Late Archaic Period	Chinchorro, Quiani
4000 BP to 1500 BP (2547 ± 171 BC to AD 500*)	Formative Period (Initial Period)	Chinchorro, Faldas del Morro, El Launcho, Alto Ramirez,
AD 500 to AD 1000	Middle Period (Middle Horizon, Middle Intermediate Period, Tiwanaku)	Loreto Viejo, Cabuza, Maytas (Maitas)-Chiribaya, Tiwanaku
AD 1000 to AD 1450	Late Intermediate Period (Desarrollo Regional)	Chilpe, Maytas (Maitas)-Chiribaya, San Miguel, Gentilar, Pocoma, Desarrollo Regional
AD 1450 to AD 1532	Late Period (Late Horizon)	Inka, Gentilar, Saxamar, Chilpe
AD 1532 to AD 1600	Andean-European contact	Inka

* No standard deviation is given for 1500 BP; therefore, the number was arbitrarily set at 500 for consistency within the chronological system. There is potentially a year of overlap in the chronology for the South Central Andes.

Early Archaic Period -- 11,000 BP to 8000 BP (~10,973 ± 117 BC to ~6903 ± 145 BC)

The chronology and cultural history of the northern Chilean coast and Azapa valley in the Archaic Period is the chronology most relevant here. It is unclear from where the early inhabitants of southern Peru and Chile came; one hypothesis suggests they traveled from the Amazonian highlands toward the coast (Arriaza, 1995a:46; Guillen, 1992:99-101). Paiján, a site in the northern Peruvian Andes is estimated, based on projectile points, to date to around 8000 BC (Chauchat, 1988; Wells and Noller, 1999). A second hypothesis states that early inhabitants of northern Chile traveled south along the coast from Peru (Arriaza, 1995a:46). There are habitation sites in southern Peru that are

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contemporary with early sites in Chile; however, there is no evidence for artificial mummification as practiced by the Chinchorro. Phenotypic, cultural, and molecular evidence suggests that those in the Chinchorro culture may be related to earlier Andean PaleoIndians (Sutter 1997, 1999:82, 2005, 2006) or Amazonian populations (Moraga et al., 2001, Rivera, 1975, 1984, 1991; Rivera and Rothhammer, 1986; Rothhammer and Silva, 1989, 1992; Rothhammer et al., 1984, 2002).

The inhabitants of this period used resources in the Andean highlands and occupied sites up to 2,000 masl. These hunters lived in the highlands from at least 11,800 cal. yrs. BP to 10,500 cal yrs. BP (11,757 ± 160 BC to 10,456 ± 205 BC) and focused their habitation and economies around lakes, rivers, springs, and seeps to gain access to water, vegetation, and animals (Núñez et al., 2002:821; Dillehay, 2002:765). Hunters killed and consumed mastodon, horses, and deer, as evidenced by butchered bones (Núñez, 1989). Artifacts from highland sites suggest they traded with coastal peoples or made trips to the ocean, rivers, and oases. Highland sites with coastal trade goods include Tojo-Tojone and Patapatane (Guillen, 1992:99-101; Dauelsberg, 1983; Santoro and Chacama, 1984). Evidence of fish bones at the site of Aragón-1, which is located in the Jazpampa Quebrada, 34 km from the coast and 1,100 masl, suggests that seasonal migrations may have occurred from highland lakes and low ravines to the coast. Charcoal from the site is dated to 8660 ± 230 BP (7816 ± 291 BC) (Gak-5965) (Núñez, 1983:184). Highland dwellers may have

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eventually migrated to the coast because of climatic fluctuations, including increasing aridity that dried the lakes (Núñez et al., 2002:821-823; Dillehay, 2002:764-765). Around 9000 cal yr. BP (8141 ± 142 BC) the climate became more arid in the highlands and the water in the lakes receded. After 8800 BP cal yr. (7934 ± 194 BC) and until 4500 BP cal yr. (3191 ± 149 BC) the sites by the lakes appear to have been abandoned.

The Peruvian sites of Quebrada Tacahuay and Quebrada Jaguay are located near Arequipa and constitute some of the earliest known evidence of coastal cultures in the New World. Quebrada Tacahuay produced charcoal hearth dates between 12,700 cal. BP ± 150 and 12,500 cal. BP ± 140 (13,131 ± 379 BC to 12,830 ± 381 BC), (Beta 108692A and Beta 95869C) (Stuiver and Reimer, 1993; Keefer et al., 1998:1833). Twelve terminal Pleistocene dates from charcoal have been obtained from occupations at Quebrada Jaguay that date from approximately 11,100 to 9900 BP ^{14}C years (11,056 ± 142 BC to 9472 ± 16 BC) (Stuiver and Reimer, 1993; Sandweiss et al., 1998:1830)

Among the earliest well-dated maritime sites in northern Chile is Quebrada de las Conchas, near Antofagasta. Samples of what is described only as “vegetable carbon from hearths” at the base of the shell midden have been dated between 9680 ± 16 BP and 9400 ± 160 BP (9204 ± 19 BC and 8760 ± 272 BC) (P-2702) (Llagostera, 1979:314; Stuiver and Reimer 1993). There is no evidence for the practice of artificial mummification at this site.

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Sometime between 9000 and 7000 BC, a small group settled in and around Arica (Muñoz and Chacama, 1993:28; Arriaza 1995a:43). Evidence of habitation at Acha-2, the oldest recognized Chinchorro site forms this estimation (Muñoz and Chacama, 1993:50-51; Muñoz et al., 1993, Arriaza, 1995a:6). Acha-2 dates between 8900 ± 150 and 6950 ± 150 BC (Llagostera, 1979; Muñoz and Chacama, 1982:28, Muñoz and Chacama, 1993). Muscle from "Acha Man," a male mummy named for this site in the Azapa valley, about 6 km from the modern city of Arica, was dated to around 7020 BC (Arriaza, 1995a:51-53). The early inhabitants of the Azapa valley were probably nomadic transhumant hunters and gathers who traveled between the valley and the highlands to the east, trading and hunting camelids for their meat, skin, and wool, which could be used for food, clothing, and the construction of homes (Santoro, 1989; Santoro 1993, Sutter, 2006). This form of exchange may have developed around 8000 BC and persisted until 1000 BC (Guillen, 1992:99-101), although, Núñez (1983:184) suggests it discontinued around 4000 BP (2547 ± 171 BC). They also engaged in fishing and shellfish collecting as evident by the remains of both at Acha-2 and Acha-3 (Arriaza et al., 1993; Standen, 1997; Sutter, 2006). Early inhabitants likely found advantages in a coastal settlement, including access to the Pacific Ocean and other water sources (small rivers, springs, and seeps). The ocean and these fresh water sources supplied food, water for drinking and washing, and reeds, grasses, sticks, and seashells to construct tools and homes.

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Middle Archaic Period -- 8000 BP to 6000 BP (~6903 ± 145 BC to ~4911 ± 125 BC)

Occupation in the coastal lowlands reflects a movement toward a maritime economy during this period. According to Núñez (1983), this happened slowly and involved multiple groups who migrated to the coast. Among Chinchorro sites from Ilo to Iquique during this period were the sites of Aragón-1, Tiliviche-1b, some strata of Morro-1, Camerones-14, and Camerones-17, (Núñez, 1976:123; Núñez and Moragas, 1978; Allison et al., 1984:163-165; Schiappacasse and Niemeyer, 1984:26; Muñoz, 1985:275; Rivera, 1991; Guillen, 1992:100; Aufderheide et al., 1993:191; Muñoz and Chacama, 1993:28; Arriaza 1995a:42-43). The adoption of a maritime economy by coastal dwellers may have involved three stages: (1) exploration of coastal resources, (2) the use of fishhooks to catch marine resources, and, (3) creation and use of rafts to collect marine resources (Llagostera, 1989). Better fishing and shellfish collecting techniques came with exploration of terrestrial resources (Llagostera, 1989).

Late Archaic Period -- 6000 BP to 4000 BP (~4911 ± 125 BC to ~2547± 171 BC)

Human habitation sites in the highlands reappear around 4500 BP (3191 ± 149 BC) (Núñez et al., 2002:821-823; Dillehay, 2002:764-765). Surrounding late glacial and early Holocene paleolakes in the highlands at 3600 m are numerous archaic sites (Núñez et al., 2002:821). They included artifacts of human habitation. Fish bones and shells dated to 5910 ± 90 BP (4797 ± 110 BC) were found at Patapatane cave in the highlands of Arica. Using Santoro's

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subdivisions, this is a Late Archaic Period site, although Santoro and colleagues (2005:329) describe it as belonging to the Middle Archaic (Arriaza, 1995a:51; Santoro and Chacama, 1982; Santoro et al., 2005). Their discovery suggests that there was continued trade between inland and coastal dwellers. Large cemeteries also exist to the north of Chile in Paloma, Peru dating to 4360 BC ± 340 BC; however, there is no evidence of secondary burials at Paloma or artificial mummies, suggesting these sites are unrelated to coastal Peruvian and Chilean sites that display the Chinchorro mortuary practice (Quilter, 1989; Arriaza, 1996:134). Sites associated with this cultural stage include the Ring site, Los Burros, and Carrizal in Peru; and Tulan, Chinchorro-1, Confluencia-1, Puripica-3/P34, Hakenasa, Puxuma, some strata of Morro-1, and Morro-1/6 in Chile (Lanning, 1967; Mujica et al., 1983; Núñez, 1983; Allison et al., 1984; Santoro and Núñez, 1987; Focacci and Chacón, 1989; Sandweiss et al., 1989; Wise, 1989; Guillen, 1992:99; Muñoz et al., 1993; Lavallée et al., 1999; Núñez et al., 2002; LeFebvre, 2004; Grosjean et al., 2007). The recently excavated Yungay site in Arica may also belong to this period; however, attempts to radiocarbon date the site failed due to insufficient collagen in the bone sample.

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The Formative Period -- 4000 BP to 1500 BP (~2547 ± 171 BC to ~AD 500):

The introduction of pottery, metallurgy, textiles, and agriculture characterize this period. The earliest evidence of these technological developments appears around Arica and extends to La Capilla in the area between Lluta and Camarones (Muñoz, 1989; Guillen, 1992:101). These advances appear around 1000 BP (1032 ± 111 BC) in the Tarapacá region and around 3650 BP (2042 ± 136 BC) in caves at Tulán within the *puna* of the Atacama Desert (Guillen, 1992:101).

This period included fiber and sand tempered ceramics contemporaneous with those seen in altiplano sites (Sutter, 2006). Their introduction probably resulted from diffusion from northern groups including Qaluyo, Chiripa, and Wankarani from the Titicaca basin (Guillen, 1992:101). Some researchers believe these artifacts with altiplano Pukara motifs represent movement from the alitplano to the coast (Muñoz, 1989); others think that the genetic and cultural origins of the Formative Period populations in northern Chile came from the proceeding Chinchorro groups (Rothhammer and Santoro, 2001; Sutter, 1997, 1999, 2000, 2005, 2006). Smoking pipes, wooden snuffing containers, and trays filled with hallucinogenic plant remains also appear in this period (Manual, 1995).

The earliest pottery in this region is associated with settlements in the Osmore Drainage in Peru (Rivera, 1991). In Chile, sand-tempered ceramics are associated with the following cultures and locations: Faldas del Morro, El Launcho, and early phases of Alto Ramírez, San Pedro de Atacama, and

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Tarapacá (Focacci, 1974; Dauelsberg, 1985; Guillen, 1992:101). Archaeological sites at Azapa dated from 1000 BC to 800 BC (AZ-70, AZ-71, and AZ-115), including those associated with the Alto Ramírez culture and dated from 800 BC to AD 500, are included in this period. The inhabitants of the Azapa valley had a mixed agropastoral and maritime economy (Muñoz, 1981, 1983, 1989; Santoro 1980a,b,c; Sutter, 2006). Chinchorro sites without pottery and metallurgy also existed, including Morro-1, Morro-1/6, and PLM-8. Coastal inhabitants continued to engage in a primarily maritime economy.

Organized village life and early horticulture began toward the end of this period. Houses were square or rectangular structures made of tortoro reeds, wooden branches, and mud erected on terraces and slopes where they received protection from the wind (Arriaza, 1995a:33-36). The average number of houses per site is unknown. Trade may have begun, via reed boats or through foot travel with other coastal cultures, and with early Tiwanaku and Cabuza cultures that came from the Bolivian highlands. Domestication of the llama may have also occurred during this period, aiding in the transport of trade goods.

Middle Intermediate Period (AD 500 to AD 1000):

This period saw the spreading influence of the Tiwanaku culture west of the altiplano basin in the South-Central Andes (Guillen, 1992:102-103; Stanish, 1992). It includes Classic and Expansive Tiwanaku cultures with the following ceramic styles: Loreto Viejo in Peru, and Cabuza, Maitas (Maytas), and Quitor in Chile (Berenguer and Dauelsberg, 1989; Guillen, 1992:102-103; Stanish, 1992;

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Sutter 2006). Tiwanaku culture was recognized for its socio-economic organization, agro-pastoralist economy, development of irrigation, and technological advances. Also well known is its religion and iconography; stone, textiles, and ceramics became an outlet for religious expression (Berenguer, 1978; Berenguer and Dauelsberg, 1989). The Tiwanaku culture spread throughout the Azapa valley. Bio-distance studies using craniometrics and mtDNA suggest to some researchers that the inhabitants of the Tiwanaku culture resettled the coastal population circa Arica during this period (Rothhammer et al., 1982; 1983; 1989; Cocilovo and Rothhammer, 1999; Varela and Cocilovo 2002:264-265; Moraga et al., 2005). However, researchers using nonmetric dental data (Sutter, 1997, 1999, 2000, 2005, 2006) and cranial data (Guillen, 1992, Sutter and Mertz, 2004) have shown that differences among the Middle Intermediate Period Azapa valley populations and earlier valley populations are not significant, suggesting that any changes are from diffusion rather than population replacement from the altiplano (Sutter, 2006:66).

The Azapa valley inhabitants lived in small communities. Study sites in include AZ-71, AZ-140, and AZ-141. Playa Miller-3, a coastal site, is also included in this period. Influenced by the Tiwanaku culture from the northeast, the Azapa valley inhabitants were hastened in their expansion and technological development by the arid soil of the valley. However, they did adopt technologies such as irrigation, herding, and new agricultural products. They also designed camelid wool textiles, looped bags, polychrome four-cornered hats, turbans,

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braided belts, and baskets (Rivera, 1991; Cordova et al., 1999; Peters, 2004). The people of this culture created textiles and baskets decorated with geometric patterns, anthropomorphic figures, and collected trophy heads, which some researchers speculate expressed aspects of a shared ideology (Rivera, 1977:43, Rivera, 1991:21; Browman, 1978:327).

Signs of status became apparent during this period, one example was the four-pointed hats designed in the Tiwanaku style and found in the Azapa sites. Tiwanaku V ceramics and textiles have also been recovered from burials and túmulos in both Azapa and coastal sites (AZ-6, AZ-71, AZ-75, AZ-141, and AZ-143) (Focacci, 1981; Muñoz, 1989; Sutter, 2006). Another possible status indicator may have been the inclusion of Loreto Viejo ceramics in Azapa valley burials (Rivera, 1991; Sutter, 2006). The inhabitants of this region created plaques, ornaments, rings, and beads of gold, silver, copper, and bronze (Rivera, 1991; Peters, 2004). Pipes, trays, tubes, spatulae and other accessories linked with the use of hallucinogenic plants and stimulants persisted into this period, and may have been associated with shamanistic ceremonies (Rivera, 1991:31; Berenguer and Dauelsberg, 1989). Masks with iconographic designs, including puma-human representations appear, and may represent divinities (Berenguer and Dauelsberg, 1989).

Late Intermediate Period (AD 1000 to AD 1450):

The decline of Tiwanaku gave rise to regional cultures between AD 1000 and AD 1450 (Guillen, 1992:103). The defining cultures of this period include

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Chiribaya, Estuquina, La Yarai in the Osmore Drainage in Peru and San Miguel, Chilipe, Gentilar, Pocoma, and Desarrollo Regional within the Azapa valley (Guillen, 1992:103). The emergence of unique ceramic traditions associated with the San Miguel, Pocoma, and Gentiliar cultures on the coast of the South-Central Andes provide further definition (Rivera, 1991; Sutter, 2006). There was a decline in the socio-economic structure of the Tiwanaku culture. Bio-distance studies suggest there was an influx of people into the Azapa valley during the Desarrollo Regional Period following the collapse of the Tiwanaku structure (Rothhammer and Santoro, 2001; Rothhammer et al., 2002; Sutter, 1997, 2005, 2006). Inhabitants of the Azapa valley likely received low levels of gene flow from people migrating from the altiplano (Rothhammer and Santoro, 2001; Rothhammer et al., 2002; Sutter, 1997, 2005, 2006). The coastal communities maintained genetic continuity with earlier peoples, but saw a gradual dispersion of various groups with a movement toward local chiefs (Sutter, 1997, 1999, 2000; Cocilovo and Rothhammer, 1999; Cocilovo et al., 2001; Varela and Cocilovo, 2002; Sutter and Mertz, 2004, Sutter, 2006). The continuous movement of people from the Lake Titicaca region to the coast led to the development of strategically located fortified settlements (Rivera, 1991:35). Villages included specific spaces for agriculture, domestic, and ceremonial activities. Homes had areas for food storage and corrals for animals (Rivera, 1991:35; Cordova et al., 1999).

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A mixed economy of agriculture, animal husbandry, and hunting developed in the valleys, whereas fishing and gathering dominated along the coast (Rivera, 1991:35-37; Cordova et al., 1999). Crops grown during this period included maize, beans, quinoa, pumpkins, jicama, squashes, potatoes, cotton, pacae fruit, guava, lúcuma, and coca (Rivera, 1991:35; Cordova et al., 1999). Log rafts and rafts made of reeds provided coastal inhabitants access to a greater variety of marine foods (Rivera, 1991:37). Playa Miller-6 is the only research site included here, although AZ-71 and AZ-140 may have extended into this period.

The Late Period (AD 1450 to AD 1532):

The Inka (or Inca) Empire occupied southern Peru and northern Chile between AD 1450 and AD 1532 (Guillen, 1992:103). It established a social, economic, and ideological order that united the Andean populations (Rivera, 1991:39-41; Cordova et al., 1999). Created during this period were elaborate textiles, ceramics, and metal objects. Various items were traded throughout the region, including fruits, coca, guano, salt, maize and aji peppers (from the coast), potatoes (from the precordellia), and llama and alpaca by-products (from the alitplano) (Rivera, 1991:39). The Inka constructed public buildings including tambos (way stations), fortified settlements, complex settlements, sanctuaries, and associated funerary buildings (Rivera, 1991:39-41; Cordova et al., 1999).

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Summary of Cultural History:

Much of the cultural history of the South-Central Andes relates to understanding how groups living in different ecosystems shared and distributed resources and ideas. The Archaic Period involved movement from the north and eastern highlands to the coast, and saw the development of a maritime economy with an elaborate mortuary process. Beginning in the Formative Period and following in the subsequent periods there was movement from the *altiplano* to the coast and valleys, and the establishment of a social organization that developed agriculture, ceramics, and metallurgy. Tiwanaku, in the Middle Intermediate Period, saw the creation of a complex form of social and economic organization. This social organization expanded with the Inkas. Elaborate funerary practices occurred from the Archaic to the Formative Period; during this time, there was little social differentiation in burial practices. The migration of people from the highlands changed the mortuary practice and burial mounds (túmulos) became popular while the mummification practices of the Chinchorro faded. Until the Inka Empire, there was little status differentiation in the burials (Guillen, 1992:104). Table 9 includes data on the sites discussed within the cultural history section. Some sites have several radiocarbon dates; not all dates are included here.

Introduction to the Chinchorro Culture

Overview:

The Chinchorro anglers and hunter-gatherers lived in small houses in settlements along the coast of northern Chile and southern Peru thousands of

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Table 9 Sites discussed in cultural history of South-Central Andes

Period	Site	Age	Material and Context	Reference
Early Archaic 11,000 BP to 8000 BP (~10,973 ± 117 BC to ~6903 ± 145 BC	Quebrada Jaguay	11,056 ± 142 BC 9472 ± 16 BC	Hearth charcoal	Sandweiss et al. (1998)
Early Archaic	Tojo-Tojone	9265 ± 2423 BC	Charcoal	Dauelsberg (1983)
Early Archaic	Quebrada las Conchas	8760 ± 272 BC 9204 ± 19 BC	Vegetable Carbon from Hearths	Llagostera (1979)
Early Archaic	Patapatane	8160 ± 160 BC	Charcoal	Santoro and Chacama (1984)
Early Archaic	Paijan	est. ~8000 BC	Based on projectile points	Chauchat, (1988); Wells and Noller, (1999)
Early Archaic	Aragón-1 (in Jazampa Quebrada)	7816 ± 291 BC	Charcoal	Núñez (1983)
Early Archaic	Acha-2	8900 ± 150 BC 7020 ± 150 BC 6950 ± 150 BC	Charcoal and Muscle	Muñoz and Chacama (1982:28); Muñoz and Chacama (1993)
Early Archaic	Tiliviche-1b	7180 ± 365 BC	Vegetal fibers	Núñez and Moragas (1977); Muñoz (1985)
Middle Archaic Period (6903 ± 145 BC - 4911 ± 125 BC)	Aragón-1	6710 ± 230 BC	Charcoal	Núñez (1976)
Middle Archaic Period (6903 ± 145 BC 4911 ± 125 BC)	Tiliviche 1b	5900 ± 280 BC; 4955 ± 65 BC;	Vegetal fibers	Núñez (1976); Núñez and Moragas (1977); Muñoz, (1985)
Middle Archaic Period	Morro-1	5860 ± 180 BC	Camelid fur	Allison et al. (1984); Rivera (1991)
Middle Archaic Period	Camarones-14	5470 ± 225 BC; 5050± 135 BC	Muscle Charcoal	Schiappacasse and Niemeyer (1984); Rivera (1991)
Middle Archaic Period	Camarones-17	4980 ± 140 BC	Wood	Aufderheide et al., (1993)
Late Archaic Period (4911 ± 125 BC - 2547± 171 BC)	Tulán-67	4827 ± 66 BC	Charcoal	Núñez et al. (2002)
Late Archaic Period	Patapatane	4797 ± 110 BC	Fish skeletal elements, shells	Santoro et al. (2005)
Late Archaic Period	Chinchorro-1	4406 ± 200 BC	Wood	Muñoz et al. (1993)
Late Archaic Period	Paloma	4360 BC ± 340 BC	Charcoal	Quilter (1989) Benfer (1984; 1986)
Late Archaic Period	Confluencia-1	4200 ± 136 BC	Charcoal	Lanning (1967)
Late Archaic Period	Purpica-3/P34	3937 ± 133 BC	Charcoal	Núñez et al. (2002)
Late Archaic Period	Hakenasa	3927 ± 95 BC	Charcoal	LeFebvre (2004)
Late Archaic Period	Ring site	3861 ± 76 BC	Charcoal	Sandweiss et al. (1989)
Late Archaic Period	Los Burros	3252 ± 105 BC	Organic material	Lavallée et. al (1999)

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Table 9 Sites discussed in cultural history of South-Central Andes, cont.

Period	Site	Age	Material and Context	Reference
Late Archaic Period	Carrizal	3104 ± 168 BC	Charcoal	Wise (1989)
Late Archaic Period	Puxuma	2822 ± 139 BC	Charcoal	Santoro and Chacama (1982)
Late Archaic Period	Morro-1	2625 ± 158 BC	Wood	Allison et al. (1984)
Late Archaic Period	Morro-1/6	2568 ± 115 BC	Human muscle	Focacci and Chacón (1989)
Formative Period (2547 ± 171 BC - 528 ± 91 BC)	Piñuta	2453 ± 148 BC and 1170 ± 139 BC	No stated	Guillen (1992); Grosjean, 2007
Formative Period	Tarapacá-18	2404 ± 245 BC	Charcoal	True and Gildersleeve (1980)
Formative Period	Morro-1	2400± 136 BC	Charcoal	Allison et al. (1984)
Formative Period	Morro1-6	2360 ± 145 BC	Human Liver	Focacci and Chacón (1989), Aufderheide and Allison (1994)
Formative Period	Playa Miller-8	2140 ± 105 BC	Wood	Núñez (1976)
Formative Period	Tulán-67	2025 ± 168 BC	Charcoal	Núñez et al. (2002)
Formative Period	Pisagua (region/site)	1300 BC - 600 AD	--	Horta (2004)
Formative Period	Azapa-70	est. 1000 BC – AD 750	--	Sutter (2006)
Formative Period	Azapa-14	est. 1000 BC – AD 750	--	Sutter (2006)
Formative Period	Azapa-115	est. 1000 BC – AD 750	--	Sutter (2006)
Formative Period	Conanoxa E-6	est. 1000 BC – AD 750	--	Agüero and Cases (2004)
Formative Period	Punta Pichalo	est. 1000 BC – AD 750	--	Agüero and Cases (2004)
Formative Period	Guatacondo	ca. 616 ± 136 BC	--	Guillen, (1992) Rivera (2002)
Middle Intermediate Period (AD 500 – AD 1000)	San Pedro-1	est. AD 300 - AD 900	--	Augustyniak (2004)
Middle Intermediate Period	AZ-75	AD 421 ± 224	Human Tissue	Aufderheide et al. (2002)
Middle Intermediate Period	AZ-71	AD 950 ±75	Human Tissue	Focacci 1981
Middle Intermediate Period	AZ-140	AD 500 – AD 1000	--	Sutter (2006)
Middle Intermediate Period	AZ-141	AD 500 – AD 1000	--	Sutter (2006)
Middle Intermediate Period	AZ-6	AD 730 ± 80	Wool	--
Middle Intermediate Period	AZ-143	AD 500 – AD 1000	--	Sutter (2006)
Late Intermediate Period (AD 1000 - AD 1450)	Playa Miller-6	AD 1000 - AD 1450	--	--
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years before the adoption of agriculture in the region. Their maritime subsistence and semi-nomadic culture changed little over 5,000 years, with only minor technological improvements to their fishing and hunting kits (Arriaza, 1995a:46-48). Two measures define their chronologies: tool technology and mortuary practice. Mummy seriation provides a diachronic record of cultural changes and better exemplifies the changes that occurred in this culture than examining the tool technology, habitation sites, or subsistence, which changed little (Arriaza, 1995b).

Artificial mummification was practiced during four periods; a small population, fishing and gathering subsistence, low mobility, and a relatively high rate of treponemal-like skeletal lesions characterize all periods (Allison et al., 1984; Arriaza, 1995a, b; Arriaza, et al., 1998). The Late Chinchorro population (1720 BC to 1110 BC) practiced natural mummification. A higher population density, natural mummification, agro-pastoralism, high mobility, trade, and a low rate of treponemal-like infections characterized this period (Allison et al., 1984; Arriaza, 1995b; Arriaza et. al., 1998). Treponemal rates generally increase with population density, trade, and travel. Additionally, the adoption of agriculture usually brings a decrease in health status (Larsen, 1999). In this case, it appears to be the opposite, suggesting that other factors like cultural practices (mortuary rituals), geography, a change in the treponemal disease, or another disease are possibilities.

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Chinchorro Settlement Patterns and Habitation Sites:

The Chinchorro lived in band level settlements of less than 100 individuals (Arriaza, 1995a:6, 33; Renfrew and Bahn, 2000:167). Documentation of the formation of houses and permanent villages, suggests that by 7000 BC the Chinchorros led a mostly sedentary life along the coast of southern Peru and northern Chile (Arriaza 1995a:33). Habitation sites associated with the Chinchorro include Acha-2 (Muñoz and Chacama, 1993) and Quiani-9 in Arica (Muñoz and Chacama, 1982:75), Conanoxa W (a) in Camarones (Schiappacasse and Niemeyer, 1969; Aufderheide and Allison, 1994), Cerro Moreno in Antofagasta, and Caleta Huelén- 42 in the drainage of the Loa River (Núñez, 1976:92). Aragón-I, Ilo, and Villa del Mar in southern Peru (Núñez and Zlater, 1976; Núñez, 1976; Muñoz, 1985:275; Rivera, 1991:17; Wise, 1991) may be associated with the Chinchorro; however, more research is needed (Arriaza, 1995a:126, 130).

Chinchorro structures were made of perishable materials; however, given the arid coastal climate, they provided adequate shelter (Arriaza, 1995a:35). With the exception of camps, there is no evidence to suggest these ancient structures were temporary residences. Homes varied in shape from circular to semi-circular to cone shaped in diameter from 1m to 3 m Some contained a hearth in the center. Cobbles and obliquely placed wooden posts formed the framework; branches, thatched reeds, and camelid or sea lion skins probably comprised the exterior (Arriaza, 1995a:33-34).

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Chinchorro settlement patterns varied from a few huts at the site of Acha-2 to about 180 at the site of Caleta Abtao in Antofagasta (Llagostera, 1989:70; Arriaza, 1995a:33-36, 47). The average number of houses per Chinchorro sites has not been determined, but it was probably less than 50. Cemetery size varies from a few individuals to greater than 100; however, it is difficult to tell in some instances if a particular cemetery is contemporaneous with a particular site (Arriaza 1995a: 33-37). Additionally, many cemeteries contain a mixture of different styles of mummies, and have radiocarbon dates spanning thousands of years (Arriaza 1995a: 33-37). Acha-2, the earliest recognized Chinchorro coastal settlement in Arica, contained 11 circular homes occupying an area about $880m^2$ (Arriaza, 1995a:34; Muñoz and Chacama, 1993:25). Assuming the homes were contemporaneous, Arriaza (1995a:34) estimated that if two to four persons lived in each, the population at the site would range from 22 to 44 persons. Numerous shell middens suggest a long occupation (Arriaza, 1995a:35).

Quiani-9 (4420 ± 540 BC and 4165 ± 280 BC) contained two semi-circular homes (Muñoz and Chacama, 1982:75). Quiani-1 and Quiani-2 are middens measuring 5 - 10 m high; they date to 3300 ± 430 BC, suggesting at least a thousand years of occupation (Bird, 1943; Mostny, 1964; Muñoz and Chacama, 1982:72; Arriaza, 1995a:35). Given the proximity of the middens to the houses, they are probably related (Arriaza, 1995a:35).

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Five hundred miles south of Arica are two circular homes at the site of Caleta Huelén-42 that date to 2830 ± 100 BC (Núñez et al., 1975; Núñez, 1976:92; Llagostera, 1989:71; Arriaza, 1995a:35). Stones placed vertically made up the low walls of the house; the roof and upper house walls were made of sticks, reeds, and skins. The floors were earthen, compact, and covered with seaweed ashes (Arriaza, 1995a:35).

In the Camarones Valley, 100 km south of Arica and 40 km inland at the Conanoxa site, are circular house foundations (Arriaza, 1995a:35; Schiappacasse and Niemeyer, 1969). The homes had stones at their base with posts extending up from them in a conical shape. The walls and roof were probably comprised of sticks, grasses, and/or animal skins. A summary of some early Chilean and Peruvian sites, including Chinchorro sites associated with habitation appears in Table 10.

Temporal Chinchorro Change:

Chinchorro structures did not change much over time. The same variations on homes appear throughout the thousands of years the Chinchorro occupied the Arica-Camarones area. The earliest inhabitants of the region, circa 8000 BC, who lived in the highlands utilized rock overhangs and caves as shelters; however, as noted, the use of caves discontinued when this population relocated to the coast (Arriaza, 1995a:50; Chauchat, 1988). The settlement pattern on the coast may have remained the same until sometime between 2000 BC and 1000 BC when the population increased, specialization occurred, and

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Table 10 Early Chilean and Peruvian archaeological sites

Site	Type	Radiocarbon Date	Material	Lab No.	Reference
Tiliviche-1b	__	9760 ± 365 BC	Charcoal	SI-3116	Núñez and Moragas (1977)
Acha-2	__	8900 ± 150 BC	Charcoal	Teledyne SR	Muñoz and Chacama (1993)
Aragón-1	__	8660 ± 230 BC	Charcoal	GAK-5966	Núñez and Zlater (1977)
Tunia, Calama	__	8257 ± 254 BC (9080 ± 170 BP)	__	__	Núñez (1976)
Acha-2	__	8127 ± 340 BC (8970± 255 BP)	Muscle	__	Aufderheide et al., cited in Guillen (1992)
Kilómetro-4	__	8030 ± 100 BC	Charcoal	Beta-77947	Wise (1999)
Tiliviche-1b	__	7850 ± 280 BC	Vegetal Fiber	GAK-052	Núñez and Moragas (1977)
Quebrada Las Cochas	midden	7730 ± 160 BC	Charcoal	__	Llagostera (1979:314)
Quebrada Las Conchas	midden	7450 ± 160 BC	Charcoal	__	Llagostera (1979:314)
Camarones-14	__	7420 ± 225 BC	Charcoal	I-999	Schiappacasse and Niemeyer (1984)
Tiliviche-1b	midden	7180 ± 365 BC	__	__	Núñez and Moragas (1977); Muñoz (1985:275)
Acha-2	__	7020 ± 150 BC	__	__	Muñoz and Chacama (1993:28)
Acha-2	Camp	6950 ± 150 BC	Charcoal	__	Muñoz and Chacama (1993:28)
Camarones-17	__	6930 ± 140 BC	Wood	GX-15081	Muñoz et al. (1993) Aufderheide et al. (1993)
Tiliviche-1b	__	6905 ± 65 BC	Charcoal	SI-3115	Núñez and Moragas (1977)
Tarapacá-14-A	__	6830 ± 270 BC	__	GAK-2432	True et al. (1971)
Camarones-17	__	6780 ± 110 BC	Wood	GX-15080	Muñoz et al. (1993) Aufderheide et al. (1993)
Aragón-1	Camp	6710 ± 230 BC	Charcoal	GAK-5966	Núñez (1976:123)
Camarones-14	__	6650 ± 155 BC	Charcoal	I-9817	Schiappacasse and Niemeyer (1984)
Camarones 14	__	6615 ± 390 BC	Charcoal	I-9816	Schiappacasse and Niemeyer (1984)
Tarapacá-14-A	__	6430 ± 390 BC	Charcoal	WSU-987	True et al. (1971)
Quiani 9	__	6370 ± 540 BC	Charcoal	GAK-8782	Muñoz and Chacama (1982)
Camarones-14	__	6300 ± 246 BC	__	__	Schiappacasse and Niemeyer (1984)
Camarones Pta. Norte	__	6270 ± 130 BC	Charcoal	GAK-7135	Alvarez (1980)
Camarones Pta. Norte	__	6240 ± 160 BC	Charcoal	GAK-7132	Alvarez (1980)
Kilometro-4	__	6220 ± 70 BC	Charcoal	Beta-77951	Wise (1999)
Quiani-1	__	6170 ± 220 BC	Charcoal and Bone	I-1348	Mostny (1964)

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Table 10 Early Chilean and Peruvian archaeological sites, cont.

Site	Type	Radiocarbon Date	Material	Lab No.	Reference
Quiani-9	—	6115 ± 280 BC	—	I-11.643	Muñoz and Chacama (1982)
Cobija-1	—	6030 ± 70 BC	Charcoal	Beta-3933	Bittmann (1984)
Chinchorro-1	—	6070 ± 285 BC	Wood	I-15084	Muñoz et al. (1993)
Tiliviche-1b	—	6060 ± 60 BC	Charcoal	SI-3114	Núñez and Moragas (1977)
Caramucho-1	—	5980 ± 120 BC	Shell	GAK-8375	Sanhueza (1980)
Tarapacá-12		5970 ± 120 BC	Charcoal	GAK-2205	True et al. (1971)
Camarones Pta. Norte	—	5950 ± 130 BC	Charcoal	GAK-7137	Alvarez (1980)
Tiliviche-1b	Midden/ Habitation	5900 ± 280 BC	Vegetal fibers	GAK-6052	Núñez (1976:123)
Camarones Pta. Norte	—	5880 ± 160 BC	Charcoal	GAK-7134	Alvarez (1980)
Villa del Mar	—	ca. 5850 BC	—	—	Wise (1991)
Ilo	Habitation	ca. 5850 BC	—	—	Wise (1991)
Tarapacá-14A	—	5753 ± 235 BC	Vegetal Fiber and Charcoal	—	Núñez (1976)
Camarones Pta. Norte	—	5750 ± 170 BC	Charcoal	GAK-7133	Alvarez (1980)
Camarones Pta. Norte	—	5670 ± 140 BC	Charcoal	GAK-7136	Alvarez (1980)
Camarones Pta. Norte	—	5640 ± 160 BC	Charcoal	—	Alvarez (1980)
Camarones Pta. Norte	—	5640 ± 160 BC	Charcoal	GAK--8645	Alvarez (1980)
Quiani-1	—	5630 ± 130 BC	Charcoal and Bone	I-1349	Mostny (1964)
Chinchorro-1	—	5560 ± 175 BC	Wood	I-15083	Muñoz and Chacama (1982)
Cobija-13	—	5510 ± 60 BC	Shell	GAK-8375	Bittmann (1984)
Camerones-14	—	5470 ± 225 BC	Charcoal	I-999	Schiappacasse and Niemeyer (1984:26)
Cobija-S1	—	5460 ± 140 BC	Charcoal	Beta-3114	Bittmann (1984)
Cobija-S1	—	5440 ± 150 BC	Charcoal	Beta-3115	Bittmann (1984)
Caleta Abtao-1	—	5350 ± 120 BC	—	Gif-1660	Boisset et al. (1969)
Quiani -9	—	5250 ± 430 BC	Charcoal and Bone	GAK-8781	Muñoz and Chacama (1982)
Tarapacá-12	—	5250 ± 340 BC	Charcoal	GAK-3895	True et al. (1971)
Morro-1	—	5240 ± 230 BC	Wood	GAK-9902	Vera (1981)
Camarones Pta. Norte	—	5230 BC	—	—	Alvarez (1980)
Pisagua Viejo	—	5220 ± 245 BC	Wood	IVIC-170	Núñez (1976)
Aragón-1	—	5170 ± 200 BC	Charcoal	GAK-5965	Núñez and Zlater (1977)
Caleta Abtao-1	—	5100 ± 130 BC	Shell	IVIC-681	Boisset et al. (1969)
Caleta Abtao-1	—	5090 ± 80 BC	Shell	IVIC-682	Boisset et al. (1969)
Cobija-13	—	5060 ± 120 BC	Charcoal	Beta-3117	Bittmann (1984)

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Table 10 Early Chilean and Peruvian archaeological sites, cont.

Site	Type	Radiocarbon Date	Material	Lab No.	Reference
Abtao-2	—	5030 ± 70 BC	Shell	IVIC-679	Boisset et al. (1969)
Morro-1	—	5010 ± 110 BC	Wood	GAK-71309903	Allison et al. (1984)
Tiliviche-1b	Midden	4955 ± 65 BC	—	—	Núñez and Moragas (1977) Muñoz (1985:275)
Camarones-Punta	—	4950 ± 210 BC	Wood	GAK-9903	Alvarez (1980)
Pisagua Viejo-4	—	4880 ± 320 BC	Wood	IVIC-170	Núñez (1976)
Cobija-SA	—	4880 ± 90 BC	Charcoal	Beta-3114	Bittmann (1984)
Tiliviche-1b	Midden	4850 ± 90 BC	—	—	Núñez and Moragas (1977) Muñoz (1985:275)
Caleta Abtao-2	—	4820 ± 70 BC	Shell	IVIC-680	Boisset et al. (1969)
Caleta Abtao-2	—	4800 ± 70 BC	Shell	IVIC-683	Boisset et al. (1969)
Tarapacá-14-A	—	4780 ± 130 BC	Charcoal	GAK-2529	True et al. (1971)
Caleta Huelén-42	Village	4780 ± 100 BC	Charcoal	GAK-3546	Núñez (1971)
Maderas Enco	—	4750 ± 155 BC	Wood	GX-17464	Arriaza (1995c)
Punta Guasilla-1	—	4730 ± 180 BC	Charcoal	Beta-3121	Bittmann (1984)
Tarapacá-12	—	4690 ± 80 BC	Charcoal	UCLA-1293	True et al. (1971)
Kilómetro-4	—	4620 ± 90 BC	Charcoal	Beta-27417	Wise et al. (1994)
Morro-1	—	4520 ± 90 BC	Wood	Beta-40956	Standen (1997)
Tarapacá-12	—	4480 ± 170 BC	Charcoal	GAK-5867	Tartaglia (1980)
Quiani-9	Camp	4420 ± 540 BC	Charcoal	GAK-8782	Muñoz and Chacama (1982:75)
Camarones-17	—	4406 ± 200 BC (5560 ± 175 BP)	Wood	—	Aufderheide, et al., cited in Guillen (1992:167)
Camarones P.N.	Midden	4320 ± 130 BC	—	—	Muñoz (1985:275)
Camarones P.N.	Midden	4290 ± 160 BC	—	—	Muñoz (1985:275)
Quiani-1	Midden	4220 ± 220 BC	Charcoal	I-1348	Bird (1943); Mostny (1964)
Quiani-9	Camp	4165 ± 220 BC	Charcoal	I-11643	Muñoz and Chacama (1982:75)
Tarapacá-2-A	—	4160 ± 80 BC	Wool	UCLA-1834A	Tartaglia (1980)
Tiliviche 1b	Midden	4110 ± 130 BC	—	—	Núñez and Moragas (1977) Muñoz (1985:275)
Playa Miller-8	—	4090 ± 105 BC	Wood	GAK-5811	Rivera (1977)
Morro-1	—	4040 ± 100 BC	Wood	I-13543	Allison et al. (1984)
Conanoxa W(a)	—	4020 ± 110 BC	Charcoal	IVIC-875	Schiappacasse and Niemeyer (1969)
Camarones P.N.	Midden	4000 ± 130 BC	—	—	Muñoz (1985:275)
Aragón-1	—	3996 ± 223 BC (5170 ± 200 BP)	Charcoal	—	Núñez (1976
Conanoxa W(a)	—	3970 ± 120 BC	Charcoal	IVIC-876	Niemeyer and Schiappacasse (1963)

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Table 10 Early Chilean and Peruvian archaeological sites, cont.

Site	Type	Radiocarbon Date	Material	Lab No.	Reference
Kilómetro-4	—	3970 ± 80 BC	Charcoal	Beta-77948	Wise (1999)
Cáñamo	—	3960 ± 80 BC	Charcoal	GAK-102	Núñez and Moragas (1983)
Camarones P.N.	Midden	3930 ± 160 BC	—	—	Muñoz (1985:275)
Tarapacá-18	—	3910 ± 170 BC	Charcoal	GAK-2433	True and Gildersleeve (1980)
Lluta-13	—	3900 ± 100 BC	Charcoal	—	Santoro (1999)
Tiliviche-2	—	3870 ± 100 BC	Human Coprolites	GAK-3772	Standen and Núñez (1984)
Patillos	—	3690 ± 60 BC	Human Muscle	Beta-113382	Llagostera (2003)
Caleta Huelén-42	Village	3780 ± 90 BC	Wood	GAK-3545	Núñez (1971)
Kilómetro-4	—	3760 ± 70 BC	Wood and Charcoal	Beta-52797	Wise et al. (1994)
Kilómetro-4	—	3750 ± 60 BC	Wood and Charcoal	Beta-52796	Wise et al. (1994)
Camarones P.N.	Midden	3720 ± 170 BC	—	—	Muñoz (1985:275)
Conanoxa w(a)	—	3740 ± 130 BC	Human Coprolites	IVIC-175	Schiappacasse and Niemeyer (1969)
Quiani-2	Midden	3680 ± 145 BC	Charcoal and Fish	I-1349	Bird (1943); Mostny (1964)
Kilómetro-4	—	3680 ± 70 BC	Charcoal	Beta-77946	Wise (1999)
Camarones P.N.	Midden	3650 ± 150 BC	—	—	Muñoz (1985:275)
Quiani-7	—	3590 ± 100 BC	Wood	GAK-5814	Rivera (1977)
Caleta Abtao-1	—	3550 ± 100 BC	Shell	Gif-1658	Boisset et al. (1969)
Kilómetro-4	—	3340 ± 70 BC	Charcoal	Beta-77950	Wise (1999)
Quiani-9	Midden	3300 ± 430 BC	Charcoal and Wood	GAK-8781	Muñoz and Chacama (1982:75)
Quiani-7	—	3280 ± 100 BC	—	I-13654	Grosjean et al. (2007)
Caramucho-1	Midden	3270 ± 130 BC	—	—	Muñoz (1985:275)
Quiani-7	—	3240 ± 100 BC	—	I-13655	Grosjean et al. (2007)
Kilómetro-4	—	3240 ± 60 BC	Charcoal	Beta-77943	Wise (1999)
Aragón-1	Camp	3220 ± 200 BC	Charcoal	GAK-5965	Núñez and Zlater (1976); Rivera (1991:17)
Caleta Abtao-1	Midden	3150 ± 130 BC	—	—	Boisset et al. (1969); Muñoz (1985:275)
Camarones-Sur	—	3060 ± 290 BC	—	RL-2055	Rivera (1994)
Camarones-15	—	3060 ± 100 BC	Wood	GAK-5813	Rivera et al. (1974)
Camarones P.N.	Midden	3000 ± 210 BC	—	—	Muñoz (1985:275)
Caleta Abtao-1	Midden	2870 ± 70 BC	—	—	Boisset et al. (1969); Muñoz (1985:275)
Caleta Huelén-42	Village	2830 ± 100 BC	Charcoal and Bone	GAK-3546	Núñez (1976:92)
Kilometre-4	Midden	2670 ± 100 BC	—	—	Wise (1991)
Bajo Molle	—	ca. 2620	—	—	Arriaza (1995a)

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Table 10 Early Chilean and Peruvian archaeological sites, cont.

Site	Type	Radiocarbon Date	Material	Lab No.	Reference
Aragón-1	Camp	2530 ± 170 BC	—	—	Núñez and Zlater (1976); Rivera (1991:17)
Conanoxa W(a)	—	2495 ± 187 BC	Animal Coprolite	—	Núñez (1976)
Carrizal	Midden	2440 ± 170 BC	Charcoal	—	Wise (1991)
Conanoxa W	Camp	2020 ± 120 BC	—	IVIC-876	Schiappacasse and Niemeyer (1969); Aufderheide and Allison (1994)
Conanoxa W	Camp	1790 ± 130 BC	Camelid Coprolites	IVIC-175	Schiappacasse and Niemeyer (1969; Aufderheide and Allison (1994)
La Capilla-1	—	1720 ± 160 BC	Bone	GAK-8778	Muñoz and Chacama (1982:10)
La Capilla-1	—	1500 ± 90 BC	Bone	I-11642	Muñoz and Chacama (1982:10)
Cerro Moreno	Habitation site	undated	—	—	Núñez (1976:92).
Hipodromo (near Antofagasta)	—	undated	—	—	Arriaza (1995a)
Hipodromo (near Arica)	—	undated	—	—	Arriaza (1995a)

rafts were possibly constructed. This late period, during the decline of the Chinchorro, saw the adoption of cotton, an increase in the transformation of natural fibers into clothing and tools, the loss of collective internments and elaborate mummification, and a reorganization of coastal communities. Alterations within Chinchorro culture and the dissemination of other cultures may have hastened these changes. Few Chinchorro structures survive into modern times. Explanations for this include: (1) structures built along the coast washed away, (2) modern Arica covers most of their settlements and cemeteries, and (3) settlement patterns require further study (Arriaza, 1995a:36).

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Chinchorro Technology

Material Culture:

The material culture of the Chinchorro indicates a focus on maritime activities including shell fishing, fishing, hunting, and scavenging sea mammals. Their maritime toolkit was simple and efficient, lasting over 5,000 years (Uhle 1922; Schiappacasse and Niemeyer, 1984; Rivera, 1991; Standen, 1991; Llagostera, 1992; Arriaza, 1995a:85). This suggests that environmental changes along the coast were not drastic enough to warrant the development of new fishing implements. A description of Chinchorro material culture and possible use for various items appears below. The Chinchorro did not create ceramics or engage in metallurgy.

Artifacts Associated With Shell Fishing and Fishing:

Chinchorro collected shellfish from shallow tide pools and from various depths. To open the shells they used a *chope,* a sea lion rib with a seaweed handle sharpened on one end to help pry the mollusk from its shell. *Chope* came in various sizes; some included two skeletal elements, one larger, and the other smaller. The average size was 16 cm (Arriaza, 1995a:85-86; Llagostera 1989:62; Schiappacasse and Niemeyer, 1984:36). Chinchorro collected shellfish and small fish in nets constructed from tortoro reeds; looped vegetal fibers formed a circular frame over several bent sticks creating a netted basket (Schiappacasse and Niemeyer, 1984:36; Llagostera 1989:62; Arriaza, 1995a:85). Lining river mouths, reeds lent themselves to easy collection; the Chinchorro

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could spin them into cord for multiple tasks (Arriaza, 1995a:85). Fishhooks were made from cactus needles, shells, bone, and wood. Collected while still green, the Chinchorro bent, then hardened the needles through heating, yielding a sharp rounded hook (Arriaza, 1995a:88). Cactus spine fishhooks were especially common between 4000 and 5000 BP (2050 BC and 3050 BC) (Núñez, 1983:197; 1994:357). A stone file was used to shape and polish large mussel shell cores or disks (*Choromytilus chorus)* into circular hooks; these hooks ranged from 10 mm to 40 mm (Schiappacasse and Niemeyer, 1984:27-35; Arriaza, 1995a:88). Around one end of the hook the Chinchorro strung fishing line; while these shells were not ideal hooks, the iridescent glimmer of their interiors made them irresistible to fish (Arriaza, 1995a:88). They plied reeds and camelid hairs occasionally wound with human hairs, into fishing line; oval-shaped stone sinkers notched at one end and attached to the line allowed the hook to sink. The Chinchorro used sharpened bone and wooden fishhooks to catch larger fish and crustaceans (Arriaza, 1995a:88).

Artifacts Associated With Hunting:

The Chinchorro hunted sea lions, birds, and camelids with harpoons, atlatls, spears, and darts. Harpoon heads measured 25 cm long on average; the ones from the site of Morro I measured approximately 20 cm long. Harpoons with their detachable points measured up to 100 cm long (Arriaza, 1995a:88-89; Llagostera, 1989:62). Lanceolate lithic points were adhered to harpoon heads with resin; lithic points and bone formed the tips of spears. Lithic tools between 5

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cm and 8 cm long formed cutting devices (Schiappacasse and Niemeyer, 1984:55; Arriaza, 1995a:90). Whale skin and skeletal elements from scavenged carcasses served as support beams, roofs, and walls for homes (Donnan, 1964; Bird et al., 1985:74; Arriaza, 1995a:88). At the Peruvian sites of Chilca (Houses 6 and 12) and Huaca Prieta, the inhabitants used whale skeletal elements in the construction of homes and as ornaments in funerary buildings (Donnan, 1964; Bird et al., 1985:74; Arriaza, 1995a:88).

Artifacts Associated With Ornaments and Clothing:

The late Chinchorro adorned themselves in headbands of spun and plied camelid fibers (z-spun and s-plied), z-plied into heavy cords and wrapped around the head (Arriaza, 1995:88). Z-spun is a counterclockwise spin of the material; s-plied refers to one piece of material twisted clockwise, likewise z-plied refers to a counterclockwise twist. Shell adornments were uncommon. Clothing came from softened reeds, animal skins, and camelid hair; males wore animal skin loincloths and females wore short-fringed skirts (Arriaza, 1995a:91). Body painting using red and black pigments, while best known in the context of death rituals, may also have beautified the living (Arriaza, 1995a:91).

General Artifacts:

The angular hollow long bones of birds made excellent lightweight storage cases for hallucinogenic powders (Arriaza, 1995a:93) Large twined reed blankets and mats were made to enclose the dead, or presumably for sitting or sleeping upon. Most mats and blankets from the Morro-I site were undecorated

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with fringed ends; however, one small mat had geometric designs in purple and yellow dyed camelid yarns embroidered upon it (Arriaza, 1995a:93; Standen, 1991). Uhle (1922:64) describes a mat with a black and red ladder-like pattern. Arriaza (1995a:93) suggests that the mats may have "served as roofing materials for their huts," but notes, "there is no direct evidence for this." There were several different techniques used in creating the mats, including simple looping, simple looping and twisting, simple looping with a knot, spaced twining, and compact twining (Schiappacasse and Niemeyer, 1984:65; Arriaza, 1995a:92-93).

While the Chinchorro utilized fire and hot ashes in their mortuary practice, no artifacts associated with cooking exist from the earlier sites, aside from hearths in a few homes. At later sites, around the decline of the Chinchorro, gourd (*Cucurbita sp.)* utensils appear (Cordova et al., 1999:17). At Morro I, there is evidence of a fire drill composed of two sticks. To operate the fire drill, one stick was held in the ground or in a log probably by a person's feet, and at the same time another stick was held perpendicular to the ground and rapidly rubbed between the hands or through a bow to produce sparks (Arriaza, 1995a:93).

Relationship of Technology to Settlement Data:

The Chinchorro could easily collect, manufacture, and sustain their technology; everything necessary for survival was within footsteps of the ocean (Arriaza, 1995a). Their technology was mostly transient in its material composition; however, given the arid environment, it could have lasted many years. While there is some indication the Chinchorro traveled inland to hunt

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camelids, there is no indication that they were nomadic; all of their technology points to a maritime lifestyle. While it is possible the Chinchorro were semi-nomadic, there is no indication of a specific technology developed for living in the Andean highlands. The majority of hunting implements and skeletal elements at the sites are for hunting sea mammals or birds, not land mammals. Most Chinchorro sites were located near the coast; others were a short distance inland. Due to their perishable nature, many artifacts have been lost over time, though others may remain but are, like the settlements, buried beneath the modern city.

Relationship of Technology to Subsistence Data:

Chinchorro technology indicated a heavy reliance on coastal and ocean ecosystems as the predominant food source. Food from the ocean and rivers was rich in protein, this supplemented with grains such as quinoa (*Chenopodium sp.),* seasonal vegetables (wild and those obtained through trade), and terrestrial fauna would have provided an adequate diet to sustain health (Cordova et al., 1999:13). Tools such as fishhooks, spears, and lithics were easy to acquire and make. The tools used for subsistence activities were simple, efficient, and easy to replace (Arriaza, 1995a).

Chinchorro Subsistence

As noted, once the Chinchorro arrived in the Arica-Camarones area, they engaged in a mostly maritime subsistence (Núñez, 1994; Arriaza, 1995a:83-88). Studies of bone chemistry (strontium and stable isotope ratio determinations,

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including nitrogen and sulfur ratios) and intestinal contents show that 89% of their diet came from marine foods (shellfish, sea lions, fish, seaweed, and other foods from the ocean and rivers) (Arriaza, 1995a:84-85). Nineteen percent of the mummies sampled had tapeworm eggs, which were probably the result of consuming raw or insufficiently heated fish or sea lions (Arriaza 1995a:67; Reinhard, 1992:236, 237; Reinhard and Urban, 2003:191-192). The few dental caries the Chinchorro had are consistent with a marine diet, as is the high degree of attrition on their teeth from consuming shellfish intermixed with sand (Kelley et al., 1991; Arriaza, 1995a:75-76).

Analysis of 31 mummies from Camarones, circa 550 BP (circa AD 1378 ± 67), showed skin lesions associated with exposure to high levels of accumulated arsenic (Arriaza, 1995b:83); the concentration of arsenic was 342 times the normal amount found in human kidneys (Figueroa et al., 1988:39). A high level of naturally occurring arsenic is endemic in the soil and water at Camarones (Arriaza, 1995a:83-84).

Butchered bone fragments in midden sites suggest they also ate camelids, such as guanco and deer (Arriaza, 1995a:88, 1995b:52). However, strontium tests on the ribs of 62 adults revealed that terrestrial meat comprised 5% of their diet, while 6% was plant-based (Aufderheide and Allison, 1992; Arriaza, 1995a:84-85).

Fishing, hunting, gathering, and food preparation implements used by the Chinchorro included fishhooks, harpoons, atlatls, spears, sinker stones, worked

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bone and wood tools, stone flakes, shaped shells from mussels (*Choromytilus chorus)* and snails (*Lica Peruviana*), fishing line from reeds, camelid and human hairs, and knotted fiber nets, bags, and mats painted with water-based natural pigments. Cooking implements and utensils are missing from the early Chinchorro sites; presumably the Chinchorro crafted such items but they either did not last, were lost, or were neglected during the excavation process (Arriaza, 1995a:85-88). Chinchorro mortuary sites have garnered greater interest over the years than their habitation sites, which may account for the scarcity of data on subsistence and habitation.

The Chinchorro saw little change over time in their subsistence. As noted, early occupation of this Andean region dates to around 8000 BC (Chauchat, 1988; Arriaza, 1995a:50); if the first inhabitants came from the highlands, they may have created tools to hunt large mammals like camelids (guanaco, alpaca, and vicuña), as well as small rodents (vizcacha and chululo (*Ctenomys fulvus*)) (Santoro and Núñez, 1987; Rivera, 1991:11-13). Around the decline of the Chinchorro, 2000 BC to 1000 BC, the population increased, specialization occurred, and burial and subsistence practices changed. Cultivation of vegetables, including maize, beans, chili peppers, sweet potatoes, various fruits, and gourds began around 1000 B.C to 500 BC, although primarily inland (Lynch, 1983:9). There was also increased interaction with regions of denser populations and the development of social complexity in the Andes, altiplano, and lowlands (Núñez, 1983:195).

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Chinchorro Population Health

Mortality:

The skeletal analysis of mummies suggests the Chinchorro had an average life span of 25 years (Arriaza, 1995a:65), but high infant mortality levels influence this figure. At Morro I approximately, 44% (42/96) of the mummies were subadults; of these 26% (N=23) were under a year (Arriaza, 1995a:64). At Camarones 14, 21% perished between birth and two years (Kamps, 1984:165; Arriaza, 1995:64). The Chinchorro did not have a higher rate of childhood mortality than most prehistoric Andean populations. At La Paloma, a contemporaneous site in Peru, 42% of the population died in childhood (Quilter, 1989:20; Arriaza, 1995a:64). In an analysis conducted at Morro I, death rates for women in their childbearing years were only slightly higher than those for males (m = 33.3%, f = 35.4%, undetermined = 31.3%) (Arriaza, 1995a:66). In the skeletons of some children, childhood health problems such as cribria orbitalia, porotic hyperostosis, and Transverse (Harris) Lines on long bones are present. Metabolic stresses link all three; the first two result from malabsorption of iron due to intestinal parasites, and the last from multiple causes -- including nutritional deficiencies and acute infections (Arriaza, 1995:66-67). Table 11 provides a list of Chinchorro cemeteries discussed in this dissertation. Radiocarbon dates for individuals appear in the Results and Discussion in Chapter 5.

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Table 11 Chinchorro cemeteries/burials

Site	Type of Site or Mummy Type	Radiocarbon Date	Material	Lab No.	Reference
Acha-2 +	--	8970 ± 255 BC	Human Muscle	KE-15082	Muñoz and Chacama (1993)
Acha-3+	--	8120 ± 90 BC	Human Muscle	Beta-40956	Standen and Santoro (2004)
Acha-2 TI	Natural Mummy	7020 ± 255 BC	Human Muscle	GX-15082	Muñoz and Chacama (1993:28)
Camarones-14	--	7000 ± 135 BC	Human Tissue	1-11431	Schiappacasse and Niemeyer (1984)
Morro-1 T7 C1	Red Mummy	5860 ± 180 BC	Camelid Fur	I-11431	Allison et al. (1984:163-165)
Ilo	Habitation	ca. 5850 BC	--	--	Wise (1991)
PLM-8 3524	Black Mummy	5744 ± 310 BC	Human Bone	DRI-3445	Arriaza et al. (2005)
Chinchorro-1 T1 C2	Black Mummy	5560 ± 175 BC	Wood	GAK-15083	Muñoz et al. (1993) Aufderheide et al. (1993)
Camarones-14	Cemetery	5479 ± 225 BC	Charcoal	I-999	Schiappacasse and Niemeyer (1984:26)
Maestranza-1 C4	Black Mummy	5438 ± 170 BC	Wood	DRI-3452	Arriaza et al. (2005)
Morro-1 T10B	Complex-style Mummy	5434 ± 59 BC	Human Bone	DRI-3450	Arriaza et al. (2005)
Morro-1 T10B	Complex-style Mummy	5414 ± 58 BC	Vegetable Matrix	DRI-3449	Arriaza et al. (2005)
Arica	Black Mummy	5240 ± 110 BC	Wood	GAK-9902	Allison et al. (1984)
Morro-1 T1 C4	Black Mummy	5160 ± 110 BC	Human Lung	I-13539	Muñoz et al. (1993); Aufderheide et al. (1993)
Camarones-14	Black Mummy	5050 ± 135 BC	Human Muscle	I-11431	Schiappacasse and Niemeyer (1984:26)
Camarones-17 T1C4	Black Mummy	4980 ± 140 BC	Wood	GX-15081	Aufderheide et al. (1993:191)
Camarones-17 T1C3	Black Mummy	4830 ± 110 BC	Wood	GX-15080	Aufderheide et al. (1993:191)
Maderas Enco-1 C1	Black Mummy	4750 ± 155 BC	Wood	GX-17464	Arriaza (1995c)
Camarones-14	Cemetery	4700 ± 155 BC	Charcoal	I-9817	Schiappacasse and Niemeyer (1984:26)
Camarones-14	Cemetery	4665 ± 390 BC	Charcoal	I-9816	Schiappacasse and Niemeyer (1984:26)
Camerones-8	--	4635 ± 90 BC	Human Tissue	GX-15079	Muñoz et al. (1993); Aufderheide et al. (1993)
Morro-1 T23 C12	Red Mummy	4633 ± 55 BC	Human Muscle	DRI-3448	Arriaza et al. (2005)
Morro-1 T25 C6	Mud Mummy	4570 ± 100 BC	Human Muscle	I-13542	Allison et al. (1984)
Morro -1 T7 C1	Red Mummy	4520 ± 90 BC	Human Muscle	Beta-40956	Standen (1991)
Morro-1 T7 C4	Red Mummy	4394 ± 54 BC	Human Muscle	DRI-3388	Arriaza et al. (2005)

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Table 11 Chinchorro cemeteries/burials, cont.

Site	Type of Site or Mummy Type	Radiocarbon Date	Material	Lab No.	Reference
Morro-1	Red Mummy	4350 ± 280 BC	Human Muscle	I-13650	Allison et al. (1984)
Morro-1-6	Natural Mummy	4310 ± 145 BC	Human Tissue	GX-18260	Focacci and Chacón (1989)
**MNHN Caja 72	Red Mummy	4297 ± 135 BC	Reeds	DRI-3455	Arriaza et al. (2005)
Morro-1	Natural Mummy	4200 ± 100 BC	Human Muscle	I-13541	Allison et al. (1984)
Morro-1 T7 C5	Red Mummy	4174 ± 86 BC	Reeds	DRI-3451	Arriaza et al. (2005)
Chinchorro-1 T1C1	Black Mummy	4120 ± 285 BC	Wood	GX-15084	Aufderheide et al. (1993:191)
Morro-1-5 (MI)	Red Mummy	4120 ± 75 BC	Human Liver	GX-17019	Guillen (1992:179)
Morro-1 T7 C5	Red Mummy	4093 ± 81 BC	Wool, Hair, and Human Muscle	DRI- 3389	Arriaza et al. (2005)
PLM-8	Red Mummy	4090 ± 105 BC	Wood	GAK-5811	Núñez (1976)
Morro-1 T23 C7	Red Mummy	4040 ± 105 BC	Wood	I-13543	Allison et al. (1984)
Camarones-15b	--	4010 ± 75 BC	Human Tissue	GX-18258	Muñoz et al. (1993); Rivera (1994)
Morro-1	Natural Mummy	4010 ± 75 BC	Human Liver	GX-18263	Focacci and Chacón (1989); Rivera (1994)
**MNHN 10955	Red Mummy	3933 ± 143 BC	Cord	DRI-3447	Arriaza et al. (2005)
Morro -6 T. 53	Natural Mummy	3895 ± 75 BC	Human Cartilage	GX-18262	Focacci and Chacón (1989)
Morro-1-6 T39	Natural Mummy	3880 ± 70 BC	Human Muscle	GX-18259	Focacci and Chacón (1989)
Morro-1 T23 C5	Natural Mummy	3818 ± 60 BC	Human Muscle	DRI-3390	Arriaza et al. (2005)
Morro -1 T28 C22	Natural Mummy	3830 ± 100 BC	Human Lung	I-13652	Allison et al. (1984)
Morro-1 T28 C8	Natural Mummy	3790 ± 140 BC	Human Muscle	I-13656	Allison et al. (1984)
Morro-1	--	3780 ± 100 BC	Human Tissue	I-14957	Allison et al. (1984)
Morro-1-6 T 27	Natural Mummy	3750 ± 140 BC	Human Cartilage	GX-18261	Focacci and Chacón (1989); Arriaza (2005)
Maestranza-1 C1	Natural Mummy	3960 ± 103 BC	Human Bone	ETH-18324	Arriaza et al. (2005)
Patillos	Complex Style Mummy	3690 ± 60 BC	Human Muscle	Beta-113382	Llagostera (2003)
Morro-1 T28 C9	--	3670 ± 100 BC	Human Muscle and Human Lung	I-13651	Rivera (1994)
Camarones-15d	--	3650 ± 200 BC	Human Tissue	RL-2054	Rivera (1994)
**MNHN 10988	Red Mummy	3618 ± 83 BC	Cord	DRI- 3446	Arriaza et al. (2005)
Chinchorro-1 T1C2	Black Mummy	3610 ± 175 BC	Wood	GX-15083	Aufderheide et al. (1993:191)

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Table 11 Chinchorro cemeteries/burials, cont.

Site	Type of Site or Mummy Type	Radiocarbon Date	Material	Lab No.	Reference
Morro-1-6 T13	Natural Mummy	3560 ± 100 BC	Human Muscle	I-14958	Focacci and Chacón (1989)
Morro-1 T1 C5	Black Mummy	3488 ± 317 BC	Reeds	DRI-3453	Arriaza et al. (2005)
MNHN 11040	Red Mummy	3296 ± 210 BC	Reeds	DRI-3454	Arriaza et al. (2005)
Arica	Black Mummy	3290 ± 230 BC	Wood	GAK-9902	Vera (1981:12)
*Piagua Viejo-4 T2	Black Mummy	3270 ± 170 BC	Wood and Vegetal	IVIC-170	Núñez (1965:23)
Morro-1 T1 C4	Black Mummy	3210 ± 110 BC	Human Lung and Vegetal	I-13539	Allison et al. (1984:163-165)
Arica	Black Mummy	3060 ± 110 BC	Wood	GAK-9903	Vera (1981:12)
*Piagua Viejo-4 T2	Black Mummy	2930 ± 320 BC	Wood and Vegetal	IVIC-170	Núñez (1965:23)
Morro-1 T25 C5	Red Mummy	2846 ± 124 BC	Wool and Hair	ETH-18326	Arriaza et al. (2005)
Maderas Echo C1	Black Mummy with Red Coloring Also	2800 ± 155 BC	Wood	GX-17464	Arriaza (1993)
Camarones-8 T1C1	Red Mummy	2685 ± 90 BC	Human Muscle	GX-15079	Aufderheide et al. (1993:191)
Morro-1 T25 C6	Mud-coated Mummy	2620 ± 100 BC	Human Muscle	I-13542	Allison et al. (1984:163-165)
Morro-1 T7 C6	Red Mummy	2570 ± 90 BC	Human Muscle	B-40956	Standen (1991:288)
Baja Molle	Complex Mummy Style	ca. 2570 BC	--	--	Llagostera (2003)
Punta Pichalo	Complex Mummy Style	ca. 2570 BC	--	--	Llagostera (2003)
Morro-1 T19	Red Mummy	2400 ± 280 BC	Charcoal and Human Organ	I-13650	Allison et al. (1984:163-165)
Morro-1-6 T9	Natural Mummy	2360 ± 145 BC	Human Liver	GX-18260	Focacci and Chacón (1989:46); Aufderheide and Allison (1994)
Morro-1 T21 C1	Natural Mummy	2250 ± 100 BC	Human Muscle	I-13541	Allison et al. (1984:163-165)
Morro-1-5 (MI)	Red Mummy	2170 ± 75 BC	Human Liver	--	Guillen (1992)
Playa Miller-8	Red Mummy	2140 ± 105 BC	Wood	GAK-5811	Núñez (1976:122)
Morro-1 T23 C7	Red Mummy	2090 ± 100 BC	Wood	I-13543	Allison et al. (1984:163-165)
Conanoxa	--	ca. 2070-1790 BC	--	--	Arriaza (1995a:130)
Morro-1-6 T23	Natural Mummy	2060 ± 75 BC	Human Liver	GX-18263	Focacci and Chacón (1989:46); Aufderheide and Allison (1994)

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Table 11 Chinchorro cemeteries/burials, cont.

Site	Type of Site or Mummy Type	Radiocarbon Date	Material	Lab No.	Reference
Morro-1-6 T53	Natural Mummy	1945 ± 75 BC	Human Cartilage	GX-18262	Focacci and Chacón (1989.46); Aufderheide and Allison (1994)
Morro-1-6 T39	Natural Mummy	1930 ± 70 BC	Human Muscle	GX-18259	Focacci and Chacón (1989:46); Aufderheide and Allison (1994)
Morro-1 T28 C22	Natural Mummy	1880 ± 100 BC	Human Lung	I-13652	Allison et al. (1984:163-165)
Morro-1 T28 C8	Natural Mummy	1840 ± 140 BC	Human Muscle	I-13656	Allison et al. (1984:163-165)
Caleta Huelén-42	Natural Mummy	1830 ± 90 BC	Harpoon Wood	GAK-3545	Núñez (1976:122)
Morro-1-6 T27	Natural Mummy	1800 ± 140 BC	Human Cartilage	GX-18261	Focacci and Chacón (1989:46); Aufderheide and Allison (1994)
La Capilla	--	Ca. 1720 BC	--	--	Arriaza (1995a:130)
Quiani-7	Cemetery	1640 ± 100 BC	Wood	GAK-5814	Núñez (1976:122); Rivera (1977:200)
Morro-1-6 T13	Natural Mummy	1610 ± 100 BC	Human Muscle	I-14-958	Focacci and Chacón (1989:46)
Quiani-7 T12	Natural Mummy	1330 ± 90 BC	Human Muscle	I-13655	Lab Record, University of Tarapacá, Chile
Quiani-7 T16	Natural Mummy	1290 ± 90 BC	Human Muscle	I-13655	Lab Record, University of Tarapacá, Chile
Camarones-15	Natural Mummy	1110 ± 100 BC	Wood	GAK-5813	Rivera (1991:17)

+ possibly Chinchorro associated
*These two samples from Piagua Viejo have the same laboratory number
** Abbreviation for Museo Nacional de Historia Natural Santiago

Diet and Subsistence Data:

The Chinchorro had less health problems than the later regional inhabitants, whose health deteriorated with the adoption of agriculture (Arriaza, 1995a:82). One researcher, however, has argued that they were poorly adapted to their environment (Núñez, 1994:359). Cited examples of maladaptation include numerous injuries (fractures), high infant death rates, and high female

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death rates due to calcium deficiency (Núñez, 1994:359). However, no studies show that calcium deficiency was a cause of death; it was more likely a contributing factor to osteoporosis.

Osteoporosis and associated fractures are common in Chinchorro women, possibly due to the demands of childbirth and from inadequate nutrition. In a study of 45 adult spines from the late Chinchorro, Arriaza (1995a:74) found that approximately 19% of adult females (1 in 5) had vertebral fractures likely related to osteoporosis, as compared to only 4% of males. Perhaps because of high infant death rates, women had numerous children often in their teenage years. Increased fecundity could lower the health status and bone density of the mother.

The Chinchorro also suffered from osteoarthritis, idiopathic and diffuse arthrides, and spondylolysis. Approximately 7% suffered from mild diffuse idiopathic skeletal hyperostosis (DISH) and 4% had inflammatory arthrides, in particular, seronegative spondylolarthropathies (SNS) (Arriaza, 1995a:69). Spondylolysis is a separation of the neural arch from the vertebral body typically around the fourth or fifth lumbar vertebra, although it can occur in the cervical vertebra. Arriaza (1995a:69) examined 28 Chinchorro males and 23 females for signs of spondylolysis and found the condition present in 18% of the men and none of the females. It results from chronic microtrauma, which leads to fractures of the vertebral arch. The presence of spondylolyis and some other diseases discussed here suggests Chinchorro men were engaged in physical tasks that demanded hyperextension of the back (Arriaza, 1995a:69).

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About 36% of Chinchorro men suffered from external auditory exostoses, chronic irritation of the ear canal from repetitive diving in cold water (Arriaza, 1995a:68-69). Even "Acha Man," from the early Acha-2 site, suffered from this condition (Arriaza, 1995a:69). Exostoses are present in only 4.3% of females, suggesting that it was an occupational hazard faced primarily by men (Arriaza, 1995a:68-69). Some Chinchorro also had a circular cranial deformation probably due to head wrapping (Llagostera, 1979).

Chinchorro suffered fewer dental caries and other ailments than later agricultural populations. Only 4.9% of the Chinchorro had dental caries (Kelley et al., 1991:207; Arriaza, 1995:75). The Chinchorro maintained most of their teeth throughout life, losing an average of 1.4 teeth and they had a rate of 1.2 dental abscesses per person (Kelley et al., 1991:207; Arriaza, 1995a:75). Abrasion, calculus accumulation, periodontia, and reabsorption of bone sockets were prevalent (Arriaza, 1995a:76).

Evidence for Conflict:

Despite research that suggests they were peaceful people (Núñez, 1994), there is evidence of trauma in 30% (27/89) of adult skeletons at the Museo Arqueológico San Miguel de Azapa in Arica (Standen and Arriaza, 2000a:239). The injuries consist primarily of skull fractures 24.6% (17/69), followed by injuries to the upper limbs 8.7% (7/80), trunk 2.9% (2/68), and lower limbs 1.1% (1/89). Skull fractures were three times more common in men than women, 34.2% (13/38) and 12.9% (4/31) respectively (Standen and Arriaza, 2000a:239).

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Healing was evident in most skull fractures. These injuries probably resulted from interpersonal or inter-group violence (Standen and Arriaza, 2000a).

Disease:

Scientists studying skeletons of the Chinchorro have suggested that they were afflicted with treponematosis, a bacterial infection caused by treponemal spirochetes (Allison et al., 1982:275-283; Verano, 1998:219; Arriaza, 1995a:76-80; Standen and Arriaza, 2000b:185-193). This dissertation focuses on four subtypes of treponematosis: syphilis, yaws, bejel, and pinta. All of them except pinta affect the bone. Treponematosis can cause cutaneous lesions, periosteal reactions, cortical thickening, osteitis, osteoperiostitis, and gummatous and stellate lesions, depending on the strain (Aufderheide and Rodríguez-Martín, 1998:154-171). Bone lesions suggestive of treponemal infection have been found in Chinchorro individuals from the coast of southern Peru and northern Chile, and in particular from archaeological sites in Arica and San Miguel de Azapa (Allison et al., 1982; Aufderheide and Rodríguez-Martín, 1998; Arriaza, 1995a, 1995b; Standen et al., 1984). Analysis of the Chinchorro skeletons is complicated because of the artificial mummification, a process that makes it difficult to obtain radiographs. Preservation efforts also present a challenge. To preserve the mummies, research is limited to those individuals in less pristine condition and/or who have lost some of their mummification materials (Arriaza, 1995b).

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In a study conducted on 31 mummies from Arica, 39% of the Chinchorros showed severe periosititis in their tibiae. A breakdown by sex indicates that 35% of Chinchorro females and 36% of males were affected (Allison et al., 1982; Arriaza, 1995b). These numbers contrast with those of later populations in the same region who rarely showed infection. In a study that examined hundreds of mummies from agropastoral populations from Arica, only six individuals had periosteal infections of the leg (Allison et al., 1982; Arriaza, 1995b).

The inflammation of skeletal elements ranged from mild to severe. The radiographs of most long bones, and in particular tibiae, showed a reduced and infected medullary cavity surrounded by new bone growth and a thickened cortex. Bone damage to the legs suggests the mummies had yaws, a form of treponematosis resulting in periosteal reactions, lytic defects in the cortex of the bone, and "saber shin," a thickening and anterior curvature of the tibia often associated with treponematosis (Arriaza, 1995a,b; Hackett, 1967; Aufderheide and Rodríguez-Martín, 1998:156). In some cases, arm bones had periosteal infections (Arriaza, 1995b). While the osseous infections were not fatal, they would have caused ulcerations of the skin, swelling, and pain. Within the Chinchorro population, mostly young adults were infected; however, there were some severe cases in subadults. A 15-year-old male showed a chronic infection with deformation of the tibia and fibula (Arriaza, 1995b).

Studies by Standen and Arriaza (2000b) suggest the disease is yaws. It is unknown whether the Chinchorro also had pinta, as it is difficult to see on

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mummified tissue and there is no DNA test for it at present. Two types of endemic treponematosis (yaws and bejel) spread differently and affect the bones, soft tissues, cardiovascular system, and nervous system to varying degrees. Yaws is associated with a humid environment and bejel with an arid environment. Treponematosis, however, results from a bacterial spirochete that affects the skin and mucous membranes.

The treponemes, in general, are spread through bodily fluids, intimate contact (open wounds, saliva, genital secretions, mucous membranes of the genitals, mouth, or anus), and depending on the subtype by flies, contaminated water, and shared drinking vessels or utensils. Yaws and bejel spread primarily among children; neither disease is congenital. Children often receive scratches and cuts during play and are likely to share items and pass on the bacteria. Cuts may also have played a factor in spread of the disease among adults. The rugged coast and slippery rocks may have led to more cuts and scrapes for coastal dwellers, and thus, the disease may have gained a greater foothold there. However, there is no direct evidence to suggest that coastal living among the Chinchorro led to increased cuts and scrapes in that population compared with highland populations. It just as easily could have been the opposite; mobile highland populations may have sustained more falls, cuts, and scrapes because they traveled farther distances. However, that would not explain the seemingly lower rate of treponematosis in the highland populations. Preservation of the bodies did not allow for an assessment of cuts and scrapes between coastal and

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inland dwellers. While data may exist on the amounts of cuts and scrapes between coastal and inland dwellers for other ancient and modern populations, the collection of this kind of data is beyond the scope of this dissertation.

Yaws is usually a disease of childhood (Aufderheide and Rodríguez-Martín, 1998:155). The treponemal infection may have been the result of the manipulation and extensive handling of the dead (Arriaza, 1995a:155). While children were probably not handling the dead extensively, women may have been (Arriaza, 1995a:155). If women were involved in cleaning the bodies, then this infectious disease may have spread from mother to child (Arriaza, 1995a:155).

The potential treponemal infection present might also be a coastal phenomenon. Standen and Arriaza (2000b) suggest there is a higher frequency of treponematosis among coastal populations (Chinchorro) than those living inland (Azapa valley); however, the reasons for this are unclear. The practice of Chinchorro-style mummification occurred primarily on the coast and this could be one reason why the frequency of treponemal infection is higher here than in inland communities, where it appears to be almost absent. Limited trade with the highland peoples may have restricted the infection primarily to the coast. Coastal dwellers may also have had more contact with groups traveling by water or foot who carried the disease.

A high frequency of treponematosis is an anomaly in a population that is small and not traveling or trading widely. One explanation is that the high rate of

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treponematosis found in the Chinchorro population is a consequence of handling decomposed cadavers and a result of the artificial mummification practice. Mortuary rituals, which included the saving of all the skin, the removal of all internal organs, the filling of the body cavities, and the painting of the dead may have allowed for the spread of treponemal infection from the deceased to the living through surface cuts (Arriaza, 1995b, Arriaza, pers. comm., 2000). Those handling the dead would be at higher risk and would be more susceptible to disease and more likely to spread disease to others whom they had contact (Arriaza 1995b).

Hapke (1996) suggested that females prepared the bodies during the creation of the most elaborate (black) mummies, and that males took over during the later period, creating the red mummies, a variation on the black style (See section on Chinchorro Mortuary Behavior). He based his hypothesis on "the universality of female cooperation versus male competitiveness" (Arriaza et al., 1998:192). Hapke (1996) thought the black style represented a communal effort and produced generic mummies whereas the red style was more individualistic. He attributed the creativity of the red style to males trying to garner prestige (Arriaza et al., 1998:192). However, there is no data to support his ideas. Females just as easily as males may have been trying to gain prestige, if that was even an issue within Chinchorro society.

Spiritual and religious practices, and in particular mortuary practices, have long been considered contributing factors in the spread of infectious

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disease (May, 1950). For example, mortuary rituals in New Guinea's Eastern Highlands played a significant role in the spread of kuru (Steadman and Merbs, 1982). The cultural practice amongst the Fore of consuming the tissue of their dead led to the prion disease kuru (Arriaza, 1995b; Gajdusek, 1973, 1977, 1996; Gajdusek and Zigas, 1957; Gajdusek et al., 1966; Rhodes, 1997; Prusiner, 1998). Other examples of disease spread through religious and cultural practices include the communal washing of bowls in Buddhist temples, which has been responsible for the spread of eye, skin, and other diseases (May, 1950). The daily ablutions of Moslems have spread disease through contaminated water. Religious pilgrimages have spread cholera and other diseases from ancient to modern times (May, 1950) The early inhabitants of the Gilbert Islands, a chain of 16 atolls and islands in the Pacific Ocean, which are part of the nation of Kiribati, followed a superstitious belief, and disposed of their feces in the ocean. While their disposal established a hygienic practice that helped the locals avoid spreading hookworm, it resulted in the spread of disease when salt from the ocean water was added to food after cooking (May, 1950).

The treatment of disease has also inadvertently resulted in the spread of bacteria and viruses. In Tonkin, a disease called sparaganosis, which results from cestode worm larvae, may have spread through poultices made from freshly killed vertebrates. A similar practice in the Mediterranean to cure headaches and meningitis involved the use of poultices made from dead pigeons and chickens, this in turn caused secondary infections with salmonella (May, 1950).

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Poor hygiene and cultural prescriptions about the treatment of the dead in Chinchorro culture may have played a role in the transmission of treponematosis; however, because there are no written records, the impact of these practices is unknown (Arriaza, pers. comm., 2000). What is clear though, is that an elaborate mummification process was practiced that involved much hands-on contact, potentially increasing the risk for disease spread. Several researchers (Hackett, 1963; Basset et al., 1994; Froment, 1994; Grin, 1956) have proposed a relationship between climate and the various treponemes. While more data are required, it is possible that there is an environmental connection among the treponeme(s) present in the Chinchorro population.

Standen and Arriaza (2002) hypothesize that the etiologic agents of treponematosis in the Chinchorro and later Andean populations changed from yaws to bejel. Considering that the Andean region has been drying out during the last 10,000 years, it is possible that the etiological agent did not remain the same throughout time (Arriaza, 1995a,b). A change in the treponemal syndrome could have led to a change in the frequencies of treponemal infection among the populations (Arriaza, pers. comm., 2000).

To help distinguish among the treponemes, Rothschild and Rothschild (1995) developed a quantitative assessment of the bone lesions specific to the various treponemes that involves examining the cortical and articular surfaces of the skeletal remains for bone changes. Metaphyseal, diaphyseal, and periosteal changes are also noted. The Rothschilds' method, abbreviated as SPIRAL,

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includes six criteria. These criteria are "Saber shin without periostitis, Prepubescence, Involvement of tibia unilaterally, Routinely affected hand or foot, Average number of bone groups affected, and Lacking periostitis but flattened." Rothschild and Rothschild (1995) have suggested, for example, that in cases of yaws, sabre tibia occurs in 33% of the population. This figure is consistent with the rate of infection seen in the Chinchorro practicing artificial mummification. Sabre tibia with periosteal reactions has been associated with all the forms of treponematosis, although most commonly yaws and bejel. One way to distinguish between yaws and bejel when looking at the tibia seems to be through the presence of tibial flattening, a characteristic of bejel, not yaws or venereal syphilis (Rothschild and Rothschild, 1995). The affected mummies did not have tibial flattening, which seems to dispute Standen and Arriaza's (2002) hypothesis about the change in treponemal strains. However, the Rothschilds' (1995) system is untested.

Chinchorro Mortuary Behavior

Who, What, and How of Mummification:

The Chinchorro manipulated the bodies of their dead purportedly to give them immutable form (Arriaza, 1995a:30, 139-141). While there are no written records detailing that it was the Chinchorro themselves who mummified their dead, it is assumed here (given no evidence to the contrary) that they were the mummy-makers. They allowed the deceased to maintain a lifelike form by stuffing the body cavities with reeds and reinforcing the body with sticks; a

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process that will be described below in detail. They created and painted facial masks and artificially preserved their bodies with great care. The practice of artificial mummification in the Chinchorro culture dates from approximately 5900 BC to about 1110 BC (Arriaza, 1995b:48; Arriaza et al., 2005). The Chinchorro mummification practice peaked around 3000 BC; it lasted over 3500 years, developing into three techniques and many styles (Arriaza, 1995a:115). These practices preceded Egyptian mummification by 2,000 years (Arriaza, 1995a:123). Below is a summary of the Chinchorro mummification techniques (Uhle, 1922: cited in Arriaza, 1995b:7):

1. **Simple Treatment:** naturally desiccated bodies
2. **Complex Treatment (includes black, red, and "bandage" mummies):** bodies artificially preserved by removal of the internal organs, filling of the cavities with human or animal hair and grass, and stitching of the incisions. Treatment includes painting of the dead, the addition of a human hair wig, and the body buried in an extended position
3. **Mud-coated mummies:** bodies covered in a thin layer of mud.

Natural vs. Artificial:

Natural mummification occurs when a body becomes desiccated, which in northern Chile occurred because of a hot dry climate and a high concentration of salts in the soil (Arriaza, 1995a:3). The dry heat and salts act as a preservative by absorbing bodily fluids and slowing decay. Mummification can be intentional or unintentional (Arriaza, 1996).

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Artificial mummification refers to the process of making a mummy by taking deliberate action to preserve the body (Arriaza, 1995a:16-17; Arriaza, 1996:131-140). The study of artificial mummification in prehistoric populations is important because mummification requires time, energy, specialized skills and, in general, an organized system to support it. Additionally, artificial mummification can be symbolic and may represent the spiritual beliefs of a group (Arriaza, 1996:131).

Age and Sex Variation:

According to Arriaza (1995a) and Núñez (1994), the Chinchorro culture is unique in that treatment of the dead appears the same regardless of age, sex and, to a lesser degree, status. However, Standen (1991, 1997), who analyzed burial patterns that include decorative artificial mummies alongside natural mummies, disagrees. She argues that the artificial and the natural mummies were contemporaneous in many instances and not, as previous researchers suggested, the result of burial re-use or cultural variation (Arriaza et al., 2005:662). Part of this controversy stems from the fact that the Chinchorro burial stratigraphy was horizontal, not vertical. Additionally, burials were less than a meter deep and included no tombs. Arriaza and colleagues (2005:662) have asked whether differential mummification reflects a family lineage or is evidence of a social hierarchy; they maintain that the various mummification forms present alongside each other represent status. If this is the case, then the complex

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painted mummies should be the minority and the natural mummies the majority. What the people equated with wealth is unknown.

Of 96 bodies exhumed at Morro-1, natural mummies comprise 37%. Artificial mummies (black, red, or mud) comprise the remainder. To resolve the debate between "equalities and evolutionary cultural changes," Arriaza and colleagues (2005:663) selected 15 mummies from four cemeteries (Morro Uhle, Morro-1, PLM-8, and Maestranza-1) to be radiocarbon-dated. The mummies selected that were found together were prepared in distinctive mummification styles. For example, naturally prepared mummies were found next to black or red artificially prepared mummies. Additionally, those prepared in a complex style (skeletal elements supported with sticks and reeds, incisions, etc.) were found next to mummies prepared in a simpler style (no supporting materials, simple molded mud).

Their results revealed that the black mummies are the oldest (ca. 4950 - 3475 cal BC) followed by the red (ca. 3200 – 2450 cal BC) and, lastly, natural mummies (ca. 2375 - 1900 cal BC) (Arriaza et al., 2005:668). However, the researchers did find some overlap between an infant prepared in the black style (4300 - median cal BC) and a male aged 14-15 years naturally mummified (4010 cal BC - median) buried next to each other (Arriaza et al., 2005:668). In addition, Allison and colleagues (1984:163-165) cite a radiocarbon date for an early red mummy from Morro-1 T7 C1 that precedes the black mummies.

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Their research raises interesting questions about differences in status, as the 14-15 year-old male previously mentioned lived during the height of the black style. His burial is located next to two individuals mummified in the black style (an adult male and a young male). It is unknown whether he is related to the others (Arriaza et al., 2005:668). In addition, three other naturally mummified individuals (two adult males and one adult female) have radiocarbon dates contemporaneous with those mummified in the red style. Complicating matters further, there are two red mummies exhibiting a very simple mummification process, at a time when red mummies involved a very complex process. A look at recently radiocarbon dated remains suggests that while there may be a trend from the creation of black to red to mud and back to natural mummies, there are several instances of overlap and inconsistencies; this all suggests that the style and techniques may have been more fluid that previously thought. Additional radiocarbon dates should help clarify the chronology of mummification techniques and styles.

Change Over Time:

Four periods of the culture practiced artificial mummification (Arriaza, 1995a:126-132; Allison et al., 1984:157-161). These periods correspond roughly to the mummification treatments listed earlier (Arriaza, 1995b:7). They are respectively:

- **Initial Chinchorro,** dated from about 5050 BC to 4980 BC
  Includes the first artificially mummified individuals

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- **Classic Chinchorro,** dated from approximately 4980 BC to 2800 BC Includes the black mummies
- **Transitional Chinchorro**, dated from about 2620 BC to 1720 BC Includes the red and "bandage" mummies
- **Late Chinchorro**, dated approximately from 1720 BC to 1110 BC) Includes the mud mummies

Simple Treatment:

Mummies receiving the simple treatment appear predominantly at the start and finish of the culture, and date to about 7020 BC and 1110 BC, respectively (Arriaza, 1995a:7-8). Still, there are several natural mummies interspersed between those receiving complex treatments; natural desiccation created these mummies. Why they did not receive a secondary treatment is unknown.

Complex Treatment:

The complex treatment is the best-known, but the process and pigments used varied. As noted, this treatment included three mummification styles: black mummies, red mummies and "bandage" mummies. Described below are the three styles.

Black Mummies:

The black mummies take their name from the black manganese pigment that colored their masks. The use of this paint began somewhere between 5900 and 5000 BC and lasted for about 2000 years (Arriaza, 1995a: 98-106; Allison et al., 1984:158). Artificial mummification of an individual in the complex treatment

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involved cleaning of the body, evisceration, and detachment of the head (Arriaza, 1995a:116-119; Núñez, 1994:359:154). Below is a description from Arriaza (1995a:98-106) of the mummification of a baby in the black style.

The first step for the person preparing the body was to use a stone knife to remove the skin, flesh, and organs, including the eyes. The person(s) would then cut into the skin retracting it to expose the skeletal elements and internal organs. The skin was soaked in water to keep it supple. The hands and feet were untouched. Some bodies appear to have been cleaned; others decomposed (Arriaza 1995a:105-106). It is unclear why some received hands-on treatment and others did not. Environmental conditions or "idiosyncratic variations" in the style of the preparer(s) may have dictated the method (Arriaza 1995a:105-106).

The majority had intact skulls when found; the foramen magnum allowed for the removal of the brains. Some mummies did not have organs or eyes. Removal of the brains allowed for filling of the skull with straw and occasionally ash (Arriaza, 1995a:99; Allison et al., 1984:158). Tortoro reeds stabilized the cranium and mandible. A straight stick placed between the skin and the skeletal elements along the back of the body gave support to the spine. This stick reattached the skull. Tortoro reeds wrapped around the stick provided support. Sticks placed between the skin and skeletal elements beginning at the ankles and rising to the chest supported the leg bones (Arriaza, 1995a:100; Arriaza, 1995b:43-45; Núñez, 1994:359). More sticks and reeds gave the skeleton

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greater support. Hot ashes dried the body cavity (Núñez, 1994:359; Arriaza, 1995b:43). Grass and a paste of ash, water, sea lion blood, bird eggs, or fish glue replaced the organs. This same paste covered the body, molded genitals, and breasts, and created a facial mask. After the paste dried, individuals replaced the skin and attached a wig of short human hair to the skull (Arriaza, 1995b:103 Núñez, 1994:359). Animal skin compensated for the shrinkage of human skin (Arriaza, 1995b:45). Once reconstructed, individuals decorated the body with black manganese pigment. Individuals used wood or pebbles to polish the painted body (Arriaza, 1995b:45).

Red Mummies:

Around 2620 BC production of red ochre-painted mummies occurred at the sites of Morro-1/5, PLM-8, Quiani, Punta Pichalo, Camarones-8, Bajo Molle, and Patillos (Arriaza, 1995a;130;1995b) (Figures 2 and 3). At the site of Morro-1 (T. 7, C1), a red mummy was found earlier; it dated to 5860 ± 180 BC (Allison et al., 1984:163-165; Arriaza, 1995b: 43, 48). The name "red mummy" refers to a mummification style change and not necessarily to the color of the pigment applied to the facial mask. Some mummies created in the red-style had masks painted black or green (Arriaza, 1995b:45). It is unknown why one color of paint was favored over another. It is possible that the symbolism changed or that certain materials became scarce. Red mummies received the complex treatment, but without dismemberment or disarticulation of the lower skeletal elements. Disarticulation of the head did occur (Arriaza 1995b:46). Sticks and

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stuffing from reeds, sea bird feathers, soil, and camelid wool supported their bodies. Incisions allowed for the removal of organs and muscles at the shoulders, pelvis, knees, and ankles, which were sewn-up with human hair threaded through cactus needles (Arriaza, 1995b:106). Reeds did not support the sticks tied to the skeletal elements in these mummies (Arriaza, 1995b:106-107, 1996; Arriaza and Standen 2002). The length of the wig was also different. Strands of black human hair as long as 60 cm were bundled with reed cords and stuck to the back of the skull with ash paste that was later painted red or other colors (Arriaza and Standen, 2002).

"Bandage Mummies":

The "bandage mummies" also received the complex treatment. Like the red mummies, they represent a variation on the black style of mummification. The reattachment of the skin in "bandage mummies" is different in three of the 27 Morro I red mummies. The three mummies were from children, and all of them had strips of skin from humans, sea lions, or pelicans wrapped around their limbs and trunk. "Bandage mummies" look like the quintessential Hollywood movie image of a mummy (Arriaza, 1995b:110; Arriaza, 1996:133; Arriaza and Standen, 2002:30-31).

Mud-Coated Mummies:

Another mummification change took place around 1720 BC and persisted until around 1110 BC (Arriaza, 1995a:114-115; Arriaza, 1995b:47-48). At this time, for unknown reasons, the Chinchorro gave up much of their mummification

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process. There are two subtypes of these mummies: (1) with evisceration and (2) without (Arriaza, 1995a:114). The bodies of the deceased during this period received only a slim coat of paste. At the site of Morro-1, 24 mud mummies were found. Burials took place before the mud hardened and mummies were stuck in the ground by the mud covering them. These mummies were only found in Arica. The abandonment of the earlier techniques may have been a result of trade, which brought new ideas. They also may have started to associate decomposition with disease. However, there is no evidence of either.

Summary of Changes in Mortuary Behavior and Relationship to Status:

The mortuary practice adapted and changed over time; however, data shows that within each style some juvenile and some older individuals of both sexes did not receive the artificial mortuary treatment that was normal for the time. It appears, however, that all infants did receive the complex treatment (black, red, or mud) (Arriaza et al., 2005:669-670). Both Arriaza (1995a:134; 1995b:50) and Standen's (1991, 1997) hypotheses about burial type and status are valid. The radiocarbon dates show a trend, albeit not a perfect linear one, in classification based on the evolutionary styles of mummification (Arriaza, 1995a39-43). However, there were natural mummies throughout all periods of mummification. Therefore, a relationship may have existed between status and mortuary treatment. Arriaza and colleagues (2005:270) have proposed that those who received the natural treatment during periods when the other treatments (red, black, mud) dominated were outsiders or persons of "low

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sociocultural position." Few grave goods were recovered from Chinchorro burials (primarily fishing tools and nets), and the ones that were, do not suggest status differentiation.

Arriaza and colleagues (2005:663) provide data on Chinchorro mummies from Arica by type and cemetery. Tabie 12 shows their results. Mummies discovered and/or dated after 2005 are not included in this table. The table shows that the majority of mummies from sites in and around Arica received natural mummification, followed by the red, mud, and black styles. These data suggest that mummification in the natural style was not fluke, but "a recognized cultural phenomenon" (Arriaza et al., 2005:670). If mummification technique was selective, then a cultural practice or belief likely affected the type of burial received (Arriaza et al., 2005).

Table 12 Chinchorro mummies by type and cemetery (Arriaza et al., 2005:663)

Site	Black and Complex Mummies	Red Mummies	Natural Mummies	Mud Mummies	Total
Morro-1	8	27	36	25	**96**
Playa Miller-8	1	9	0	0	**10**
Morro-1/6	0	0	69	0	**69**
Morro-1/5	0	17	1	0	**18**
Morro Uhle	0	9	1	2	**12**
Arica	2	0	0	0	**2**
Chinchorro-1	2	0	0	0	**2**
Maestranza-1	7	0	4	0	**11**
Quiani	0	1	0	0	**1**
Maderas Enco-I	3	0	0	0	**3**
Total as of 2005	**23**	**63**	**111**	**27**	**224**
Total dated as of (Arriaza et al., 2005)	10	13	13	3	**39**

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Implications for Understanding Social Organization:

While there is no evidence as to who within the Chinchorro settlements mummified the dead, Arriaza (1995a:117-119) has hypothesized that the Chinchorro had a well-defined division of labor that guided their mortuary practice. According to him, this division of labor may have involved elders in the creation of the mummy. Apprentices, youth, or women may have been involved in the collecting of the reeds, feathers, sticks, and camelid fibers used to decorate and help the body maintain its shape (Arriaza, 1995a:117-119). Women and children may also have assisted in the creation of the wigs, grass skirts, and in the collection of ashes, clay, and various pigments (Arriaza, 1995a:117-119). Elders may have passed the art of mummification to their descendants (Arriaza, 1995a:117-119). Specialists may have been involved in the creation of the mummies, and through their travels to neighboring villages spread the practice (Arriaza, 1995:116). However, the only evidence of organizational complexity is their dedication of formal cemetery areas. There is no physical evidence suggesting that a particular sex or age group, or group of individuals was responsible for the creation of the mummies.

Implications for Understanding Ideology:

Chinchorro mortuary practices reveal important aspects about their culture and their spiritual beliefs (Arriaza, 1995a,b; 1996, Bloch and Perry, 1982; Chapman et al., 1981) Arriaza (1995a:84) believes that the Chinchorro felt that there was "a spirit of reciprocal altruism between the living and the dead: by

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displaying and caring for the mummies, the Chinchorro secured protection." The Chinchorro may have made offerings of food and regularly visited their mummified dead, although there is no evidence for this. Sturdily constructed, it is possible relatives moved mummies for special occasions, although there is no way to know if this actually occurred. Encasing the mummies in woven shrouds and placing them in a shallow grave, sometimes in groups of six lying adjacent to one another with personal belongings, probably signaled the end of the specified mourning period. These group burials may indicate a relationship between the deceased (Arriaza, 1995a).

Chinchorro spirituality encourages speculation; it is impossible to know about the personal beliefs of a long-dead culture, without written or pictorial records. Science trains for skepticism, and most scientists are uncomfortable with hypotheses that cannot be tested; however, "a great deal of our consciousness involves guessing well" (Libet, 2004:185). It is human nature to "make a coherent story out of fragments" (Libet, 2004:185). Our attempts to understand the Chinchorro mortuary practices are no exception.

Through their veneration and preservation of the dead, the Chinchorro may have set the stage for later groups who would worship their dead. While the practice of artificial mummification ceased before the Spanish arrived, the idea appears to have influenced later Native American groups (Reid, 1999). Sixteenth century texts, kept by Spanish explorers, may inadvertently provide insight into the Chinchorro culture. These texts, which detail the beliefs of later South

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American native groups, discuss *huacas* (sacred objects) that included natural mummies. Considered gods by some Andean groups, these mummies are associated with fertility. These texts describe relatives caring for the *huacas.*

Summary of Chinchorro Mortuary Practices:

The Chinchorros elaborate mortuary practice, which over time encompassed several styles and techniques, expressed their artistic side. All deceased were mummified regardless of age or sex; however, the methods used may be indicative of status in some cases. It is impossible to say what spiritual beliefs directed their care of the dead. Their practice of artificial mummification began around 7020 BC and continued in some form until around 1110 BC (Rivera, 1991:17; Muñoz and Chacama, 1993:28; Arriaza, 1995a:125-132; Arriaza, 1995b:35). The adoption of tuber horticulture inland may have taken time away from the mortuary process, and this may explain why, in the later Quiani phase, artificial mummification ceased (Arriaza, 1995a:132). Additionally, if they were able to connect the manipulation of cadavers with the spread of infectious disease in the population, they may have chosen to scale back or end their mortuary practice.

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Chapter III.

Review of the Treponemal Literature

Introduction

Treponematosis is a bacterial infection caused by spirochetal gram-negative microorganisms from the genus *Treponema* and the species *pallidum* (Thomas, 1989; Aufderheide and Rodríguez-Martín, 1998:154-171; Wilson, 1998). *Treponema pallidum* is a complex bacterium, in that it causes multiple stages of infection and, in the case of *T. pallidum* subsp. *pallidum* (syphilis), results in a debilitating and potentially fatal disease in a third of untreated individuals (Cameron et al., 2000). *Treponema pallidum* can spread topically and/or hematogenously to any tissue, and can live for decades in spite of an active host immune response (Norris et al., 1998).

This dissertation focused on three clinically distinct but antigenically related treponematoses that affect the skeletal elements: *Treponema pallidum* subsp. *pallidum*, the etiological agent of venereal syphilis; *T. pallidum* subsp. *pertenue,* the etiological agent of yaws; and *T. pallidum* subsp. *endemicum,* the etiological agent of bejel (Aufderheide and Rodríguez-Martín, 1998; Wilson, 1998; Singh and Romanowski, 1999; Centurion-Lara et al., 2006). This dissertation will not focus on *Treponema denticola*, a form of treponemal infection that causes periodontal disease, or *Treponema carateum* (Pinta), which affects the skin.

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The origin and distinctiveness of the pathogenic human treponemes has long been a subject of debate. Before molecular research on *Treponema* began, many researchers believed that *Treponema pallidum* was the sole etiological agent of syphilis, bejel, yaws, and pinta. Some believed that the clinical differences among the treponemes were due to factors involving the environment and host, which were the result of adaptations by the same microorganism to different epidemiological conditions (Hudson, 1963, 1965:891; Aufderheide and Rodríguez-Martín; 1998:166); others thought more than one organism might have been responsible (Hackett, 1963, 1967). Where the first treponemal infection originated remains a mystery; many believe it was in Africa and that the infection spread with early humans during their migrations. How those humans, or perhaps their ancestors, were initially infected is also a source of debate. Some believe an animal, possibly a primate that had an infectious disease from which the mutations arose, infected humans (Cockburn, 1963; Hackett, 1963). Others think that the spirochetes came from treponemes living in water that humans picked up as commensal saprophytes (Wilcox, 1972). Saprophytes are parasitic organisms that subsist on living or decaying organic substances. Regardless of how and where, once the treponemes found human hosts, they appear to have adapted to their new environment (Wilcox, 1972).

The only known animal treponeme, *T. canicula,* leads to a venereal syphilis in a species of English rabbits (Wilson, 1998). While *T. canicula* is morphologically indistinguishable from the other treponemes, phylogenetically, it

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"is not as closely related to the other human treponemes, as they are to one another" (Wilson, 1998:101). The rabbit treponeme occurs naturally and does not allow cross-immunity to the human treponemes (Hackett, 1963; Wilson, 1998). *Treponema pallidum* is a natural pathogen only in humans, or so most believe (Musher and Knox, 1983). However, Rothschild and Turnbull (1987:61-62) report that they have identified *T.p. pallidum* antigen in a Pleistocene bear *(Arcdotus simus)* from Indiana dated to 11,500 BP (11,449 ± 140 BC), but this has been disputed. Neiburger (1988) has written that the lesions on the vertebrae and long bones of the bear skeleton are more likely to be the result of a fungal infection common to wild animals in the area. He also questions the accuracy of the positive immunological test, stating that the results are from only one sample and may represent a false positive, and/or may be indicative of contamination from ground soil or handlers (Nieburger, 1988:603). He suggests further tests be conducted to confirm the results.

Understanding the treponemes is difficult because bacteria must be isolated from infected mammalian tissue. Additionally, the scarcity of nonvenereal strains hampers comparative studies of the treponemes (Harper et al., 2008). Inducement of experimental infection has occurred in several species, including apes and rabbits (World Health Organization, 1970). The standard way to study *T. pallidum* is through intratesticular infection of rabbits (Norris et al., 1998). Infected rabbits develop erythematous lesions (Lukehart, 1983). Chimpanzees infected with *T. pallidum* develop antibodies to the infection, but no

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lesions (Brown et al., 1970). Hamsters (Schell et al., 1980), guinea pigs (Wicher et al., 1976: 284-297; Wicher et al., 2000), and irradiated mice (Klein et al., 1980) have a different reaction to treponemal infection than either humans or rabbits. It is difficult to induce infection regularly in guinea pigs and hamsters; however, when infected they develop chronic skin infections, enlarged lymph nodes, and gain weight (Schell and Musher, 1983). Mice develop antibodies to treponemal infection, but do not develop lesions (WHO Report, 1970). Animal experiments working with the human treponemes have shown that there is minimal cross-immunity between yaws and syphilis and even less among pinta, syphilis, and yaws (Hackett, 1963; Wilson, 1998:101). A study using guinea pigs injected with *T.p. pertenue* (yaws) demonstrates that there is "a low incidence and short persistence of the pathogen in internal organs" (Wicher et al., 2000:3219). These findings show that yaws has a greater affinity for the skin than the internal organs (Wicher et al., 2000:3219). This dissertation also demonstrates that there is no transplacental transmission of the yaws pathogen, unlike the syphilis pathogen (Wicher et al., 2000:3219; Antal et al., 2002:84).

Etiology of Treponemal Infections

Treponema pallidum infection is acquired in the following ways: (1) from an infected human through sexual contact and/or via bodily fluids; (2) by blood transfusion; (3) by transplacental passage (syphilis only); (4) by skin-to-skin contact; (5) through fomites (objects or substances that carry infectious organisms) and infected laboratory animals (Musher and Knox, 1983), and (6)

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possibly through flies, for example, the *Hippelates* species (yaws only) (Howe, 1981). Transmission of yaws via an insect vector has been claimed but has not been proven (Hunter et al., 1966; Howe, 1981; Powell and Cook, 2005:13).

The various treponemes may require different population demographics to survive. Syphilis, for example, probably needs a large population density compared with bejel, yaws, and pinta (Larson, 1997:87; Larson and Harn, 1994). Venereal syphilis results from direct human contact, whereas the others, while spread person to person can spread through inadequate sanitation or other means. Non-venereal forms of treponematosis commonly spread in childhood through contact with infected lesions or items touched by the lesion (Wilson, 1998). Yaws, for example, usually spreads by skin-to-skin contact in childhood, and bejel through shared clothing or items exposed to lesions (Wilson, 1998).

Yaws:

Yaws is a non-venereal infection, which usually occurs among children in hot and humid climates where little clothing is worn, open lesions on the skin are common, and hygiene is inadequate (Musher and Knox, 1983). Secondary yaws is a systemic infection caused by the spread of the spirochetes in the blood stream. Periosteal reactions (formation of new bone in response to injury) and lesions in the skeletal elements of the hands, feet, wrists, and tibia are common. Cortical rarefaction (decreased bone density) appears in radiographs (Musher and Knox, 1983). Gummatous lesions (masses or nodes that are chronically infected) of the soft tissues, skeletal elements, and cartilage of the nasal septum,

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and palate when seen, occur in the tertiary stage (Musher and Knox, 1983). Usually, the tibia is the most affected bone, followed by the fibula, medial clavicle, femur, ulna, and the skeletal elements of the hand and foot (Aufderheide and Rodríguez-Martín, 1998:156). The diaphyses (shaft of the long bone) and metaphyses (growth plate between the diaphysis and rounded end of the bone or epiphysis) are the areas that are most affected. Dactylitis (swelling of the fingers and toes) occurs with yaws (Aufderheide and Rodríguez-Martín, 1998:156). In yaws, the skull is not usually involved; however, when it is, concave depressions mark the cranial vault (Aufderheide and Rodríguez-Martín, 1998:156). Gangosa (ulceration of the nose and hard palate) may occur (Thomas, 1989; Brothwell, 1981:137). Gummatous osteomyelitis (usually a bacterial infection of the bone and bone marrow) of the long bones is common (Aufderheide and Rodríguez-Martín, 1998:156). There is no cardiovascular or central nervous system involvement as seen in tertiary syphilis (Musher and Knox, 1983).

Bejel or Endemic Syphilis:

Bejel is associated with low socioeconomic conditions and warm and arid climates (Aufderheide and Rodríguez-Martín, 1998:157). It exists in places with poor sanitation and dense living conditions (Grin, 1953). Endemic syphilis commonly spreads among children. The late manifestations are similar to venereal syphilis. The symptoms include gummatous lesions that may involve the skin and nasopharyngeal area. In bejel, the skeletal elements and skin are sometimes affected. The disease may affect the tibia, fibula, ulna, radius,

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clavicle, and phalanges. Lesions may occur on the metaphyses and diaphyses; periosteal reactions occur primarily in the center of the diaphysis, and there may be tibial flattening (Rothschild and Rothschild, 1995; Aufderheide and Rodríguez-Martín, 1998:157). Sabre shin may also be present (Aufderheide and Rodríguez-Martín, 1998:157). Sabre shin is a condition in which the "anterior border of the tibia is marked with sharp convexity" (Thomas, 1989:1621). The cardiovascular system and neural system may be affected (Aufderheide and Rodríguez-Martín, 1998:157). However, this occurs less often than in venereal syphilis and when it does occur, the symptoms are "mild to moderate" (Wicher and Wicher, 1983:150).

Venereal Syphilis:

Treponema pallidum subsp. *pallidum,* or syphilis, spreads primarily through genital contact or through bodily fluids; exposure is usually through vaginal or anal intercourse. Syphilis is associated with a higher population density than with yaws or bejel. Of those exposed to the spirochetes, only 10% to 25% will develop a venereal syphilis infection (Aufderheide and Rodríguez-Martín, 1998:158). Many researchers theorize that the changes that led to improved hygiene, sanitation, economic conditions, and the wearing of more clothing, also led to this form of treponemal infection (Hudson, 1946). Syphilis is the most virulent of the four treponemal infections and, if left untreated, affects fertility by causing spontaneous abortions, stillbirths, and neonatal deaths. This infection also results in cardiovascular and neurological lesions (Wilson, 1998).

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The frequency of the bone lesions in venereal syphilis ranges from 10% to 20% (Aufderheide and Rodríguez-Martín, 1998:158). The skeletal elements most frequently affected in late syphilis are the tibia, frontal, parietal, nasal-palatal region, sternum, clavicle, vertebrae, femur, fibula, humerus, radius, and ulna, although any bone or several areas of the bone may be infected (Musher and Knox, 1983; Aufderheide and Rodríguez-Martín, 1998:158-159). Bone lesions indicative of syphilis include gummas, stellate (star-shaped) lesions, caries sicca (characterized by irregular thickening, necrotic bone tissue, bony defects, and gummas on the skull), osteoperiosteal reactions, spiculated (irregular edges) on periosteal reactions, and osteitis (generalized inflammation of bone) (Aufderheide and Rodríguez-Martín, 1998:158-163). Venereal syphilis may also involve narrowing of the medullary cavities (central cavity of the bone shaft) in long bones causing pitting, gummatous osteomyelitis, as well as bone lesions on the vertebrae, crania and joints (Aufderheide and Rodríguez-Martín, 1998:158-161). Hackett (1976, 1983) developed diagnostic criteria with a syphilis sequence for caries sicca, and a second syphilis criterion for the development of nodes and cavitations within the long bones.

Congenital Syphilis:

Congenital syphilis, because it spreads directly through the blood stream, from mother to fetus, results in early systemic disease (Powell, 1992). In non-venereal treponematosis, women usually contract the infection before menarche. Because infection occurs during youth, the spirochetes in their blood are usually

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too low for congenital transmission to take place (Wilson, 1998). In congenital syphilis, periosteal reactions and osteochrondritis (joint disease where bone and cartilage loses its blood supply) may appear at birth (Musher and Knox, 1983).

Moon's molars and Hutchinson's incisors may occur during the first week of an infected infant's life and are a sign of congenital syphilis (Cook, 1994). Described by Henry Moon in 1884, Moon's molars refers to a deformity of the first (six-year) molar in which the tooth develops with "a broad base, small crown, and multiple small enameled cusps" (Dennie and Pakula, 1940:32). Hutchinson's incisors, named in 1857 for Jonathon Hutchinson, are "pumpkin-seed shaped with a crescent-shaped notch on the cutting edge" (Dennie and Pakula, 1940:29). Twenty-four percent of infants with congenital syphilis have bone, joint, or skin infections (Dennie and Pakula, 1938; Wilson, 1998). Late signs of congenital syphilis usually begin at age two and include enamel defects and symptoms like those of tertiary venereal syphilis and/or yaws (Aufderheide and Rodríguez-Martín, 1998:165-166; Wilson, 1998)

Hypotheses on the Origin of Treponematosis

There are four predominant hypotheses as to the origins of treponematosis. They are the Unitarian hypothesis, the NonUnitarian hypothesis, the Columbian hypothesis, and the Pre-Columbian hypothesis (Aufderheide and Rodríguez-Martín, 1998). Included also is a fifth hypothesis, Livingston's Alternative Hypothesis (Livingstone, 1991). Discussed briefly below are the five hypotheses.

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The Unitarian Hypothesis:

According to the Unitarian hypothesis, there is only one treponemal disease, which changes its epidemiology to adapt to its host's environment and socio-cultural conditions (Hudson, 1965; Aufderheide and Rodríguez-Martín, 1998:166). These different manifestations comprise the treponematoses (Hudson, 1965). This hypothesis names Africa as the origin of treponematosis (Cockburn, 1963; Hudson, 1965; Aufderheide and Rodríguez-Martín, 1998:166). Some have the disease beginning as yaws in central Africa and subsequently spreading via *Homo sapiens* 100,000 years ago (Hudson, 1965; Aufderheide and Rodríguez-Martín, 1998:166; Wilson, 1998). Cockburn (1963) has suggested that there may be a relationship among the treponemal forms. Researchers employed a method developed to distinguish among the treponemes using osteology, disease patterning, and demographics to examine skeletal remains at the National Museum of Nairobi in Kenya (Rothschild et al., 1995). A reanalysis of periosteal patterns in KNM-ER 1808 and other *Homo erectus* suggests that the disease in these specimens was most likely polyostotic yaws (yaws involving multiple skeletal elements) (Rothschild et al., 1995). This evidence, if correct, sets an origin date for yaws somewhere in the middle Pleistocene (~1.5 million years ago) and suggests that *Homo erectus* carried the disease to Europe (Rothschild et al., 1995); however, other scientists have not affirmed these assertions.

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Regardless of how the treponemes spread, it is probable that environmental and cultural conditions affected their epidemiology. This hypothesis stresses alterations in behavior, as opposed to mutations in the spirochete, as the cause of differences among the treponemes (Hudson, 1946, 1958a, 1958b, 1965). In 1927, Butler and Peterson used the term "treponematosis" to describe an infection found in Haiti that resembled something between yaws and syphilis (Hudson, 1965). They believed that behavioral changes resulted when people with yaws moved from a humid environment to a cooler, dry environment. In the cooler environment, they hypothesized people began to show lesions typical of bejel. Hudson (1965) surmised that with increased density, bejel lesions in children would decrease, and syphilis lesions in children would increase. The similarity of the lesions seen in modern cases of bejel and yaws in Senegal, Niger, and the Ivory Coast suggest that a "transitional state" exists among the diseases (Basset et al., 1994). Hudson (1965) hypothesized that a person's immune system could account for the different lesion patterns seen in various individuals. A neurosyphilis study, which showed that heredity might determine whether a person infected with venereal syphilis develops neurological symptoms, supports this idea (O'Leary, 1938; Wilson, 1998).

According to Crosby (1972:143), clinically and osteologically, the "syndromes of the 'different' diseases of the treponematosis group are not sharply contrasted from the other." A 17th century English doctor, Thomas

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Synderham, wrote that syphilis was a kind of yaws that made its way to Europe and America on boats transporting slaves from Africa (Crosby, 1972). The book, Every Man His Own Doctor or Poor Planter's Physician suggested that syphilis and yaws be treated the same because the symptoms are similar and one is probably is an extension of the other (Crosby, 1972).

However, this hypothesis is problematic. If one believes that the treponemes share a common ancestor, the question then becomes, how did the treponemes travel from central Africa to the Americas? Two ideas have been proposed. The first states that recent immigrants carried the treponemes to the Americas (Cockburn, 1963; Aufderheide and Rodríguez-Martín, 1998:166). The other states that the disease traveled with early humans from Asia to America when they crossed the Bering Strait (Cockburn, 1963; Aufderheide and Rodríguez-Martín, 1998:166). However, there is insufficient evidence to substantiate these hypotheses. Also proposed, is a nonhuman origin in the African apes. In this scenario, the disease was transmitted from apes to our hominoid ancestors, and was spread to the New World via early human migrations (Cockburn, 1963).

The NonUnitarian Hypothesis:

The NonUnitarian hypothesis states that treponemal infections took different clinical patterns due to mutations in the various strains (Hackett, 1963, 1967; Aufderheide and Rodríguez-Martín, 1998:166). Hackett (1963) described

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a scenario, in which the original treponemal infection was pinta, which he hypothesizes was present throughout the world in the Pleistocene.

Pinta as the first treponematosis fits with its geological distribution and with the increasing virulence seen from yaws to bejel and finally to syphilis. The disease Pinta may have begun in Africa, moved to Asia, and then traveled to the Americas during the last glaciation, 10,000 to 15,000 years ago (Hofreiter et al., 2004:12963; Remington, 1968). The first mutation may have occurred 12,000 years ago (Hackett, 1963, 1967; Aufderheide and Rodríguez-Martín, 1998:166; Gray et al., 2006); It may be due to a humid environment, which caused yaws in Africa, Southeast Asia, and possibly in the Pacific Islands and Australia.

The second mutation probably happened around 9,000 years ago, and caused yaws to turn into bejel (Hackett, 1963; 1967; Aufderheide and Rodríguez-Martín, 1998:166; Gray et al., 2006). At this time, there was a change from a warm, humid climate to a dry cool climate. This second form of treponematosis was supposedly then seen in northern and sub-Saharan Africa, southwestern and central Asia, and central Australia (Hackett, 1963, 1967; Aufderheide and Rodríguez-Martín, 1998:166; Gray et al., 2006). According to this theory, bejel did not replace yaws; yaws changed into bejel under the drier, cooler environmental conditions (Hackett, 1963, 1967). Yaws was still active in warm, humid environments; bejel became an adaptation to a new ecological niche (Hackett, 1963, 1967).

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The third proposed mutation took place around 5,000 years ago (Hackett, 1963, 1967; Gray et al., 2006). It was a reaction to increasing population density and the wearing of more clothing. This form of treponematosis occurred around the Mediterranean Sea and southwestern Asia. In these locations, bejel changed into venereal syphilis (Hackett, 1963, 1967; Aufderheide and Rodriguez-Martín, 1998; Gray et al., 2006). By the beginning of the century, venereal syphilis was present in villages near the Mediterranean Sea, but its form was less virulent than today's.

The final alteration, which happened in Europe in the late 15th century, led to venereal syphilis due to changing environmental, social, and cultural conditions (Hackett, 1963, 1967). This last form was not isolated to one environment, and was susceptible to cultural and demographic conditions (Hackett, 1963, 1967; Aufderheide and Rodríguez-Martín, 1998:166). A problem with this hypothesis is that pinta would have had to change into yaws in more than one place and at more than one time. Pinta may have appeared in Africa 12,000 years ago, but the ethnohistorical evidence suggests it stayed in Central and South America (Hackett, 1963; Ortner and Putschar, 1981). It is more likely that the treponematosis changed due to ecological isolation (Bogdan and Weaver, 1992). Other researchers have suggested that the treponemes were in the Old and New Worlds but developed differently due to isolation (Stewart and Spoehr, 1952).

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To understand how treponematosis may have changed over time, the treponemes can be discussed in regard to Mayr's definition of a species, which states, "species are a group of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr, 1996:262-267). Bacteria can reproduce by mating – that is they exchange conjunctive plasmids -- therefore, Mayr's definition of a species is applicable (Lederberg and Tatum, 1946; Cockburn, 1961). Under this definition, the various treponemes fall under one genus, *Treponema.* People in Central America were isolated for the longest time; therefore, an evolutionary perspective could explain how humans and pinta came to co-exist, and why it persists in isolated parts of South America (Guyana, among Amazonian tribes) (Cockburn, 1961; Antal et al., 2002; Harper et al., 2008).

Gray and colleagues' (2006) molecular study of the evolution of six genes from the *Treponema pallidum* repeat (*tpr*) gene family does not necessarily consider venereal syphilis to be the most recently evolved of the treponemes; however, it does suggest that there was not an enormous time difference between the development of yaws and syphilis. *Treponema pallidum* repeat genes encode potential virulence factors and are useful in understanding the pathogenesis and evolution of the genus *Treponema;* a bacterial genome which has evolved via gene duplication and gene conversion events (Gray et al., 2006:2220). Their studies indicate a more coincident development for three of the treponemal infections (syphilis, bejel, and yaws) (Gray et al., 2006:2231),

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which could fit with this hypothesis and possibly with the Pre-Columbian hypothesis. There are no genetic tests for pinta yet, which is why molecular biologists have not reached a conclusion about the timing of its origination.

Columbian Hypothesis:

The Columbian hypothesis stems from the belief that a syphilis epidemic occurred in Europe in the years following Columbus' return (~1493). The proponents of this hypothesis cite historical records that document the spread through Europe of syphilis, which suggests the introduction of a virulent disease to a previously unexposed population (Dennie, 1962; Aufderheide and Rodríguez-Martín, 1998:167-169). However, the explorers of the New World did not debate in print whether Columbus had brought back syphilis from the New World until 40 years after his return (Steinbock, 1976; Aufderheide and Rodríguez-Martín, 1998:167-169). Many Europeans believed that Columbus was responsible for syphilis (Aufderheide and Rodríguez-Martín, 1998:167-169). The discovery that guaiacum, a type of wood found in the New World, helped treat syphilis encouraged this belief.

The Doctrine of Divine Clemency also boosted the Columbian Theory (Steinbock, 1976; Aufderheide and Rodríguez-Martín, 1998:167-169). The population at the time in Europe was religious and believed that "God always provides a remedy where he inflicts a disease" (Aufderheide and Rodríguez-Martín, 1998:167). Guaiacum was a Native American Indian medicine used to treat treponematosis (Steinbock, 1976). The first publication describing this

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wood was written by Nicholas Pol, a doctor for Charles V. of Spain in 1517 and it was titled, The cure of morbus gallicus by the wood of Guaiacum (*De Cura Morbi Gallici, per lingnum Guayanum, libellus*) (Holcomb, 1934). The author writes that 3,000 Spaniards had been cured of "*morbus gallicus*" (syphilis) by the wood; however, there is no scientific evidence that guaiacum alleviated any of the symptoms of syphilis (Holcomb, 1934:403). In 1518, Leonard Schmaus described how natives on an island in Spain used an Indian (Native American) wood for the treatment of venereal disease (Holcomb, 1934:403; 1938:12; Wilson, 1998:56). This guaiacum plant grows today in tropical parts of the Americas, including Florida and California in the United States. Schmaus titled his publication, *"Lucubratinculade morbo gallico et cura ejus noviter repeat cum lingo indico"* (Holcomb, 1934:403; 1938:12). Considered a holy wood, guaiacum sold well. A third book written by Ulrich von Huttten in 1519, was entitled, "*De Guaiaci Medicina & Morbo Gallico"* or "On the Medicine Guaiacum, and the *Morbus Gallicus*" (Holcomb, 1934). This book was published in German, Latin, French, English, and other languages (Holcomb, 1934). The English version, written by Thomas Paynell in 1533, was titled "Of the wood called guaiacum, that healeth the Frenche-pockes, and also helpeth the goute in the feet, stone, the palsy, lepree, dropsy, fallynge evyll, and other diseases" (Holcomb, 1934).

A recent phylogenetic study by Harper and colleagues (2008) has bolstered this hypothesis with molecular research that supports the idea that Columbus and other explorers brought back a treponemal variant around 1493

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that mutated into venereal syphilis. Prior to the completion of their study, the only evidence for this hypothesis was the uncanny timing of the syphilis epidemic in Europe, which corresponded roughly with the return voyages of Columbus and his crew. Harper and colleagues (2008) examined the evolutionary relationships among 26 geographically distinct strains from the *Treponema* genus (23 from the *Treponema pallidum* species) by identifying areas of mutation or single nucleotide polymorphisms (SNPs), or insertions and deletions (indels). Their results suggest that the *T.p. pallidum* (syphilis) strain of treponematosis originated most recently, and is most closely related to the *T.p. pertenue* (yaws) strain found in South America than to other nonvenereal strains, *T.p. endemicum* and *T. carateum* (bejel and pinta). Their study suggests that there may have been more than one variety of yaws present in the historical (and perhaps) ancient Americas. The South American *T.p. pertenue* strain, which was isolated from two samples from recently afflicted children in a remote region of Guyana, appears to be distinct from the Old World strains of *T.p. pertenue* (Harper et al, 2008). It occupies a phylogenetic position between Old World *T.p. pertenue* (yaws) strains and modern *T.p. pallidum* (syphilis) strains (Harper et al, 2008). Based on the analysis of 17 regions of mutation in the treponemal strains, the authors constructed a phylogenetic tree of linked-together sequences and concluded that the Old World yaws strains occupied a basal position on the phylogenetic tree, which would indicate they were the first treponemal strains to

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arise in humans (Harper et al, 2008). A simian strain from Africa was indistinguishable from the Old World human yaws strains (Harper et al, 2008).

Their study, however, is not without its critics; Mulligan and colleagues (2008) are suspicious of a New World origin for venereal syphilis based on the sequence similarity between the yaws strains from Guyana and the syphilis strains studied. Harper and colleagues' (2008) conclusion relies on the homology of four SNPs in the Guyana samples analyzed with the group of *pallidum* strains (Harper et al., 2008). However, the Guyana samples were degraded and they came from only one location in Guyana (Harper et al., 2008). Admittedly, modern South American yaws strains are scarce in the laboratory, and are limited primarily to Guyana's interior (Scolnik et al., 2003; Harper et al., 2008). However, the SNPs from the South American yaws strain cause nonsynomous changes and occur in a short region (~15 amino acids) of the TprI protein, a member of the family of *tpr* genes (Mulligan et al., 2008). These *tpr* genes show antigenic changes among the treponemal subspecies (Gray et al., 2006). Mulligan and colleagues (2008) state, "This is an extraordinarily high rate of evolutionary change in a genus that has been characterized by very little change."

This particular area of variation (*tprI*) is an area thought to be associated with changes in disease expression, and therefore would be subject to natural selection, thus violating assumptions built into the phylogenetic analysis. Harper and colleagues (2008:7) claim that their phylogenetic tree using maximum

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likelihood and parsimony methods showed that all the *T. pallidum* (syphilis) strains lie within a single clade. Within this clade, the authors identified multiple *T. pallidum* (syphilis) clades with bootstrap support greater than 90% (Harper et al., 2008). The substrains of *T.p. pallidum* (syphilis) and *T.p. endemicum* (bejel) formed groupings distinct from *T.p. pertenue* (yaws) (Harper et al., 2008).

Mulligan and colleagues (2008) see a potentially different interpretation of the tree. According to them, if the tree is redrawn to show only branches with minimal 50% bootstrap support, the *T.p. pertenue* (yaws) cluster disappears, and the three subspecies, plus the simian isolate, branch off the most basal branch together, suggesting no evolutionary order can be determined (Mulligan et al., 2008; Harper et al., 2008). Mulligan and colleagues (2008) also question the length of the branches, suggesting that the *pallidum* (syphilis) strains have longer branches not because they evolved most recently but because they have been subject to greater recombination or selection (e.g. something other than evolution by mutation only). They suggest caution "when drawing conclusions about the evolution of a 'subspecies' that may represent a biological continuum, rather than discrete agents" (Mulligan et al., 2008).

Gray and colleagues (2006) also pointed out the dangers of drawing evolutionary conclusions from the use of the *tpr* family of genes, which are prone to high levels of recombination and have undergone multiple gene conversion events. Their research does not support a much earlier origin for yaws relative to the other subspecies, as proposed by proponents of the Columbian hypothesis

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(Baker and Armelagos, 1988; Rothschild, 2003) and Harper and colleagues (2008). Gray and colleagues (2006:2231) also point to the fact that the Nichol's strain of *T.p. pallidum* (syphilis) collected in the early 1900's is identical at the loci examined to several later *T.p. pallidum* strains collected in the 20th century. This similarity in the strains suggests that the mutation rate was not high enough to have created variants within this period. Whatever the origin of the treponemes, Harper and colleagues (2008) study has rekindled much needed interest into the historical path of treponemes.

Pre-Columbian Hypothesis:

The Pre-Columbian hypothesis states the opposite of the Columbian hypothesis. This hypothesis states that syphilis was present in the Old World before Columbus, but was confused with leprosy, gonorrhea, and/or other diseases (Holcomb, 1941; Aufderheide and Rodríguez-Martín, 1998:167; Wilson, 1998). In this hypothesis, the European epidemic beginning around 1493 was due to doctors' recognizing syphilis as a new disease (Wilson, 1998). Advocates of this hypothesis included Italian physicians Niccolo Leoniceno and Niccolo Massa in 1532 (Aufderheide and Rodríguez-Martín, 1998:167-168). There are many references to "venereal leprosy," which was supposedly present in the 13th and 14th centuries in Europe (Bogdan, 1989:18). There are also many references to "hereditary leprosy;" however, leprosy, or Hansen's Disease as it is referred to today, probably does not spread sexually or congenitally (Bogdan, 1989:18; Aufderheide and Rodríguez-Martín, 1998:142-143,167). The mode of

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transmission for Hansen's Disease is unknown, but it is thought that *Mycobacterium leprae,* its etiological agent, is spread from person to person in respiratory droplets (CDC, 2008a; WHO, 2008). It is also possible that it spreads via insects (WHO, 2008).

Ethnographic records support the idea that 12th and 13th century Europeans may have confused an endemic treponemal infection or syphilis for Hansen's disease (leprosy) (Aufderheide and Rodríguez-Martín, 1998:167-169). These ethnographic records include stories of the Crusaders returning from the "Holy Land" (ancient Palestine) carrying "Saracen Ointment" (Aufderheide and Rodríguez-Martín, 1998:167). This ointment contained mercury and cured "leprosy." However, mercury does not cure Hansen's Disease (leprosy). Mercury has however, demonstrated some success in lessening the symptoms of syphilis over the past 400 years (Aufderheide and Rodríguez-Martín, 1998). Mercury was prescribed by injection, administered orally, and given as an ointment or plaster to be applied to the secondary rashes of syphilis, and over enlarged lymph nodes. Multiple injections, ingestions, or applications were required to receive benefit. The addition of potassium iodide, and later arsenic and bismuth mixtures, to mercury injections suppressed superficial symptoms of the disease, including lesions and condylomata, and killed treponemal bacterial cells (Brooks, 1910). However, these "remedies" were poisonous neurotoxins that also killed normal cells and interfered with the ability of red blood cells to take up oxygen. Not only did these drug combinations not prevent relapses, they

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caused fatalities (Shelton, 1962). It is unlikely that syphilis was confused with gonorrhea. The Bible and many other books recognized by early doctors, describe the symptoms of gonorrhea (Stiller, 1974).

Livingstone's Alternative Hypothesis:

Livingstone's Alternative Hypothesis (Livingstone, 1991) states that there is no reason to believe the treponemes were undergoing the same adaptations or mutating in a similar manner (Livingstone, 1991; Aufderheide and Rodríguez-Martín 1998:167); he suggests that geographic isolation led to the subspeciation of *Treponema* (Aufderheide and Rodríguez-Martín, 1998:167-168). Most importantly, this hypothesis considers the transport of Africans to the New World by Europeans and suggests that an Old World form of treponematosis, presumably yaws, may have transformed either within the New World or as a result of European contact with Africans and/or New World populations (Livingstone, 1991; Rodríguez-Martín, 2000). He notes that the Portuguese had established a trading fort in Ghana 15 years before Columbus' voyage to the New World, providing opportunity for the nonvenereal treponemal infection to adapt to the Americas (Livingstone, 1991; Aufderheide and Rodríguez-Martín, 1998:168). Livingstone (1991) assumes that by 1493, European living had improved to a point where the transmission of the *Treponema* species by skin-to-skin contact was difficult (Livingstone, 1991; Baker and Armelagos, 1988; Rodríguez-Martín, 2000). For *Treponema pallidum* to survive, a venereal form (*T.p. pallidum*) replaced the previous treponemal yaws and bejel subspecies

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(*T.p. pertenue and T.p. endemicum*) (Cockburn, 1961; Baker and Armelagos, 1988; Aufderheide and Rodríguez-Martín, 1998:167-168). This theory is consistent with the idea that social, cultural, and environmental factors produce different types of treponematosis (Hudson, 1965).

Treponematoses in the New World: A Brief Introduction

The many reports of Pre-Columbian treponematosis suggest that the infection was present in the New World prior to Columbus's voyage (Bruhl, 1890; Lamb, 1898; Hrdlička, 1922; Steinbock, 1976; Hackett, 1983; Griffin, 1984; Baker and Armelagos, 1988; Powell, 1988 a,b; Guerra, 1990; Bogdan and Weaver, 1992; Rothschild and Rothschild, 1996). However, it is necessary to differentiate "the antiquity of New World syphilis from the issue of historic epidemiology" when trying to determine the treponemal epidemiology of Native Americans (Dobyns, 1989:342). It is also important to take a geographical approach to treponematosis in understanding how it interacts with its human hosts in the Americas. It is possible that there are degrees of differences between Pre-Columbian treponematosis and Post-Columbian treponematosis (Wilson, 1998). It is also possible that there are treponemal-like diseases, or unrecognized strains of treponematosis that affected past populations that may have gone unrecognized. Changing biological, environmental, and social conditions experienced by the people treponematosis infected influenced its expression.

The evidence for Pre-Columbian treponematosis in the New World comes mostly from skeletons, and it should be evaluated for two possibilities: "(1)

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unambiguous identification of treponemal disease and, (2) unambiguous determination of pre-Columbian archaeological age" (Ortner and Putschar, 1981:205). Infectious diseases present in the New World before 1492 include the following: treponematosis, tuberculosis, hepatitis, leishmaniasis, oroyo fever, and staphylococcal infections, to name a few (Merbs, 1992).

This research reports on evidence of pre-Contact and circa Contact treponematosis from Meso-America, South America, and Mexico.

Treponematoses in Meso and South America:

There have been several reports of skeletal remains with signs of treponematosis from Central and South America (Ashmead, 1896, Gann, 1901; MacCurdy, 1923; Weiss, 1984; Wilson, 1998). The treponemal diseases seen in these groups are yaws or bejel, according to the researchers (Verano, 1998). However, many early reports describe the lesions as being consistent with venereal syphilis. These reports deserve inclusion because many have not been re-examined, and because the form of treponematosis in South America may have represented some kind of unknown intermediary between yaws and syphilis (Harper et al., 2008). Much of the confusion in determining the treponemal subtype results from different interpretations of cranial lesions. While caries sicca is most commonly associated with syphilis, it also occurs in skeletons described as having yaws or bejel. The exact infection present in these specimens is unknown. In the future, DNA may clarify the osteological profile. The analysis of treponemal DNA holds the potential to correct errors in

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osteological and/or X-ray analysis. However, the molecular study of ancient human remains is difficult to achieve because most specimens will not lend themselves to DNA analysis because of taphonomic effects, including those induced through curation. Even if a sample can be subjected to molecular analysis, there is nothing to guarantee the recovery of treponemal DNA. Additionally, funding for high-risk research is limited, and the costs are substantial.

Pinta likely affected ancient populations, just as it has past populations in Mexico and Colombia where it was once endemic (Falabella, 1994), and remote modern populations such as in Guyana and the Amazon where it persists today (Antal et al., 2002; Scolnik et al., 2003). However, because it only affects the skin, and there is no DNA specific test for its presence, the evidence for it lies in ethnographic reports (May, 1950). Several researchers have said that pinta was common in forest-dwelling and rural populations in arid and semi-arid parts of Central and South America. Historically it has affected poor populations lacking adequate sanitation (Antal et al., 2002).

Skeletons from Central American sites with suspected treponemal disease have been reported in Guatemala (Zeculen and Alar de Sacrificios), Belize, Antilles, Santo Domingo (Cucamama, Costanza, and Samana), and Honduras (Ricketson, 1925; Aufderheide and Rodríguez-Martín, 1998:170-171). From Zeculen in Guatemala, two prehistoric skulls dated from AD 900 to AD 1000 have lesions thought to represent venereal syphilis. Non-venereal forms of

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treponematosis have been reported in the Carib Indians from Belize (Black 1980; Garruto, 1981). In Honduras, at the ancient site of Tipu, a skeleton found in the center of an earthen mound had lesions on the diaphysis of the femur thought to be from syphilis. A second skeleton found at this site showed deformities of the femur and humerus suggesting the individual had an infectious disease (Ricketson, 1925). None of the skeletons at Tipu had cranial lesions, which suggests that if treponematosis was the cause of the long bone lesions, it was probably a non-venereal form.

There are reports of three skeletons possibly with yaws from Aguazuque, Colombia (Soacha and Cundinamarca) at sites dated from 5025 BP and 4030 BP (3827 ± 106 BC and 2604 ± 164 BC) (Urrega, 1989; Aufderheide and Rodríguez-Martín, 1998:170-171; Rodríguez-Martín, 2000). Samples with signs of treponemal infection have also been found in Argentina (Rio Negre, Valle del Rio Chubut and Calchaqui) (Aufderheide and Rodríguez-Martín, 1998:170-171) and Venezuela (Hackett, 1957).

Pre-conquest populations in Ecuador were afflicted with at least two treponemes, the most common of which was pinta, which sufferers described as producing bodily rashes of blue, pink, yellow, and violet. Ethnographic records suggest that syphilis, of varied virulence, was widespread after the Spanish conquest of Quito in 1534. These records are unclear as to who had the more virulent form: the Spanish, the natives, or both. This uncertainty could indicate multiple forms of treponematosis. It could also suggest that one or more groups

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were newly exposed to the infection. Spanish naval officers Jorge Juan and Antonio de Ulloa, who were in Quito in the late 1730's, noted, "few persons are free of it [syphilis], tho' its effects are much more violent in some than others" (Alchon, 1991:103). Spanish explorer Francisco Javier Eugencio de Santa Cruz y Espejo wrote a report entitled "Reflexiones sobe el contagio transmisión de las viruelas," in which he described the malady as affecting both men and women (Alchon, 1991:103).

Reports describe treponematosis in many South American samples from Peru, including early Nazca samples from Paracas; Canete Valley; Machu Pincho; Urumbamba Valley; Paucarcancha, and Patallacta (Allison et al., 1982; Aufderheide and Rodríguez-Martín, 1998:170-171). There are reports of skeletons from the Chilca I site in central Peru describing skeletal elements showing curvatures and other pathologies indicative of a treponemal infection (Engel, 1976). Another report describes evidence of syphilis in three skulls, as well as the tibiae and humeri of five Pre-Columbian individuals from Paucarcancha (MacCurdy, 1923). A Pre-Columbian tibia from the southern Peruvian and northern Chilean Tacna-Arica region was found in 1936, and was dated from AD 900 to AD 1350 (Krumbhaar, 1936). Examined visually and by X-ray, the tibia shows "syphilitic osteoperiostitis" (El-Najjar, 1979:601). Additionally, there are signs of chronic periosteal infections (especially tibial infections), and a few cranial lesions in the Chinchorro population from the Arica-Camarones area, and in other northern Chilean mummies (San Miguel de Azapa and environs)

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(Allison et al., 1982; Standen et al.,1984; Rogan and Lentz, 1994; Arriaza, 1995a,b; Standen and Arriaza, 2000b). The infection in the northern Chilean mummies is probably yaws, according to the researchers (Allison et al., 1982; Standen et al., 1984; Rogan and Lentz, 1994; Arriaza, 1995a,b; Standen and Arriaza, 2000b).

In addition to skeletal and ethnographic data, there are also archaeological data that suggest that a form of treponematosis was present in ancient populations. Goundou is a periostitis of the nasal skeletal elements and maxillae caused by yaws or syphilis. Depicted in Peruvian pottery, its appearance suggests that the disease was present in early Peru (Moodie, 1929). The pre-Inca Chimu pottery of Peru includes anthropomorphic pots that show persons with deformities of the mouth, nose, and both. Various theories have been put forth to explain the pots, ranging from Salaman's (1937) hypothesis that the figures pay homage to potato spirits, to Linne's (1939) hypothesis that the pots show deformities consistent with syphilis (Salaman, 1939). Some pots depict children with facial deformities, which may represent congenital syphilis (Salaman, 1939). Others have identified the deformities depicted as being representative of various diseases including the protozoan *Leishmania braziensis,* yaws, leprosy, cancer, *uta* (skin tuberculosis), *espundia* (mucosal leishmaniasis), and fungal infections caused by blastomyces (Tamayo, 1908; Linne, 1939; Salaman, 1939).

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Leishmania braziensis is a form of leishmaniasis, a disease spread by ectoparasites, such as ticks, fleas, sand flies, and mosquitoes that causes cutaneous, mucocutaneous, and viscerai (liver, spleen, anemia) infections. *Uta* derives from the Quechuen word *huta*, which refers to a gnawing disease; it represents a probable tuberculosis infection that caused cutaneous ulcerations. *Espundia,* also from the Quechuen language, refers to mucocutaneous ulcerations usually associated with a form of leishmaniasis (Salaman, 1939). Blastomyces refer to a variety of fungal infections caused by inhaling airborne spores into the lungs, from contaminated soil disturbed by activities such as digging, wood clearing, or animal husbandry. Untreated, it can cause pulmonary infections, cutaneous lesions, bone lesions, and genitourinary infections, and can spread to the central nervous system.

Hrdlička doubted that syphilis was present in pre-Columbian South America; he claims to have found no syphilitic lesions in his studies of Peruvian skeletal remains (Salaman, 1939). While yaws existed in the Amazon, early investigators mistakenly believed that it did not affect the mucous membranes; it does. Others have suggested that the pots depict intentional mutilations or early surgical treatments (Tamayo, 1908; Tello, 1922). Supporting this last idea, archaeological evidence suggests pre-Inka populations used a variety of bronze instruments and were skilled in primitive surgical techniques (Dietschy, 1957).

An outbreak of smallpox soon after the arrival of the Spaniards (from the Caribbean) in central Mexico in AD 1520 suggests that they carried that disease

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to the region. Other diseases may have been introduced by Spanish expeditions to Mexico and the Pacific coast of South America during the 1500's (Alchon, 1991). However, skeletal and ethnographical evidence suggest that yaws was already present in several areas in Central and South America (Arriaza, 1995b; Aufderheide and Rodríguez-Martín, 1998:170-171).

Modern tribes with minimal contact with outsiders provide valuable information about diseases in the past. There is past immunological evidence of infection with endemic treponematoses in three isolated northern Brazilian Indian tribes: the Xikren, Kuben Kran Kegan, and Mekronti (Black, 1975). Evidence was obtained through the florescent treponemal antibody absorption test (FTA-ABS) or the *Treponema pallidum* immobilization (TPI) test. These tests are used to confirm whether a positive screening for *Treponema*, for example, the venereal disease research laboratory test (VDRL) or rapid plasma regain test (RPR), represents a true infection. A positive result indicates prior or present infection with syphilis or one or more of the endemic treponematosis (Black, 1975). Three Kayapo tribes in Brazil showed a high prevalence of treponemal infection, with 60% of adult members testing positive by both the VDRL and the FTA-ABS test. Another 19% were found to be positive by only one of the two tests. No positive results were obtained for children under seven years of age, suggesting that the infection was an endemic treponematosis and not syphilis (Black, 1975). A clinical examination of those who tested positive revealed no signs of venereal syphilis or congenital syphilis. The absence of clinical

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symptoms indicates that the infection was mild and well tolerated by the population. Black (1975) has suggested that the infection among the Kayapo was of low-virulence or that the inhabitants possessed an unusual resistance.

Evidence from Mexico:

Sites with human remains showing lesions indicative of treponemal disease have been found in the pre-Columbian Mexican sites of Tula (Hidalgo), Cueva de la Candelaria (Cohuila), Santiago Tlateloco and Ochicalco (Morelos) and Valle de Tehucan, where remains with syphilis have been dated to 1000 BC (McCaa, 1997; Aufderheide and Rodríguez-Martín 1998:170-171). The caves in the Candellaria Mountains of Mexico have skeletons dated from the 6th to the 16th centuries AD that show signs of treponematosis, including osteitis on the skull indicative of syphilis, and lesions on the long bones indicative of yaws or syphilis (Goff, 1967). The incomplete skeleton of a two-year-old from the 17th - 18th centuries AD with congenital syphilis was found in a coffin at San Jeronimo's Church in Mexico City (Mansilla and Pijoan, 1995). Skeletal lesions include bilateral osteochrondritis, diaphyseal osteomyelitis, and periosteal reactions on the long bones (Mansilla and Pijoan, 1995). Radiological analysis shows symmetrical osteomyelitic foci, especially on the tibiae (Mansilla and Pijoan, 1995). The skull has signs of hydrocephaly (abnormal accumulation of cerebrospinal fluid in the ventricles of the brain) and periosteal changes on the vault (Mansilla and Pijoan, 1995). There are also signs of dental hypoplasia and Hutchinson's incisors (Mansilla and Pijoan, 1995). In Mexico City, skeletons from

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the Metropolitan Cathedral's ossuary were analyzed and found to have lesions on the long bones indicative of syphilis (Marquez, 1994). One of the most interesting specimens is a skull from Tehuacán, which dates to about 300 BC, in which the cranial vault shows extensive destruction from treponemal lesions (Goff, 1967).

Ethnographic records suggest that pinta was present among the Aztecs who lived in the south-central region of modern Mexico (Antal et al., 2002). Individuals who had contracted pinta were referred to as "*pintados*," or people with paint, so named for the colored patches on their skin (Arriaza, 1995b:78). These people were considered special because of their unique appearance (Thomas, 1989; Arriaza, 1995b).

Summary of Treponematoses in Meso and South America:

Treponematoses-endemic foci, in particular yaws and pinta, remain on some Pacific coast islands in Guyana, and in northwestern South America (among Amazonian tribes and in adjacent regions) (Antal et al., 2002; Scolnik et al., 2003; Harper et al., 2008). While treatment campaigns have lessened the incidence of disease, pockets of endemic infection remain. The fact that these diseases persist today emphasizes the need to understand the evolutionary history of these spirochetes. Prior evidence for treponematosis in the Americas has come from the examination of skeletal remains. However, these determinations of treponematoses are problematic because many diseases look similar, and skeletal markings left by the treponemes that affect the skeletal

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elements (teeth excepted) are distinguishable only by differences in distribution, morphology, and frequency of lesions. Additionally, analyses for treponematoses are best accomplished on multiple and complete skeletons, which eliminate many archaeological samples. Mummified human tissue samples hold great potential for the recovery of genetic evidence of ancient treponematosis.

A Brief Introduction to Ancient DNA

The study of ancient DNA (aDNA) is complex and controversial. Like all new technology, it has had false starts (Nicholls, 2005). While the analysis of ancient DNA holds promise for the understanding of ancient phylogenies, population genetics, and host-pathogen interactions, it is not without its difficulties; these include, most importantly, a lack of viable DNA and issues involving contamination (Zink et al., 2002; Nicholls, 2005). To complicate matters, many scientists polarize on the issue of ancient DNA. At one extreme are researchers who dismiss all ancient DNA results as questionable; they maintain that any results are due to contamination by researchers. At the other end, are researchers who believe in the viability of ancient DNA and its potential, and are actively working to develop protocols to address issues of contamination and validity (Nicholls, 2005). Contamination is one of the biggest concerns for any scientist working with ancient DNA, and there are good reasons for this.

One of the most publicized failures in ancient DNA analysis involved the gene sequencing of an 80-million-year-old dinosaur (Woodward et al., 1994; Nicholls, 2005). Unfortunately for the investigators, when their work was

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reviewed it was found that they had sequenced human DNA (probably their own). A phylogenetic analysis would have caught this error; however, there are time and costs associated with analyzing phylogenies (Nicholls, 2005). Despite harsh criticism, many people involved in ancient DNA study at the time could have made this mistake. Mishaps have resulted in the creation of a "criteria of authenticity," a set of standardized protocols for future projects (Cooper and Poinar, 2000:1139; Nicholls, 2005:192). These criteria include "stringent laboratory controls; cloning of products amplified by PCR; replication of results from a second, independent extract; and, for really new or stringent results, replication of results by an independent research group" (Nicholls, 2005:192). Researchers strive to adhere to these principles, but receiving funding for both the initial project and the checks and balances is often hard to do. Granting agencies want accurate results, but they do not want to invest too heavily in high-risk projects; it is something of a Catch-22. Some say, "do it right, or not at all" (Cooper and Poinar, 2000), but that kind of thinking, while presumably creating more accurate studies, also creates an elitist field.

As with any methodology, there are limits to ancient DNA. The question is -- what are those limits? While there is no cut-off date, there are environmental conditions that help predict success. The limit is presently around 2-million-years ago, even for frozen material in the ground (Cooper and Poinar, 2000; Nicholls, 2005). However, evolutionary anthropologist, Svante Pääbo, has narrowed the range by stating that it is "optimistic" to think that one might amplify one million-

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year-old molecules (Hoftreiter et al., 2001a,b; Nicholls, 2005). The technology in this area of research is changing rapidly; what is impossible one day may be possible the next. However, as exemplified by Frederika Kaestle (2005) and the United States National Park Service in their attempts to analyze the remains of Kennewick Man – ancient DNA analysis may be as much about science as it is luck. Scientists were unable to analyze, successfully, samples from Kennewick Man's remains except at very high extract concentrations, which can and usually do result in contamination (Kaestle, 2005). Yet, at the same time, others succeeded in analyzing older samples (Stone and Stoneking, 1996; Kaestle, 1997, 1998, 2005; Krings et al., 1997; Naumova and Rychkov, 1998; Green et al., 2006; Noonen et al., 2006).

In 1997, Krings and colleagues described the extraction and analysis of the D-loop sequences of mitochondrial DNA (mtDNA) from an ancient Neandertal specimen. However, questions remain as to whether it was contamination-free. Cooper and colleagues (1997) tested faunal samples from a range of Neandertal sites and found contaminated mtDNA. However, no data exists on the preservation of their specimens either. Contaminants used in the preservation process of both could have caused mutations in the sequences (Caldararo and Gabow, 2000).

Despite the setbacks, there have been many successes in very old humans, animals, and plants. Successes with nonhuman remains include the sequencing of a weevil (beetle from the superfamily *Curculionoidea*) trapped in

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amber, and dated to 120 to 135 million years ago (Cano et al., 1993). DNA was also extracted from plants persevered in crystallized rat urine collected from an 11,700-year-old rodent midden (11,633 ± 157 BC) from the Atacama Desert in Chile (Kuch et al., 2002).

The most recent accomplishment has been the sequencing of one million base pairs of Neandertal DNA in a fossil (Vi-80) dated to 38,310 ± 2130 years BP (40,621 ± 1659 BC) (Serre et al., 2004:317) from Vindija Cave, Croatia; it required massive parallel 454 sequencing (Marguiles et al., 2005) conducted by Green and colleagues (2006:330-331). This method is a kind of high-throughput DNA sequencing, which uses a parallel sequencing-by-synthesis approach (Marguiles et al., 2005). The sequencing and analysis of 62,250 base pairs of Neandertal genomic DNA by Noonan and colleagues (2006) via the development of a Neandertal metagenomic library and high-throughput sequencing analysis is also significant. Their results allowed them to estimate the human-Neandertal divergence time using multiple randomly distributed autosomal loci (Noonan et al., 2006:1113). Both projects required the development of new technology, and large amounts of time, money, repetition, and perseverance; however, both have provided data that will be invaluable in understanding human and Neandertal biology (Noonan et al., 2006:1118; Green et al., 2006:335-336).

From the more recent past, Pääbo (1985a) was able to sequence nuclear DNA from the skin of a naturally mummified child in Egypt dated to 2430 ± 120 BP (572 ± 152 BC), although he only recovered a low amount of DNA.

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Promising results have also been obtained from bodies recovered from bogs. Researchers recovered DNA from a 7,000-year-old body (5879 ± 97 BC) from an excavation at Little Salt Spring, Florida and from a 6,000 to 8,000-year-old (4911 ± 125 BC to 6903 ± 145 BC) body at Windover, Florida (Pääbo et al., 1988). Unlike most bogs, there is a near neutral pH level from limestone at these sites, which prevents acidity and alkalinity from destroying the molecules (Pääbo et al., 1988; Lawlor et al., 1991; Brown and Brown, 1992).

Hagelberg and colleagues (1989) were able to amplify DNA from human skeletal elements, the oldest of which was dated to 5450 BP (4267 ± 125 BC). Other samples analyzed include the recovery and sequencing of DNA for sex identification (alphoid repeats on the sex chromosome) from two small specimens of bone from two 9,400-year-old human skulls from Cayönü Tepsi, Turkey (Matheson and Loy, 2001). Also successful was the sequencing of mtDNA and amelogenin (used in sex determination) genes from the tibia of an 8,000-year-old Native American skeleton in the southern Rocky Mountains in Western Colorado (Stone and Stoneking, 1996). A 2,000-year-old rib and a 5,000-year-old mandible from two sites in China were also successfully sequenced (Yang et al., 1998). While these cases hold promise for future research, they are subject to the standard ancient DNA shortcomings, including potential sources of error in sampling and analysis.

To understand the difficulties in extracting ancient DNA, it is necessary to understand how organisms decompose. When an organism dies, nucleases

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digest DNA. Freezing or high salt concentrations can slow the nucleases; however, even in these cases DNA damage may occur from radiation, oxidation, and/or hydrolysis (Zink et al., 2002; Nicholls, 2005). In fact, most ancient DNA sequences have damage and fragmentation (Lindahl, 1993). What remains is often low copy number (LCN) DNA, and this requires a protocol sensitive enough to analyze just a few cells (Gill, 2001). This kind of DNA is prone to problems, one of which is allelic dropout, where one allele of a heterozygote locus amplifies at the expense of the other. Stutter (false alleles) is also an issue. Additionally, chemical changes or damage to the DNA can lead to the insertion of inappropriate bases or modification of bases (Lindahl, 1993; Cipollaro et al., 2005; Nicholls, 2005). Interstrand crosslinks also appear at higher frequencies in damaged DNA and interfere with the PCR process (Pääbo, 1989; Cipollaro et al., 2005). Crosslinks are connections formed between reducing sugars and amino acid groups (Nicolls, 2005). Bacterial cloning can also introduce error, and is problematic in that it is here that initial template molecules are often lost in transformation. Problems in amplification and sequence analysis include template competition and nucleotide conversions and misincorporations.

One of the toughest problems, however, is contamination. Before attempting to amplify DNA, scientists following the established criteria (Cooper and Poinar, 2000) usually assess the copy number of target DNA using quantitative competitive PCR (QC-PCR). This method involves adding known quantities of PCR competitor to a PCR reaction containing samples. A

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comparison of the PCR products, as well as the quantity of the target gene products, is then assessed (Maxim Biotech, Inc., 2003). If the number of starting templates is <1,000, sporadic contamination is a real threat, especially when working with human DNA (Cooper and Poinar, 2000). With LCN DNA if there is even a hint of contamination, amplification of it will likely occur (Gill, 2001). The saying goes, "if you try hard enough to amplify something, you probably will" (Cipriano, pers. comm., 2006). Following these standard procedures is expensive in both time and money, but necessary for the acceptance of, and the accurate reporting of results in reputable scientific journals (Cooper and Poinar, 2000). The costs involved in ancient DNA analysis are prohibitive for most individual researchers, thus necessitating collaboration, generally on a large scale at well-funded scientific research institutions.

The problems outlined above are daunting, and explain why many scientists working with ancient DNA prefer mtDNA to nuclear DNA (nDNA). Mitochondrial DNA has more copies, which increases the chances of successful amplification. Intergenetic (among genes) areas of DNA, in particular the hypervariable regions (e.g. D-loop, HV1, HV2), are used to create lineages and molecular clocks (Hagan, 2005). Nuclear DNA has fewer copies making working with it inherently more difficult. One way to address the difficulties with ancient DNA is to identify damaged DNA and try to fix it. Purified repair enzymes can fill in and rejoin gaps in ancient DNA sequences to recreate original sequences (Lindahl, 1993; Hofreiter et al., 2001b; Pääbo, 1989). Crosslinks can block PCR

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fragments by reducing denaturation and preventing amplification of endogenous templates, which can increase contamination; however, these links can sometimes be broken by chemicals such as N-phenacylthiazolium bromide (PTB) that target crosslinks caused by glycosylation end products (Poinar et al., 1998; Hofreiter et al., 2001b; Nicholls, 2005; Willerslev and Cooper, 2005; Hansen et al., 2006). High fidelity polymerase enzymes can also limit sequence errors (Willerslev and Cooper, 2005:6).

Addressing contamination is harder than dealing with decay, because it usually results from human error. An example of the difficulties in avoiding contamination comes from research on a bacterium culture contained in a 250-million-year-old salt crystal (Vreeland et al., 2000). Replication of this research revealed that the same primers that permitted the amplification of the bacterium also allowed for amplification of numerous kinds of halobacteria, including dust bunnies from shelves in the natural history museum in Oxford (Nicholls, 2005). The primers were not specific enough. This, in addition to contamination is a very common, often hard-to-avoid problem. Further amplification and analyses of several types of bacteria suggest that the bacterium in the original culture was a modern contaminant and not ancient (Nicholls, 2005). While most believe bacteria could not go so many years without evolving, it is not impossible and therein lays the controversy (Nicholls, 2005). Vreeland, the microbiologist who conducted the above study, still believes that he cultured and amplified DNA from an ancient bacterium in a salt crystal (Vreeland et al., 2000). He argues that if

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you cannot prove contamination, you cannot prove it was contaminated (Nicholls, 2005). There is truth to this argument; this is why independent replication is so vital. Adherence to the criteria to protect against contamination has its pluses and minuses -- it can prevent the publication of good research that does not stick to the standards and it can permit spurious research that does (Nicholls, 2005). The price tag that comes with adherence to these criteria also limits who can conduct this kind of research. Additionally, granting agencies are reluctant to fund research when the odds are against its success.

Another problem is that individuals evaluating these projects often have no experience working with ancient DNA, and therefore are not qualified to judge. Social and physical scientists are reluctant to admit a lack of knowledge in a particular area of their discipline. One of the mistakes made by these individuals is to use the criteria as a "checklist" instead of evaluating each project on its own protocols (Nicholls, 2005:195).

The announcement that DNA from Cro-Magnon samples was similar to DNA from modern humans exemplified the difficulty in evaluating ancient DNA (Caramelli et al., 2003). Like the earlier Neandertal study (Krings et al., 1997), the authors of the Cro-Magnon research (Caramelli et al., 2003) said nothing about the handling of the samples before they arrived at the laboratory. Contamination by modern humans could have occurred (Nicholls, 2005). While Cooper and Poinar's (2000) criteria are not perfect, throwing out the standards is not the answer. For comparative purposes and for legitimacy, there needs to be

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guiding protocols. The larger question is how to create a design that ensures that those doing and evaluating the research study under people who specialize in ancient DNA and have published (Hofreiter et al., 2001b;Nicholls, 2005).

The study of ancient DNA is, by nature, multidisciplinary, including the fields of anthropology, geology, volcanology, histology, biology, chemistry, and physics just to name a few (Cipollaro et al., 2005). Because no one person or department can know all things, it is necessary that the disciplines work together. Despite difficulties, the study of ancient DNA holds great promise.

To achieve a better understanding of humans and their pathogens, mummies would appear to be excellent candidates for ancient DNA analysis. Recovered DNA exists from mummy's 2,400 and up to 5,000-years-old, from countries including Egypt, Peru, Chile, and Greenland (Pääbo, 1984, 1985a, 1985b, 1989; Del Pozzo and Guardiola, 1989; Thuesen and Engberg, 1990; Rogan and Salvo, 1994). Scientists have obtained nucleotide sequences from several mummies and mummy fragments in Egypt from the Sixth Dynasty (~2370 BC - 2160 BC) (Pääbo, 1985a) and from the liver of an Egyptian priest from the 12th Dynasty (Pääbo, 1989). Scientists today are analyzing ancient DNA for many purposes, among them: to understand migration patterns, genetic relationships, and disease. The discovery of the Human T-cell Leukemia Virus Type-1 (HTLV-1) in the genetic material of ancient mummies from the Atacama Desert in Chile blends all of the above. This disease affects modern Japanese; finding it in ancient Chileans suggests that Asians who presumably migrated

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across the Bering Straits land bridge over 20,000 years ago may have settled in that region. The strains the researchers found in 1,500-year-old Chilean mummies were very similar to the modern strains in Japan (Hong-Chuen et al., 1999). This research is especially valuable because it not only investigates disease in an ancient population, but it also contributes information about population movements.

The identification of ancient pathogens in mummies also includes studies of tuberculosis and Hansen's Disease (leprosy). Scientists have identified *Mycobacterium tuberculosis* (tuberculosis) from the lungs of a 1,000-year-old pre-Columbian Peruvian mummy (Salo et al., 1994). They have also recovered *Mycobacterium leprae* (leprosy/Hansen's Disease) from Norse bone fragments dated to the 13th century AD in Orkney, United Kingdom (Bosch, 2000).

Ancient DNA and Treponematoses

Recent advances in molecular technology, an increase in syphilis rates, and the recognition of syphilis as a co-factor in human immunodeficiency virus have sparked a renewed interest in the analysis of treponematoses in modern and ancient populations (Greenblatt et al., 1988; Cameron et al., 2000; Stamm and Bergen, 2000). Fritz Schaudinn and Erich Hoffmann identified the etiological agent of venereal syphilis in 1905. Many researchers, including, Elie Metchinikoff, Karl Landsteiner, and Paul Erhlich (Norris et al., 1998) subsequently studied it. Speculation about the treponemes as a group, however, did not begin until later. In 1928, Japanese bacteriologist Hideyo Nogouchi

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claimed that the four treponematoses (syphilis, bejel, yaws, and pinta) were indistinguishable (Hovind-Hougen, 1983). Partial cross-immunity among the treponemal syndromes also suggested they were very similar (Baker and Armelagos, 1988). Part of the problem in distinguishing among them lay in the fact that the treponemes were at that time, morphologically indistinguishable with >95% DNA homology (Miao and Fieldsteel, 1980; Noordhoek et al., 1989; Singh and Romanowski, 1999). Later, use of the electron microscope would demonstrate that *T. carateum*, the etiological agent of pinta, has slightly longer cells than *T. pallidum* (Noordhoek et al., 1989); much later molecular biology would elucidate genetic differences among the treponemes (Centurion-Lara et al., 1998, 2006; Gray et al., 2006).

In the late 80's and early 90's, when molecular research into the treponemes accelerated, the only other difference known among the subtypes was between a strain of yaws and of syphilis. The difference consisted of an amino acid in one protein; this gave geneticists one of their first tools to distinguish among them (Noordhoek et al., 1989, 1990a,b).

While *T. p. pallidum* has had a long infectious history, little is known about it in comparison to other bacterial pathogens (Norris et al., 1998; Fraser et al., 1998; Cox et al., 1992) and treponemal subspecies (Harper et al., 2008). What is known about *T. pallidum* is that it has 12 possible membrane proteins and several agents that destroy red blood cells (Fraser et al., 1998). Its genome has little variation, making the reporting of single nucleotide polymorphism (SNP)

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significant (Noordhoek et al., 1990b; Cameron et al., 1999; Centurion-Lara et al., 1999; Harper et al., 2008). In comparison to the other treponemes, most of the variation found between the subspecies *pallidum* and *pertenue* exists within the *Treponema pallidum* repeat genes or *tprA-L* family of 12 genes, which comprise approximately 2% of the genome. What is interesting about these paralogs is that multiple copies of *tpr* genes represent areas of antigenic variation from which scientists can distinguish clinical isolates of *T. pallidum* (Fraser et al., 1998; Pillay et al., 1998; Singh and Romanowski, 1999; Gray et al., 2006). While the *tpr* family of genes is widely discussed in the treponemal literature now, the discovery of the SNPs within them that are used in identifying the treponemal subtypes took place only in the last decade. A few of the studies most relevant to this dissertation are summarized briefly below.

Both ancient protein research and ancient DNA research are used to detect treponematosis. Tuross and Owsley (Ortner et al., 1992) identified IgG antibodies to treponema antigens by immunological detection in a Native American skeleton dated to 1240 BP (AD 795 ± 102). Individuals infected with treponematosis are immunologically positive to treponemal-specific tests for decades. The authors hypothesized that ancient skeletons might retain IgG antibodies that would bind with treponemal antigens. Thirty skeletons, from different geographical regions over a range of 2,000 years were tested for these antibodies. All but one of the skeletons was negative. The one that tested positive had a strong positive reaction to the antibody test (Ortner et al., 1992).

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The inability to differentiate among the treponemes began to change with the sequencing of *T.p. pallidum,* which provided information on the biological structure of the bacterium and its genome (Fraser et al., 1998). Spirochetes are from an ancient phylum and represent a unique bacterial group (Fraser et al., 1998*)*. *Treponema pallidum* are "helical to sinusoidal bacterium with outer and cytoplasmic membranes, a thin peptidoglycan coat, and flagella that lie in the periplasmic space that stretch from both ends toward the middle of the organism" (Fraser et al., 1998:375).

Treponema pallidum requires most of its nutrients from its host, whom it is dependent on for the synthesis of fatty acids, amino acids, purines, and pyrimidines (Norris et al., 1998). It contains systems for DNA replication, transcription, translation, and repair. It has limited catabolic and biosynthetic activities. In experimental research with tissue culture, *T. pallidum* uses glucose, maltose, and mannose for replication (Fraser et al., 1998). It has few transporters and no phosphoenolpyruvate (PEP) (for example, phosphotransferase or PTS carbohydrate transporters). It does not have a respiratory electron transport chain. It produces adenosine triphosphate (ATP) through substrate-level phosphoralization (Fraser et al., 1998).

The genome of *T.p. pallidum* (Nichol's strain from rabbit testicle) is a small spherical chromosome with only 1,138,006 base pairs and 1041 open reading frames (ORF) (Fraser et al., 1998). An open reading frame is any DNA or RNA

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that can be translated into a protein; it is a stretch of nucleotides that is not interrupted by a codon (TAA, TGG, TGA) (Micklos et al., 2003).

A better understanding of the biology of *T.p. pallidum* and the unveiling of its genetic map made it easier for researchers to identify genes present in its metabolic pathways allowing for better identification, prevention, and treatment of disease. While further research is needed to comprehend the surface proteins of this bacterium that stimulate the immune response, the sequencing of the genome was a step toward vaccine development. It has also proved to be instrumental in helping molecular biologists and physical and molecular anthropologists begin to piece together the evolutionary history of the treponemes. One of the ways this is happening is through the study of single nucleotide polymorphism (SNPs) or insertions and deletions (indels), both representing areas of variation in the genetic sequence.

In 1998, Pillay and colleagues developed a molecular subtyping method for *T.p. pallidum* isolates using PCR amplification of *tpr* and acidic repeat protein (*arp*) genes and restriction fragment length polymorphism (RFLP). Proteins, like DNA, can provide genetic information. These particular membrane proteins are useful in discriminating among strains and are important in developing a vaccine for syphilis -- a difficult task given that the outer membrane of *T. pallidum* has insufficient surface proteins to create an antibody. Toward this goal, two new recombinant antigens have been found that provide a degree of protection from experimental syphilis; these include *tprK* (a member of the *tpr* gene family) and

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the enzyme glycerophophodiester (Gpd) (Stebeck et al., 1997; Cameron et al., 1998; Cameron 2000; Pillay et al., 1998).

Centurion-Lara (1998) and colleagues also identified a single base pair change or SNP in the *tpp15* gene that introduced an *Eco*47III restriction site that allowed for the identification of *T.p. pallidum* from the other subspecies by Restriction Fragment Length Polymorphism (RFLP) analysis of PCR-amplified products (Centurion-Lara et al., 1998). Restriction sites are sequences of nucleotides that are recognized by restricting enzymes.

The recognition of genetic signatures in the 5' and 3' flanking regions of the 15kDa lipoprotein gene (*tpp15*) in *T. pallidum* (Centurion-Lara et al., 1998) led to the identification of *T.p. pallidum* (syphilis) in a 200-year-old skeleton from Easter Island (Kolman et al., 1999). A comparison of the DNA sequences at the *Eco*47III restriction site appears below (Kolman et al., 1999:2062).

***Eco*47III**

T.p. pallidum	CTCAG**C**GCTTTA
T.p. pertenue	CTCAGTGCTTTA
T.p endemicum	CTCAGTGCTTTA

The substitution of a "C" in the place of a "T" in *T. p. pallidum* identifies it from the nonvenereal treponemes yaws and bejel (*T.p. pertenue* and *T.p. endemicum)* (Kolman et al., 1999). The identification of this SNP and the subsequent finding of *T.p. pallidum* in an historical skeletal sample have spurred scientists to investigate disease through the analysis of ancient DNA. Sampling

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from the lesions, Kolman and colleagues' (1999) were able confirm an initial treponemal infection by purification of immunoglobulin that reacted with *T. pallidum* (syphilis) antigen. A SNP in the extracted DNA allowed for the identification of *T.p. pallidum* (syphilis) from the other treponemes (Kolman et al., 1999).

Kolman and colleagues (1999) research involving the analysis of *T.p. pallidum* (syphilis) in a 200-year-old skeletal sample is particularly relevant to this dissertation. Their research provided an experimental protocol for detecting treponemal infection in bone; the samples analyzed came from Easter Island and showed suspected treponemal lesions, including "anterior curvature and subperiosteal thickening of the diaphyses of the tibiae" (saber shins) (Kolman et al., 1999:2061; Owsley et al., 1994). The bone collagen of these samples was dated by accelerator mass spectrometry (AMS) radiocarbon measurement to 240 years ± 50 years BP (AD 1673 ± 118) (Kolman et al., 1999).

The study involved two forms of analysis: immunoglobulin (IgG) analysis and DNA sequencing (Kolman et al., 1999). The IgG purification was conducted on a sample from the femur, which was pulverized for analysis. Fifteen grams of powdered bone were collected for the experiment. The bone sample was purified to remove inhibitors. The purified extractions were tested in an Enzyme-Linked ImmunoSorbent Assay (ELISA) against *T. pallidum* antigen (Clark Laboratories, Jamestown, N.Y.) multiple times. An ELISA assay is a test where an antibody or antigen is linked to an enzyme to determine if there is a match

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between the antibody and antigen. For example, to test for treponematosis, the sample is tested with an antigen; in this case the treponemal bacteria, to see if the immune system recognizes it. Kolman and colleagues (1999) tested two skeletal samples; one was used as a negative control. If the negative control were to amplify through PCR, then the experiment would have been contaminated. High-and-low-positive controls were run to delineate a range of positive immunoreactivity. Positive controls are samples that are certain to amplify. By running multiple strengths of control substances, Kolman and colleagues (1999) were able to determine how great the exposure to treponematosis had been.

The second part of their experiment involved DNA sequencing. To conduct this experiment, DNA was taken through an organic (phenol-chloroform) extraction method. The extraction method separates proteins and other cellular components from the DNA molecules. Extraction protocols are constantly being improved to better eliminate inhibitors and improve DNA yields. A more extensive organic extraction to remove cellular proteins that protect the DNA in the cell has been developed by Ye and colleagues (2004).

To replicate any experiment, it is essential to know exactly what primers were used; for that reason, they are usually published. The amplification of the DNA samples was done using primers L243 (5′ GAGCAGGATGTCTCTATGAGTTATAAAGAG3′) and H123 (5′-GAAGCCACTACCGATGTGTGCG-3′) to amplify a segment of the 5′ flanking

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region of *tpp15* (Kolman et al., 1999:2061). New DNA taq polymerases used in the PCR process to amplify DNA have also been developed since Kolman and colleagues (1999) completed their research. These new enzymes, buffers, and additives will help aid future ancient DNA studies by making samples cleaner (less inhibitors), increasing DNA yields, and by amplifying small quantities.

The results of the first test, the purified IgG fraction, were positive and showed "significant antigen binding," indicating that a treponemal infection was present (Kolman et al., 1999: 2062). The authors also had success in their second experiment, the DNA sequencing. The fragment that was produced was the expected 120 bp. The digestion of the *Eco*47III restriction site produced a fragment of 92 bp., also within the expected size. The sequence was determined from amplification fragments and from cloned amplicons. All the sequences identified the 5′ flanking region of *tpp15*. Additionally, the cytosine at position -150 (identifies *T.p. pallidum*) was in all of the sequences (Kolman et al., 1999:2061-2062). The fact that everything was within the expected size and in the right genetic locations suggests that the results Kolman and colleagues (1999) obtained are a true representation of treponematosis in this skeletal sample.

Centurion-Lara and colleagues (2000a) have identified two *tprD* alleles associated with *T.p. pallidum*: *tprD2* is found in seven of 12 *T.p. pallidum* isolates and in seven of eight non-pallidum isolates. They also found that *tprD3* is found in a *T.p. pertenue* isolate. These isolates also led to the identification of a SNP

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that distinguishes *T.p. pallidum* (syphilis) from the nonvenereal treponemes (yaws and bejel) (Centurion-Lara et al., 2000a). The TprD2 antibodies are found in individuals with syphilis, which means that this isolate is produced during infection (Centurion-Lara et al., 2000a). The surface proteins of *T. pallidum* can help in the identification of strains of treponematosis. Comparing ancient strains of treponematosis with modern strains can help researchers understand how the disease evolved, where it mutated, and how both relate to its geographical distribution (Centurion-Lara et al., 2000a).

Bouwman and Brown (2005) also conducted a study relevant to this dissertation to look for treponematosis in archaeological skeletal samples from Britain; unfortunately, they were unsuccessful. Their inability to generate treponemal products could be mulifactorial. Concentrating specifically on venereal syphilis, the authors selected 46 skeletal elements of various ages for analysis, the majority of which displayed pathological lesions. The skeletal elements were drawn from seven English cemeteries dating from the 9th to the 19th centuries AD. Of the skeletal elements, 12 consistently yielded mtDNA sequences that could be reproduced through PCR. Another 13 yielded mtDNA that could not be reproduced consistently. The authors were able to assign haplogroups to nine of the skeletal elements that yielded mtDNA, and these groups were consistent with the geographical origins of the skeletal elements. Negative mtDNA PCRs were obtained from 21 skeletal elements, suggesting that

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there was minimal modern contamination. Of those 21 skeletal elements with positive results, each gave only one sequence (Bouwman and Brown, 2005).

Suggesting that the samples were good was the fact that seven of the skeletal elements yielded *Mycobacterium tuberculosis* (tuberculosis). It is important to note, though, that regardless of degradation, the tuberculosis bacterium is much easier to sequence than the treponemal spirochete due to its biological structure. The authors noted that while they were able to obtain mtDNA and tuberculosis sequences, there was significant evidence of DNA degradation. Regardless, some of the skeletal elements were good candidates for ancient DNA analysis. All 46 skeletal elements were tested with nine different treponemal primer sets, each of which was optimized to detect <5 genomes (Bouwman and Brown, 2005:706). The primers included *tpr* genes (*tprJ*, *tprGJ*, *tprCDEFGIJ*, *tprCDFI*, *tpr*E*GJ*, and *tprK*) and the 15kDa lipoprotein gene (Bouwman and Brown, 2005:706, 710). While these primers can identify a treponemal infection, when aligned with the various treponemal strains downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (a public database for molecular biology information), they do not appear to be diagnostic for subtypes other than *T.p. pallidum* (syphilis) (Bouwman and Brown, 2005:706, 710). Some of their PCRs gave sizes in the expected range; however, when sequenced it was shown that none of the products represented treponemal amplicons (Bouwman and Brown, 2005).

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More often than not, in the experience of the author, when experiments fail, sampling and extraction protocols are the prime suspects. Contamination is another issue that often affects results. In this author's experience, other issues that could have led to their failure include insufficiently removed inhibitors (in extraction or in water), poor quality DNA, low copy number (LCN) DNA, little or no signal in the raw data, degraded or failed synthesis primer, or strains significantly altered from the modern ones. Admittedly, these researchers could have done everything right and still failed for reasons unknown. Additionally, it is always possible that the disease present was not treponematosis, and that is why it was not sequenced. While the authors published their primer information in their article (Bouwman and Brown, 2005:705, 710), they did not enter their primer sequences into the NCBI GenBank database; therefore, there are no accession numbers.

Bouwman and Brown (2005) have suggested that it is not possible to recover molecular evidence of ancient treponemal bacteria; this may be true, but it is too early to be certain. Molecular treponemal research is in its infancy and there are too many variables at this point for anyone to suggest that the entire field be laid aside. Moreover, there is a certain responsibility among scientists to work together and build upon one another's research -- successes and disappointments. Damage to DNA is not consistent, which means that each case should be evaluated as to the extent of its damage and to other relevant factors. Their research also suggests that ancient DNA scholars might be well-

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served by spending more time focusing on the difficulties with nucleic acids in amplification procedures and DNA repair than in treponemal specific research (Goodman and Tippen, 2000; Cipollaro et al., 2005).

Recent studies of six members of the *tpr* gene family (*tprC,D, I, K, G*, and *J*) have found additional areas of variation, but have also noted a high rate of intra-gene conversion events, including homogenizing gene conversions involving the entire length of the sequence and site specific conversions affecting smaller areas (Gray et al., 2006). This research is especially relevant to the study of the evolution and divergence of the treponemes. It confirms that there are greater levels of variation among the human treponemal subspecies than within them, supporting grouping them into three subspecies. Their research also shows that intragenomic (gene inside a gene) recombination played a significant role in the evolution of the *tpr* genes, which makes their use in determining evolutionary relationships among the treponemes troublesome. Their research indicates that there is too much variation in the *T. pallidum* sequences to support Baker and Armelagos' (1988) modification of the Columbian Theory of treponemal evolution. However, it is more consistent with the co-evolution of three treponemal subspecies (syphilis, bejel, and yaws) as proposed by Hackett (1963), though contra Rothschild who envisioned different dates for the emergence of venereal syphilis and yaws.

Toward molecular identification of the treponemes, Centurion-Lara and colleagues (2006) recently identified two *tpr* genes (*tprC* and *tprI*) which when

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used in combination with their previous research (Centurion-Lara et al., 1998) identifying the 5' flanking region in the *tpp15* gene allows scientists to distinguish the *T. pallidum* subspecies, including the simian treponeme. Harper and colleagues (2008) recent phylogenetic study should spur more research into unresolved questions, such as how the limited divergence between the treponemal species and subspecies generated the differences seen in disease development and expression (Mulligan et al., 2008).

Previous Molecular Research in Northern Chile

Rogan and Salvo (1990a,b) conducted molecular studies of mummies from South America, including those from the northern coast of Chile. They were able to isolate, identify, and clone human DNA sequences from some of the remains. They concentrated on the Maitas (Maytas) (700 BP) (AD 1292 ± 79) and Camarones (500 BP) (AD 1432 ± 100) sites (Chinchorro culture) in their preliminary analysis, and were able to recover high molecular weight DNA (>1200 bp) from nine individuals. Southern blot hybridization showed that each of the samples contained both genomic and human mitochondrial DNA (Rogan and Salvo, 1990a,b). By repairing the damaged ancient DNA sequences, they were able to amplify specific target sequences by the use of polymerase chain reaction (PCR). Using this approach gave them several amplified genomic sequences, which they proposed to use for kinship studies and to reveal the causative agents in early pathologies.

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In 1994, Rogan and Lentz attempted to identify the etiological agent of the lesions seen on the skeletal elements of the coastal northern Chilean mummies. They analyzed muscle from the remains of four individuals excavated from El Morro and Morro sites, Arica (Chinchorro culture) and from San Miguel de Azapa (Gentilar and Alto Ramírez cultures), Chile. Their work is described in an unpublished manuscript draft, which was provided by Rogan to this author to give an idea of what their research encompassed. The results of their study were presented at the American Association of Physical Anthropology (AAPA) meeting in 1994 and they are discussed below. As the manuscript is an unfinished work, Rogan has asked that any discussion of it refer to the citation for the American Journal of Physical Anthropology (AJPA) paper he and Lentz gave at the AAPA meeting in 1994. The samples from the El Morro cemetery (Mo-1/6) are described as late phase Chinchorro and are dated from 4000 BP to 3000 BP (2547 ± 171 BC to 1225 ± 139 BC) (Rogan and Lentz, 1994). The samples from the cemetery in San Miguel de Azapa (AZ-71; Gentilar culture (possibly also Cabuza and Tiwanaku cultures) and AZ-8; Alto Ramírez culture) are dated to the Middle Horizon Period (Middle Intermediate Period). Rogan and Lentz (1994) did not give specific dates for these samples, but that period is considered to be around AD 500 to AD 1000 (Torres-Rouff, 2002).

Their study used ancient bacterial DNA sequences to try to identify the etiological agent of the presumed tertiary treponemal infections. While the persistence of spirochetal evidence in ancient mummified remains was unknown,

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the preservation of infectious agents, such as *Mycobacterium tuberculosis* (tuberculosis) had been recorded microscopically (Allison et al., 1973; 1981), and in DNA sequences with transposable elements present in modern prokaryotic genomes (Salo et al., 1994; Rogan and Lentz, 1994).

Using sterile procedures, Rogan and Lentz (1994) collected internal tissues (primarily skeletal muscle) to minimize contamination. One gram to 2 g was sampled from nine mummified individuals. All molecular experiments were conducted at a dedicated ancient DNA laboratory, which with the exception of controls for *E coli*, were absent modern human DNA or cultured microorganisms. Samples were extracted and purified from approximately 1g of skeletal muscle (Rogan and Salvo, 1994).

The samples were finely powdered and extracted for 5 days in 0.2 M EDTA (pH 7.0) and subjected to a solid-phase radioimmunoassay that used aliquots of rabbit antiserum against killed *T. pallidum* (Lee Laboratories) (Rogan and Lentz, 1994). The bound *T. pallidum* antibodies were then detected with radioactive ^{125}I—labeled goat and rabbit gamma globulin, which was measured with a scintillation counter. They employed both positive and negative controls.

In their experiments, Rogan and Lentz (1994) identified segments of the 16S ribosomal DNA (rDNA) useful in the taxonomic analysis of ancient treponemes. They used 16S because the genes that encode it are "orthologous, polymorphic among species, and monomorphic and multicopy within each species;" this helps with inter-species comparison (Rogan and Lentz, 1994;

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Woese, 1987; Holt, 1984). To identify 16S segments for their project, they analyzed information from 106 aligned 16S rDNA genes derived from various bacterial species. A computer program called PILEUP (Devereaux et al., 1984) was used to create the alignment, which allowed them to identify "three highly conserved sequences 20-30 nucleotides long adjacent to two intervening divergent regions of less than 250 bp" (Rogan and Lentz, 1994). These segments were then used to create primers to amplify rDNA from various bacterial species. To recover DNA from a wide spectrum of templates, primers with invariant 3' terminal nucleotides were used (Rogan and Lentz, 1994). They chose two sets of universal primers developed from three highly conserved segments. Primers 2 and 3 separated PCR reactions and were complimentary to one another. The first product generated was predicted to be a 155 bp fragment, and the second was predicted to be a 170 bp fragment from primers 3 and 4.

Before amplification, a pre-extension PCR was conducted to address presumed damage caused by oxidation, potential embalming treatments, and/or storage conditions (Rogan and Lentz, 1994; Rogan and Salvo, 1994). Replicates were synthesized in a random-prime reaction with Klenow DNA polymerase and AMV-reverse transcriptase, and served as templates (Rogan and Lentz, 1994). In the PCR, 2 µl to 4 µl of template was used. The sample was run in a thermocycler for 40 cycles (92°C , 1'; 50°C , 1'; 72°C , 2') (Saiki et al., 1985). Degenerate primers, those with multiple options at several positions in the sequence to allow annealing to and amplification of related sequences, were

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designed based on portions of the sequence logo by Genosys Biotechnologies (now Sigma Genosys, Woodlands, TX). Modern DNA templates and negative controls were amplified beside each other and analyzed through agarose gel electrophoresis.

These products were then shotgun cloned into pCR™II (Invitrogen Corporation, Carlsbad, CA.) and transformed into *E. coli.* (Rogan and Lentz, 1994). Shotgun cloning involves cutting the DNA to be cloned with a restriction enzyme or otherwise smashing it. These fragments are then gathered and cloned into a vector. Its purpose is to generate small segments of DNA for sequencing. Genomic DNA from contemporary treponemes, and other bacterial species including: *T. pallidum, T. phagedenis, T. denticola, T. pertenue, T. refrigens, T. carateum,* and *Chlamydia trachomatis* were used to evaluate the 16SrRNA primers. Also used were universal primers designed to amplify a segment of the 28S rRNA gene to show that human DNA was present in the replicates (Rogan and Lentz, 1994). The PCR products correspond to the following: the 5' to position 2698 and the 3' termini to position 2849 (NCBI GenBank locus HUMRGM, accession number M11167) (Rogan and Lentz, 1994). By searching the complete nonredundant sequence database with the Basic Local Alignment Search Tool (BLASTn) they were able to determine what sequences in the database most closely matched those from the mummies (Altschul et al., 1990; Rogan and Lentz, 1994). Significance values were calculated using a method developed by Karlin and Altschul (1990).

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For the phylogenetic analysis, they created dendograms of treponemal sequences from maximum parsimony (DNAPARS) and distance matrix methods (NEIGHBOR, UPGMA) with Phylogeny Inference Package (Phylip software package, v.3.5:Felsenstein, 1977). They further created a majority rule consensus tree from 100 bootstrapped phylogenies to validate the topology (Rogan and Lentz, 1994).

They had several important findings. First, they showed that the16S rDNA sequences found in the pre-Columbian mummies with presumed tertiary treponematosis were related to contemporary pathogens and other bacteria (Rogan and Lentz, 1994). Second, they demonstrated that the primer sets they developed worked, in that they generated products of the expected size 16S rDNA from modern treponemal extracts of six pathogenic and nonpathogenic species (Rogan and Lentz, 1994). The pre-extension amplification of the ancient DNA prior to normal PCR amplification generated 16S products in two of four mummy samples tested (Morro-1, T. 28 C-9 and Morro-1/6, T. 7) (Rogan and Lentz, 1994). No amplification was seen for AZ-8, T. 6B and Morro-1, T. 22 (Rogan and Lentz, 1994). Fragmentation, degradation, and/or inhibitors may have resulted in the failure of the other two. From the 28S rDNA, they were able to generate sequence products from all of the DNA isolates from both the El Morro and the San Miguel de Azapa sites (Rogan and Lentz, 1994). Distinct 16Sr DNA sequences were generated from Morro-1, T. 28 C-9 with *T. phagedenis* and *T. pallidum* as the positive controls. Third their research

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indicated that two clones generated from samples Morro-1, T. 28 and Morro-1, T. 7 showed a match of 98% sequence identity over a 143 bp interval (Rogan and Lentz, 1994). These sequences were more conserved when compared to a number of eubacterial 16S rDNA sequence identities, which suggests the clones derive from similar or identical organisms (Rogan and Lentz, 1994). These two clones show 87% identity with *Sprichaeta zuelzerae*, a spirochete with antigenic cross-reactivity to *T. pallidum* (DeBruijin, 1960; Rogan and Lentz, 1994). They also showed similarity with *T. phagedenis*, *T. denticola*, *and T. pectinovorum* at about 80% to 85% (Rogan and Lentz, 1994).

While their results indicate that they were able to recover prokaryotic recombinant DNA sequences from two of the nine individuals tested, neither was identical to modern *T. pallidum,* based on molecular genetic and immunologic studies (Arriaza, 1995a; Rogan and Lentz, 1994). Additionally, the primers for the gene encoding the 47kd major coat protein of *T. pallidum* also failed. Rogan and Lentz (1994) have offered several possible explanations for this, including: 1) that particular gene was absent in the ancient sequence, 2) the gene was conserved among treponemal species, or 3) there was a fault in the sensitivity of their assay. Another setback was that the radioimmunoassay using polyclonal antisera against *T. pallidum* antigens did not recognize antigens in extracts of the mummy tissue (Rogan and Lentz, 1994).

Two different forms of phylogenetic analysis were done, although, the results were not consistent. The neighbor-joining analysis showed that the

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sequences derived from Morro-1, T. 28 (clone B5) and Morro-1/6, T. 7 (clone B8) formed a common branch within the genus *Treponema*, which suggests that there is a common ancestor between these sequences and the treponemal lineage (Rogan and Lentz, 1994). However, the tree created using the UPGMA method showed the mummy sequences to be closest to *L. interrogans*, a pathogenic spirochetal bacterium (Rogan and Lentz, 1994). A consensus tree, conducted to clarify the results of two phylogenetic approaches showed that the single branch with the two mummy sequences was most likely derived from the treponemes associated with humans; the results showed 60% significance. Results greater than 50% are considered reliable (Rogan and Lentz, 1994; Hillis, 1993). The consensus tree separated the ancient samples from the *Borrelia* and *Leptospira* genuses (Rogan and Lentz, 1994).

In summary, their comparison of cloned ancient 16S rDNA sequences against all known sequences found that eight of the 10 closest relatives were spirochetes, with five belonging to the genus *Treponema*. They found that the sequences were most closely associated with *T. phaedenis*, *T. denticola*, *S. zuelzerae* and *T. pallidium*, rather than other spirochetes (Rogan and Lentz, 1994). The first three can be products of the decomposition process, but the last is a similar strain to modern treponematosis.

Two additional clones from Morro-1, T. 28 (clone A1) and Morro-1/6, T. 7 (clone B1) were also amplified in their study. The results showed that the clone A1 was most closely related to rDNA from *Spiroplasma* or *Clostridia* at 90%

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identity (Rogan and Lentz, 1994). Clone B1 showed some resemblance to *Helicobacter cinaedi* 16S rDNA at 70% (Rogan and Lentz, 1994). *Spiroplasma* is a genus of *Mollicutes* -- small bacteria without cell walls found in the hemolymph (blood) of insects, and in plants. *Clostridum* is a genus of gram-positive bacteria belonging to the *Firmicutes*. They exist primarily in soil, aquatic environments, and the intestinal tract of mammals, and are responsible for a variety of anaerobic bacterial diseases in humans. These bacteria are likely associated with decomposition because *Spiroplasma* species have been found in the guts of some beetle species (Rogan and Lentz, 1994). *Helicobacter* are gram-negative bacteria often found in the gastrointestinal tract, and are associated with gastritis and cellulitis.

Rogan and Lentz's (1994) attempts to amplify two different modern *T. pallidum* structural genes were unsuccessful. Their failure to amplify these genes could suggest that the ancient and contemporary sequences either had become separate species or had undergone mutation in the genes that disrupt annealing of the oligonucleotide primers to the ancient template. Their research suggests that the organism might be either a "contemporary unknown infectious agent" or an "extinct relative of a modern treponeme." Rogan and Lentz (1994) have suggested, that "while it may not represent a contemporary pathogen, this relationship indicates a plausible evolutionary step toward a common, possibly infectious ancestor that produced contemporary *T. pallidum."*

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This idea is especially interesting in light of recent phylogenetic work by Harper and colleagues (2008:7-9), that identified a basal position on their phylogenetic tree for *T.p. pertenue* (yaws) showing an ancestral position for yaws in the *T. pallidum* family. Their research further suggests that New World *T.p. pertenue* (yaws) strains belong to a distinct group from Old World *T.p. pertenue* strains, "occupying a phylogenetic position between Old World nonvenereal strains and modern *T.p. pallidum* strains" (syphilis strains) (Harper et al, 2008:9). Given the scarcity of modern nonvenereal *T.p. pertenue* (yaws) samples from South America; one or more strains may have come and gone unnoticed. However, this is a very new study based on limited SNP data, so caution is needed, as is the analysis of more modern and ancient specimens (Harper et al., 2008; Mulligan et al., 2008). Additionally, Rogan and Lentz's (1994) work has never been replicated.

Conclusion

Ongoing treponemal research at multiple institutions ensures that this line of research will continue. Major leaps in knowledge have advanced the field since the sequencing of the genome for *T.p. pallidum* in 1998 by Fraser and colleagues. Phylogenetic and molecular studies are raising interesting questions and providing insights that will stimulate discussion and further investigation into the origin and history of the treponemes. Scientists are already advocating new collaborative methods to analyze archival DNA specimens; techniques, including whole genome identification, the sequencing of DNA from bacterial cells with

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laser capture microscopy, and new dissection, extraction, and amplification techniques (Mulligan et al., 2008; Marcy et al., 2007; Zhang et al., 2006).

Nucleic acids research is at the heart of ancient DNA studies and it is from here that many advances in this field are coming. The optimization of extraction protocols and the synthesizing of new chemicals will aid scientists in reducing inhibitors, improving yields, and ultimately in succeeding to amplify ancient DNA products (Cipollaro et al., 2005; Yang et al., 2003). Comparative genomics and bioinformatics will also provide new aids to researchers via software programs that allow scientists to better compare nucleotide sequences and explore the evolution of pathogens (Cipollaro et al., 2005). The continued analysis of skeletons from archeological sites will provide insight into the pathogenic treponemes and their history. Still, perhaps the best chance for future molecular success and advancement in ancient treponemal research will come from the soft tissue and skeletal elements of mummies. With perseverance, hard work, and luck these new ideas and techniques may allow for the further identification and molecular subtyping of ancient treponemal pathogens (Mulligan et al., 2008).

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Chapter IV.

Materials and Methods

Curation of Samples

All inspections, recordings, and sampling of skeletons and mummies used in this dissertation took place at the Museo Arqueológico San Miguel de Azapa, an affiliate of the Departmento de Arquelogía Y Museología, Universidad de Tarapacá. The museum is located approximately 12 km east of the city of Arica in northern Chile. Work at the museum took place under the guidance of physical anthropologist Dr. Bernardo Arriaza, and with the help of lab technician Letica Latorre Orrego. Maria and Anita Flores and Raúl Rocha also provided assistance. Contact information for the museum and other facilities used in the production of this research appear in Appendix A.

Sample Description

The skeletal and mummified human remains of 47 of the 51 individuals identified by Standen and Arriaza (2000b) as exhibiting osteological changes (lesions, periostitis) consistent with treponemal infection were examined to determine if the disease was likely treponemal, and to identify individuals for DNA sampling. Sixty-eight individuals with no recorded signs of treponematosis were also examined as part of the differential diagnosis, bringing the total number of individuals studied to 115. These 68 individuals came from various sites (Camarones, Morro-1, Morro-1/6, Quiani, Yungay, PLM-3, PLM-4, PLM-6,

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and PLM-8). They were chosen because they were from the same region and, simply, were the only specimens made available for examination. Four individuals previously identified to have treponematosis by museum researchers were not analyzed because they could not be located by museum staff and/or were otherwise inaccessible. Radiographs, as well as archaeological, autopsy, and physical anthropology reports were reviewed when available.

Twenty-two mortuary samples in 15 individuals from 11 Arica and Azapa Valley archaeological excavations in northern Chile were collected for the molecular portion of this dissertation. Each sample received a unique identifiler beginning with "CHI" to designate the project name followed by a sample number and the last two digits of the year laboratory analysis began.

Of the 15 individuals, three had no signs of treponematosis on the skeletal elements or tissues (Designations: CHI-2-06, CHI-5-06, and CHI-16-06); the remainder did. The unaffected individuals were collected as negative controls. As noted, the sites and their associated cultures, as identified by the archaeologists who excavated them, are: Morro-1 (Chinchorro), Morro-1/6 (Chinchorro, Faldas Morro), Yungay-372 (probable Chinchorro), PLM-3 (Desarrollo Regional), PLM-6 (Desarrollo Regional), PLM-8 (Chinchorro), AZ-70 (Alto Ramirez), AZ-115 (Alto Ramirez), AZ-71 (Cabuza/Tiwanaku), AZ-140 (Maitas or Maytas-Chiribaya/San Miguel), AZ-141 (Cabuza) (See Chapter 2). When archaeologists listed the associated culture on their original recording forms, the individuals had not been radiocarbon dated; therefore, there may be

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some discrepancies between the associated culture and chronology in which that culture was placed.

Four reasons necessitated the collection of samples from multiple sites. First, most sites (excluding Morro-1 and Morro-1/6) had a limited number of individuals exhibiting osteological changes consistent with treponemal infection. Second, some sites are, in fact, a single extended-use cemetery. Third, it was hoped that collection of samples from both coastal (Morro-1, Morro-1/6, PLM-3, PLM-6, PLM-8) and later inland sites (AZ-70, AZ-71, AZ-115, AZ-140, and AZ-141) would allow for temporal and geographic comparison of the frequency of treponemal infections, as well a comparison of DNA preservation among sites. Fourth, curatorial constraint and a strong desire to preserve the mummies determined the number, specific individuals, and anatomical sites that could be sampled.

The majority of the individuals sampled were mummified naturally, because that is all that was made available (11/15). Regardless, in most cases, it was problematic to determine which mummification technique was used due to preservation issues, and unfamiliarity of the author in distinguishing techniques based on preserved tissues. The sample is biased toward natural mummies; however, for purposes of DNA collection, this bias is beneficial because minimal manipulation resulted in better preservation and lower chances of contamination. The sample is also biased toward adults (14 adults, 1 subadult), and males rather than females (potentially 10 males and 5 females). It is unknown if the

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age of the individual might affect the recovery of treponemal DNA from ancient bone. It is unlikely that the sex of the individual would have any bearing on DNA recovery.

Previous Osteological Analysis

Several researchers have analyzed and/or discussed the Chinchorro and the Azapa valley mummies (Dauelsberg, 1969; Santoro, 1980b; Muñoz, 1981; Allison et al, 1982; Standen et al., 1984; Arriaza et al, 1984; Muñoz and Focacci, 1985; Guillen, 1992; Arriaza, 1995a,b; Goldstein, 1995; Sutter, 1997; Cocilovo and Rothhammer, 1999; Sutter, 1999; Standen and Arriaza, 2000a,b; Rothhammer and Santoro, 2001; Rothhammer et al., 2002; Aufderheide et al., 2002; Varelo and Cocilovo, 2002; Sutter and Mertz, 2004; Sutter, 2006). Standen and Arriaza (2000b) compiled the osteological data used in this report from their studies of treponematosis in the populations of the Atacama Desert in Northern Chile. The expertise of both researchers in the area of skeletal identification and recognition of pathologies is well established. Arriaza provided raw data from their work and previous museum researchers. These data include detailed information on the site, tomb, locus, culture, age, sex, skeletal elements affected by treponematosis, conservation, suspected disease/pathology, and their general observations during analysis. This research also included data on numbers of individuals infected within three periods for both coastal and inland populations (Standen and Arriaza, 2000b). Skeletal remains include right and left tibia, fibula, femur, ulna, radius, humerus, clavicle, scapula, pelvis, cranium,

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sternum, vertebrae, and ribs. Their classification of treponematosis was as follows: (1) affected; (2) normal; (3) absent; and (4) bone with tissue. Under observations, the researchers listed the degree to which treponematosis affected bone (minor, intermediate, or severe). A spreadsheet with these data is included in Appendix C.

Observation and Recording of Lesions

The author re-examined all available skeletons identified by Standen and Arriaza (2000b) as having treponematosis; this examination was conducted solely to ensure that there was general agreement about what pathologies represent possible treponematosis, the degree of skeletal elements affected, and to determine which individuals and areas were suitable and permissible for molecular analysis.

Methods used in the examination of skeletal pathologies for this dissertation included gross observation and a detailed description of the pathological element(s). All skeletal and mummified elements present were examined for evidence of periosteal reactions, osteitis, and observable lesions. The descriptions recorded for each specimen included the location of the pathology within the skeleton and within the specific bone (unilateral or bilateral, on the diaphyses, metaphyses, epiphyses, etc.), the areas involved, the size, and shape of the pathology and the distribution pattern, if any of the pathology/ies.

Criteria developed in previous studies, in particular Hackett's (1976) list of diagnostic criteria for treponematosis, and Bogdan's (1989) criteria for the

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analysis of human remains with signs of treponmatosis were used here to describe the range of expression of treponematosis found in the individuals examined. The osseous changes commonly seen in treponemal infections are described in Chapter 2. Hackett's diagnostic criteria are briefly described below.

Hackett's diagnostic criteria (1976) were utilized in this dissertation to help determine those skeletal elements that were affected with treponematoses, and for this reason, a description of Hackett's criteria are described here. According to Hackett (1976), the outer table of the skull is more susceptible to cranial lesions than the interior. Additionally, in treponematosis the lesions rarely cross cranial sutures. Treponemal related cranial changes usually occur on the frontals and parietals. Hackett (1976) created a diagnostic criterion for both crania and calvarium and a separate criterion for other skeletal elements. The first part of his diagnostic criteria refers to "Focal Bone Destruction" and the second part refers to "Massive Bone Destruction" (Hackett, 1976). The third section refers to lesions seen on other skeletal elements. According to Hackett, (1976:30) eight main groups comprise the caries sicca sequence in crania and calvaria. These eight groups are broken into three main divisions: The "Initial Series," which includes *"(1) clustered pits and (2) confluent clustered pits;"* the "Discrete Series," which includes *"(3) focal superficial cavitation, (4) circumvellate cavitation, and (5) radial scars;"* and the "Contiguous Series," which includes *"(6) serpiginous cavitation, (7) nodular cavitation, and (8) caries sicca."* There are four other categories for describing massive bone destruction of the calvarium

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and face, they include *"(9) sequestra, (10) depressions, (11) perforations, and (12) naso-palatine destruction."* Categories for other skeletal elements include *"(13) plaques, (14) general bony thickening and (15) bony excrescences."*

Hackett (1976:75) has also drawn up a set of criteria for diagnosing lesions on long bones. He created 14 main groups for the specimens he examined and divided these into four main subgroups. The divisions are as follows: *"(A) 'Normal' Bone with Surface Changes, which includes (1) 'Normal' Bone with Plaques, (2) 'Normal' Bone with Fine Striation, (3) 'Normal' Bone with superficial Sequestra (B) Nodes/Expansions with Surface Changes, which includes (4) Nodes/Expansions with Plaques, (5) Finely Striate Nodes/Expansions, (6) Coarsely Striate and Pitted Expansions, (7-9) Rugose Nodes/Expansions (Slight Changes, Moderate Changes and Gross Changes); (C) Nodes/Expansions with Destruction, which includes (10) Sequestra and Expansions (11) Nodes/Expansions with Superficial Cavitation, (12) Metaphyseal Expansion and Cavitation (D) Expansion and Deformity, which includes (13) Platforms, and (14) Bowing and Expansion."*

Hackett (1976) describes these subdivisions as a "continuous series of changes that occur in the bone." He classifies "nodes" as enlargements of the bone involving only one or two surfaces (Hackett, 1976:79). These changes are a result of the remodeling of new bone in the periosteum or superficial layers of the cortex (Hackett, 1976:79). "Expansions," according to Hackett (1976:80-87), involve most of the bone and may show rough patterns that encircle the shaft.

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"Rugosity" as described by Hackett (1976:87) causes a mixed pattern of "rippling and trabeculation with some striation and pitting." When a bone displaying "rugosity" is sectioned, the cortex may show signs of "the medullary canal narrowing, a thickened and compact cortex or a finely cancellous cortex" (Hackett, 1976:87).

According to Hackett (1976), unlike the presence of caries sicca on the calvarium, there is no one factor that positively identifies syphilis in the long bones. In the long bones, superficial cavitation may occur, but there are no holes in the cortex like that which occurs in the calvarium (Hackett, 1976:75). Depressions are unusual; however, bony deposits on the surface of the bone that cause nodes and expansions are much more common (Hackett, 1976:75). Bowing of the long bones and sequestra caused by pyogenic osteomyelitis may occur. Multiple sequestra may be present in the calvarium, but in the long bones, the sequestra are mostly single and may be accompanied by an involucrum (Hackett, 1976:75). Hackett's (1976) full diagnostic criteria appear in Appendix D.

Age and Sex Estimation

Previous researchers recorded the age and sex for most individuals however, in a few cases it was absent or undetermined. For these cases, age and sex were estimated as an indicator of which members of the population had treponematosis and whether there were differences related to sex in the mummification rituals. Sliding and spreading calipers were used to take

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measurements of lesions and metric measurements for age and sex when necessary. Data collection utilized standard osteological recording forms (Buikstra and Ubelaker, 1994). Additionally, a cover sheet from the physical anthropology lab at the Museo Arqueológico San Miguel de Azapa was used to summarize information on the excavation and osteological analysis. Examples of all recording forms used in gathering data for this dissertation appear in Appendix E.

The author made drawings of lesions of interest, and took digital photographs for reference purposes. Molecular analysis requires sampling the bone or tissue; because this process is inherently destructive, this author and Dr. Arriaza used separate cameras to take photographs of all skeletal elements prior to sampling. The drawings and photographs provide a photographic record of all elements damaged or lost to the research process. These photos are available through Dr. Arriaza and this author. A few of these photos appear in Appendix F.

Sample Selection

Sampling from Soft Tissue:

In selecting tissues for sampling, the author used the following guidelines, as approved by the curatorial staff. The goal was to collect endogenous DNA (mtDNA and treponemal DNA). Endogenous DNA or host DNA refers to DNA produced or growing from within. The treponemal sampling methods presented herein came from discussions with Dr. Carney Matheson, from the Department of Anthropology at Lakehead University. When possible, tissue came from the

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following: the extremities of limbs (fingers and toes), internal samples, from lesions, the periphery of lesions, and from lymph nodes, lips, mouths, breasts, and genitals.

In an arid depositional environment, the extremities will dry out first, which potentially means less hydrolytic damage to the DNA. Internal sampling is advantageous because it reduces the chance for contamination from handlers. The skin lesions and the periphery of the bone lesions are full of spirochetes in life, and therefore may be a good choice for recovering treponemal DNA. However, it is difficult or impossible to identify skin rashes of secondary syphilis on mummified tissue. Treponemes can move hematogenously, and therefore, they are potentially found in almost all tissues, especially in tertiary stage of syphilis. However, gummas that form in tertiary syphilis have very few treponemes; therefore, Matheson (pers. comm., 2005) hypothesized that the periphery of lesions may be a better place to sample for spirochetes.

Sampling from Bone:

Bone samples for endogenous DNA analysis (mtDNA and treponemal DNA) came, when possible, from skeletal elements with a protected interior to reduce contamination and increase the yield of DNA. Teeth usually produce high yields of DNA because enamel protects them. Unfortunately, the curators did not permit the collection of teeth from any of the mummies analyzed here, as they felt their removal would have affected their aesthetic appeal, or caused damage to the mandible or maxilla. In general the harder a bone or tooth surface, the

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greater the protection from degradation and chance of recovering viable DNA (Kaestle and Horsburgh, 2002). Enamel is less affected by autolysis (cell destruction), cell lysis (cell death), and bacterial and fungal degradation (Smith et al., 1993; Hillson, 1996: 230, 294; Parsons and Weedn, 1996; Alonso et al., 2001).

From the skeleton, the best area to sample for endogenous human DNA is the cortex of the long bones (Alonso et al., 2001, 2003). Cortical bone has the highest concentration of osteocytes (living bone cells), thus the highest concentration of DNA. The skull is also a good area to sample, providing the following criteria are met: (1) the cortex on both sides is intact to reduce the amount of contamination reaching the inner bone (2) there is little contaminating soil permeating the skeletal elements (for example, few signs of discoloration), (3) the piece is big enough to allow a buffer of bone to reduce contamination on the far interior of the bone, (4) the piece has little value for anthropometric analysis by physical anthropologists, and (5) it is trabecular bone. Ideal skeletal elements for sampling include the mastoid process, followed by the ascending ramus, and finally by any thick cranial fragments.

The third choice after the cranium would be a vertebra, providing the main body of the vertebra is intact and there is a little soil contamination (Matheson, pers. comm., 2005). There is less anthropological value placed on vertebra because there is occasionally a vertebra or two missing in the archaeological

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record through differential preservation or through the recovery process (Matheson, pers. comm., 2005).

The fourth choice is the patella, if the cortex is still intact, and there is little soil contamination (Matheson, pers. comm., 2005). The fifth choice would be the small skeletal elements of the foot and phalanges of hand or foot (Matheson, pers. comm., 2005). The sixth choice would be bone from the ball joints especially of the femur, if the piece is big enough and has a large proportion of the cortex intact (Matheson, pers. comm., 2005).

Sampling hard tissue for syphilis is difficult because macrophages remove treponemes during phagocytosis, and as such, they may be absent or in very low concentrations in the lesions. The lesion represents an area of reactive bone formation and may have more macrophages than treponemes. The spirochetes can localize at active sites of endochondral ossification in the metaphyses of long bones in developing individuals.

Sample Collection

The author collected samples from the external border of skeletal elements with focal lesions. A 1 cm square was removed using a Dremel® drill equipped with a modified hole saw fitted with a small narrow diameter pilot bit to reduce heating and increase the volume of bone recovered. In collecting samples from long bones, especially those with soft tissue, the author used the following procedures and equipment.

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Standard aseptic collection protocols were used. A clean area for the drill press and Dremel® tool was arranged by wiping all surfaces with a 10% commercial bleach (7 mM sodium hypochlorite solution), followed by a 70% ETOH solution. All materials and equipment including scalpels, scissors, clamps, mandrils (shaft to mount a working tool), and hole saws were either pre-sterilized and disposable or sterilized in 0.6% hypochlorite solution and wiped with 70% ETOH, and then flame sterilized before use. Flame sterilization involves dipping the instrument into ETOH, lighting it on fire, letting the flame burn out, and repeating the process for a total of three times. After the flame is extinguished the final time, the instrument is used to collect the sample.

A sterilized scalpel blade was used to cut a 1 cm square into tissue to allow a flap of skin to be lifted, but not completely removed (so that the skin could be replaced after the sample was removed). Next, using a drill press and a Dremel® high-speed cutter (model #117) core samples were drilled from long bones and crania. The cutter was fitted with a Bahco Snap-on® bi-metal hole saw measuring 21mm (model #3830-21-US) with a modified Bahco Snap-on® mandril, or pilot bit (model # 3834-ARBR-930-US). Soft tissue was carefully removed with the use of sterilized scalpels and blades, scissors, and clamps.

Separately labeled 50 ml sterile centrifuge tubes, sealed with parafilm were used for the collection of samples. Packaging in labeled, quart size Ziploc® bags provided an extra measure of protection for the transport of the tubes. A chain of custody form accompanied all samples. Doctor Arriaza, head of

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Physical Anthropology at the museum, Dr. Cordova, director of the museum, and Dr. Calogero Santoro Vargas, director of the Department of Anthropology at the University of Tarapacá acknowledged in writing, all samples. The museum and university retain a written record of all samples taken.

Molecular Analysis

All DNA extractions and polymerase chain reaction (PCR) amplifications took place at the Paleo-DNA Laboratory at the Northern Ontario Technology Center at Lakehead University in Thunder Bay, Ontario, Canada; guidance was provided by Dr. Carney Matheson, technical manager Stephen Fratpietro, and operations supervisor Renée Fratpietro. Post-PCR processing (cloning and sequencing) took place at the Conservation Genetics Laboratory at San Francisco State University in San Francisco, CA., under the guidance of laboratory director Dr. Frank Cipriano. All pre-amplification work took place in the ultra-clean Paleo-DNA Laboratory to prevent contamination. The Paleo-DNA Laboratory is a BioSafety Level II laboratory, with dedicated areas for the analysis of ancient DNA, and is accredited by The Standards Council of Canada accredits it (ISO/IEC 17025). No modern treponemes have been analyzed there.

For pre-PCR manipulations, laboratory personnel wear nitrile and latex gloves, a hair net, facemask, protective eyewear, hooded Tyvek® Biohazard suit, and Tyvek® boots and sleeves. Body surfaces are completely covered at all times. All personnel must pass through an air shower before entering the clean rooms, and upon exiting the pre-amplification areas. Each area of the laboratory

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(extraction, amplification, and post amplification) is separated and everything flows in one direction. Within the pre-amplification rooms, pass-through boxes built into the walls allow for the sterile transfer of materials from one room to the next. Counters and laboratory hoods were cleaned before and after each experiment with 10% commercial bleach (7mM sodium hypochlorite solution), and 70% ETOH. Latex gloves were changed between rooms, before and after conducting each phase of an experiment, between handling samples from different individuals, and at any other times deemed necessary or prudent. Nitrile gloves worn underneath the latex gloves remained on at all times. Reagents, (excluding some primers) used in the pre-amplification experiments at the Paleo-DNA Laboratory, were purchased as complete products and sent directly from the manufacturers. Matheson provided the primers, with the exception of those designed by Cipriano and Kaye. The primers designed by Cipriano and Kaye based on research by Centurion-Lara and colleagues (2006) were sent directly to the laboratory from the manufacturer, Elim Biopharmaceuticals, Inc. (Hayward, CA.). Anyone who entered the laboratory (for any reason) was required to submit to a buccal swap for the collection of mtDNA. These HV1 and HV2 DNA samples were sequenced and their results were stored in a computer database to be used for exclusionary purposes.

Sample Preparation:

Samples were surface cleaned with 10% commercial bleach (7mM sodium hypochlorite solution), and rinsed with water and 70% ETOH. They were left to

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dry overnight in a plastic weigh boat under a UV light in a hood in a dedicated extraction area. Bone samples were pulverized in a Spex SamplePrep, (Metuchen, NJ) mixer mill and aliquoted into 1.5 mL or 2.0 mL tubes. All samples received a unique identifiler that was recorded on the sample, in a laboratory notebook, and on a computerized form at the Paleo-DNA Laboratory.

DNA Extraction:

An SDS/Proteinase K lysis buffer and an organic (phenol-chloroform) DNA extraction method were used to extract DNA from the bone and tissue samples. The 1X extraction buffer was prepared using the following: 290 µL of TNE (Tris HCl; NaCl; EDTA), 40 µL, of 20% SDS (10%), 40µL DTT (0.39M), 2µL of Proteinase K (20 mg/mL final concentration of 100 µg/mL), and 28 µL of ddH_20 for a total volume of 400 µL. Sodium dodecyl sulfate and Proteinase K were included to encourage solubolization and degrade DNAses. To begin this process, 500 µL of 1X extraction buffer was added to an aliquot of bone or soft tissue. This tube was then placed on an Eppendorf (NY, NY) thermomixer at 37°C overnight. In the morning, aliquots of organic extraction chemicals were prepared: 16:15:1 phenol-chloroform/isoamyl alcohol, 2 tubes per sample of 1mL total, and 15:1 chloroform/isoamyl alcohol, one tube per sample of 1 mL total. One tube (1mL) of aliquoted phenol/chloroform/isoamyl mixture was added to the lysis buffer/sample mixture, which was then vortexed briefly. The sample was then centrifuged for five minutes at 13,000 rpm. The top layer (aqueous phase) was removed and placed into another aliquot of

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phenol/chloroform/isoamyl mixture, where it was vortexed briefly. The sample was then centrifuged again for five minutes at 13,000 rpms. Next, the aqueous phase was again removed and placed in a previously aliquoted tube of chloroform/isoamyl alcohol. The organic layer was discarded. The sample was then vortexed briefly, and centrifuged for five minutes at 13,000 rpm.

The phenol-chloroform/isoamyl mixture absorbs proteins and degradation from the DNA (Butler, 2001). Centrifugation separates the organic from the aqueous phase where the DNA is most soluble, allowing the DNA to be retained for molecular analysis (Butler, 2005). Ethanol precipitations to further purify and concentrate the sample, and passage through Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns (Bio-Rad Laboratories, Hercules, CA.) (to remove inhibitors) followed the steps described below.

Ethanol precipitation involved the following: the top layer of the aqueous phase was removed and placed in a new 1.5 mL tube. Added to this tube was 3 M sodium acetate (pH 5.2) to make a final concentration of 0.3 M sodium acetate (1/10 of aqueous phase volume). The sample was vortexed briefly; after which, 2.5 volumes (ethanol to sample ratio) of 100% ice-cold ethanol was added. This mixture was vortexed briefly and placed on ice. After 30 minutes, the samples were centrifuged for five minutes at maximum acceleration, and the supernatants were discarded. Following this, 500 pL of 1 mL of 95% ice-cold ethanol was added, and the samples were again vortexed. Samples were centrifuged for 10

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minutes at 14,000 rpms, after which the supernatant was discarded. The pellet was dried and resuspended in 70 µl ddH_20 and left at 37°C for 10 to 15 minutes.

The resuspended pellet was purified with Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA.). Extracts were purified using Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns two times, and then the purified extracts (in Tris-HCl buffer) were frozen in a -86°C freezer; samples that were to be used immediately were stored in either a 4°C fridge or -20°C freezer to prevent nuclease activity.

Other extraction protocols tried in this dissertation included the recovery of DNA by complete demineralization based on a modified description provided by the Armed Forces Institute of Pathology (AFDIL), and Loreille and colleagues (2007). A few experiments were also conducted using silica beads. These protocols were developed after the bulk of the dissertation research had been completed, so experimentation with them was limited.

For total demineralization, 0.200 g of powdered bone was placed into a sterile 2.0 mL tubes. Added to each tube were the following: 1.5 mL 0.5M EDTA, 75 µL 20% laureyl sarcosinate, 40 µL Proteinase K (20 mg/mL). These contents were vortexed until completely mixed, and the tube was sealed with a small piece of sterile parafilm to prevent leakage and contamination. The tubes were then placed into a 56°C incubator and rocked at 100 rpm overnight. The next morning the protocol for the phenol-cholorform/isoamyl purification was conducted as

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described previously. This was followed by ethanol precipitation and size exclusion column purification with Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns.

In some cases, silica beads were used after total demineralization. In this protocol, sample tubes were removed from the incubator the next morning, and were then centrifuged for one minute at 13,000 rpm, after which, the supernatant was transferred to a sterile 15 mL falcon tube. Into each sample tube was placed 3 mL of Guanidine Thioisocyanate (GuSCN) and 15 µL of silica beads. This mixture was vortexed for 30 seconds. Next, the sample tubes were placed on ice for three hours. After such time had passed, the sample tubes were centrifuged for one minute at 3,500 rpm, and the supernatant was carefully removed and discarded. To each tube was added 500 µL of working wash buffer. The silica beads were resuspended and vortexed for one minute, and the mixture was transferred from the falcon tubes to sterile 1.5 mL tubes. An additional 500 µL of working wash buffer was added to each of the now empty falcon tubes to resuspend any remaining silica; this was transferred to the 1.5 mL tubes. The tubes were centrifuged at for one minute at 3,500 rpm, after which the supernatant was again carefully discarded. The remaining air pellet was left to dry overnight. In the morning, 100 µL of ddH_20 was added to the tubes and they were vortexed briefly. The tubes were placed in a 56°C incubator and rocked at 400 rpm for at least one hour. Next, the samples were removed from the incubator and centrifuged for one minute at 13,000 rpm. This protocol was

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followed by size exclusion purification with Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns. The protocols for the extractions, precipitation, and use of the Bio-Rad Micro Bio-Spin® P-30 Tris Chromatography Columns appear in Appendix G.

PCR Amplification and Primers Tested:

Extracted samples were amplified with mtDNA primers for human hypervariable regions 1 and 2 (Anderson et. al., 1981; Vigilant et al.,1989; Andrews et al., 1999; Kolman and Tuross, 2000; Gabriel et al., 2001), with primers for treponematosis (including venereal syphilis, yaws, and bejel), and with primers for tuberculosis (IS 1 and 2) (Eisenach, 1989; Marchetti et al., 1998; Kotlowski et al., 2004) and (Hansen's Disease) leprosy (Lep 1 and 2) (Cole et al., 2001; Donoghue et al., 2001). As noted, bone damage from the latter diseases mimics some aspects of treponematosis. Thirteen treponemal primer sets were utilized. Kolman and colleagues (1999) designed one set based on research by Centurion-Lara and colleagues (1998, 1999); Bouwman and Brown (2005) designed eight, and Cipriano and Kaye designed four sets based on research by Centurion-Lara and colleagues (2006). Tables 13 and 14 list the primers used, their sequences, expected product sizes, annealing temperatures, and references.

The primers designed by Cipriano and Kaye were developed based on sequences presented in an article by Centurion-Lara and colleagues (2006) that

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Table 13 Human mtDNA primers and primers for tuberculosis and Hansen's Disease

Target Locus	Primers	Product Length (bp)	Annealing Temperature (°C)	Reference
(a) Human mitochondrial DNA				
HVI and HV2	5'-CCCAAAGCTAAGATTCTAAT-3' 5'-TGGATTGGGTTTTATGTA-3' 5'-CACCCTATTAACCACTCACG-3' 5'-TGAGATTAGTAGTATGGAG-3'	342 268	60	Anderson et al., 1981; Andrews et al., 1999; Gabriel et al., 2001
16210F	5'-CCCATGCTTACAAGCAAGTA-3'	___	60	Kolman and Tuross, 2000
16420R	5'-TGATTTCACGGAGGATGGTG-3'	___	60	Vigilant et al., 1989
16096F	5'-GTACATTACTGCCAGCCACC-3'	___	60	Kolman and Tuross, 2000
16322R	5'-TGGCTTTATGTACTATGTACTG-3'	___	60	Kolman and Tuross, 2000
15971F	5'-TTAACTCCACCATTAGCACC-3'	___	60	Designed by AFDIL
16208R	5'-CTTGCTTGTAAGCATGGGGA-3	___	60	Designed by Paleo-DNA Laboratory
16301F	5'-CAGTACATAGTACATAAAGCCA-3'	___	60	Paleo-DNA Laboratory
Mt15F	5'-CACCCTATTAACCACTCACG-3'	___	60	Designed by AFDIL
Mt152R	5'-AGGATGAGGCAGGAATCA-3'	___	60	Designed by Paleo-DNA Laboratory
Mt274	5'-TGTGTGGAAAGTGGCTGTGC-3'	___	60	Designed by AFDIL
Mt155F	5'-TATTTATCGCACCTACGTTC-3'	___	60	Designed by AFDIL
Mt429R	5'-CTGTTAAAAGTGCATACCGCC-3'	___	60	Designed by Paleo-DNA Laboratory
Mt164F	5'-CACCTACGTTCAATATTACAG GCG-3'	___	60	Paleo-DNA Laboratory
Mt280R	5'-GATGTCTGTGTGGAAAGTGG-3'	___	60	Designed by Paleo-DNA Laboratory
Mt1F	5'-GATCACAGGTCTATCACCC-3'	___	60	Designed by Paleo-DNA Laboratory
Mt187R	5'-CGCCTGTAA TATTGAACGTAGG-3'	___	60	Designed by Paleo-DNA Laboratory
Mt389R	5'-CTGGTTAGGCTGGTGTTA GG-3'	___	60	AFDIL
Mt247F	5'-GAATGTCTGCACAGCCAC-3'	___	60	Designed by Paleo-DNA Laboratory
(b) *Mycobacterium tuberculosis*				
IS1 and IS2	5'-CCTGCGAGCGTAGGCGTCGG-3' 5'-CTCGTCCAGCGCCGCTTCGG-3'	123	60	Eisenach. 1989, 1990
(c) *Mycobacterium leprae*				
Lep1 and Lep2	5'-TGCATGTCATGGCCTTGAGG-3' 5'-CACCGATACCAGCGGCAGAA-3'	129	60	Donoghue et al., 2001; Cole et al., 2001

* Mitochondrial DNA, Tuberculosis, and Leprosy (Hansen's Disease) primers were supplied by Dr. Carney Matheson, Associate Professor of Anthropology at Lakehead University. Due to fragmentation, mini primer sequences from the mtDNA human variable regions were used. The product length of these varies depending on which 5' (F) or 3' (R) primers are used.

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Table 14 Treponemal primers

Target Locus	Primers	Product Length (bp)	Annealing Temperature (°C)	Reference
(d) *Treponema pallidum* subspecies *pallidum* DNA				
15 kDa 1 & 2 lipoprotein gene	5'-GAGCAGGATGTCTCTATGAGTTATAAAGAG-3' 5'-GAAGCCACTACCGATGTGCGT-3'	118	55	Koiman et al., 1999
15 kDa 3&4 lipoprotein gene	5'-TGAGCAGGATGTCTCTATGAGTT-3' 5'-AAGAGAAGCCACTACCGATGT-3'	122	50	Bouwman and Brown, 2005
tprJ 1&2	5'-AACACCATCCGAAGCTGTGG-3' 5'-TGTACAGGTCATGGGTGAGG-3'	142	55	Bouwman and Brown, 2005
tprJ 3&4	5'-TGCACAGCTGCGTGCTGGT-3' 5'-GCCGATTCCCTGGGTGAGG-3'	130	50	Bouwman and Brown, 2005
**tprGJ* 1-3	5'-TGCTAAACCTACCGGTAAGG-3' 5'-GAAGGTGTTCATTACCGACC-3' 5'-TACCTGCTCAAACCCCTTCA-3'	157 124	58	Bouwman and Brown, 2005
tprCDEFGIJ 1&2	5'-AACTTTGCCCAGCTGTGGAA-3' 5'-GTCAGTACTATCCCAGGCACC-3'	189	58	Bouwman and Brown, 2005
tprCDFI 1&2	5'-CTTTGTCACCCGTGCCTATT-3' 5'-TACTCCGCTGTTTCCCACAT-3'	109	60	Bouwman and Brown, 2005
tprEGJ 1&2	5'-AGGAGGATGCGGGTACAGTAT-3' 5'-CAAAGGAGAGAAACCCCACA-3'	117	56	Bouwman and Brown, 2005
tprK 1&2	5'-ATTAAAGTGGAGCTCACCGG-3' 5'-GCTTCATACTCACCTTAGCC-3'	112	58	Bouwman and Brown, 2005
tprC outers 1&2	5'-CCAGGTGGGACTGACGTTC-3' 5'-GGAGGTGGAATCGAACAGC-3'	189	55	This paper
tprC inners 1&2	5'-AGGTGGAGCTCAGCGCCC-3' 5'-CAAAGGTGCCGAAGTGGCG-3'	102	55	This paper
tprI outers 1&2	5'-CTCCAGTACCAGGTGGGAC-3' 5'-CTCTGGCCCACACGACAAC-3'	191	55	This paper
tprI inner 1&2	5'-CGTTCAGTCCCTTCGAGAAG-3' 5'-TTCACCCCACAGATAAACGTG-3'	133	55	This paper

The *tprGJ* primers were used in combination; tprGJ 1 and 2 yields a 157 bp product, and tprGJ1 and 3 yields a 124 bp product.

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identified diagnostic genetic signatures in two *tpr* genes (*tprC* and *tprI*). However, sequencing of both products is required to identify the variants. Doctor Charmie Godornes at the University of Washington Harborview Medical Center in the laboratory of Dr. Sheila Lukehart tested these primers and were able to amplify successfully samples from all the treponemal strains tested. Aliquoted primers were mailed to Dr. Lukehart's laboratory.

The primers were tested against the following strains: Chicago, Nichols, Seattle 81-4, Bosnia A, Iraq B, Simian, Samoa D, Mexico A, Seattle 81-3, and Gauthier. All PCR experiments conducted at the Harborview Medical Center used Go-Taq Flexi DNA Polymerase Kit in 50 µL reactions (Promega Corporation, Madison, WI., cat. no. M8295). The reagents, volumes, and final concentration (in parentheses) were as follows: 10 µL of 5x Green Go Taq Flexi Buffer (1 x); 4 µL of 2.5 mM dNTPs (200 µM); 3 µL of 25 mM $MgCl_2$ Solution (1.5 mM); 1.5 µL of each primer (0.3 µM); 3 µL of template; 0.35 µL of DNA GoTaq Polymerase (1.75 U); and 26.65 µL of molecular water. Godornes tested two different thermal cycling conditions: (1) 94°C for two minutes; 94°C for 30 seconds; 55 °C for 30 seconds; 72°C for 30 seconds; for 40 cycles with a hold at 14°C; and (2) 94°C for two minutes; 94°C for one minute; 60°C for two minutes; 72°C for one minute; for 40 cycles with a hold at 14°C.

PCR amplification experiments at the Paleo-DNA Laboratory were performed. Several different PCR mixtures and thermal cycling profiles were used. Master-mixes were made in 1.5 ml tubes and distributed into 200 µL (0.2

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mL) tubes. Strip tubes were not used to avoid contamination that can occur during removal of the lid. Various taq polymerases were used in the experimental process, including GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI., cat. no. M8295), Expand High Fidelity ^{Plus} PCR System (Roche Molecular Systems, Brussels, Belgium, cat. no. 03 300 242 001), and Platinum® Taq Polymerase (Invitrogen Corporation, Carlsbad, CA., cat. no. 10966). The manufacturer supplied buffers were used with each DNA polymerase. Hemi-nested and nested PCRs attempted to address issues of low copy number DNA results from degradation and/or fragmentation. Amplification reactions were run in an MJ Research Tetrad thermal cycler (PTC-225) (Bio-Rad, Hercules, CA.).

Three different PCR mixtures and thermal cycling profiles were tested and used with a variety of primers. The regents, volumes, and final concentration (in parentheses) are presented below; all experiments were 25 µL reactions. The thermal cycling profiles used with each appear below. The annealing temperatures were dependent on the primers used.

The PCR recipe included: 2.5 µL of 10X Platinum PCR Buffer (1X); 0.5 µL of 2.5 mM dNTP mix (0.2 µM); 1.0 µL of 50 mM $MgCl_2$ (2.0 mM); 0.5 µL of 10 µM of each primer (0.2µ M.); 0.2 µL of Platinum® Taq Polymerase (1 unit) (Invitrogen Corporation, Carlsbad, CA); 9.8 µl of ddH_20; and 10.0 µL of template. The following thermal cycling conditions were used: 94°C for two minutes, 94°C

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for one minute, annealing temperature for one minute, 72°C for two minutes, for 45 cycles with a hold at 4°C.

The second PCR recipe included: 2.5μL of 10X Platinum PCR Buffer (1X); 0.5 μL of 2.5mM dNTP mix (0.2 μM); 1.0 μL of 50 mM $MgCl_2$ (1.5 mM); 0.25 μL of 10 μM of each primer (0.2 μM); 0.15 μL (2.5 units/μL) of Platinum® Taq Polymerase; 10.35 μL of ddH_20; and 10.0 μL of template. The following thermal cycling conditions were used: 94°C for two minutes, 94°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds, for 35 cycles for external primers and 25 cycles for internal primers with a hold at 4 °C.

The third PCR recipe included: 5.0μL of 5X Expand High Fidelity ^{Plus} PCR Buffer (1X); 1.5 μL of 2.5 mM dNTP mix (0.3 μM); 1.0 μL of 50 mM $MgCl_2$ (1.5 mM); 0.625 μL of 10 μM each primer (0.25μM); 0.2 μL of Expand High Fidelity ^{Plus} PCR System (2.5units/μL) (Roche Molecular Systems, Brussels, Belgium); 6.05 μl of ddH_20 for outer primers and 10.05 μL of ddH_20 for inner primers; and 10.0 μL of template for outer primers or 6 μl of template for inner primers. The following thermal cycling conditions were used: 94°C for two minutes; 94°C for 30 seconds; annealing temperature for 30 seconds; 72°C for 30 seconds; for 35 cycles for external primers and 25 cycles for internal primers with a hold at 4 °C.

Gel Electrophoresis:

Polyacrylamide gel electrophoresis (PAGE) of PCR producers took place at the Paleo-DNA Laboratory; this process allows for better separation of small molecules (i.e. DNA fragments of 100 bases or less). Compared to agarose

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mini-gels, the gels used here were 6% PAGE, and run at 118 volts for 45 minutes. Gels were stained with ethidium bromide for 15 minutes. The PAGE was made by laboratory staff from the following reagents 5X TBE, Acrylamide/bis-Acrylamide 29:1 40% stock, water, Temed, and 10% Ammonium Persulfate. The gel cassettes were purchased from Invitrogen. GeneRuler™ Low Range DNA Ladder (Fermentas, Glen Burnie, MD., cat. no. SM1193) and MiniSizer Plus 50 bp Ladder (Norgen, Thorold, ON., cat. no. 13511) were used. Extraction blanks and PCR negatives were run alongside each of the PCR products. No positives were run due to the high risk or contamination.

The only analysis of modern treponemes was done in the Lukehart Laboratory for testing of the nested *tprC* and *tprI* primers. There, 15 pL of PCR product was run on 1.5 NuSieve 1% agarose gels at 70 volts. A 100 bp ladder was used. Polymerase Chain Reaction products were run on agarose gels (EMB Chemicals®, Gibbstown, N.J.). Depending on the size of the products expected, either 1.8% agarose or 2.8% agarose gels were poured. A 1.8% full-size gel was made by adding 1.5 g agarose to 85 mL of 1X TBE and microwaving the mixture in a glass flask for approximately 1.5 to 2.0 minutes, with mixing after the first minute. A 2.8% gel was made by adding 2.4 g agarose to 85 mL of 1X TBE following the above procedure. Gels ran at 100 volts for between 50 minutes and 1.5 hours.

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Cloning:

Cloning allows for the isolation of individual PCR-amplified molecules, in order to separate multiple products formed during the PCR, so that each can be screened independently. All cloning used the TOPO TA Cloning® Kit for Sequencing (Invitrogen Corporation, Carlsbad, CA., cat. no. K4575). Manufacturer's protocols were followed. Detailed protocols for the ligation, transformation, and screening appear in Appendix H.

The cloning process consists of three main steps: (1) ligation, where the amplified product is inserted into a vector, (2) transformation, where vector molecules are inserted into bacterial cells, and (3) screening, which involves the PCR amplification of inserted PCR products and the identification of colonies that contain the desired insert. In addition to the above steps, 3' A-tailing was performed to add 3' adenines to blunt end fragments before cloning. There are two reasons for this procedure: (1) loss of A-overhangs due to a time lag between the initial amplification and cloning; and (2) use of proofreading taq polymerases for some reactions, which remove the 3' overhang necessary for TA cloning (Invitrogen, 2003). A-tailing was accomplished by adding 0.5 µL of TaKaRa Ex Taq™ (Takara Bio. Inc., Shiga, Japan, cat. no. RR001A) to 5 µL of PCR product in a 200 µL. tube and incubating at 68°C for 30 minutes.

For the ligation, a master mix was made for "n" reactions consisting of n x 1.0 µL vector, n X1.0 µL salt solution and 2.0 µL sterile water. The reagents, their measurements, and components are in the manufacturer's instruction

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manual. Four microliters of the master mix was aliquoted into 0.5 mL tubes, and 2.0 µL of PCR product was added to the individual tubes. Tubes were incubated at room temperature for 30 minutes before transforming. During transformation, tubes of TOP 10 cells (1 tube per ligation) were removed from the -80°C freezer and placed in an ice container to thaw. The tubes were then labeled with the corresponding sample numbers. After that, 2 µL of ligation mix was added to the appropriately labeled tube of cells and gently stirred with a pipette. All remaining ligation products were stored in a -20°C freezer, as this can be used to repeat the transformation later. This vector/cell mixture was then incubated on ice for 30 minutes, heat shocked for exactly 30 seconds in a 42° C water bath, and placed on ice for two minutes. Following this, 250 µL of SOC medium (thawed to room temperature) was added to each tube. The tubes were then placed on a tilted angle in a shaker at 37° C for one hour at 225 rpm.

Before plating out cells in liquid medium LB-agar, plates were warmed in an incubator at 37°C for 10 minutes. For blue-white screening, 40 µL of X-gal stock solution was added to the center of the plate and spread evenly with a sterile spreader (Fermentas, Glen Burnie, MD.). The spreader was sterilized via flash sterilization. The solution was allowed to soak for 10 minutes. After the hour of shaking, 175 µL and 75 µL of the ligand medium was spread onto two separate plates with a flash sterilized metal spreader. The cells were allowed to soak in for 10 to 15 minutes, after which plates were incubated in a 37°C oven

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overnight. In the morning, the plates were placed in a 4°C refrigerator for one to three hours to allow color development before screening.

After color development, plates were placed on a light table to better visualize the colonies. The colonies to be screened were circled and labeled on the bottom of the petri dish. Additional agar plates were warmed in the incubator for restreaking selected colonies. PCR master mix was made using the following recipe per sample: 2.5 µL of 10x of Ex buffer (Takara Bio. Inc. Shiga, Japan); 2.5 µL of dNTP (Σ10mM); 2.0 µL of $MgCl_2$ (25 mM); 1.25 µL of M13 Forward (5'-GTAAAACGACGGCCAG-3') and 1.25 µL of M13 Reverse (5'-CAGGAAACAGCTATGAC-3') primers; and .10 µL of TaKaRa Ex Taq™ (Takara Bio. Inc., Shiga, Japan, cat. no. RR001A) for a total volume per reaction of 25 µL. The master mix was aliquoted into 200 µL PCR tubes, enough for the selected colonies plus a negative control. Circled colonies were touched with the tip of a sterile toothpick, then the agar restreak plate was gently touched, and finally the toothpick was twirled in the PCR tube. The PCR reactions were placed in a thermal cycling machine with the following profile: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds for 25 to 30 cycles, with a 4°C hold. After sampling, the screened plates were placed back in the incubator to grow for another two to three hours, and then were returned to the refrigerator to arrest growth. The restreaking plates were placed in the incubator overnight. The next morning, those plates were sealed with paraffin and placed in a 4°C fridge.

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PCR Clean-up:

Polymerase chain reactions sequenced at the at the Paleo-DNA Laboratory were purified for sequencing using the Qiagen QIAquick Purification Kit Protocol following the manufacturer's instructions (Valencia, CA.. cat. no. 28104). To elute the DNA, 50 mL of Buffer EB (10 mM Tris-Cl, pH 8.5) or H_20 was added to the center of the QIAquick membrane and centrifuged for one minute. Alternatively, to increase DNA concentration, 30 µL of elution buffer was sometimes added to the center of the QIAquick membrane and the column was let to stand for one minute before it was centrifuged.

At the Conservation Genetics Laboratory, PCR products were purified for sequencing using Exo-SAP-IT® following the manufacturer's protocol (USB Corporation, Cleveland, OH.). Exonuclease I cleaves unused primers by hydrolyzing phosphodiester bonds of single strand polynucleotide molecules in a 3 'to 5' end direction. Shrimp Alkaline Phosphatase (SAP) degrades unused dNTPs at the 5' end. Once the reaction was finished, the cleaned PCR was stored in a -20° C freezer until the cycle sequencing reaction was done. The protocols for both methods appear in Appendix I.

Cycle Sequencing:

The BigDye® 3.1 Cycle Sequencing Kit was used for all sequencing reactions (Applied Biosystems, Foster City, CA.). For each set of samples, a master mix with the following reactant volumes per sample was assembled: 0.5 µL of BigDye 3.1 pre-mix, 2.2 µL of 5X buffer (comprised of 400 mM Tris, pH 9.0;

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10 mM $MgCl_2$), 1.0 µL of primer, 2.0 µL of template, and PCR water up to a total volume of 12 µL. After aliquoting the master mix into individual 200µL tubes, adding the template, vortexing and centrifuging, the products were cycled with the following parameters: 94° C for 90 seconds for initial denature; 94° C for 20 seconds; 50+° C for 30 seconds (higher temperatures used for greater precision; (i.e., the annealing temperature used in the particular PCR); 60° C for four minutes for 25 cycles, followed by a rapid cool and a hold to 14° C. The protocol appears in Appendix J.

Cycle Sequencing Clean-up:

An ethanol/sodium acetate precipitation was used for dye terminator removal at the Paleo-DNA Laboratory. The ethanol/sodium acetate solution was prepared in a 1.5 mL tube by adding the following reactant volumes per sample to be analyzed: 3.0 µL of 3M sodium acetate, pH 5.4, 62.5 µL of 95% ethanol, and 14.5 µL of sterile water. The mixture was placed in a vortexer, centrifuged, and aliquoted into individual 1.5 mL tubes for enough reaction to be precipitated. Sequencing reaction volume tubes were brought to 20 µL by adding sterile water, and individual sequencing reactions were added to tubes containing the ethanol/sodium acetate mixture. The tubes were vortexed and left to sit in the dark for 20 minutes. The tubes were centrifuged at 13,000 rpm for 20 minutes; following this, the supernatant was aspirated and discarded. Then, 250 µL of 70% ethanol was added to each tube and they were vortexed briefly. The tubes were placed in the microcentrifuge and spun for five minutes at 13,000 rpms.

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The supernatant was again aspirated, after which the samples were dried in a vacuum centrifuge (Savant, Fullerton, CA.) for 10 minutes with no heat.

At the Conservation Genetics Laboratory, cycle sequencing reactions were precipitated by adding 1.25 µL of 65% isopropanol to each sample, mixing well and transferring into a 1.5 mL tube, then spinning at 15,000 rpm for 30 minutes in a TX-160 Centrifuge (Tomy Tech USA, Inc., Fremont, CA.). After centrifugation, the isopropanol was aspirated out and another 125 µL of 75% isopropanol was added. The samples were vortexed then centrifuged for five minutes at 15,000 rpm, after which the isopropanol was aspirated off. Samples were then dried in a vacuum centrifuge CentriVap Concentrator for 10 minutes with no heat (LabConco Corporation, Kansas City, MO.). The protocols appear in Appendix K.

Samples were resuspended in 15µL of Hi-D™ formamide (Perkin-Elmer, Waltham, MA; cat. no. 4311320). They were denatured at approximately 85°C for two minutes, and then were placed in a -20°C freezer until ready to load onto a plate.

Sequencing:

Samples were sequenced on an Applied Biosystems 3100 Genetic Analyzer (Foster City, CA.) at both the Paleo-DNA Laboratory and the Conservation Genetics Laboratory. Sequencing was done in both directions to minimize PCR artifacts, ambiguities, and base calling errors using the same primers as in the original PCR, or for sequencing cloned materials, using T3

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Forward (5'-ATTAACCCTCACTAAAGGGA-3') and T7 Reverse (5'-TAATACGACTCACTATAGGG-3') primers (Invitrogen, 2003: vi). Applied Biosystems (Foster City, CA.) 3100 Data Collection Software, Version 1.1 was used.

Sequence Analysis:

Nucleotide sequences were checked and edited with Sequencher® Version 3.1 (Gene Codes Corporation, Ann Arbor, Mich.) sequence analysis software. Improved alignment of DNA sequences was performed using European Molecular Biology Lab - European Bioinformatics, Inc., (Cambridge, UK.) ClustalX® (Thompson et al., 1997), a general-purpose multiple-alignment program was also utilized. MacClade 4.08 (Maddison and Maddison, 1992) and PAUP * 4.1 (Swofford, 2003) were used for further refining alignments and to generate figures for presentation of final sequences.

Radiocarbon Dating

Samples from five individuals were radiocarbon dated via accelerator mass spectrometry. Three samples (AZ-70, T. 7 C2, Morro-1, CH. 22, and PLM-3, T. 142) were tested at Beta Analytic Inc., and two (AZ 71, T. 601 and Morro-1/6, T7) were tested at the Center for Accelerator Mass Spectrometry (CAMS), Lawrence Livermore National Laboratory (LLNL, Livermore, CA). Collagen extraction of the two samples, sent to LLNL (AZ-71, T. 601 and Morro-1/6, T.7), was conducted at the Fossil Bone Lab, Alaska Quaternary Center, University of

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Alaska Fairbanks (UAF) by Dr. Daniel Odess, Dr. Paul Matheus, and Ms. Andy Krumhardt; personnel at Beta Analytic Inc. extracted the remainder.

For AZ-71, T. 601 and Morro-1/6, T.7 collagen extraction was performed using a modified Longin method of gelatinization (with alkali extraction) on ground bone. The Morro-1/6, T.7 sample was more problematic due to poor collagen preservation. To avoid contamination and address preservation issues, the collagen extraction from this sample was hydrolyzed and extruded through non-ionic exchange resins (formerly referred to as XAD-2), which absorb short-chain, non-polar species of organic contaminants. This process removes bone contaminants such as soil humates and their derivatives. In addition to radiocarbon dating, aliquots of the respective extracts were analyzed for carbon and nitrogen stable isotope levels on a ThermoElectron Delta V Plus mass spectrometer at the Alaska Stable Isotope Facility. Conventional radiocarbon age was calibrated using either Calib. 5.0.1 Radiocarbon Calibration Program with the Southern Hemisphere ShCal04 curve (Stuiver and Reimer, 1993; Stuiver et al., 2005) or the Cologne Radiocarbon Calibration and Paleoclimate Research Package calibration curve CalPal2007_HULU (See Appendix B for the reports).

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Chapter V.

Results and Discussion

Treponemal Results

The null hypothesis that treponemal spirochetes could not be recovered and amplified from ancient bone, tissue, and/or organs using molecular techniques were tested for this dissertation. Four samples generated PCR positives in the expected size; however, none of the sequenced products for the skeletal elements or tissues was consistent with the expected sizes. Testing was done by sequencing of cloned products in three of the four positive samples; the fourth was direct sequenced, as no original extract remained. Cloning was attempted on PCR products with multiple bands when it was not clear if there was a positive in the expected size. Cloning attempts were made in a few cases where a band was not seen, as reduced template product or enzyme inhibitors may have resulted in low amplification, resulting in insufficient product to visualize on a gel (O'Rourke et al., 2000).

Potential treponemal DNA was recovered from the bone of one individual (CHI-9-06) using the *tprJ1* and *tprJ2* primers (Bouwman and Brown, 2005:706). The recovery of potential treponemal DNA allows for the tentative rejection of the null hypothesis, pending replication. The individual, designated in this research as CHI-9-06, was a 40 to 45-year-old male from the inland site of Azapa 71, T. 601 from the pre-Columbian Cabuza period dated to 2140± 30 BP (222 ± 95 BC)

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(LLNL CAMS 133582. See Table X in Chapter X). Standen and Arriaza (2000b) described this individual as having severe treponematosis, with signs of infection in the tibiae, left fibula, left clavicle, and left ulna. G. Focacci excavated this individual in 1980. Paleopathologist Marvin Allison examined him in 1983. The remains were in good condition, with all skeletal elements present except the right foot and some teeth. The individual even had a preserved beard and mustache. Associated artifacts included a pair of sandals. Pathologies noted on this individual by Allison included an umbilical hernia, possible osteomyelitis in the tibiae, reabsorption of the alveolar bone in the maxillae, and arthritis (extensive lipping and osteophytes on the vertebrae). There was also mild periostitis on the clavicles (toward the acromial ends), and mild periositis on the diaphysis of the left ulna, primarily on the anterior and medial aspects of the bone. Mild osteoperiostitis was also present on the fibulae, especially along the distal one-third of the bone. The most severe infection included a large involcrum and areas of uneven bone along the diaphysis of the left fibula characteristic of osteomyelitis. The left tibia had extensive osteoperiostitis on the distal half of the bone, primarily along the anterior and medial aspects but also extending to the posterior. Bisected by a previous researcher, the right tibia displayed extensive osteoperiostitis on the distal half, and had a narrowed medullary cavity with cortical thickening and sclerotic trabeculae. There was some dentin exposure on the mandibular molars and premolars.

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The sample tested consisted of 307 mg of bone collected from the interior of the intact corpus sterni (sternum). Powdering of the sample occurred in a Spex SamplePrep, (Metuchen, NJ) mixer mill. The recovery of DNA occurred via an SDS/Proteinase K lysis buffer and an organic (phenol-chloroform) extraction method. The 51 bp sequence, of the expected 142 bp sequence, recovered was not from a region diagnostic for the treponemal subspecies, so the analysis only distinguished the infection as belonging to *T. pallidum.* While the sequence was less than the expected size, the flanking primers matched in this conserved region. Once potential compression, artifacts, and extra bases were deleted, there were found to be five mismatches, an insertion, and a deletion. There were also three mismatches for a particular reference sequence that did not match the others; however, this is probably due to differences among the strains. The fact that this section of the sequence was contiguous with the primers that were used in the amplification suggests this may be a viable treponemal sequence, and not an artifact caused by contamination. Additionally, when the sequence generated was searched using the Basic Local Alignment Search Tool (BLAST), a utility that searches NCBI GenBank (a database of nucleotides and proteins) the entire sequence, including the portion generated by the primers, most closely resembled *T. pallidum,* and importantly, did not match any other sequences in the database well. It is unlikely that these treponemal primers would generate a sequence fragment this long from something that is not *T. pallidum.* Direct sequencing of ancient DNA products

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does not yield an especially clean sample. Given the antiquity of the sample, degradation, fragmentation, and base pair changes could occur.

These differences between the potential treponemal sequence generated here and the known treponemal sequences in the NCBI GenBank database could be representative of several factors, including: DNA damage, sequence artifacts, natural variability in the strain generated, variability in the ancient strain, uncaptured variability in the modern strains, or a sequence from something else (not *Treponema pallidum*). The kind of changes seen in the sequence generated here are consistent with results reported by others conducting ancient DNA research (Rogan and Salvo, 1994; Krings et al., 1997; Hofreiter et al., 2001a,b). Hofreiter and colleagues (2001a) have reported nucleotide substitutions, misincorporations, and jumping DNA (gaps in the sequence), which are explained by damaged or ancient DNA. Multiple amplifications, and sequence determination from multiple clones are precautionary measures that help interpret misincorporated nucleotides and other changes that commonly result from working with low molecular weight ancient DNA extracts. Figure 6 shows the sequence generated here (named PDL 284) aligned with the reverse primer and three *T.p. pallidum* (syphilis) strains and two *T.p. pertenue* (yaws) strains downloaded from NCBI GenBank. These represent only a few of the many *T. pallidum* strains. This figure also shows the chromatogram or raw data generated from the sequencer. Figure 7 shows the same data with both the

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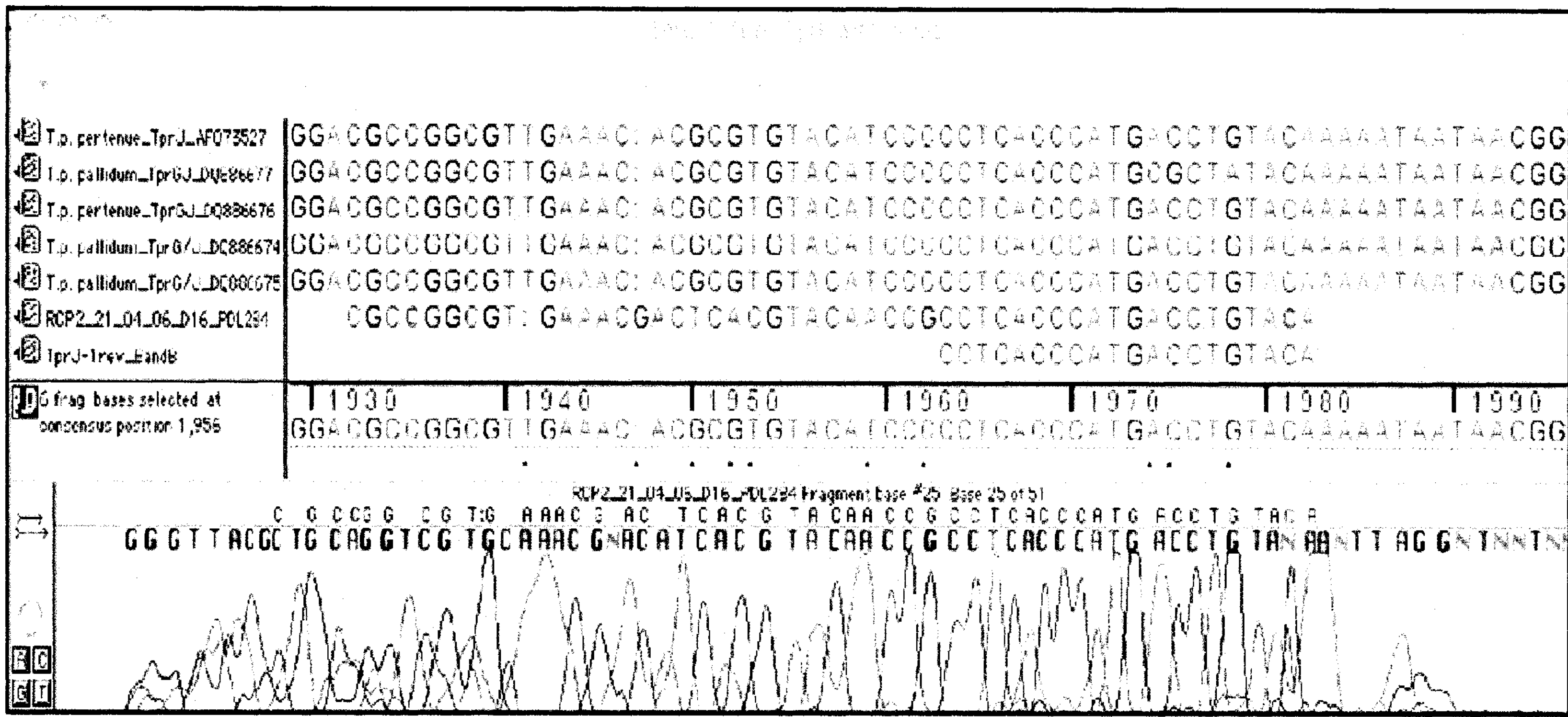

Figure 6 Potential treponemal positive PDL284

```
                                          10        20        30        40
                                           .         .         .         .
T.p._pertenue_TprJ_AF073527    GGGTGTTAGATAACACCATCGCAAGCTGTGGCGACTTTGC
T.p._pallidum_TprGJ_DQ886677   GGGTGTTAGATAACACCATCGCAAGCTGTGGCGACTTTGC
T.p._pertenue_TprGJ_DQ886676   GGGTGTTAGATAACACCATCGCAAGCTGTGGCGACTTTGC
T.p._pallidum_TprGJ_DQ886674   GGGTGTTAGATAACACCATCGCAAGCTGTGGCGACTTTGC
T.p._pallidum_TprGJ_DQ886675   GGGTGTTAGATAACACCATCGCAAGCTGTGGCGACTTTGC
TprJ_1_BandB                              AACACCATCCGAAGCTGTGG->

                                          50        60        70        80
                                           .         .         .         .
T.p._pertenue_TprJ_AF073527    CGGATTCCTCAAACTCGAAACTAAGAGCGGTGACCCCTAC
T.p._pallidum_TprGJ_DQ886677   CGGATTCCTTAAGCTCGAAACTAAGAGCGGTGACCCCTAC
T.p._pertenue_TprGJ_DQ886676   CGGATTCCTCAAACTCGAAACTAAGAGCGGTGACCCCTAC
T.p._pallidum_TprGJ_DQ886674   CGGATTCCTCAAACTCGAAACTAAGAGCGGTGACCCCTAC
T.p._pallidum_TprGJ_DQ886675   CGGATTCCTTAAGCTCGAAACTAAGAGCGGTGACCCCTAC

                                          90       100       110       120
                                           .         .         .         .
T.p._pertenue_TprJ_AF073527    ACCCACCTGCTCACCGGCCTGGACGCCGGCGTTGAAAC-A
T.p._pallidum_TprGJ_DQ886677   ACCCACCTGCTCACCGGCCTGGACGCCGGCGTTGAAAC-A
T.p._pertenue_TprGJ_DQ886676   ACCCACCTGCTCACCGGCCTGGACGCCGGCGTTGAAAC-A
T.p._pallidum_TprGJ_DQ886674   ACCCACCTGCTCACCGGCCTGGACGCCGGCGTTGAAAC-A
T.p._pallidum_TprGJ_DQ886675   ACCCACCTGCTCACCGGCCTGGACGCCGGCGTTGAAAC-A
RCP2_21_04_06_D16_PDL284       -----------------------CGCCGGCGT-GAAACGA

                                         130       140       150       160
                                           .         .         .         .
T.p._pertenue_TprJ_AF073527    CGCGTGTACATCCCCCTCACCCATGACCTGTACAAAAATA
T.p._pallidum_TprGJ_DQ886677   CGCGTGTACATCCCCCTCACCCATGCGCTATACAAAAATA
T.p._pertenue_TprGJ_DQ886676   CGCGTGTACATCCCCCTCACCCATGACCTGTACAAAAATA
T.p._pallidum_TprGJ_DQ886674   CGCGTGTACATCCCCCTCACCCATGACCTGTACAAAAATA
T.p._pallidum_TprGJ_DQ886675   CGCGTGTACATCCCCCTCACCCATGACCTGTACAAAAATA
RCP2_21_04_06_D16_PDL284       CTCACGTACAACCGCCTCACCCATGACCTGTACA
TprJ_1rev_BandB                            <-CCTCACCCATGACCTGTACA
```

Figure 7 Treponemal positive PDL284 with primers

forward and reverse primers and with the same strains. The numbers that follow the strains are their NCBI GenBank accession numbers. The primers are identified as "TprJ1-1_BandB" and "TprJ1-1rev_BandB," and the sample is designated as "RCP2_21_04_06_PDL284."

The PCR product from this particular bone sample was also positively amplified using primers *tprJ3* and *tprJ4* (Bouwman and Brown, 2005:706). Cloning of the sequenced product generated 68 bp, but only the reverse primer matched the flanking region. This second experiment used 264 mg from a

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separate aliquot of the same powdered bone. Slight variation in the extraction processes might give results not completely reproducible. It is also possible that different primer sets would behave differently. Unfortunately, there were insufficient funds to replicate these promising samples, or to try them with the newly developed *tprC* and *tprI* primers (Centurion-Lara et al., 2006; Gray et al., 2006). Future plans include independent replication with the *tprJ* primers and analysis with the new, *tpr*C and *tpr*I, primers. Any validated sequence data generated from this project will be submitted to NCBI GenBank.

Discussion of Treponemal Results

This research tests the hypothesis that treponemal DNA cannot be recovered from ancient bone, tissue, and or organs using molecular techniques. The results presented here indicate the potential recovery of treponemal DNA from ancient Pre-Columbian bone. The word "potential" is used because while the results are suggestive of treponemal DNA, they have not yet been replicated. For this reason, the conclusion should be tempered by the need for further study, including the sequencing of cloned products, and replication at independent laboratories. These results remain unreplicated not due to ignorance or willful disregard for Cooper and Poinar's (2000:1139) authenticity criteria. Several proposals were written to secure funds to replicate this research; however, they were not successful. Attempts to secure funding for future research are underway. In the interim, the results and the molecular methods used in this study should aid future researchers in their attempts to recover treponemal DNA

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from ancient samples. This research will also serve as a guide to future northern Chilean researchers, in helping them select their samples and prepare for the difficulties they will encounter when attempting molecular studies on mummies and skeletons from this region.

The recovery of potential treponemal DNA from only one individual is not a reason to doubt that treponematosis existed in these populations. Degradation sharply limits the amount of amplifiable DNA, as do organic PCR inhibitors, which are often extracted alongside viable DNA (O'Rourke et al., 2000). Additionally, the curation of most of the mummies and skeletons in rooms that were not temperature controlled, and in one case, in a very hot lorry trailer, might also have affected the preservation of the DNA. Most inhibitors to PCR are products of soil degradation (O'Rourke et al., 2000). A phenol-chloroform or a phenol-chloroform-isoamyl extraction was done to try to remove these inhibitors, which are mostly phenolic (O'Rourke et al., 2000). However, despite these efforts several of the DNA extractions resulted in a dark gold or brown-colored product, even after they were twice filtered with Bio-Rad Micro Bio-Spin® P-30 purification columns. This extract coloring was likely the result of humic acid, fulvic acid, or Maillard products (by-products of sugar reduction). The inclusion of a silica based extraction protocol, rather than a Proteinase K extraction protocol may help in lowering PCR inhibitors (Boom et al.,1990; Hoss and Pääbo, 1993; Davoren et al., 2007). However, guanidiunium thioyanate (GuSCN) is not without problems. Its use is less likely to co-extract PCR

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inhibitors, but silica itself is a strong inhibitor (O'Rourke et al., 2000). Additionally, it has an affinity for DNA, which can cause it to become contaminated with nucleic DNA (O'Rourke et al., 2000). Both methods were tried here, neither was found to be more successful than the other was.

Most ancient specimens contain only a small proportion of DNA that can be amplified by PCR; this is why many researchers use high-throughput screening techniques. These techniques detect samples that are poorly preserved, reducing amplification attempts that are unlikely to succeed (Hofreiter et al., 2001b:354). Quantification is another method that is used to estimate the number of ancient DNA template molecules from which the PCR will draw from. This process can identify which samples are most promising for analysis, reducing errors in the first cycles from few or single strand DNA, which would otherwise be incorporated into the final PCR products. Quantification is a useful discriminatory step, which can be done through Competitive PCR (Handt et al., 1996) and the use of Real-Time Quantitative PCR (Morin et al., 2001). Quantification was not done here, primarily because of the associated costs. Mitochondrial DNA analysis was used instead to assess the degree of fragmentation. This method, while not ideal, was acceptable given the number of samples. Ideally, some form of biochemical assay for macromolecular preservation should be done to determine the degree of DNA preservation (Cooper and Poinar, 2000; Hofreiter et al., 2001b).

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It was unknown when beginning this research whether bone or tissue would be a better material. Matheson (pers. comm., 2005) had hypothesized that mummified tissue might be a better medium for recovering treponemal DNA. As treponemal bacteria spread hematogenously, they could potentially be anywhere in the body. Pääbo (1989) has suggested that soft tissue might be a better source material than bone for DNA in general, as desiccation might protect DNA from hydrolytic damage, although not oxidative damage. Oxidation is a process in which water-based hydroxyl or superoxide radicals modify the DNA bases and/or alter the helix (O'Rourke et al., 2000:218). Hydantoins (oxidized pyrimidines) probably cause most of the damage to DNA; unsuccessful outcomes correlate with their presence (O'Rourke et al., 2000:218). O'Rourke and colleagues (2000) note that ancient DNA yields from desiccated soft tissues, are generally lower than those recovered from bone, and are more likely to have inhibitors in the extraction. This research supports their statement.

Bone is considered an excellent source of ancient DNA because the binding of DNA to hydroxyapatite slows molecular degradation (O'Rourke et al., 2000). Studies done by Tuross (1994) support this conclusion, although others have said that amplification success is independent of tissue type (Hoss et al., 1996). There was some question here as to whether bone would be a good source of treponemal DNA; Bouwman and Brown (2005:703) noted in their attempts to amplify treponemal DNA that, "treponemal DNA is not preserved in human bone and that it is therefore not possible to use ancient DNA analysis to

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study venereal syphilis." However, Kolman and colleagues (1999) succeeded in identifying treponemal DNA from a 200-year-old skeleton, which while not exactly ancient, is not modern. However, their research was not independently replicated. Previous ancient DNA researchers have suggested that sampling from lesions (of any kind) should be avoided, as they can be a source of contamination (O'Rourke et al., 2000). However, this study was seeking to find pathogens that might reside in or around lesions. An attempt was made to amplify treponemal DNA from osseous lesions, and from the periphery of lesions, however neither attempt was successful. It is unknown whether macrophages remove most of the treponemal DNA from lesions. Regardless of the results, the sample size was insufficient for statistical purposes to draw conclusions as to whether lesions or their periphery, are best sampled for treponemal DNA. Within this research, only a few samples could be divided into the two categories: lesion and periphery of lesion. This was primarily because curatorial restraint dictated how many and from which areas of the body samples could be collected.

Amplification methods included the use of nested PCRs to increase the sensitivity and specificity of the assays, and to reduce nonspecific priming or mispriming. It is not clear from the results whether nested primers work better than non-nested primers. Bovine serum albumin (BSA) was tried as an additive to help bind nonspecific enzyme inhibitors and/or to increase enzyme concentration, but no additional benefit was obtained. Bovine serum albumin is thought to increase PCR efficiency and yield (O'Rourke, 2000). Kolman and

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colleagues (1999:2062) suggested the use of the additive Spermidine. However, Dr. Matheson advised against the testing of Spermidine. He believed that given limited finances, it was better to concentrate on extraction methods that would reduce inhibition, rather than focus on PCR additives, which he believed would not significantly improve the outcome. Future tests, however, will include this additive. Spermidine is a PCR additive said to stimulate the enzyme T7 RNA polymerase. High-fidelity polymerases were used, and PCR cycles were run from 35 to 45 cycles; however, no significant improvement was noted with increased cycling. It is unknown why the majority of the PCRs failed. Possibilities include enzyme inhibitors, low copy number DNA, degradation, and a lack of treponemal DNA.

The results generated suggest that treponemal DNA can be recovered from bone contra Bouwman and Brown (2005). However, many of the same frustrations and difficulties encountered by them were also encountered here.

This project generated PCR products of the expected size that, upon sequencing, were found not to represent treponemal DNA amplicons. Many PCRs also generated products not in the expected size. There are several explanations for why treponemal DNA may not have been amplified in these cases. First, as Bouwman and Brown (2005:711) have suggested, the skeletal elements or tissues may have been unsuitable for DNA analysis because they did not contain treponemal DNA or because they contained sufficient inhibitors to prevent amplification of treponemal DNA. Without quantification of the samples

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tested in this research, the number of template molecules from which the PCRs began was unknown. Presumably, the numbers were very low. The coloring of the extraction products after purification suggests that inhibition may have played a significant role in PCR failure. Degraded and/or fragmented DNA, as indicated by the short sequences generated, may also have played a role. If intact DNA strands at the primer binding sites were not available to serve as a template, the PCR may have failed for this reason (Butler, 2005:146). Additionally, the more degraded the sample, the greater the number of potential breaks in the template, which in turn results in fewer DNA molecules that contain the full length needed for a successful PCR (Butler, 2005:146). The cloning of PCR products for this research was also difficult because the PCRs generated only small amounts of starting material, often less than 100 bp, primarily due to problems resulting from inhibition and oxidation. Additionally, chemically damaged DNA does not easily lend itself to restriction/ligation cloning; its prior damage increases its mutagenic potential, in particular, for sequence errors and cloning artifacts (Pääbo and Wilson, 1988; Brown and Brown, 1992). Rogan and Salvo (1994) who analyzed nucleic acids (using ribosomal DNA) derived from Atacamanian mummified tissue also encountered significant difficulties with chemically-modified DNA and potential misincorporations. They were, however, successful in amplifying most of their samples, including mummies from the Morro sites in Arica and the inland sites of San Miguel de Azapa. Their work remains unreplicated.

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Bouwman and Brown (2005:711) suggested that they might not have been able to recover ancient treponemal DNA because their PCR systems "were insufficiently sensitive to detect this DNA." However, given the sensitivity of assays (detection limits of 2-5 genomes or less per PCR), they find this unlikely. An additional problem encountered here using their primers, was that they were not sufficiently specific. This is one of the reasons new diagnostic primers were developed for this research based on Centurion-Lara and colleagues' (2006) research. Also of concern here, and for Bouwman and Brown (2005:711), is the fact that treponemal DNA was not detected, because it was not there to detect. The treponemal DNA may not have been in the skeletal elements or tissue because there was no treponemal infection. Alternatively, it may have been there but in insufficient quantities to detect (removed by phagocytosis).

Finally, as Rogan and Lentz's (1994) research suggests, the ancient and contemporary sequences may have become one or more separate species or undergone mutation; therefore, the pathogen was not detected by the primers designed to recognize the modern strains. Rogan and Lentz's (1994) findings are especially interesting when compared to the results generated here. Using different methods, and with over a decade among studies, similar results were obtained by Rogan and Lentz using molecular studies and 16S ribosomal DNA and by this author utilizing recent research on *tpr* genes. The similarity of the findings suggests that the ancient pathogen affecting the northern Chilean mummies, if treponemal, may be a little different from modern strains.

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Their data suggest that the ancient and contemporary sequences either had become separate species or had undergone mutation in the genes that disrupt annealing of the oligonucleotide primers to the ancient template (Rogan and Lentz, 1994). Based on this, they hypothesized that the treponemal-like organism affecting the Chilean mummies might be a "contemporary unknown infectious agent" or an "extinct relative of a modern treponeme" (Rogan and Lentz, 1994:171-172). This is an intriguing hypothesis, given the potentially positive treponemal results here that show changes from the modern pathogen.

The potential treponemal positive generated here, in addition to Rogan and Lentz's (1994) molecular research, and Standen and Arriaza's (2000b) osteological research, could be interpreted to suggest either that an unrecognized strain of treponematosis existed in South America, or that the pathogen was a relative of treponematosis. The fact that the osseous disease pattern in the samples examined diverges from that typically expected for the endemic treponematosis could be interpreted as evidence of this.

While the frequency of osseous treponemal infections is low for the total populations studied, only 8% (Standen and Arriaza, 2000b), it is much higher in the Archaic coastal period. In the early coastal periods, the higher rate is something of an anomaly (20%); yet, it drops sharply thereafter on the coast, when it might be expected to increase, such as is common with increased trade, greater density, and the adoption of agriculture (Standen and Arrriaza, 2000b). While the wearing of more clothing and a change in the mortuary ritual might

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explain the reduced frequency of infection in the later populations, an unrecognized pathogen or variant might also offer an explanation.

Rogan and Lentz (1994) have suggested that the disease present among the ancient Chilean mummies might have been a precursor to contemporary *T. pallidum.* This is of interest when considering the results of Harper and colleagues (2008) recent phylogenetic study. This study suggests that the New World subspecies of *T.p. pertenue* (yaws) belong to a group distinct from the Old World subspecies of *T.p. pertenue* (yaws), and hold a phylogenetic position between Old World nonvenereal strains and modern *T.p. pallidum* (syphilis) strains (Harper et al., 2008). According to their hypothesis, venereal syphilis is more closely related to the "yaws-causing strains from South America, than to other nonvenereal strains." Their hypothesis supports the Columbian hypothesis (Harper et al., 2008), as does this research. However, as previously mentioned, Harper and colleagues (2008) research is based on a few single nucleotide polymorphisms (SNPs) taken from a single location in Guyana.

Mulligan and colleagues (2008) have reconstructed Harper and colleagues (2008) phylogenetic tree to show that all three subspecies (*pallidum, pertenue,* and *endemicum*) derive from the lowest branch simultaneously. They suggest that the branch sizes vary due to greater recombination or selection, and are critical of Harper and colleagues (2008) hypothesis.

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Development of New Treponemal Primers

As noted, new primer sets targeting the *tprC* and *tprI* gene families were designed by Cipriano and Kaye, based directly on research by Centurion-Lara and colleagues (2006), and Gray and colleagues (2006). Used together these primer sets can distinguish among strains of treponemal subspecies *T.p. pallidum* (syphilis), *T.p. pertenue* (yaws), and *T.p. endemicum* (bejel) that affect the skeletal elements. This method of subtype identification varies from the technique presented by Centurion-Lara and colleagues (2006:3377). They used Restriction Fragment Length Polymorphism (RFLP) analysis of the *tprC* and *tprI* amplicons, in combination with the *Eco*47III digestion of *tpp15* gene flanking region amplicons to differentiate among the subspecies. This new approach was developed to deal specifically with ancient or damaged DNA, which can generate faint or multiple bands that can be unsuitable for RFLP analysis. The technique developed by Centurion-Lara and colleagues (2006), while faster and more straightforward, is better suited to fresher materials. Their protocol provides an excellent screening technique, and is ideal for testing multiple samples. This new process, while more time-consuming, may be better suited to ancient and rare templates; additionally it provides all the data via sequencing. This method should aid future researchers, especially anthropologists, in helping identify the treponemal subspecies in ancient and historical human remains.

The *tprC* and *tprI* primers are nested, so there is both an outer set and inner set. The target area of one set of primers (the inners) is located within the

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target sequence of the second set of primers (the outers). The PCR is run first with the outer primer set, and then a second PCR reaction is run with the inner primer set with the product of the first reaction as the amplification template. Using nested primers increases the sensitivity of the assay through reamplification of the first reaction in the second reaction. The specificity is increased because the inner primers only amplify if the PCR with the outer primers yields a specific product. When there is limited starting material (low copy number DNA), nesting primers can build in more specificity. Increasing specificity is important when trying to amplify rare template because it helps ensure you actually get what you are trying to amplify. Nested primers can also potentially reduce the effect of contamination in products due to spurious amplification of unexpected binding sites, or unwanted primer misbinding.

The sequences for the *tprC* and *tprI* primers are presented in Table 14 in Chapter 4. The *tprI* primers distinguish *T.p. endemicum* (bejel) strains from *T.p. pallidum* (syphilis) and *T.p. pertenue* (yaws) strains. While the *tprI* primers can identify a strain of the *T.p. endemicum* (bejel), they cannot distinguish between *T.p. pallidum* (syphilis) and *T.p. pertenue* (yaws) strains. If the infection is not a strain of *T.p. endemicum* (bejel), then the *tprC* primers must be used to distinguish between *T.p. pallidum* (syphilis) and *T.p. pertenue* (yaws) strains. To identify which of the three treponemal subtypes is present, four experiments must be run -- two with each primer set, and the results generated from the inners of both *tprC* and *tprI* must be sequenced. To avoid contamination and loss of

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product, it is probably best to not purify in between sets or run the results of the experiments with the outer primer sets on a gel before proceeding with amplification with the inner primers. Figures 8 through 11 demonstrate how the *tprC* and *tprI* primers are used.

The *tprC* and *tprI* primers were tested by Godornes at the University of Washington Harborview Medical Center in the Lukehart Laboratory. The primers were tested against several treponemal strains and were proven successful in that they generated products of the expected size. The PCR protocol and thermal cycling conditions appear in Tables 15 and 16. The results of their testing are shown in Figures 12 through 19. Godornes provided these images.

Mitochondrial DNA Results

Samples from all individuals were tested with primers targeting mitochondrial (mtDNA) in the human variable region I (HV1); limited testing was also done with primers targeting HV2. Mitochondrial DNA sequences were recovered from 17 experiments representing 10 of the 15 individuals; however all the sequences generated were extremely fragmented, most between 62 and 111 bp requiring the use of multiple, nested, and hemi-nested PCRs. Nine of these samples were generated from cloned PCR products; direct sequencing of PCR products generated the remaining five samples. Inhibition and degradation were among the biggest challenges. Mitochondrial DNA analysis showed that fragmentation varied among sites, and even samples from the same site.

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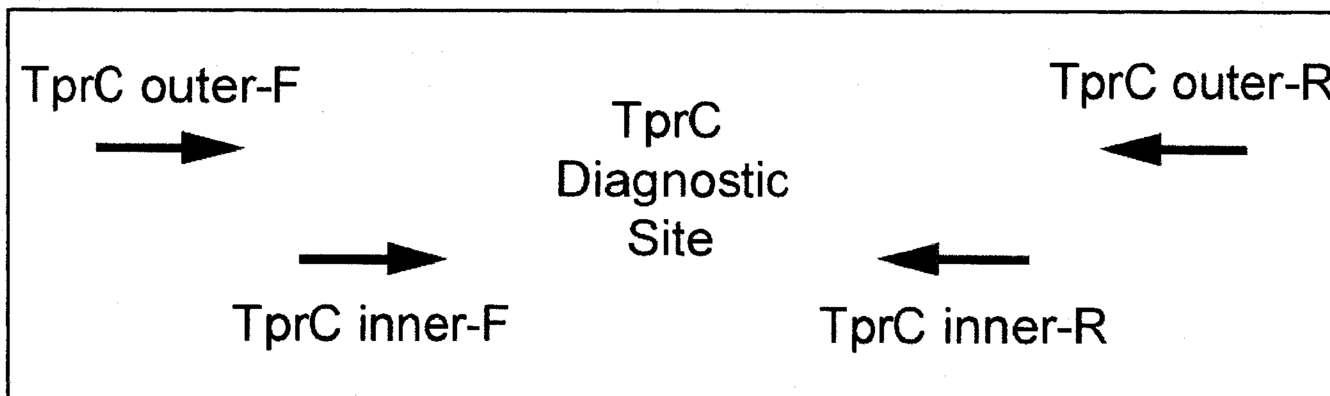

Figure 8 Diagram 1 *tprC* primers

Sequence	Alignment
T.p. pallidum_TprC_AY542157	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. pallidum_TprC_AY542155	TCCTTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. pallidum_TprC_AY536646	TCCTTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. pallidum_TprC_AY550204	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. pallidum_TprC_AY536645	TCCTTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. pallidum_TprC_CS148325	TCCTTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p.endemicum_TprC_DQ886673	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p.endemicum_TprC_AY590560	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p. endemicum_TprC_AY590563	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT
T.p.pertenue_TprC_DQ886671	TCCCTACATGGGTATTACTCAGAGCATCTGGTCCGAACGCCACTTCGGCACCTT
T.p. pertenue_TprC_AY550206	TCCCTACATGGGTATTACTCAGAGCATCTGGTCCGAACGCCACTTCGGCACCTT
T.p. pallidum_TprC_AY542154	TCCTTACATGGGTATTACTCAGAGCATCGGGTCCGACCGCCACTTCGGCACCCT
T.p. pallidum_TprC_AY542153	TCCTTACATGGGTATTACTCAGAGCATCGGGTCCGACCGCCACTTCGGCACCCT
	1910 1920 1930 1940 1950
	TCCCTACATGGGCATTGCCGAGAGCATCTGGTCCGAACGCCACTTCGGCACCCT

Figure 9 Diagram 2 *tprC* primers

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TprI outer-F

TprI Diagnostic Site

TprI outer-R

TprI inner-F

TprI inner-R

Figure 10 Diagram 1 *tprI* primers

```
T.p.pallidum_TprI_AY533510      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pallidum_TprI_AY533509      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pallidum_TprI_AY533511      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pallidum_TprI_AY533512      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pallidum_TprI_AY533513      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pallidum_TprI_AY533514      GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.pertenue_Tpr_DQ386682       GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGGGTATGTGGT
T.p.pertenue_Tpr_DQ386680       GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
T.p.endemicum_TprI_DQ886679     GAACAGGGGGTGCTCTCCGATGTTCCCTACATGGGCATTGCCGAGAGTATGTGGT
T.p.endemicum_TprI_DQ886678     GAACAGGGGGTGCTCTCCGATGTTCCCTACATGGGCATTGCCGAGAGTATGTGGT
                                      |1770     |1780     |1790     |1800     |1810
                                GAACAGGGGGTGCTTGCTGACGCTCCTTACATGGGTATTGCCGAGAGTATGTGGT
```

Figure 11 Diagram 2 *tprI* primers

Table 15 PCR protocols used in testing *tprC* and *tprI* primers

Component	Final Volume	Final Concentration
5x Green GoTaq Flexi Buffer	10 µL	1x
2.5 mM dNTPS (Promega)	4 µL	200 uM
25 mM $MgCl_2$ Solution	3 µL	1.5 mM
5' (F) Primer	1.5 µL	0.3 µM
3' (R) Primer	1.5 µL	0.3 µM
Template DNA	3 µL	x
GoTaq Polymerase (5 U/ µL)	0.35 µL	1.75 U
Molecular water	26.65 µL	

Table 16 Thermocycling conditions used in testing *tprC* and *tprI* primers

55°C	60°C
94°C X 2 minutes	94°C X 2 minutes
94°C X 30 seconds	94°C X 1 minute
55°C X 30 seconds	60°C X 2 minutes
72°C X 30 seconds	72°C X 1 minute
40 cycles, hold for 14°C	40 cycles, hold for 14°C
*Loaded 15 ul of PCR products and were run on 1.5 NuSieve gels 1% agarose at 70V	

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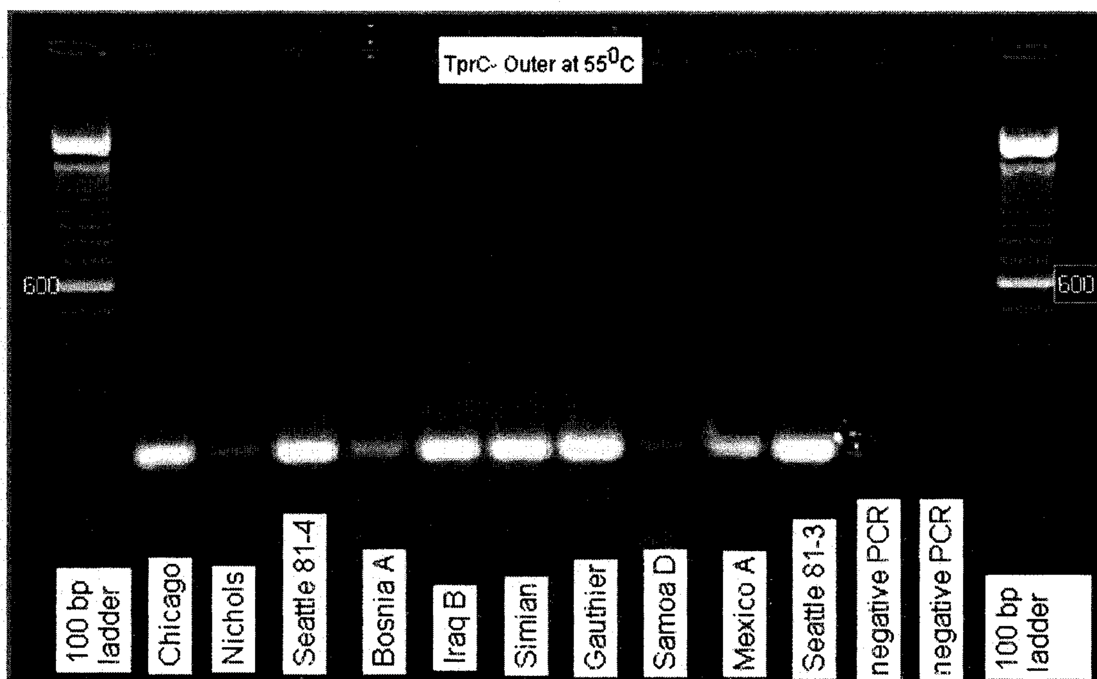

Figure 12 Testing of *tprC* outer primers at 55°C

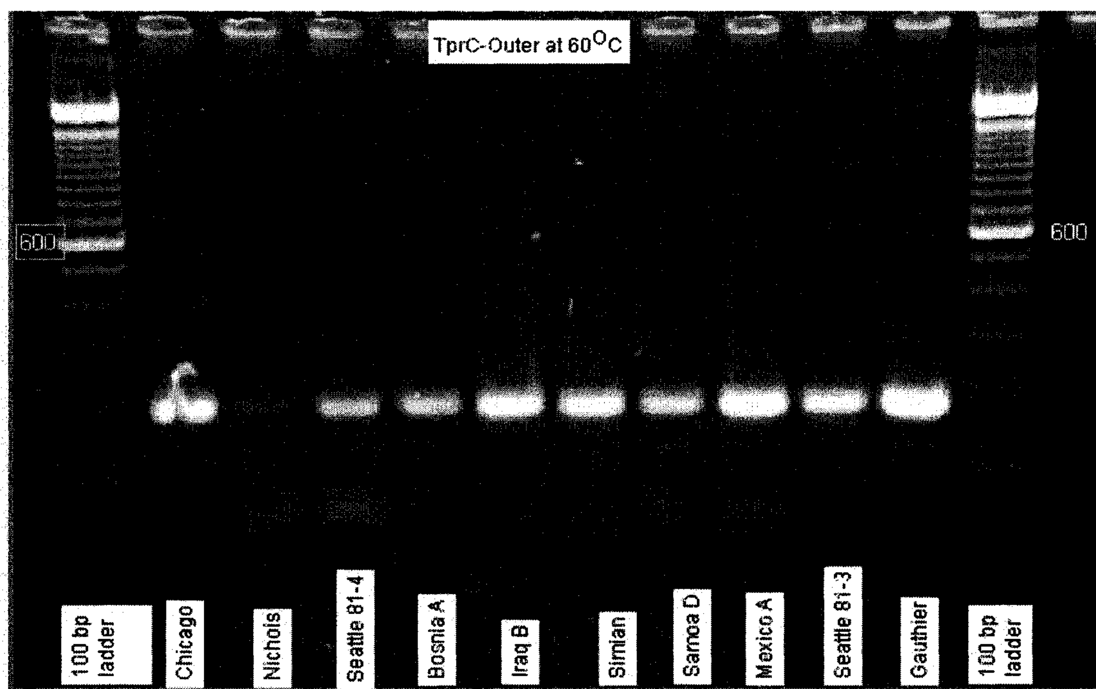

Figure 13 Testing of *tprC* outer primers at 60°C

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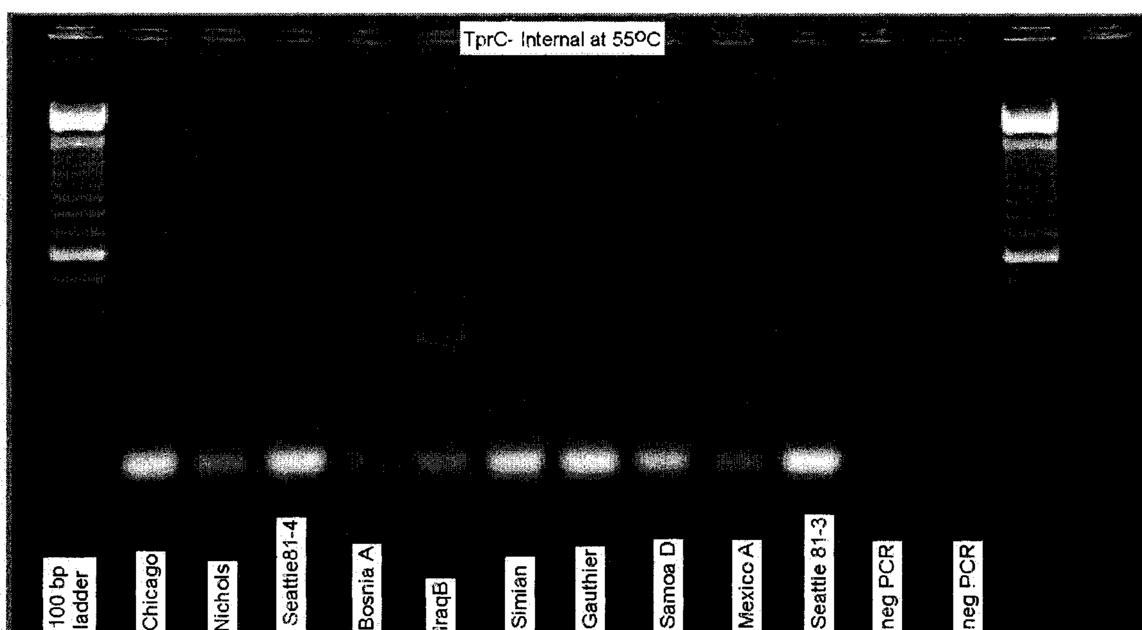

Figure 14 Testing of *tprC* internal primers at 55°C

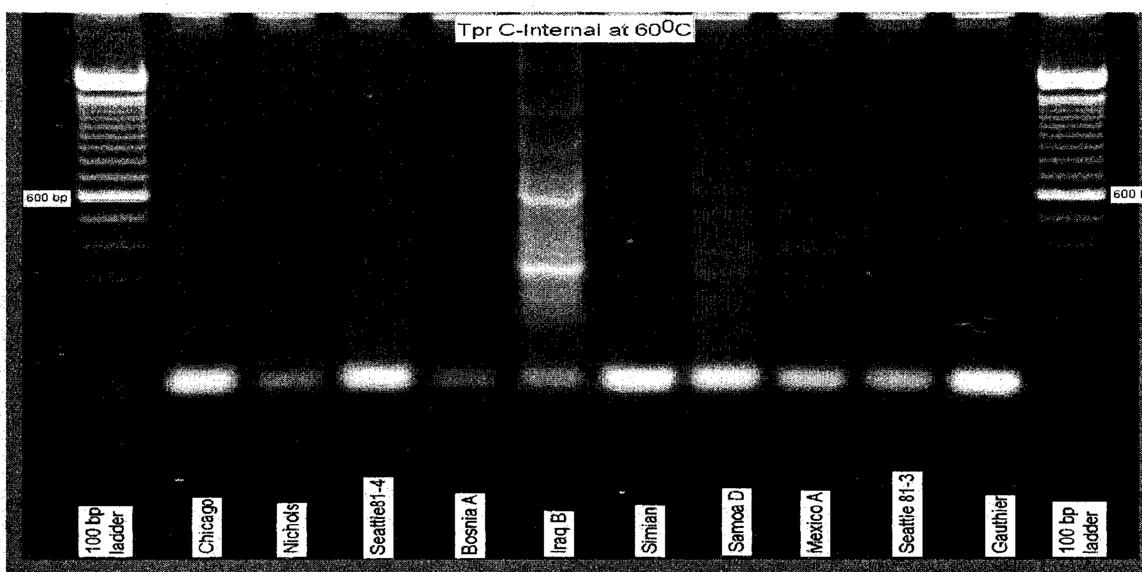

Figure 15 Testing of *tprC* internal primers at 60°C

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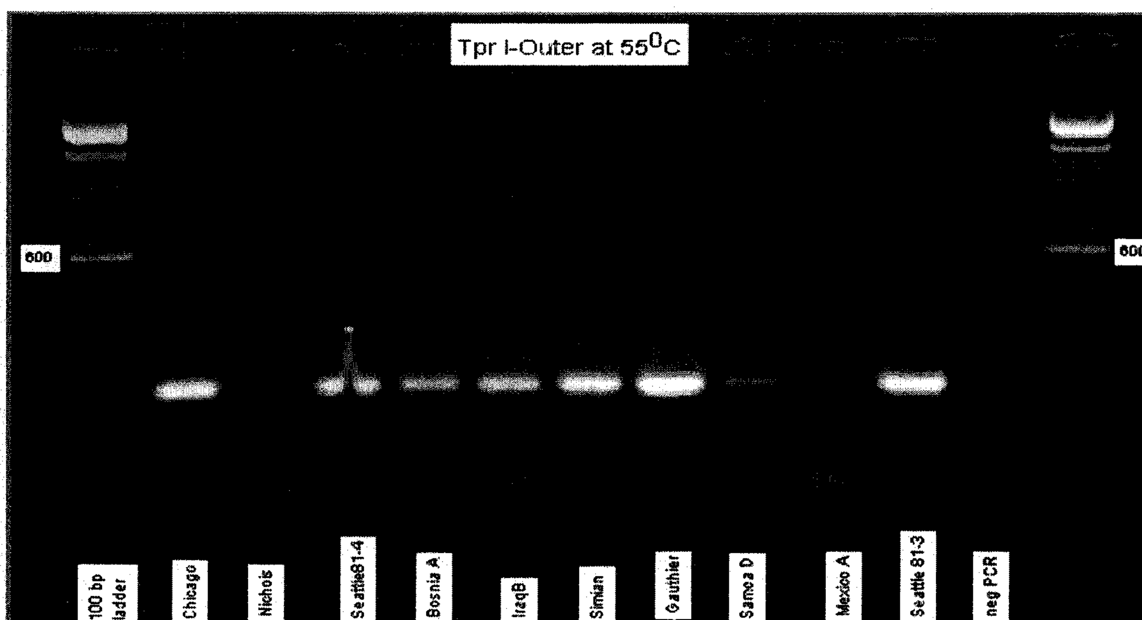

Figure 16 Testing of *tprI* outer primers at 55°C

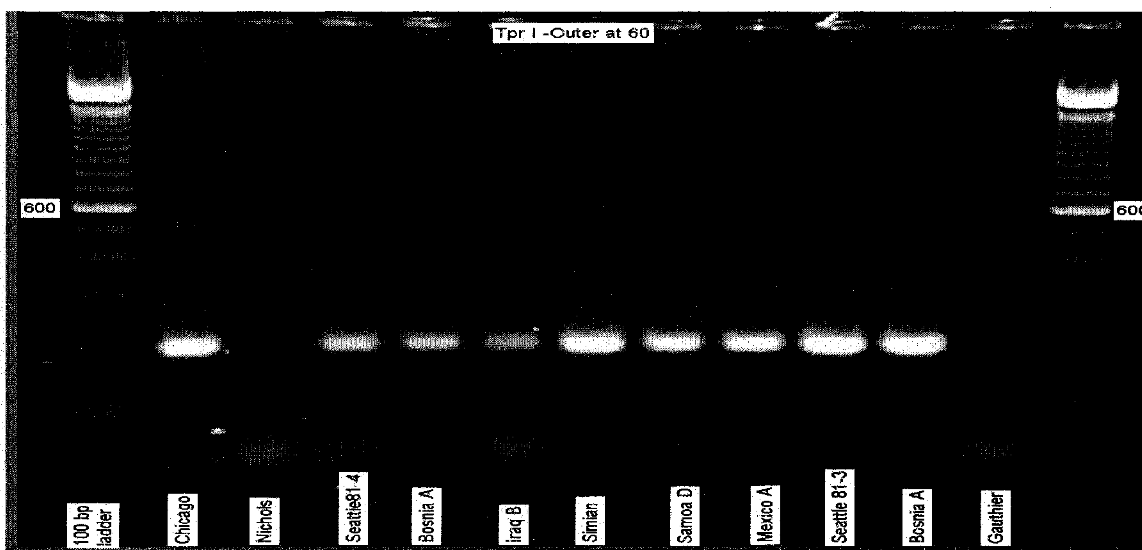

Figure 17 Testing of *tprI* outer primers at 60°C

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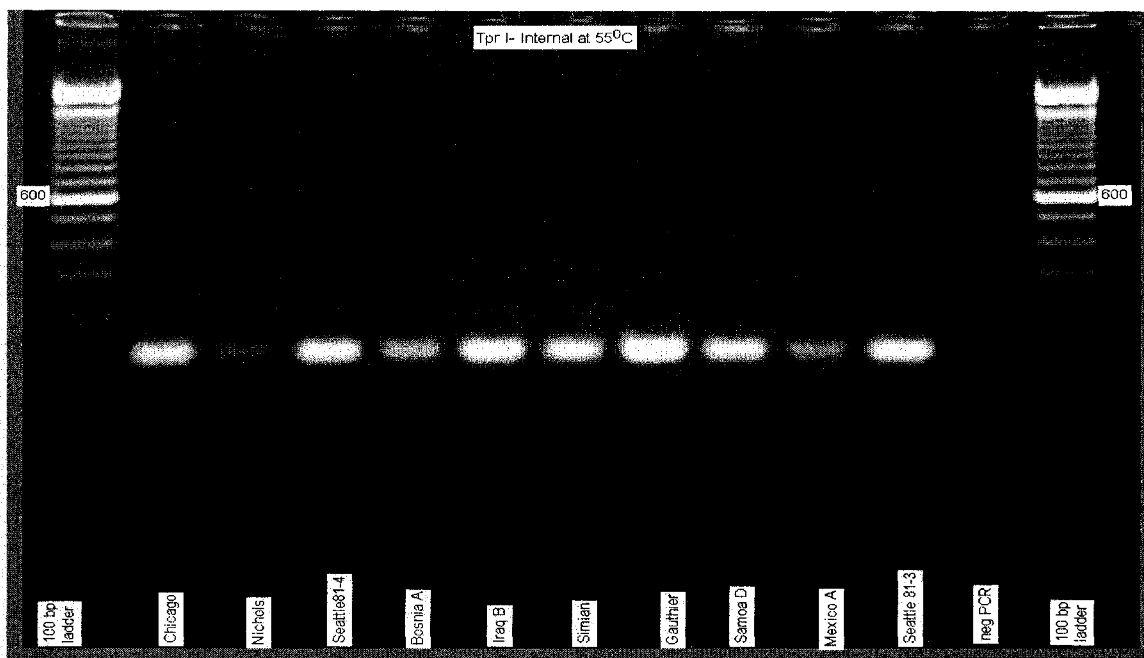

Figure 18 Testing of *tprI* internal primers at 55°C

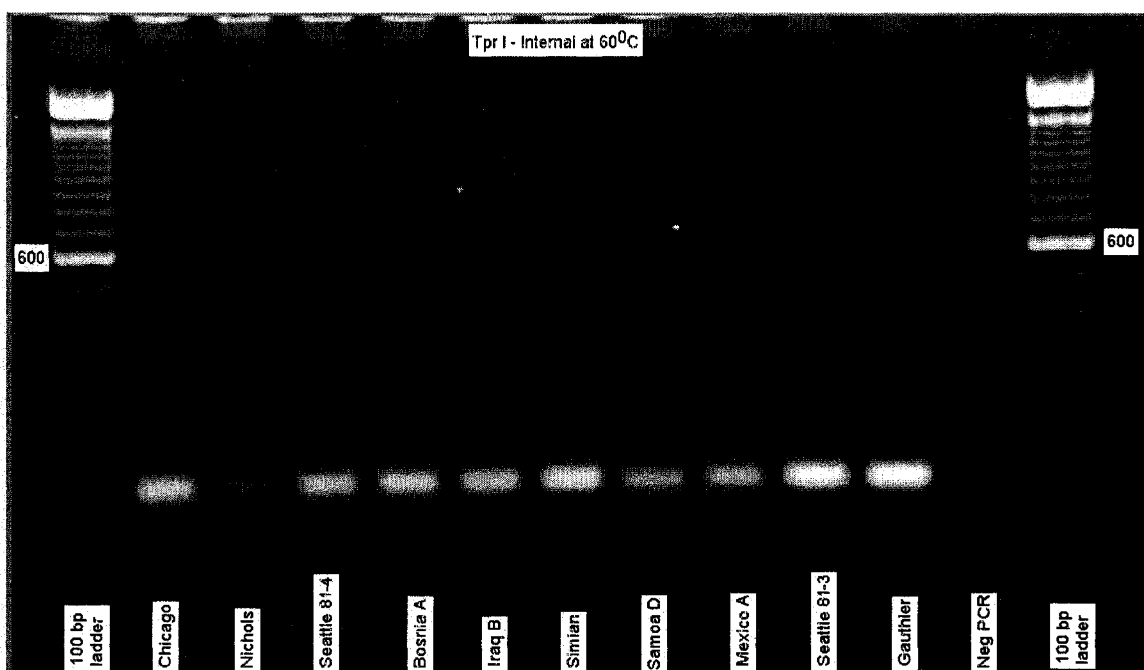

Figure 19 Testing of *tprI* internal primers at 60°C

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Contamination was a big concern with the mitochondrial data, as the mummies were excavated by Chilean and European archaeologists, and were handled by Chilean and European researchers. Not all researchers who worked with these mummies are alive or known today, which precluded collecting exclusionary samples from them. All precautions to avoid contamination were taken. Despite precautionary measures, contamination may have occurred, and affected the analysis of the mtDNA.

Three forms of contamination affect PCR: "sample contamination with genomic DNA from the environment (excavators, handlers, and curation); contamination among samples during preparation; and contamination of a sample with amplified DNA from a previous PCR reaction" (Lygo et al., 1994; Butler, 2005:152). As negative controls were run and with two exceptions (that were excluded) remained negative, a likely source of the possible contamination in these samples was the environment in which they were collected (field and laboratory). This would help explain the presence of mixtures, the contribution of two or more individuals in a sample being tested (Butler, 2005:154). These mixtures turned out to be impossible to separate. As the majority of the mitochondrial sequences generated came from muscle, this would also suggest that soft tissues versus skeletal elements were more vulnerable to environmental or handler contamination. Despite this, the commonality of the sequences generated cannot rule out the possibility of carry over, or cross sample mixing via aerial contamination, and contamination by this author in the laboratory. Only

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short sequences were generated in the HV1 region, and in this region, no variation was seen between this author's genetic sequence and the sequences generated for the samples. This does not necessarily mean the samples are contaminated; however, it cannot be proven by exclusion that there are not. The human variable region, while variable, is not always as much as people think. For example, in HV1 (from nucleotide 15971 to nucleotide 16420) this author has only one polymorphism, or variation from the revised Cambridge Reference Sequence (rCRS), the standard reference used in analyzing variations in human mtDNA (Anderson et al., 1981; Andrews et al., 1999). Given the short sequences generated, and the lack of variability, this could suggest the sequences are valid. However, this reference sequence belongs to the common predominantly European haplogroup H.

To try and determine if contamination was to blame for the lack of variation, a table was constructed comparing polymorphic mtDNA D-loop sites in HV1 with rCRS, the two researchers involved in mtDNA analysis (Michelle Kaye and Stephen Fratpietro) and haplogroups associated with Native Americans, and in particular those from Chile. Nucleotides from the following haplogroups were included: A, B, C, and D (Moraga et al., 2000:23; Maca-Meyer, 2001:1471-1472; Henriquez, 2004:663-664). Haplogroup X has also been associated with Native Americans; however, there was insufficient data on polymorphisms in HV1 to include this haplogroup. The results of this experiment also did not allow for exclusion of Michelle Kaye. These results appear in Tables 17 and 18. The

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Table 17 Polymorphic mtDNA D-loop sites 16210-16420

d-LOOP	8117	8123	8147	8154	8155	8161	8171	8178	8184	8190	8191	8198	8220	8225	8227	8263
rCRS	T	C	A	A	G	C	T	C	A	C	C	T	C	T	C	T
SF	T	T	A	A	A	C	T	T	A	C	C	T	C	T	C	T
MK	T	C	A	A	G	C	T	C	A	C	C	T	C	T	C	C
HapA	T	T	A	A	G	C	T	C	A	T	C	T	A	T	C	C
HapB	C	C	G	A	G	T	T	C	A	C	Y	T	C	T	C	T
HapC	T	T	A	A	G	C	T	C	A	C	C	C	C	C	T	T
HapD	T	T	A	A	G	C	C	C	A	C	C	T	C	C	C	C
CHI-12-06	T	C	A	A	G	C	T	C	A	C	C	T	C	T	C	C
CHI-13-06	-	-	-	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 6-06	-	-	-	-	-	C	T	C	A	C	C	T	C	-	-	-
CHI 3-06	-	-	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 1-06	-	-	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 4-06	-	-	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI13-06	-	-	-	-	-	-	-	-	-	C	C	T	C	-	-	-
CHI-10-06	-	C	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 13-06	-	-	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 14-06	-	-	-	G	G	C	T	C	N	C	C	T	C	-	-	-
CHI 15-06	-	-	-	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 14-06	-	-	-	G	G	C	T	C	N	C	C	T	C	-	-	-
CHI 15-06	-	-	A	A	G	C	T	C	A	C	C	T	C	-	-	-
CHI 16-06	T	C	A	A	G	C	T	C	A	C	C	T	C	-	-	-

- Y = Pyrimidine C or T
- N = any base
- To convert to nucleotide position in HV1 add 8100

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Table 18 Polymorphic mtDNA D-loop sites 15971-16114

D-Loop	8011
rCRS	G
SF	G
MK	G
HAPA	A
CHI 3-06	G
CHI 14-06	G
CHI 14-06	G

- To convert to nucleotide position in HV1 add 8100
- Haplogroups B-D did not have known polymorphisms at this location

results shown are from multiple experiments testing various individuals. Some sequences are shorter or longer depending on the mtDNA primer set(s) used, and the degree of DNA fragmentation.

Discussion of Mitochondrial DNA Results

In the molecular DNA data, there are legitimate concerns for contamination. As Hofreiter and colleagues (2001b:354) have noted, "DNA is particularly prevalent in the environment of laboratories and museums, and cannot be easily distinguished from the DNA endogenous to ancient human remains." Recovering treponemal DNA is easier, only in that there is a reduced risk of contamination. Providing there is enough specificity built into the primers, the primers should not falsely detect treponemal DNA unless the excavators or handlers were infected with a form of treponematosis. It is not clear how diseases closely related to the treponemes, such as Lyme's disease, might influence the results if introduced as a contaminating factor. The recovery of mtDNA suffered from many of the same issues previously discussed with the treponemal DNA, including low copy

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number DNA, degradation, and fragmentation. Oxidation may also have been a factor in mtDNA recovery. Mitochondrial DNA is in the center of oxygen metabolism, therefore oxidation has a stronger effect on mtDNA than on nuclear DNA. Most of the samples studied here were powdered in a mixer mill or ball-bearing shaker. Another concern is that in powdering the samples, the extra manipulation might have increased the sample surface area for binding with contaminating molecules (O'Rourke et al., 2000). For any future experiments on these products, demineralization (Loreille et al., 2007) may be a better approach. It might also increase DNA yields (Loreille et al., 2007).

The number of things that can go wrong in the analysis of ancient DNA analysis remains daunting; however, recent studies of ancient DNA from museum collections, Late Pleistocene animals (Leonard et al., 2000), and Neandertals (Krause et al., 2006; Green et al., 2006) have provided insight to the evolutionary past of several species and populations (Hofreiter et al., 2001b:358). More methodological research is needed to address damage to DNA, and how best to optimize, recover, remove and repair it. Moreover, better verification methods are needed to ensure the authenticity of the sequences generated (Hofreiter et al., 2001b:358).

Radiocarbon Dating Results

Radiocarbon results were obtained for five individuals. All of the dates are pre-European contact (see Table 19).

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Table 19 Radiocarbon dates generated

Site	Museum File No.	Associated Culture	Sample ID	Lab Number	Conventional Radiocarbon years BP	Calibrated Age (2 sigma)	δ13C/12C	Material
Morro-1/6, T.7	Fila A186	Chinchorro/ Faldas del Morro	CHI-1-06	LLNL CAMS 134138	3955 ± 30 BP	2475-2215 cal BC	-15.0‰	Human bone (R, acromion pr. scapula)
AZ-70, T. C2, Túmulo 7	Fila C131 (orig. recorded as B181)	Alto Ramirez	CHI-4-06	Beta -231777 AMS	2380 ± 40 BP	720-390 cal BC	-18.3‰	Human trunk muscle/skin tissue
Morro-1, CH 22	Fila A147-148	Chinchorro	CHI-6-06	Beta -231779 AMS	3830 ± 40 BP	2460-2140 cal BC	-17.4‰	Human trunk muscle/skin tissue
Az-71, T. 601	Fila B51	Cabuza/ Tiwanaku	CHI-9-06	LLNL CAMS 133582	2140 ± 30 BP	202 cal BC-cal AD 3	-13.0‰	Human bone - Corpus sterni
Morro-1, T28/C9	Fila 131, Caja 131	Chinchorro	CHI-10-06/ CHI-11-06	Lab no. I-13651	3670 ± 100 BP	2281-1698 cal BC	unreported	Human muscle/lung tissue
PLM-3, T.142	Fila A74	Desarrollo Regional	CHI-14-06	Beta- 231778 AMS	1130 ± 40 BP	Cal AD 780-1000	-10.1‰	Human muscle/skin from pelvic region

Discussion and Critique of Standen and Arriaza's (2000b) Osteological Data

The results of Standen and Arriaza's (2000b:185-192) study of 636 individuals from prehistoric groups that lived in the Atacama Desert in northern Chile showed that an endemic form of non-venereal treponematosis, probably yaws, was present. They reached this conclusion after carefully analyzing skeletal and mummified material from groups from both the coast and valley, representing three different periods in each. These statistics are displayed with permission from Arriaza, in Tables 20 and 21.From the coast, 178 individuals were examined, and from the valley, 458 individuals were examined (Standen and Arriaza, 2000b:186-187). There are 12 km between the valley and coast. (Standen and Arriaza, 2000b:186-187). From the coast, the following periods and sites were examined: (1) Archaic Period (5200 – 3700 BP) (4027 ± 148 BC - 2114 ± 144 BC), which included 150 skeletons (70 adults and 80 subadults) from the sites of Morro-1, Morro-1/6, and PLM-8, (2) Formative Period (3700 – 3600 BP) (2114 ± 144 BC - 1967 ± 144 BC), which included 24 individuals (20 adults and 4 subadults) from the site of Quiani-7, and (3) Desarrollo Regional Period (1000 – 600 BP) (AD 1032 ± 111 – AD 1351 ± 59), which included the skeletons of 2 adults and 2 subadults from the site of PLM-3, and 66 crania (40 adults and 26 subadults) from the site of PLM-4 (Standen and Arriaza, 2000b:186-187).

From the valley, the following periods and sites were examined: (1) Formative Period (2500 – 2000 BP) (616 ± 136 BC - 26 ± 122 BC), which included 127 individuals (60 adults and 67 subadults) from the sites of AZ-71, AZ

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Table 20 Numbers and percents of individuals in the coastal sites with treponematosis. (Courtesy of Standen and Arriaza, 2000b:186)

Coast	Archaic Period 5200 -3699* BP** [1](4027 ± 148 BC - 2113 ± 143 BC)		Formative Period 3700 – 3600 BP** [1] (2114 ± 144 BC - 1967 ± 144 BC)		+Desarrollo Regional Period 1000- 600 BP** [1] (AD 1032 ± 111 – AD 1351 ± 59)	
	n	(%)	n	(%)	n	(%)
Positives	30	(20.0)	2	(8.3)	1	(-)
Negatives	120	(80.0)	22	(91.6)	3	(-)
Total	150		24		4	

* Chart was modified to include a date for the Archaic Period. Date from Standen and Arriaza, 2000b:186. **Dating system was translated into English (a.P. to B.P.). +Chart also includes eliminated previous abbreviation of Desarrollo. [1] Dates were calibrated from BP to BC/AD using Cologne Radiocarbon Calibration and Paleoclimate Research Package calibration curve CalPal2007_HULU with an arbitrary standard deviation of 100.

Table 21 Numbers and percents of individuals in the valley sites with treponematosis. (Courtesy of Standen and Arriaza, 2000b:187)

Valley	Formative Period 2500 – 2000 BP** [1] (616 ± 136 BC - 26 ± 122 BC)		Middle Period 1700 – 1100 BP** [1] (AD 335 ± 121 – AD 907 ± 107)		+Desarrollo Regional Period 1000- 600 BP** [1] (AD 1032 ± 111- AD 1351 ± 59)	
	n	(%)	n	(%)	n	(%)
Positives	3	(2.3)	11	(4.1)	4	(6.3)
Negatives	124	(97.6)	256	(95.8)	60	(93.7)
Total	127		267		64	

Date from Standen and Arriaza, 2000b:186. **Dating system was translated into English (a.P. to B.P.). +Chart also includes eliminated previous abbreviation of Desarrollo. [1] Dates were calibrated from BP to BC/AD using Cologne Radiocarbon Calibration and Paleoclimate Research Package calibration curve CalPal2007_HULU with an arbitrary standard deviation of 100.

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115, and AZ-75, and 77 crania (60 adults and 17 subadults) from sites AZ-70 and AZ-14, (2) Middle Period (1700 – 1100 BP) (AD 335 ± 121 – AD 907 ± 107), which included 267 individuals (150 adults and 117) subadults from sites AZ-6, AZ-71, AZ-141, and AZ-140, and (3) Desarrollo Regional Period (1000 – 600 BP) (AD 1032 ± 111- AD 1351 ± 59), which included 64 individuals (41 adults and 23 subadults) from site AZ-8 and adjacent cemeteries (Standen and Arriaza, 2000b:186-187). It is unknown how subadults were defined. Additionally, the crania from sites PLM-4, AZ-70, and AZ-14 are not included in the tabled statistics. No other information was provided. Unfortunately, neither the coast nor the valley samples include any groups dated from 3600 – 1000 BP (1967 ± 144 BC - AD 1032 ± 111). Yet, this is the time where the majority of the samples collected for this research lie -- those thought to have treponematosis based on osteological examination. Perhaps in a small way this research can help fill in the gap between the Formative and Desarrollo Regional Periods.

In their study, Standen and Arriaza (2000b:186-187) found that 8% (51/636) of individuals showed skeletal evidence attributable to yaws. They noted the highest incidence in the coastal fishing, hunting, and gathering groups, where the total frequency of infection for all three periods examined was 18.5% (33/178). This frequency compares to a total incidence of 3.9% (18/458) in the three valley agriculturalist periods. The highest rate of infection, 20% (30/150) was seen in the Archaic coastal samples.

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The rate of infection among the coastal inhabitants decreases after the Archaic Period, to a negligible rate in the coastal Desarrollo Regional Period. There was no evidence of infection in the crania examined from the coastal Formative Period. Cranial infections were seen in three individuals, one from PLM-8, (T. 3) which is an Archaic coastal site, and two from AZ-140 (T. 140 and T. 112A) from the valley Middle Period.

In the valley, there is a slight increase beginning with a 2.3% rate of infection for the Formative Period, and rising to 4.1% in the Middle Period, and finally to 6.3% in the Desarrollo Regional Period. The rates of infection among the valley agriculturalist periods are also lower than those infection rates seen in the Archaic Period on the coast. No crania from the inland individuals from sites AZ-70 and AZ-14 were affected. Standen and Arriaza (2000b:191) attributed the differences in the frequency of infections between the coastal and inland inhabitants to minimal clothing worn in the early periods, and to the Chinchorro's mummification practice.

Of those 8% of individuals (51/636) with skeletal indicators of treponematosis, 82% (41/50) had tibial infections. The skeletal elements most affected after the tibiae were the ulna 19/45 (42.2%), radius 14/42 (33.3%), fibula 16/49 (32.7%), femur 11/46 (23.9%), ribs 7/42 (16.6%), clavicle 6/36 (16.6%), humerus 6/42 (14.3%), crania 3/29 (10.3%), os coxa 4/48 (8.3%), feet 2/34 (5.9%), sternum 2/34 (5.9%), and vertebra 1/43 (2.3%). Figures 20 and 21 are based on a table by Standen and Arriaza (2000b:189), which provides a

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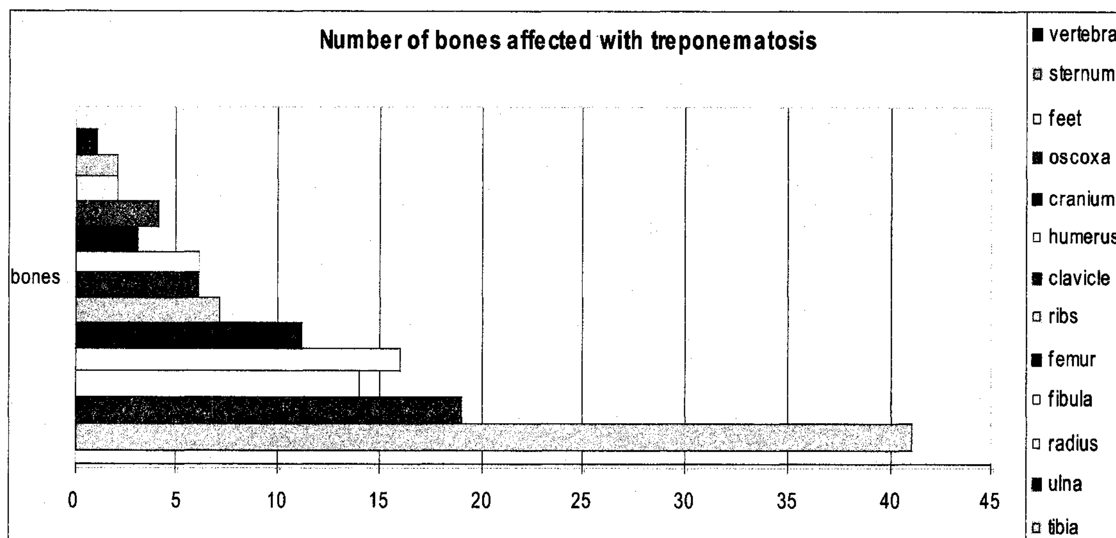

Figure 20 Number of bones affected with treponematosis. (Data courtesy of Standen and Arriaza, 2000b:189)

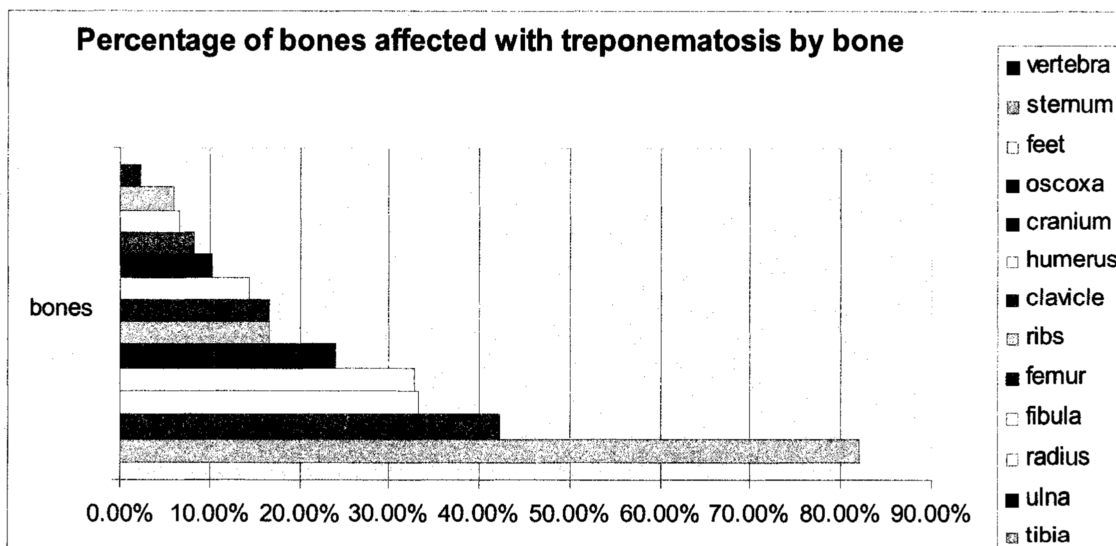

Figure 21 Percentage of bones affected with treponematosis. (Data courtesy of Standen and Arriaza, 2000b:189)

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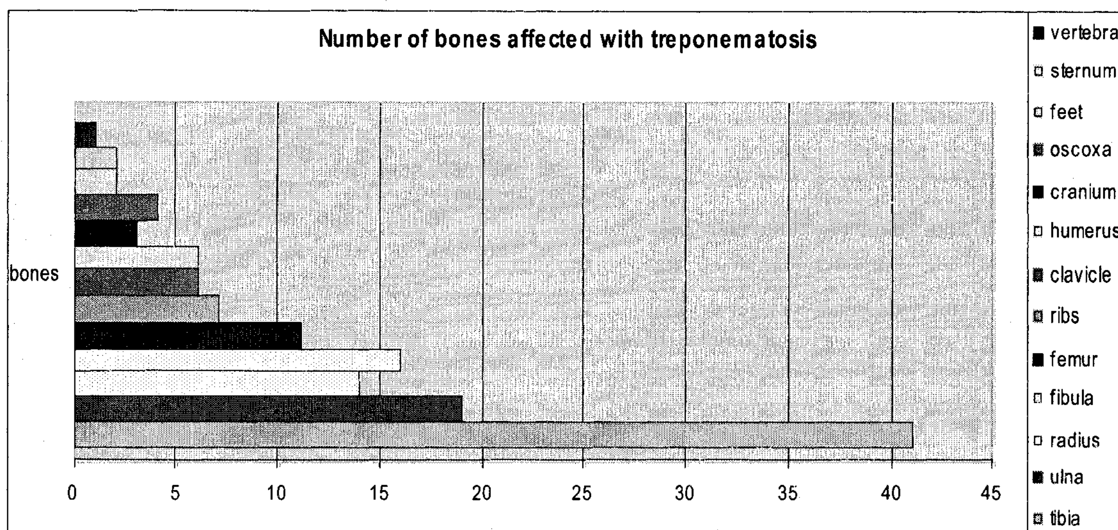

Figure 20 Number of bones affected with treponematosis. (Data courtesy of Standen and Arriaza, 2000b:189)

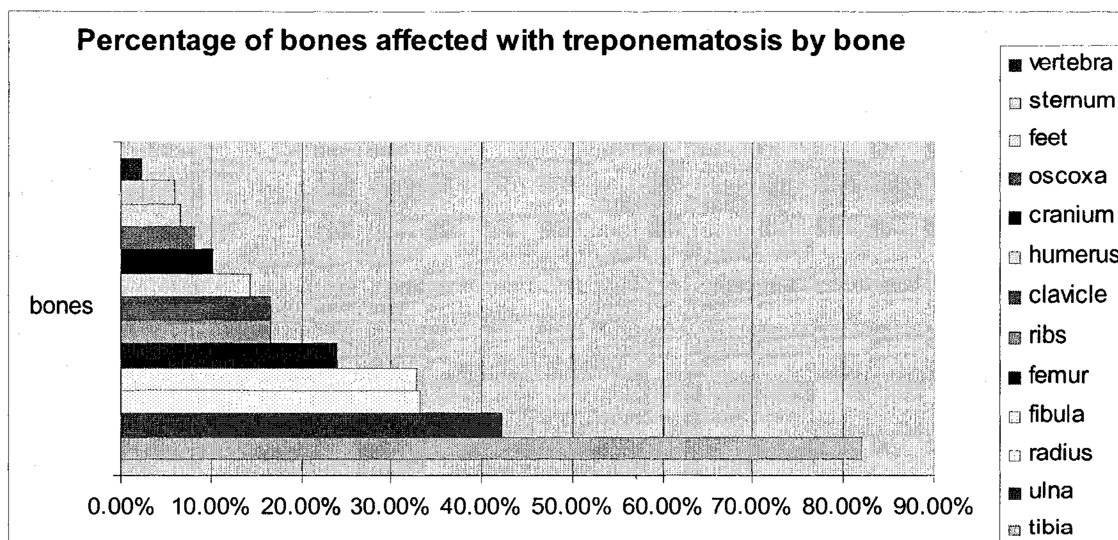

Figure 21 Percentage of bones affected with treponematosis. (Data courtesy of Standen and Arriaza, 2000b:189)

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representation of the numbers and percentages of affected individuals. In cases where the infection was bilateral in a bone type, the count includes only one bone. Several individuals had more than one type of bone affected with treponematosis; therefore, they were represented by each bone type. While the results appear high for affected tibia, they also indicate that only 41 individuals out of 636 or 6.4% had tibial infections. A better understanding of the completeness of the mummies and skeletons would provide a more accurate picture of the disease.

In 41% of the affected individuals (21/51), three sets of paired bones were affected bilaterally, in 27% (14/51) two sets of paired bones were affected bilaterally, and in 31% (16/51), one set of paired bones was affected bilaterally. Included within these numbers, nine tibiae were identified as being affected bilaterally. Identified infections included periosteal reactions, which primarily affected the diaphyses, and were marked by rugosity and woven, remodeled, or remodeling bone. Additionally there was enlargement of cancellous and cortical bone and filling of the medullary cavity with newly formed trabecular bone in a few tibiae. Three crania had healing depressed lytic lesions that caused damage to the external table and affected either the frontal, parietal, and occipital bones, or in one case, all three.

Of the 51 individuals described by Standen and Arriaza (2000b) as affected by treponematosis, 35.3% (18/51) had a severe infection, 11.8 % (6/51) had a moderate infection, and 41.2% (21/51) had an incipient or mild infection.

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It is unknown how these infections were defined, and further inquiries were unanswered. No distinction was made among osteoblastic, osteolytic, and mixed reactions. Infections were seen more frequently in adults 88.2 % (45/51), than in subadults 11.8% (6/51) (Standen and Arriaza, 2000b:189). There were no differences between the sexes reported in regards to treponemal infections.

An unpublished data sheet of affected individuals was provided by Arriaza to the author; unfortunately, it does not directly correspond with their cited study. While data from this study are very likely included within the data sheet, Arriaza could not recall which samples were used in their study. These data appear in Appendix C. Specifically, in the data sheet there are 30 cases from the Archaic Period; however, cases from two sites (Acha-3 and Cam-17) not listed in their study are included. According to Standen and Arriaza (2000b) only sites Morro-1 Morro-1/6, and PLM-8 were included in the coastal Archaic data. In addition, in the valley Middle Period there are 14 cases listed on the data sheet, and only 11 cited in their study. Because these 14 cases are from sites described as being part of their study, it is not clear who was excluded or why. Very likely, additional cases were found at a later time and added to the original data sheet. Additionally, they describe only crania from site AZ-70; however, the data sheet includes an individual from AZ-70 in the valley Formative Period with no cranium. No tomb numbers are provided in their study, so it was unclear if the individual on the data sheet was used in their study. As such, it is impossible to determine whether there are differences among coast versus valley inhabitants in severe,

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moderate, and mild infections using their study samples. However, looking at the data sheet, which included three extra individuals for a total of 54 treponemal affected individuals, the following can be ascertained about the degree of infections in coastal and valley samples.

Of those affected, 55% of coastal inhabitants sampled had mild treponemal infections compared with 38% of the valley inhabitants sampled. Moderate infections were slightly higher in the valley samples than within the coast samples (9.5% versus 9.0%), and severe infections during the comparable Desarrollo Regional Period were higher in the valley than in the coast (38% versus 27.2%). Infections described between one or more categories and absent data were noted separately within the tables.

In considering Standen and Arriaza's (2000b) statistics (see Tables 22 and 23) and the rate of treponemal infections they represent, it is important to consider the sample size and type of samples included. According to Table 20, 24 individuals represent the Formative Period in the coastal samples. Four

Table 22 Coastal samples – Infection by severity

Coast	Mild	Moderate	Severe	Moderate-Severe	Unknown	Total Cases
Archaic	16	3	9	1	0	30
Middle	2	0	0	0	0	2
Desarrollo Regional	0	0	0	0	1	1
Total #	18	3	9	1	1	33
Total %	55%	9.0%	27.2%	6.0%	3.0%	---

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Table 23 Valley samples – Infection by severity

Valley	Mild	Moderate	Severe	Mild-Moderate	Unknown	Total Cases
Formative	1	0	1	1	0	3
Middle	5	2	5	1	1	14
Desarrollo Regional	2	0	2	0	0	4
Total #	8	2	8	2	2	21
Total %	38%	9.5%	38%	9.5%	4.8%	---

individuals represent the Desarrollo Regional Period along the coast. Both samples sizes are small compared to the others. Additionally, while crania from two sites (66 crania from PLM-4 from the coastal Desarrollo Regional Period and 77 crania from AZ-70 and AZ-14 in the valley Formative Period) were described, they were not included in the tabled counts. As yaws shows a predilection for long bone infection, an analysis of isolated crania might have led to inaccurate results.

Lastly, there are few subadults in two of the coastal periods studied. There are four from the coastal Formative Period, and two from the coastal Desarrollo Regional Period. Osseous symptoms of yaws often appear in subadults, so this may affect the interpretation of results. Subadult remains are also more likely to be incomplete or fragmentary, as the skeletal elements are smaller and potentially more fragile; as such, they are more likely to be overlooked in excavation or carried away by animals. The small sample sizes and subadult count in the coastal Formative and Desarrollo Regional Periods,

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along with the exclusion of crania analyzed from PLM-4, AZ-70 and AZ-14, could alter the infection rates for these periods.

Standen and Arriaza's (2000b) study suggests that the rate of infection was higher in the coastal Archaic inhabitants than in later groups – coastal and inland. However, keeping the above noted caveats in mind, there may not have been a large decline in the rate of treponemal infections in the later coastal periods. This gap in the data could have implications for determining whether clothing and mummification contributed to the rate of infection. A larger osteological study might help determine whether the frequency of infection is lower in these two later coastal periods. This kind of study, however, may not be feasible because of the early curatorial practice of preserving only the crania. Additionally, total population numbers for all of the samples/sites is needed. For this dissertation, data on the affected versus total population numbers could be obtained only for the following Archaic sites, chronologically defined by Standen and Arriaza (2000b). (See Table 24).

Table 24 Affected versus total population for three sites

	Affected	Total Population	% Affected
Morro 1	18	96	18.75%
Morro-1/6	10	69	14.49%
PLM-8	1	10	10%

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Standen and Arriaza (2000b) cannot adequately compare all of the coast and inland groups they describe because they have no comparable archaic data from the valley -- in particular, pre-2500 BC (See Table 25). There are few data

Table 25 Missing data needed from Standen and Arriaza (2000b) for comparisons

	Archaic Period (coast)	Formative Period (coast)	Formative Period (valley)	Middle Period (valley)	Desarrollo Regional Period (coast and valley)
Coast	5200-3700 BP [1] (4027 ± 148 BC - 2114 ± 144 BC)	3700-3600 BP [1] (2114 ± 144 BC - 1967 ± 144 BC)	?	?	1000-600 BP [1] (AD 1032 ± 111-AD 1351 ± 59)
Inland	?	?	2500-2000 BP [1] (616 ± 136 BC -26 ± 122 BC)	1700-1100 BP [1] (AD 335 ± 121 –AD 907 ± 107)	1000-600 BP [1] (AD 1032 ± 111-AD 1351 ± 59)

Date from Standen and Arriaza, 2000b:186. [1] Dates were calibrated from BP to BC/AD using Cologne Radiocarbon Calibration and Paleoclimate Research Package calibration curve CalPal2007_HULU with an arbitrary standard deviation of 100.

before the Formative Period for the inland Azapa valley; most studies simply note movement of colonists from the altiplano region via Lake Titicaca to the northern Chilean coast, where the Chinchorro settled between around 8000 BC. It would seem that during the Archaic Period, populations were divided between the coast and highlands (Sutter, 2006). Sometime in the Formative Period between 1000 BC and 500 AD groups, including the Azapa and Alto Ramirez, practicing a mixed economy of fishing, animal husbandry, and agriculture settled into villages in the valley. These groups practiced natural mummification and created sand-tempered ceramics (Sutter, 2006). The main changes that occurred between the

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Archaic and Formative Periods were the re-establishment of highland sites, trade between the coast and inland valley inhabitants, the introduction of agriculture, pottery, metallurgy and textiles, and migration from the altiplano to the valley; the latter may have genetically influenced inland Azapa valley populations (Muñoz, 1989; Guillen, 1992; Núñez et al., 2002; Sutter, 2006).

As noted in Chapter 2, there are multiple ideas about the relatedness of coastal and inland groups; however bio-distance studies using craniometrics and mtDNA suggest to some that the Tiwanaku from the north and east resettled the coastal area in Arica during the Middle Intermediate Period (AD 500-1000) (Rothhammer et al., 1982; 1983; 1989; Cocilovo and Rothhammer, 1999; Varela and Cocilovo 2002:264-265; Moraga et al., 2005). Other researchers using nonmetric dental data and cranial data do not see significant differences between the early Arica and later Middle Intermediate Period groups (Sutter, 1997, 1999, 2000, 2005, 2006). Most do agree, though, that bio-distance studies show a clear influx of people from the north and east to the valley in the Desarrollo Regional Period (AD 1000 – AD 1450 (Rothhammer and Santoro, 2001; Rothhammer et al., 2002; Sutter, 1997, 2005, 2006). Cultural traditions (i.e., burial practice, subsistence, technology) vary from coast to inland, especially in the early periods and do not suggest relatedness. As noted in Chapter 2, ceramics from the Formative and later periods in the inland valley do, however, suggest relatedness with populations from the altiplano (Muñoz, 1989).

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As to the later periods, data show a 2-6% occurrence of treponematosis in the valley versus 25% on the coast. Therefore, the only adequate comparison that can be made is between 1000 BP and 600 BP (AD 1032 ± 111 and AD 1351 ± 59), not the earlier periods. In this period, the rate is higher in the valley. However, at this time the practice of artificial mummification was declining on the coast; the practice was never present in the valley, where only natural mummification occurred. Future researchers examining the cultural aspects of disease spread might want to try to address these gaps, if it is possible given the curation practices. Finances were insufficient to address these issues here.

One of the hypotheses addressed in this study was the idea that mortuary ritual practiced by the Chinchorro was not the likely cause of a higher rate of treponemal infection relative to inland groups who did not practice elaborate forms of artificial mummification. The Chinchorro mortuary practice involved prolonged, hands-on contact with the dead, which could perhaps facilitate the transmission of this disease. Inland valley cultures, as noted, did not practice artificial mummification. No data were collected on groups outside the Azapa valley with treponematosis who did not practice artificial mummification.

Mortuary practices in other cultures have resulted in unintentional disease -- the best known of which, as noted, is Kuru in the Fore of Papua New Guinea (Gadjudsek and Zigas, 1957; Steadman and Merbs, 1982; Rhodes, 1997). There is no indication of who in the Chinchorro community might have been responsible for mummification. It was thought that one way to estimate the effect

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of the mortuary practice on the spread of disease might be to examine the relationship among types of mummification, and the mummies identified with treponematosis. For example, the more complex styles (red/black) required more contact and therefore, may have been more likely to result in the spread of disease.

Unfortunately, with the exception of Morro-1, Morro-1/6, and PLM-8 (Arriaza et al., 2005), the only data on mummification type and affected individuals from Chinchorro sites was from Standen and Arriaza's (2000b) study of treponematosis in the northern Atacama Desert. These data are not suitable for statistical purposes, as no knowledge can be gained regarding the number of individuals without treponematosis by mummification type (excluding the above-named sites). Arriaza and colleagues (2005) detail mummification type data for a Quiani site, but the number of that site is not listed; there is more than one Quiani site, so no assumptions were made as to which was described in the 2005 article.

Using statistics from only those mummies identified by Standen and Arriaza (2000b) as having treponematosis also requires making an assumption that there was no bias in the selection of mummies analyzed. Arriaza suggested that a bias may exist, in that the more pristine and prized black and red mummies were not dissected or handled extensively; therefore, they could potentially have unrecorded treponemal infections. According to Arriaza, "the absence or presence of treponematosis in black and red mummies is problematic because

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many of these mummies cannot be dissected because they are unique." He adds, "thus [it] is hard to say if all prepared mummies have trep. However, we know some of them did." Unfortunately, the number of these mummies potentially infected with treponematosis is unknown.

In addition, there are inconsistencies in the temporal trends of the mummification styles and techniques that did not always occur in a linear fashion. While Uhle (1917, 1919, 1922) recorded three mummification types (simple treatment, complex treatment and mud-coated mummies), Arriaza (1995a:7) notes that radiocarbon dating techniques and new discoveries at the archaeological site of Morro-1 and others have shown that naturally mummified bodies occurred primarily at the beginning and end of Chinchorro culture (7020 BC and 1100 BC, respectively). The complex artificially mummified bodies appear in between these dates. Mud-coated mummies are found after the complex style, and mark the decline of artificial mummification. Temporal trends and the interspersing of natural mummies among the other techniques make it impossible to link conclusively the transference of disease with any particular technique. Given these limitations, I have identified areas where further research is needed, summarized the data that are available and, instead of presenting conclusions, offer some thoughts on the question.

It is important to note, that lacking the data on the number of individuals without treponematosis (Morro-1, Morro-1/6, and PLM-8 excepted) for each mummification category would incorrectly skew any percentages. For these

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data, tables, and figures presented below I am relying primarily on the raw numbers from those 54 affected individuals indentified in the aforementioned data sheet. The research revealed that most individuals with bone infections were naturally mummified 66.6% (36/54). Only one was treated in the red style, and one in the black style. In one instance, the note accompanying the mummy listed it as "red/black," suggesting characteristics of both styles or that the style could not be determined. In six cases, the mummification style was listed as unknown. Tables 26 through 28 show the styles for affected individuals and the percents for sites Morro-1, Morro-1/6, and PLM-8. Table 30 lists the count of each mummification type for affected individuals. Figure 22 provides an illustration of these counts. Again, the chart of mummification types is based on raw numbers, not percentages, so the statement about increased infection rates by natural versus complex mummification should be viewed with caution. With the above caveats in mind, these data could suggest that those mummified in the red or black styles, with rare exception, did not have treponematosis or demonstrable signs of it. However, referring back to Table 12, Chapter 2, those Chinchorro sites for which mummies are listed by type indicate that the highest frequency was the natural style. Again, it may be possible that individuals with obvious lesions received less elaborate treatment. If this were the case, there may have been less handling of the dead and fewer opportunities for disease transmission. Still, the only evidence for this hypothesis is a higher rate of

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Table 26 Mummification type of affected individuals Morro-1 (Arriaza data sheet)

Morro-1	Count	Affected Count	Percent Affected per Mummy Type
Red	27	1	3.7%
Black	8	0	0%
Mud	25	9	36%
Natural	36	3	8.3%
Unknown	0	5	---
Red/Black	0	0	0%
Total	96	18	18.75%

Table 27 Mummification type of affected individuals Morro-1/6 (Arriaza data sheet)

Morro-1/6	Count	Affected Count	Percent Affected per Mummy Type
Red	0	0	0%
Black	0	0	0%
Mud	0	0	0%
Natural	69	10	14.49%
Unknown	0	0	0%
Red/Black	0	0	0%
Total	69	10	14.49%

Table 28 Mummification type of affected individuals PLM-8 (Arriaza data sheet)

PLM-8	Count	Affected Count	Percent Affected per Mummy Type
Red	0	0	0%
Black	0	0	0%
Mud	0	0	0%
Natural	0	0	0%
Unknown	0	0	0%
Red/Black	1	1	100%
Total	1	1	100%

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Table 29 Mummification type of affected individuals only (Arriaza data sheet)

	Count	Percent
Red	1	1.9%
Black	1	1.9%
Mud	9	16.7%
Natural	36	66.7%
Unknown	6	11.1%
Red/Black	1	1.9%
Total	54	~100%
Percents are rounded to tenth		

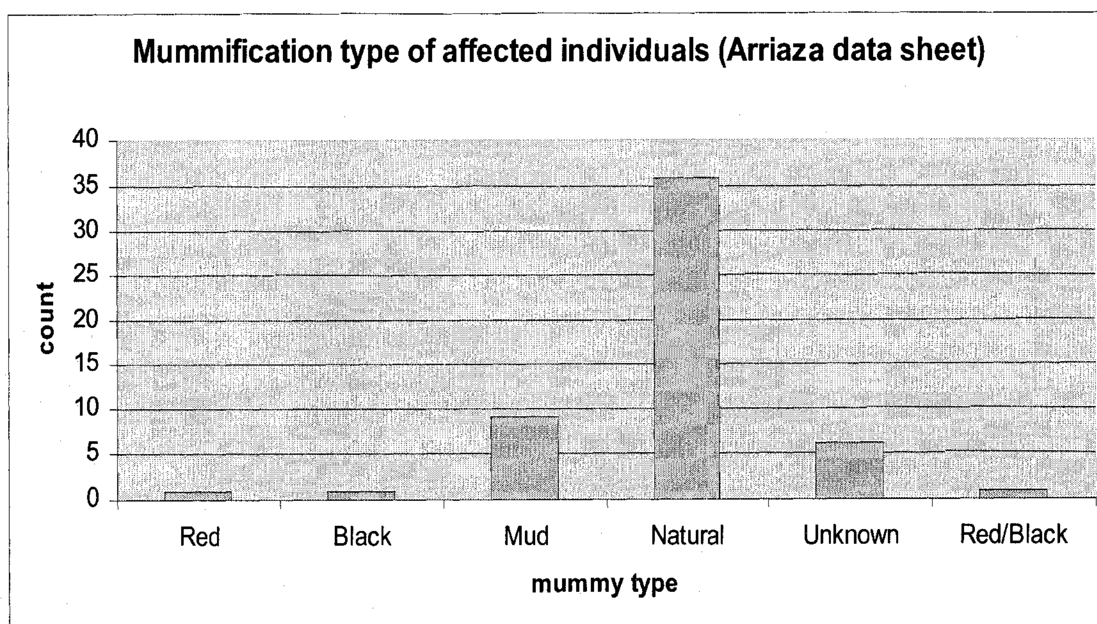

Figure 22 Mummification type of affected individuals (Arriaza data sheet)

in infection seen in those receiving less complex treatments based mostly on the raw numbers.

Of the individuals with treponematosis, the re-examination found that 55.6% (30/54) were partially mummified and 31.5% were now completely

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skeletonized (17/54); only 5.5% (3/54) were fully mummified. The degree of mummification was unknown for four individuals (i.e., 7.4% or 4/54). Given the gaps in understanding who was involved in the mummification process, why different styles were used at various overlapping times, and how many individuals received each treatment, it is impossible to say that the process did not cause the higher rate of infection in the coastal Archaic inhabitants. On the other hand, there is no evidence that it did cause the higher rate of infection. Unfortunately, this question appears to be unanswerable at present. Future research may yield more comprehensive data -- if some of the pristine mummies are subjected to radiographic or other non-invasive analyses to determine if they have diagnostic bone lesions.

Although speculative, the fact that most affected individuals identified by Standen and Arriaza (2000b) were naturally mummified may suggest that the group was cognizant of the infectious nature of the disease. Likewise, it could be the result of sample bias. Still, the contagion in a disease that has a prolonged interval between infection and visible symptoms may go unrecognized, as it is difficult to link causation with outcome with symptoms that may invisible in the early stages (Cochran et al., 2000:406).

If the infection in the coastal Archaic inhabitants were treponemal, it would not be expected to decrease significantly over time unless some cultural or environmental factor changed. In this case, both the mortuary practice and the amount and type of clothing changed. In the Early and Middle Archaic Periods

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little clothing was worn. What little there was worn was made from animal hides, fur, and plants, including skin breech clothes for men from the hides and hair/fur of various mammals, and grass or fringed reed skirts, made from macerated tortoro reeds and other plants (Arriaza, 1995a:91). Cotton textiles did not appear until 1500 BC – 1225 BC, and this occurred farther north in Peru (Arriaza, 1995a:56). However, there is a third alternative, and that is the rate of infection did not decline along the coast. That is, researchers may not yet have found affected individuals.

The decline of elaborate mummification by the Chinchorro corresponds roughly with the transformation of natural resources into textiles, including clothing; this occurred in the late Archaic/early Formative Periods (Lynch, 1983; Arriaza, 1995a:88). Modern populations afflicted with yaws are also populations where minimal clothing is worn, especially by children. The increase in clothing type and amount worn, and the decline of the mortuary ritual may have resulted in a reduction in the rate of infection, especially if risk factors were recognized.

Cultivation of vegetables on the coast, the development of ceramics and decorative metal objects, and increased trade with people from other cultures followed the clothing changes. If yaws were present, these factors would likely have increased, not decreased the rate of infection. The adoption of agriculture usually results in a decrease in health status (Larsen, 1999). Horticulture may not have caused a similar deterioration.

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A direct comparison cannot be made between the earliest inland groups (2500 – 2000 BP (616 ± 136 BC - 26 ± 122 BC)) and archaic coastal groups (5200 – 3700 BP (4027 ± 148 BC - 2114 ± 144 BC)) because of the time span between them. Whatever caused the increase and subsequent decrease in infection frequency happened on the coast. This suggests that environment, in addition to the cultural factors, deserves additional consideration. It is unclear why the inland inhabitants were less affected, at least in the Formative Period.

Northern Chile and southern Peru while warm, is not presently humid and/or tropical. It is a dry, arid desert that receives only minimal humidity. While the discovery of reeds and dry springs suggests the area was wetter in the past (Arriaza, pers. comm., 2005), it is unlikely that it was humid like equatorial West Africa, tropical Latin America, the Caribbean, Western Pacific, or Southeast Asia -- places where yaws has been confirmed (Scolnik et al., 2003; CDC, 2005; WHO, 2007). Nor is the climate like that of the eastern and southern United States, with high humidity during the summer, where there are treponematoses, including yaws (Griffin, 1984; Wilson, 1998; Powell, 1988a,b; Bogdan, 1989; Kaye, 2003).

The climate in this region more closely fits that described for bejel, i.e., as noted, warm, dry, and arid like that in the Balkan states, eastern Mediterranean, Saharan West Africa, and parts of East Africa (Aufderheide and Rodríguez-Martín, 1998; Antal et al., 2002; CDC, 2005). The question then is -- does the disease pattern better fit a diagnosis of bejel or yaws? The sequence results in

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this study were in an area of the *tprJ* gene not diagnostic for the treponemal subspecies. Thus, the area amplified only identifies the disease as a likely form of *Treponema pallidum*; it does not reveal subspecies (syphilis, yaws, bejel).

Standen and Arriaza (2000b) described the infections as being mostly osteoblastic and remodeled, or in the process of remodeling. Diaphyses were affected with osteoblastic reactions. Diaphyseal bowing is evident on anterior and medial aspects of some tibia and, in a couple cases, the apposition of new bone on the subperiosteal margin significantly narrowed the medullary cavity. In several cases, there were both osteolytic and osteoblastic reactions. Very few gummatous, and some nongummatous lesions were present. Most osteolytic lesions seen are superficial, although some larger defects may have been due to periosteal gummatous osteomyelitis. Many elements with osteoperiostitis had a rough, uneven, and in some cases, eroded appearance. A better understanding of the osseous patterns of disease expression in their samples may contribute to an even better understanding of the disease, or perhaps diseases present.

Buckely and Tayles (2003a,b) emphasized the need for an objective way to evaluate skeletal lesions that allows for cross-comparison osseous lesion recording, quantification, and interpretation. The tibia is most commonly affected in many of the differentials for treponemal infection. They suggest, based on studies by several researchers (Buikstra and Cook, 1980; Ortner and Putschar, 1981; Klepinger, 1983; Lovell, 2000), that in considering differentials three additional questions should be asked: 1) "how many individuals were affected in

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two or more bones, including the tibia, 2) how many individuals were affected in the tibia only? and 3) how many individuals were affected in any single bone that is not the tibia?" (Buckley and Tayles, 2003a:310). These questions address several issues, including whether the disease is likely systemic, which individuals might have an earlier manifestation of disease, and which display a different etiology (Buckley and Tayles, 2003a:310). These questions were applied to Arriaza's data sheet, and the answers appear within Table 30; they cannot be applied to Standen and Arriaza's (2000b) data due to inconsistencies with the data sheet.

Table 30 An evaluation of lesions in skeletal elements

	Affected in 2 or more bones	Affected in 3 bone pairs	Affected in 2 or more bone groups	Affected in the tibia only	Affected in any single bone that is not the tibia	One or both tibia affected
Standen and Arriaza (data sheet) affected groups	83.0% (42/54)	45.5% (23/54)	68.5% (37/54)	31.4% (17/54)	12.9% (7/54)	90.7% (49/54)
Some skeletal elements may have been missing. Data on the completeness of skeletons from the entire sample was unavailable. The word bone is used in this chart for consistency with terms used by Buckley and Tayles (2003a:310).						

The number of individuals infected in two or more skeletal elements, including the tibia, suggests the disease is probably systemic. The high rate of tibia infection, i.e., 31.4% (17/54) had an infection in one or both bones and no other skeletal elements, suggests the pathogen prefers the tibiae. And 12.9% (7/54) of the affected individuals show infection in a single skeletal element that is not the tibia. Together, these data suggest a treponemal disease, not an alternative pathogen.

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As noted, 83% of the affected individuals had lesions and/or periostitis in multiple skeletal elements, including the tibia. Both sexes showed this pattern, with 52.5% of females and 42.9% of males affected. Two individuals were of indeterminate sex (4.8%). All but two (14-15 yrs) of the affected subadults had signs of treponematosis in multiple skeletal elements. Only one potential subadult (age 16-19) had unaffected tibiae. Surprisingly, subadult lesions were mostly remodeled, whereas adult lesions were a mix of remodeled, or active. There does not seem to be a relationship between number of skeletal elements affected and age.

For most elements, there is no significant relationship between sex and age; cranial lesions are seen only on females, but they are few in number, (3/54). The re-examination of the 54 affected individuals on the data sheet showed that 52.9% (27/54) had osteoblastic reactions and 43.1% (22/51) had a combination of osteoblastic and osteolytic infections. No data on the type of osseous reaction were available for five individuals. Additionally, no individuals had only osteolytic, or primarily osteolytic, infections. About half of the affected elements, 48% (26/54), were remodeled, and half, 48% (26/54), showed one or more active lesions. Many individuals had both remodeled and active lesions.

Because the osteological analysis suggests the infection is treponemal, a potential treponemal positive sequence has been generated, and radiocarbon dating shows the mummies to be Pre-Columbian, the null hypothesis that treponematosis was not present in the New World before European contact can

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be rejected. Osteological and molecular analyses by previous researchers strengthen this claim (Allison et al., 1982; Allison, 1984; Standen et al., 1984; Rogan and Lentz, 1994; Arriaza, 1995a,b; Standen and Arriaza, 2000b).

As for the type of treponematosis, the paucity of: 1) lesions in the frontal and parietals, and 2) congenital syphilis indicators like Wimberger's sign (Jaffe, 1972), Hutchinson's incisors, and mulberry molars, suggest the disease is likely not syphilis. While long bones of affected subadults evidenced osteoperiostitis, there is no indication of osteochrondritis, which if present, would be supportive of syphilis. Unlike yaws and bejel, with a 1:1 ratio of males to females affected, syphilis has a 3:1 ratio -- with more males affected (Ortner, 1998). In the groups studied, both sexes were equally affected, which is consistent with endemic treponematosis (yaws and bejel). However, there are signs of infection in the vertebrae and sternum, which are uncommon in endemic treponematosis, although these numbers were low. Other indicators that suggest the disease in the sample examined by Standen and Arriaza (2000b) is a non-venereal form of treponematosis are summarized in Table 31. A brief comparison of each of the treponemal subspecies also follows.

In syphilis, the frequency of bone infection ranges between 10-20% (Steinbock, 1976), and it usually produces periosteal reactions in 5-14% of affected populations (Jostes and Roche, 1939; Freedman and Meschan, 1943; Rothschild and Rothschild, 1994). In the sampled individuals, the frequency of infection was 8% for all groups, which is low for syphilis (Standen and Arriaza,

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Table 31 Epidemiology and pathology of treponemal diseases that affect the bones

	Venereal Syphilis	Bejel	Yaws	Unknown Disease (Standen and Arriaza 2000b)
Causative organism	*T.p. pallidum*	*T.p. endemicum*	*T.p. pertenue*	Unknown
Associated climate	Worldwide [3]	Temperate, subtropic [3]	Tropic, Humid [3]	Dry, arid
Predominant Age of Infection	Adult (except congenitally) [3]	Childhood[3]	Childhood[3]	Childhood?
Predominant Mode of Infection	Venereal contact[3]	Nonvenereal contact[3]	Nonvenereal contact[3]	Nonvenereal contact
Capacity for Congenital Transmission	High[3]	Low or nonexistent[3]	None[3]	Unknown
Sex Ratio (m-f)	3:1 [7]	1:1 [7]	1:1 [7]	1:1
Bones most affected (in descending order)	tibia, the frontal, and parietal, nasal-palatal region, sternum, clavicle, vertebrae, femur, fibula, humerus, ulna, and radius [3]	tibia, fibula, ulna, radius, clavicle, phalanges and calcaneous [3]	tibia, fibula, clavicle, femur, ulna, radius, and bones of the hands and feet [3]	tibia, ulna, radius, fibula, femur, ribs, clavicle, humerus, cranium, os coxa, hands and feet, sternum, vertebrae
Skeletal involvement	5-20% [4,6]	1-5% to 20-40% [4,6,8,9]	1-5% to 20-40% [4,6,8,9]	8% (51/636) 18.5% (33/178)*
Avg. # of bone grps. affected	1-2.5 [4,5]	2 [4,5]	3+ [4,5]	2.7
Prevalence in Populations/Groups w/o Medical Treatment	5-14% [5]	25-75% [5]	25-75% [5]	8% (51/636) 18.5% (33/178)*

Source: Modeled after Powell, 1989:176-177 and Rothschild and Rothschild, 1995:1405
* Total coastal sample; [1] total affected sample; [2] excludes affected individuals missing either a tibia, fibula, or both, unless the tibia was affected and one fibula was present;
[3] Aufderheide and Rodriguez-Martín,1998:154-171; [4] Rothschild and Rothschild, 1995:1405; [5] Rothschild and Rothschild, 1996:559; [6] Steinbock, 1976; [7] Ortner, 1998;[8] Molto et al., 2000; [9] These numbers vary among authors.

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2000b:185). However, it was 18.5% (33/178) for the coastal groups, which is high for venereal syphilis, and is about on par for the endemic treponemes. Tibial involvement is usually unilateral in syphilis, and cases of saber shin often appear smooth after remodeling (Molto et al., 2000). As noted, neither was the case here.

As described, bejel is characterized by skull and nasal-palatal destruction and saber tibia. Unlike yaws, bejel is considered "pauci-ostotic," and generally it affects an average of two bone groups (Hershkovitz et al., 1995; Rothschild and Rothschild, 1995a; Molto et al., 2000:4). In bejel, the tibiae account for more than 60% of affected bone groups, which compares to 18-33% in yaws (Rothschild and Rothschild, 1995). Additionally, tibial infection is usually bilateral and sabre shin remodeling does not remove all surface evidence of periosteal reaction (Molto et al., 2000).

In yaws the average amount of bone groups affected is three or greater (Rothschild and Heathcote, 1993; Rothschild and Rothschild, 1994, 1995; Aufderheide and Rodríguez-Martín, 1998); the average was slightly lower here. As in syphilis, there can be skull involvement, although it is less common. In 7-8% of cases, there is extensive nasal-palatal destruction. Perforation of the palate occurs in 5% of cases with osseous involvement. Yaws is characterized by numerous periosteal reactions and gummatous osteomyelitis of the long bones (Aufderheide and Rodríguez-Martín, 1998).

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While infection of the tibia is common to all three treponemal infections, fibula infection is common only to bejel and yaws. While Standen and Arriaza's (2000b) article does not include these data, the data sheet shows that 38% (19/50) of the affected individuals exhibit signs of infection in at least one fibula. Individuals missing fibulae were excluded. This frequency suggests the disease is bejel or yaws. Infection of the humerus is uncommon in bejel, but is seen in yaws. Infection of the humerus also occurs with syphilis, but usually affects the epiphyses; looking at Arriaza's data sheet, the diaphyses were primarily affected. In the affected individuals, 15.0% (6/40) evidence infection in the humerus, suggesting that the disease is more likely to be yaws than bejel. Individuals missing humeri were excluded.

The infection present in the northern Chilean groups shares some characteristics with all three treponemal infections (syphilis, bejel, and yaws). Figure 23 illustrates six areas used to discriminate among treponemal infections. The criteria are present with a "yes" and not present with a "no." A "yes" is represented by a cone and a "no" is represented by a flat circle. The key to understanding the figure is to look at matching patterns of flat circles and cones. This method of discrimination was derived from a table presented by Rothschild and colleagues (2000:937).

Looking at these categories and the table, the best match for Standen and Arriaza's (2000b) sample is bejel, with five out of six categories matching; yaws is next with four of six categories, and syphilis is last with two out of six

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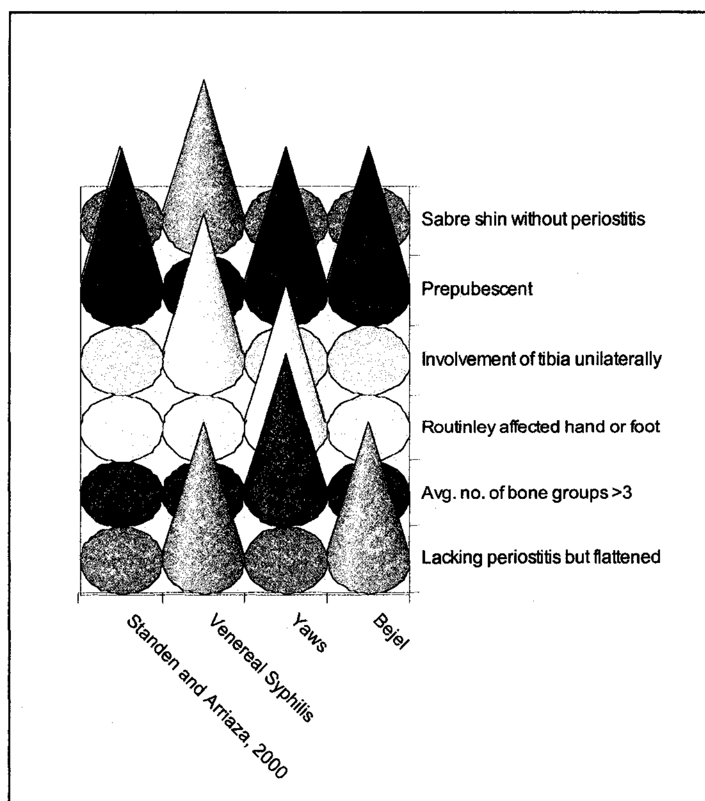

Figure 23 Discriminating factors for treponematosis

categories matching. Bejel and yaws are closer matches than syphilis, and both affect subadults; syphilis rarely has extensive hard tissue involvement, excluding the teeth, in prepubescents (Rothschild and Rothschild, 1995).

Differential Diagnoses

The differential diagnoses considered most relevant after re-examination of the affected individuals included, *Leishmania braziliensis*, tuberculosis, streptococcal and staphylococcal infections (incl. pyogenic osteomyelitis), brucellosis, paracoccidioidomycosis (South American blastomycosis), coccidioidomycosis, and other mycotic infections (Aufderheide and Rodríguez-

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Martín, 1998). A brief description of the categories is also provided in Table 32. Table 33 summarizes these diseases.

Leishmaniasis can cause mucocutaneous and destructive bone lesions, resulting in extensive disfigurement in its mucosal form called espundia (Aufderheide and Rodríguez-Martín, 1998:257). The skull from a 45 to 50-year-old female mummy from the Azapa valley dated to AD 1100 showed extensive destruction of the nasal areas, right orbit, and paranasal sinus walls, which is strongly suggestive of leishmaniasis (Allison et al., 1981; Allison et al., 1982; Aufderheide and Rodríguez-Martín, 1998:257). While some individuals in

the sample may have contracted leishmaniasis, it is unlikely that it was the main cause of the infections, as visceral leishmaniasis has a high fatality rate (CDC, 2008b). Furthermore, children are at greater risk for leishmaniasis than

Table 32 Discriminating factors for treponematosis

Discriminating Factors	Venereai Syphilis	Yaws	Bejel	Unknown Disease (Standen and Arriaza, 2000b)
Lacking periostitis but fiattened	Yes	No	Yes	No
Avg. no. of bone groups >3	No	Yes	No	No
Routinely affected hand and foot	No	Yes	No	No
involvement of tibia unilaterally	Yes	No	No	No
Prepubescent	No	Yes	Yes	Yes
Sabre shin without periostitis	Yes	No	No	No

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Table 33 Differential diagnosis summary

	Leishmaniasis	TB	Grp. A Strep & Staph	Brucellosis	Coccidioidomycosis & Paracoccidioido-mycosis
How transmitted?	Bite of female sand fly transmits protozoan [1]	Airborne bacteria, primarily from other infected humans [2]	Trauma, open wound [2]	Zoonotic – exposure to animals/ secondary products – ingestion, cuts, aerosol [2]	Thought to be from inhalation of contaminated soil [2]
Skin & subcutaneous involvement?	Common [2]	Yes, in uta [2]	Yes [2]	Occasional [2]	Yes
Oronasal mucocutan-eous Involvement?	Common [2]	Yes, in uta [2]	Depending on type [2]	Occasional [2]	Yes
Pulmonary Infections?	No [1]	Yes [2]	Depending on type [2]	Occasional [2]	Yes
Skeletal involvement?	Yes (late stage) Cranial elements esp., but can affect long bones[2]	In 1-7%- epiphyses and metaphyses --verts, ribs, tibia, ulna, radius, femur, and fibula [2,6,7]	In 5-25% of cases -- knee, tibia, femur common w/ Grp A strep. Osteomyelitis in long bones seen w/ systemic staph infec.[2]	In 10% of cases; some studies say 70-75% cases -- vertebrae, long bones, flat bones [2]	In 25% of cases. -- epiphyses, metaphyses, bony prominences-- clavicles, ribs, vertebrae, hands & feet [2]
Associated w/ periosteal reactions in the tibia?	Rarely [1]	Can affect tibia, mostly lytic lesions [2]	Can affect tibia; mostly lytic lesions [2]	Can affect tibia; mostly lytic lesions [2]	Rare, minimal [2]
Disease reported in Chile/Peru?	Yes (some forms) [1,3,4]	Yes (but not reported in Chinchorro) [3,4,5]	Yes [5,6]	Yes [2]	Yes, Paracoccidioidomyco-sis [2,8]
Affect multiple skeletal elements	Yes [1,2]	Yes [2]	Yes [2]	Yes [2]	Yes [2]
Can be fatal?	Visceral form, 75-95% of cases [1]	Yes, 35-40% within 5 yrs in antiquity [2]	Yes [2]	Rarely, can be chronic [2]	Often [2]
Sex	2:1 (m-f) [1]	Usually men more than women [2]	Mostly males [2]	2:1 (m-f) [2]	15:1 (m-f) [8]
Affect children?	Yes, but risk varies w/type [1]	Yes [2]	Yes [2]	Yes, but more common in adults [2]	Primarily no (more common in adults) [2]

Sources:[1] CDC, 2008; [2] Aufderheide and Rodríguez-Martín, 1998:133, 172-179, 257; [3]Allison et al., 1981; [4]Allison et al.,1982; [5] Arriaza, 1995:80;[6] Davidson and Horowitz, 1970; [7] Zimmerman and Kelley, 1982;[8] Steinbock, 1976 [6] Arriaza et al., 1995; Allison et al., 1979:673; [8] Schoefield, Hospenthal, and Boxwalla, 2006.

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adults (CDC, 2008b).

Bacterial infections are a form of infectious disease that can cause osseous lesions and, depending on the pathogen, pulmonary infections; for that reason they were considered in the differential diagnosis. No data have been gathered on how many individuals possess both osseous lesions and pulmonary infections, but the number may be significant.

Tuberculosis and uta, a form of skin tuberculosis in Peru that causes localized facial lesions were considered. In the late Arica groups, 2% of had tuberculosis infections (Arriaza et al., 1995). When tuberculosis attacks skeletal elements, the bacilli travel hematogenously and focus first on hematopoietic marrow (trabecular bone), instead of the cortex or medullary cavity (Aufderheide and Rodríguez-Martín, 1998:133). This explains why the vertebrae are affected in individuals of all ages. However, the vertebrae and ribs studied by Standen and Arriaza (2000b) were either affected less frequently -- 2.5% (1/40) for vertebrae or 11.6% (5/43) for ribs, or the pathologies in them were not treponemal and possibly not noted. However, when comparing the long bones predominantly affected by tuberculosis and those in the sample, the results were similar, and if the fibulae and femora were reversed, it would be exact. Reasons to exclude tuberculosis include the lack of pathology predominance in epiphyses and metaphyses, and the greater number of proliferative periosteal and osteoblastic reactions versus osteolytic reactions (Aufderheide and Rodríguez-Martín, 1998:134-137).

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Streptococcus pyogenes (Group A *Streptococcus*) can lead to joint and bone infections, and *Staphylococcus aureus* can cause ulcers and osteomyelitis. Both pathogens are infectious bacteria that can spread hematogenously, and have the potential to cause destructive bone lesions. Bacterial infections, such as osteomyelitis were seen on the skeletons and mummies from this region. These bacteria can cause large and multiple sequestra and involcrum, which are sometimes perforated by cloacae from draining abscesses (Aufderheide and Rodríguez-Martín, 1998:172-177). The osseous effects of these bacteria should be distinct enough to be separated from treponemal infections. However, the tertiary effects of treponemal infections can be confused with sclerosing osteomyelitis of Garré (chronic nonsuppurative osteomyelitis/cortical idiopathic sclerosis), as both cause deposition of bone and the narrowing of the medullary cavity (Aufderheide and Rodríguez-Martín, 1998:179).

The pattern of infections in these particular pathogenic bacteria when systemic, is different from that seen in treponematosis. The osteomyelitis-causing bacteria primarily affect the knee region first, followed by the distal third of the tibia, and proximal third of the femur (Calandruccio, 1967; Aufderheide and Rodríguez-Martín, 1998). Multiple skeletal elements are affected in only 5-25% of cases, while in the remainder only a single bone is represented (Aufderheide and Rodríguez-Martín, 1998:172-177). Infection of multiple skeletal elements, and primarily the same skeletal elements, as is seen in the Chilean sample, is uncommon in osteomyelitis.

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Additionally, these pathogens predominately affect males, possibly, because they are more prone to trauma (Aufderheide and Rodríguez-Martín, 1998:172). While chronic infection can occur, acute osteomyelitis can be fatal (Aufderheide and Rodríguez-Martín, 1998:172). There was only one apparently fatal case of what appears to be a severe disseminated osteomyelitis from an Arica skeleton thought to be contemporary with the Chinchorro (Yungay-372, Str.1, Cuerpo 1B). This skeleton has not yet been dated, and it was not included in Standen and Arriaza's (2000b) study. This skeleton, a young adult female, showed signs of osteomyelitis in every bone of the body, with few signs of healing. The skeletal elements had a worm-eaten appearance, with thick sclerotic involcrum and numerous large cloacae.

Brucellosis was considered because these groups engaged in horticulture and may have lived with or near animals and consumed their secondary products. Incubation of the disease ranges from a month to several months (Aufderheide and Rodríguez-Martín, 1998:192). Adults show clinical affects more often when compared to subadults (Aufderheide and Rodríguez-Martín, 1998:192), which is consistent with the study results. Brucellosis spreads hematogenously and skeletal involvement occurs. Long bone infection resembles pyogenic osteomyelitis with a pattern of bone destruction, abscess formation, and prominent periosteal reactions. The long bone infection in the individuals studied here is similar to this; however, there were more osteoblastic than osteolytic reactions, and the vertebrae were rarely affected.

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Fungal infections were considered, again because as noted, pulmonary adhesions were present in several individuals with osseous infections. Blastomycosis, including coccidioidomycosis and paracoccidioidomycosis, are inflammatory granulomatous diseases that have been found in South America, and are caused primarily by inhalation of contaminated soil (Aufderheide and Rodríguez-Martín, 1998:214-219). These individuals lived in a very dusty environment. Coccidioidomycosis can cause skeletal involvement in 25% of untreated cases; in 50% of those cases, it spreads to the central nervous system (Aufderheide and Rodríguez-Martín, 1998), and when it does, death usually results. Additionally, when coccidioidomycosis affects the long bones, it is usually in the epiphyses and metaphyses, and in the bony prominences. Other skeletal elements affected by coccidioidomycosis include those of the hands and feet, the vertebrae, and the ribs.

Paracoccidioidomycosis, when disseminated, shows a preference for the clavicle, ribs, vertebrae, and extremities. It can cause round, lytic lesions in areas that receive a good blood supply (Aufderheide and Rodríguez-Martín, 1998:219). In both diseases, periosteal reactions are minimal, and are not predominant as seen in the individuals studied here. Paracoccidioidomycosis has been reported in a 56-year-old woman from northern Chile dated to AD 290, who had both pulmonary and renal lesions (Allison et al., 1979:673; Aufderheide and Rodríguez-Martín, 1998:219). These lesions were examined on autopsy and through light and electron microscope (Allison et al., 1979:681). Allison and

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colleagues (1973) think the disease may have been imported, as the woman was found buried with tropical bird feathers probably obtained through trade.

Summary of Bone Infections and Differential Diagnoses

Of the diseases reviewed, treponematosis best fits the profile. The fact that the disease affects multiple bone groups, has an affinity for the tibia, and results occasionally in gummatous lesions suggests the disease is treponemal. The skeletal elements most commonly affected in the individuals studied are most consistent with an endemic treponemal infection, either bejel or yaws.

The patterning of the lesions could be interpreted as being indicative of yaws or bejel. The descriptions of the pathological manifestations of yaws, however, include more involvement of the diaphyses of the humerus; this infection pattern is evident in the individuals sampled. However, this one bone is not a reliable indicator of whether the disease is yaws or bejel. The incidence of bilateral periostitis in the tibia is high in the individuals sampled, which is consistent with both yaws and bejel. However, the rate of infection in the tibia within the entire sample is low.

Items contrary to a diagnosis of endemic treponemal disease, include the higher frequency of infection in adults versus subadults, the potentially coincident pulmonary infections, the lack of nasal-palatal involvement, the lack of gondou (tumor-like expansion of the maxillae seen in yaws) and of dactylitis (bone changes in the hands of young individuals commonly seen in yaws), the low

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frequency of infections in the total sample size, and possibly the decrease of this infection in the groups that followed the inhabitants of the coastal Archaic Period.

It is possible that the northern Chilean individuals had multiple infections. As Powell (1988b:179), quoting a paleopathologist acquaintance of hers reminds, "Remember that a dog may have both ticks and fleas," which is to say that more than one pathogen may be involved. A higher rate of pulmonary infections might have made some individuals more susceptible to disease, and might account for the rib infections. Agriculture in the inland valleys may have reduced soil-borne diseases through the introduction of irrigation, which after initial construction may have reduced dust. What seems clear is that the disease was systemic, spread hematogenously once in the body, affected multiple bone groups, had a long and variable delay between transmission and onset of symptoms, and while presumably debilitating, was probably not fatal.

The question remains as to why, the disease appears to have declined on the coast and increased inland around the same time agriculture was adopted. The coastal groups continued to maintain a maritime subsistence in addition to adopting other food productions methods, such as horticulture. The valley groups relied more heavily on agriculture, but perhaps more importantly they were affected, unlike the coastal inhabitants, by people moving from the north and the east into fertile valley. As an infectious disease, treponematosis should disappear if the infectious agent is eliminated, but the low rates in the later coastal groups suggest it was not eliminated (Cochran et al., 2000). If not

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eliminated, then why did the frequency of infection drop? After all, studies suggest that damaging diseases can persist over time indefinitely, providing they do not kill their hosts quickly (Cochran et al., 2000:418-419). Something must have changed to affect the long-term stability of the treponemal infection, which in the coastal Archaic Period was relatively high. The wearing of additional clothing and the loss of the mortuary practice could have reduced disease loads on the coast. Additionally, migration from the north and east toward the valley, instead of the coast may have reduced their contact with affected individuals. It is also possible that natural selection reduced the negative effects of the disease, but it is unclear if significant variation existed for natural selection (variation in environment, variation in vulnerability) to have occurred (Cochran et al., 2000:418)

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Chapter VI.

Summary and Conclusions

The primary focus of this research was to determine using molecular methods, whether the ancient inhabitants of northern Chile were afflicted with treponematosis. To accomplish this, attempts were made to recover treponemal DNA sequences from ancient bone and/or tissue from individuals in this region. The osteological and statistical analyses conducted by Standen and Arriaza (2000b), and the molecular research conducted by this author, indicate that the individuals comprising these groups did suffer from an endemic form of treponematosis, likely bejel or yaws.

This research reports on the discovery of potential treponemal DNA from a mostly skeletonized male from the inland site of AZ-71, T. 601 from the pre-Columbian Cabuza period. This individual was at one time naturally mummified. The DNA was recovered from the interior of the thick sternum bone and was tested using *tprJ1* and *tprJ2* primers (Bouwman and Brown, 2005:706). The sequence recovered was in a non-diagnostic region for the treponemal subspecies, suggesting only that the pathogen is *T. pallidum.* This sequence includes five mismatches, an insertion, and a deletion. Importantly, the sequence was contiguous with the flanking region of the primers used in the amplification, which suggests that this is potentially a viable treponemal sequence. Additionally, the individual from which this sample was collected

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showed signs of systemic bacterial infection. Both the osteological and molecular data support a pre-Columbian treponemal infection in this northern Chilean individual.

The molecular results here are consistent with earlier research in this region by Rogan and Lentz (1994) who recovered DNA sequences, resembling modern syphilis (*T.p. pallidum*), from two Chinchorro mummies. The sequence generated here shows a few, but not unexpected, changes from the modern pathogen. These changes could be due to factors associated with low copy number DNA, or they could represent legitimate changes from the ancient to the modern pathogen. Without replication, it is impossible to say which is true. Taxonomic and phylogenetic studies conducted by Rogan and Lentz (1994) show that the disease present in the northern Chilean groups from Arica and San Miguel de Azapa is most likely a form of treponematosis associated with humans. Their research suggests that the organism might be either a "contemporary unknown infectious agent" or an "extinct relative of a modern treponeme" (Rogan and Lentz, 1994). This idea is given credibility by recent phylogenetic research by Harper and colleagues (2008).

The molecular and osteological analysis of the northern Chilean mummy samples supports aspects of all the treponemal origin and spread hypotheses, except the Unitarian hypothesis. Harper and colleagues (2008) research suggests that the New World yaws strain occupied a phylogenetic position between Old World nonvenereal strains and modern syphilis strains (Harper et

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al., 2008). Others have suggested that the mutation rate was not high enough to have created variants within this period (Gray et al., 2006:2231; Mulligan et al., 2008). However, a variant with characteristics of both syphilis and the nonvenereal treponemes could perhaps explain some of the unusual aspects of the frequency of infection and osseous patterns seen in these groups. These include: (1) the number of bone groups affected, (2) the paucity of subadults affected, (3) the total frequency of infections, (4) the frequency of each bone affected, and (5) frequency of infections within the various periods studied.

Harper and colleagues' (2008) hypothesis states that *T. pallidum* first arose in Africa as yaws (and remained there as yaws, and also possibly as a form of bejel), and then spread with early humans to the Middle East and Eastern Europe where it changed into (or remained as) bejel. This form of bejel was then transferred into the Americas with early migrations, where it changed into a New World yaws strain. This New World strain of yaws was then reintroduced to the Old World possibly via European explorers, "becoming the progenitor" of venereal syphilis, which then spread worldwide (Harper et al., 2008). The authors hypothesize that the New World yaws strain from South America possessed characteristics similar to syphilis while remaining nonvenereal (Harper et al., 2008).

The means of bacterium transmission from Europe to the New World is unclear. Furthermore, the data on bejel is even less clear than that for yaws due to a lack of strains available for research. Therefore, it is possible that a strain of

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treponematosis more closely related to syphilis existed in pre-Columbian South America, and was transmitted through sex. However, because the osseous symptoms of modern syphilis often do not appear until the tertiary stage, and the early inhabitants of northern Chile did not live long, it is very difficult to determine at what age the disease may have been contracted. It is not clear whether a venereal form of treponematosis could have existed in South America and the Caribbean before Columbus' voyages, and it seems unlikely that it could have existed as early as the Archaic Period in coastal northern Chile and southern Peru, and in these small groups. However, no one knows if this New World strain manifested itself in the same manner as the modern treponemal strains. Therefore, it remains possible that Columbus could have imported a venereal strain of endemic syphilis (yaws or bejel) from the New World, or its progenitor that rapidly changed into the venereal form (Harper et al., 2008). This research cannot answer this question.

Osteological analysis of 636 individuals from three periods from the coast and inland valleys of northern Chile (Arica/San Miguel de Azapa) revealed that the individuals studied were likely afflicted with an endemic treponemal infection. The osseous reactions included cortical thickening and bone expansion, lytic defects in the cortex, cavitations, cortical destruction, gummas, and saber shin with proliferative periosteal reactions. On average, less than three bone groups were affected. The skeletal elements were primarily affected on the diaphyses, and the tibia was the most frequently affected bone. There were no bone

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reactions indicative of venereal syphilis; however, the patterning of lesions was suggestive of venereal syphilis when examined for the frequency of infection, number of subadults affected, and hands and feet affected. Cranial lesions were rare, as was nasal-palatal destruction, and there were no signs of congenital syphilis. The disease affected both men and women equally. Subadults in the coastal groups are under-represented, but given their numbers in the later groups studied, the disease appears to have affected adults more often.

Of the skeletal elements with osseous lesions and signs of healing, it can be said that the affected individuals' immune systems were unable to rid the body of infection; yet, they were not fully overcome by it, as there is evidence of healing (Roberts and Manchester, 1995:125; Ortner, 1991; Powell, 1988a,b). It is also important to note that any osteological assessment looked at only one stage of disease in any individual, or in some cases, element (for example: the crania). Most bacterial infections, treponematosis included, do not affect the skeleton unless there is long-term survival (though with a short life span this is relative) (Ortner, 1992b:5-12). The Chinchorros' life expectancy was, on average, only about 24 years. Of those skeletal elements that appeared healthy or unaffected, it can only be said that they did not show signs of tertiary treponematosis, which is different than saying the skeletal elements came from healthy individuals (Ortner, 1992b:5-12; Powell, 1988a,b).

The frequency of infection for Standen and Arriaza's (2000b) sample was 8%, with the highest frequency, 20% (30/150), in the coastal Archaic groups.

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This latter high rate of infection is unusual for groups that were small, in relatively good health, and not traveling or trading widely. Their diet likely provided them with sufficient nutrition to remain active despite the disease load. Parasite loads, from consumption of raw or undercooked fish, shellfish, and sea lions may have affected the health of the earlier coastal inhabitants, making them more susceptible to disease than their inland counterparts. However, inland inhabitants also traveled to the coast for food. The later inland groups had a mixed economy. They practiced maize and other forms of agriculture, which likely increased their nutritional stress; yet, any stress brought about by this, does not appear to have increased the rates of treponematosis significantly (though they did rise slightly). This is not to say that either group, coastal or inland, was not afflicted with health problems.

Both the coastal and inland individuals sampled suffered from several nontreponemal conditions caused by a variety of environmental and cultural factors, including: osteomyelitis, arthritis, osteoarthritis, osteoporosis, caries (87% in post-Chinchorro groups), dental abrasion, periodontia, tooth loss and alveolar resorption, diffuse idiopathic skeletal hyperostosis or DISH, zoonotic infections (tapeworms), iron deficiency anemia, external auditory exotoses, spondylosis, developmental abnormalities, tuberculosis (later groups only), trauma, and the effects of arsenic consumption (Arriaza, 1995a:66-82; Arriaza, pers. comm., 2007). Without written records, or an abundance of artifacts or

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habitation structures, their skeletal and mummified remains are the best remnants of their past behavior.

Standen and Arriaza (2000b) attributed the higher rate of infection among early coastal groups to two factors: 1) wearing minimal clothing, which facilitated the spread of disease from infected cutaneous lesions, and 2) the practice of an elaborate mortuary ritual that necessitated extensive hands-on contact. These researchers do not elaborate on how they determined what clothing the inhabitants wore, or how they came to describe it as minimal; it is assumed that this assessment is based on the preserved clothing found on the early coastal inhabitants.

If clothing is a major factor, inhabitants of the coast probably wore less, as earlier groups wore loin clothes and grass skirts, and presumably hides, hair and/or fur from animals. The only indication of a concern for sanitation is the use of shell middens along the coast. Few structures survived, especially those on the coast, as they were made of perishable or partially perishable materials (Arriaza, 1995a:35). It is unknown how closely homes were located together, or if there were special areas for disposal of human waste or for bathing.

A re-analysis of the raw numbers of affected human remains identified by Standen and Arriaza (2000b), including those in a data sheet provided by Arriaza, and excavation notes suggest that most individuals from the early coastal groups did not receive mummification in the complex red and black styles; this should, reduce the potential for disease spread through the mortuary

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practice. However, given that the percentages of overall mummification techniques are lacking for some of the coastal sites they used (Quiani-7, PLM-3, PLM-4), and Arriaza is unclear, in a few cases, which individuals were sampled, it is impossible to say with certainty that the majority of individuals were mummified in a non-complex style.

That said, presuming the practice of artificial mummification primarily occurred in the Archaic Period, and as there are data, including percentages on overall mummification for the sites of Morro-1, Morro-1/6, and PLM-8, it seems likely that most affected individuals were mummified in a non-complex style. However, this is complicated by the fact that there is only percentage data from three sites, one of which (PLM-8) has a small sample size (10 individuals), and another (Morro-1/6), that only included natural mummies. The extent to which the sample is biased in that natural mummies may have been sampled over red or black mummies is unknown. As most affected individuals appear to have been mummified in a non-complex style, it is possible that the early inhabitants recognized these lesions as a source of contagion in their communities, and deliberately chose natural mummification for them to avoid the spread of disease. However, without further data on exactly who was included in Standen and Arriaza's (2000b) sample, and in what style and technique all 636 individuals in their sample were mummified, it is impossible to say if the complex mummification practices contributed to the spread of disease. Both the mortuary practice and the amount and type of clothing worn changed over time; these

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factors may account for the significant decrease in the rate of treponemal infections between the Archaic coastal groups and the later inhabitants.

While the statistics presented by Standen and Arriaza (2000b) suggest the incidence of disease decreased to negligible rates among the coastal groups sampled over a period of approximately 4,000 years, it is alternatively possible that the disease did not decrease significantly along the coast. The rate of infection for inland groups sampled remained low through all periods, with an average frequency of 3.9%, compared with 18.5% for all coastal groups (Standen and Arriaza, 2000b:186). To consider the disease in perspective, it would be useful to know more about skeletal completeness of the individuals examined. A lack of tibiae or fibulae, for example, could significantly skew the figures.

From the data gathered, it is not clear whether the infection in these samples was yaws, as Standen and Arriaza (2000b) suggest, or bejel. The osseous lesions are indicative of either, with perhaps a slight preference toward yaws based on skeletal elements affected. However, bejel is a better match for the environment, and a variety of discriminating factors for treponematosis (Rothschild et al., 2000:937).

The first null hypotheses tested stated that treponemal spirochetes could not be recovered and amplified from ancient bone, tissue, and or/organs, using molecular techniques. The recovery of potential treponemal DNA from bone suggests, that while difficult, it is possible to recover treponemal DNA from ancient bone. In this case, the null hypothesis can be rejected, at least

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tentatively, pending independent replication. In this research, bone proved to be a better starting material than soft tissue. No treponemal DNA was recovered from muscle or organ tissue. This does not suggest that it is impossible to recover treponemal DNA from soft tissues only that it did not happen here. Muscle tissue during this project showed a higher rate of contamination. While it was thought that mummified tissues might be a better material for the recovery of ancient treponemes, this was not shown to be the case here. However, the mummies studied had been handled over the years, and some of their mummification materials were lost to natural processes, and/or to autopsies and osteological studies. Tissues from recently excavated mummies or those less frequently handled may hold greater promise.

The second hypothesis tested asked whether the pathologies present on the northern Chilean mummies represented a probable treponemal infection. It was determined that the patterning of the osseous lesions and periosteal reactions seen on the individuals examined do represent a treponemal infection, and it is probably bejel or yaws. The molecular data, which identified a *T. pallidum* infection, also supports a treponemal infection. Therefore, the null hypothesis is rejected.

The third hypothesis tested was that treponematosis was not in the New World before European contact. The potential treponemal sequence generated, the molecular and phylogenetic research conducted by Rogan and Lentz (1994), the osteological indicators of treponemal disease and, most importantly, the pre-

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Columbian radiocarbon dates generated, allow for the rejection of the null hypothesis, and provide convincing evidence that the disease present in these individuals sampled was treponemal and pre-European contact.

The fourth hypothesis, which states that the mortuary practice was not the likely cause of a higher frequency of treponemal infection in coastal groups sampled, cannot be properly evaluated without more data on mummification style of all 636 individuals analyzed by Standen and Arriaza (2000b). If just the 51 affected individuals are considered, most of which were naturally mummified, the hypothesis could not be rejected. However, given the paucity of data, this question is best left for a time when complete information can be compiled and radiographs and/or digital images of the skeletal elements of the red and black mummies can be recorded.

It is not clear to what degree other factors, such as clothing, status, other cultural behaviors, or even how the statistics were gathered, might be related to the observed frequency of infection. Regardless, the high rate appears to be unique to the Archaic coastal inhabitants. Still, there is no evidence to indicate, other than the fact that the elaborate mummification practice faded on the coast around 1100 BC and did not occur in the non-Chinchorro cultures (coastal and inland), that the Chinchorro mummification ritual contributed to their higher rate of treponemal disease. The missing data on mummification techniques, as well as the absence of evidence, in light of the few infections in the small number of red and black mummies in Standen and Arriaza's (2000b) count of affected coastal

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individuals, is not convincing enough to reject the null hypothesis. It appears that the majority of the individuals sampled (affected and nonaffected) are naturally mummified, thus sampling bias is a possibility. However, considering only the data presented here, it is possible that either an increase in clothing worn or some as yet unrecognized factor played a role in the high frequency of infections in the coastal Archaic groups. It could be that more complex mortuary treatment does reflect higher status individuals and vice versa. If this were the case, then perhaps socioeconomic differences did play a role in differential infection rates.

To address properly this question, all Chinchorro sites and mummies from those sites, including those of the complex black and red styles, would need to be evaluated for treponematosis. Total population numbers, and percentages of those affected and unaffected would be required. Comparing these data with non-Chinchorro groups, however, is complicated by the fact that later groups did not practice artificial mummification. Additionally, a temporal component cannot be addressed. For much of the time the Chinchorro inhabited the coast of northern Chile; there were no or few similarly sized settlements inland in the Azapa valley. Settlements during the same period were primarily to the north and east in Peru and Bolivia – particularly in the highlands. The differences in environment, subsistence, and culture could be significant and might affect the dynamics of disease spread; they, therefore, do not lend themselves easily to comparison. The best bet may be an internal comparison between Chinchorro mummified in the complex black and red styles, and those in less complex styles;

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a study of this kind, if possible, might offer some answers not only to the spread of treponematosis but also to status, and perhaps to who in the community was responsible for mummifying the dead.

This research has described an infection that affected the ancient northern Chilean and southern Peruvian mummies (Standen and Arriaza, 2000b:186-187). The potential treponemal sequence generated from an individual from the later inland Azapa valley suggests the disease that caused their osseous lesions and periostitis was treponematosis. The analysis of these individuals supports the research of Rogan and Lentz (1994) and possibly Harper and colleagues (2008). Pending replication, it contradicts Bouwman and Brown (2005) who said treponemal DNA could not be recovered from ancient bone, and supports Kolman and colleagues (1999) who did recover treponemal DNA from bone.

Suggestions for Future Ancient DNA Research

This addendum could be titled, "if I knew then, what I know now," as it seeks to address alternative methods that have been tested, developed, or recognized as beneficial in the analysis of ancient, degraded, or low copy number DNA since the start of this project. Perhaps the most useful publication addressing the analysis of ancient DNA is by Rohland and Hofreiter (2007), which addresses an obvious gap in the ancient DNA and forensic literature by comprehensively comparing extraction techniques. Many methods are in use in ancient DNA and forensic laboratories today; however, few researchers have the time, money, and samples necessary to conduct side-by-side comparisons of

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protocols. Rohland and Hofreiter (2007) used eight cave bear (*Ursus spelaeus*) bone samples and two cave bear teeth samples to do just this (Rohland and Hofreiter, 2007:343). All of their samples were dated by accelerator mass spectrometry to the Pleistocene (age estimates between 27,000 years and 44,000 years) (Hofreiter et al., 2004; Noonan et al., 2005; Rohland and Hofreiter, 2007:343). The samples originated from nine caves in Austria, Switzerland, Slovenia, Croatia, Russia, and Germany (Rohland and Hofreiter, 2007:343). The goal of their project was to compare these different protocols to see which recovered the most DNA. They assessed a variety of factors, including chemical additives to the extraction buffer, varying incubation time, and fluctuating incubation temperature (Rohland and Hofreiter, 2007:344). All samples were powdered consistently in a Spex 6750 freezer mill (Spex SamplePrep, Metuchen, NJ). Negative controls were run with all experiments. The DNA yields were compared to the best method in each series to determine their performance, and applied paired Student t-tests helped in this assessment (Rohland and Hofreiter, 2007:344).

The results of the first set of their experiments using 40 mg samples showed that there were “large differences both within and among methods.” Their results of quantitative PCR comparing the extraction methods using 40 mg showed the best performing method was the DNA IQ system (Promega, Fitchburg, WA) (Rohland and Hofreiter, 2007).

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In their next set of experiments, they used 200 mg samples and a 1:10 dilution, in which they found that an extraction based on digestion of the powdered sample in a buffer with EDTA, proteinase K, dithiothereitol (DTT), polyvinylpyrrolidone (PVP), N-phenacyl thiazolium bromide (PTB), and N-lauryl-sarcosine sodium salt (sarcosyl), and purification of DNA via silica binding to high concentrations of guanidinium thiocyanate (GuSCN) worked best.

Their best optimized method used a buffer consisting entirely of EDTA (minimum concentration of 0.2M) and proteinase K for bone digestion and binding DNA to silica via GuSCN for purification – this straightforward method bested all others tested on average (Rohland and Hofreiter, 2007:343, 346). They also found that grinding the samples to a fine powder was very important, as fine powder gave higher yields (more surface area for binding). They also found that incubation temperatures (room temperature at 56 °C) and incubation times (1- 4 days) resulted in only minor effects to DNA yields (Rohland and Hofreiter, 2007:346: table 4). They recommended overnight digestion.

As to the purification results, they found that sodium chloride (NaCl) performed better than all the chaotrophic salts they conducted (Rohland and Hofreiter, 2007:347-348). However, both NaCl and GuSCN have been associated with the co-purification of inhibitors, and as such, may not be right for all samples. As for the silica suspension, they found that the pH of the binding buffer had a significant effect on DNA yields, with a pH of 4.0 giving the best results (Rohland and Hofreiter, 2007:347). The incubation time for the binding

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step was also important, up to three hours was found to increase yields (Rohland and Hofreiter, 2007:347).

The authors also tested the effect of PCR additives with varying amounts of taq DNA polymerase. They found that BSA was helpful in some instances, and recommended the use of 1 mg/mL in PCRs known to have inhibitors, and a minimum of 0.25 U taq DNA polymerase. The authors acknowledged that the results they present might not work best for every sample or for every type of inhibitory substance, as taphonomic effects vary among sample collection sites and could influence results (Rohland and Hofreiter, 2007:351). Their study does however, begin to address a gap in the methodological literature, and points out the need for more empirical data.

Recent research from several other authors has also offered suggestions for the analysis of ancient and LCN DNA. Davoren and colleagues (2007) engaging in forensic research using skeletal remains from the mass graves of victims killed in the Balkans between 1992 and 1995, also recommended a silica binding method using GuSCN and chaotrophic salts (Davoren et al., 2007:479) in their experiments generating profiles with short tandem repeats (STRs). Their results indicate that they extracted on average 1.94 ng of DNA per gram of bone (range 0.259.58 ng/g), compared with 0.68 ng/g from the standard organic phenol-chloroform method alone (range 0.0016-4.4880 ng/g) (Davoren et al., 2007:478, 492).

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Loreille and colleagues (2007) recommend DNA extraction via a total demineralization protocol, which results in full physical dissolution of the bone sample, in a way that retains and concentrates the reagent volume. This method helps address the loss of DNA when the supernatant is collected (from the lysis buffer), and the undissolved powder (with remaining DNA) is discarded. DNA is also discarded in this same manner in wash solutions when EDTA is used prior to separation of the bone powder for extraction (Loreiile et al., 2007). Their study compared the DNA yield recovered using a complete demineralization with the DNA yield recovered using a standard protocol that involves the disposal of undissolved quantities of skeletal material (Loreille et al., 2007). Samples tested included 14 human skeletal elements with varying degrees of preservation ranging from five to 100 years post-mortem. Six of these skeletal elements had been previously extracted by the Armed Forces DNA Identification Laboratory (AFDIL), and were sequenced successfully (Loreiile et al., 2007; Edson et al., 2004). Experiments were conducted to compare the AFDIL casework protocol with a total demineralization protocol (Edson et al., 2004). The results indicated that the total demineralization method yielded higher amounts of DNA than the standard AFDIL protocol. On average total demineralization resulted in about 4.6 times more DNA recovered (Loreille et al., 2007).

Fundamental in ancient DNA analysis is the ability to test multiple extraction methods. However, an inherent problem with this, is that it requires the destruction of multiple samples, something that should be done with much

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forethought and great hesitation given what is known about the bacterium's ability to survive in bone and tissues, and under various environmental conditions (von Hunnius et al., 2007). Future efforts on the remaining aliquots will concentrate on extraction techniques. Multiple aliquots will be used to test the demineralization methods as described by Loreille and colleagues (2007), the silica binding method using GuSCN and chaotrophic salts described by Davoren and colleagues (2007), and the DNA IQ system (Promega, Fitchburg, WA) among others (Rohland and Hofreiter, 2007). Experiments will also be conducted using varying amounts of starting material to determine the least amount of sample that can be used to obtain replicable results. Experiments will also concentrate on methods to identify, limit, and develop new chemical protocols to counter the effects of inhibitors. Future experiments will also include more extensive testing of PCR additives such as BSA and Spermidine, as well as various taq polymerases. Pre-treatment repair methods and PCR enhancing additives that improve sensitivity and specificity during amplification of genomic DNA or RNA templates will also be investigated. Phylogenetic studies using 16S, and replication of ancient nucleic acids using 18S, 5.8S, and 28S ribosomal DNA genes may also be attempted. These analyses will allow for a better assessment of the integrity of the DNA, the amplification process, and any potential contamination (Rogan and Salvo, 1994:184). Collaborations with established researchers in the field, particularly those associated with ancient DNA laboratories, will be sought for future projects.

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The potentially positive treponemal sample identified in this research, holds great promise. If this sample can be replicated at two independent laboratories, it will be the first validated evidence of treponematosis in a sample older than 200 years. As such, it will be the first validated molecular evidence of treponemal infection in Pre-Columbian America. While Rogan and Lentz (1994) found evidence of potential Pre-Columbian treponemal infections, their work was never validated. If an additional sample from this individual can be amplified with the *tprC* and *tprI* primers, the subspecies of treponemal infection may be identified. Validation of treponematosis in this sample may open up the door for molecular anthropologists to test samples previously identified with treponematosis based on osteological analysis alone. This identification would help biologists map the spread of *T. pallidum* infections, and would help them to understand how and why treponemal bacteria diversified over time. Understanding *T. pallidum's* past contacts will open a window toward sequencing the genomes of the remaining pathogenic treponemes, and toward preventing treponemal disease in modern populations.

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Appendix A

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Appendix B

Radiocarbon Dating Reports

Note: Data presented here is from original reports from Beta Analytic, Inc. (Miami, FL.), and Lawrence Livermore National Laboratory Center for Accelerator Mass Spectrometry (Livermore, CA.).

BETA

BETA ANALYTIC INC.

DR. M.A. TAMERS and MR. D.G. HOOD

UNIVERSITY BRANCH
4985 S.W. 74 COURT
MIAMI, FLORIDA, USA 33155
PH: 305/667-5167 FAX: 305/663-0964
E-MAIL: beta@radiocarbon.com

REPORT OF RADIOCARBON DATING ANALYSES

Ms. Michelle Kaye

Report Date: 7/18/2007

Material Received: 6/19/2007

Sample Data	Measured Radiocarbon Age	13C/12C Ratio	Conventional Radiocarbon Age(*)
Beta - 231777	2270 +/- 40 BP	-18.5 o/oo	2380 +/- 40 BP

SAMPLE : AZ70-T7C2
ANALYSIS : AMS-Standard delivery
MATERIAL/PRETREATMENT : (muscle - skin): acid/alkali/acid
2 SIGMA CALIBRATION : Cal BC 720 to 700 (Cal BP 2670 to 2650) AND Cal BC 540 to 390 (Cal BP 2490 to 2340)

Sample Data	Measured Radiocarbon Age	13C/12C Ratio	Conventional Radiocarbon Age(*)
Beta - 231778	890 +/- 40 BP	-10.1 o/oo	1130 +/- 40 BP

SAMPLE : PLM3-T142
ANALYSIS : AMS-Standard delivery
MATERIAL/PRETREATMENT : (muscle - skin): acid/alkali/acid
2 SIGMA CALIBRATION : Cal AD 780 to 1000 (Cal BP 1160 to 950)

Sample Data	Measured Radiocarbon Age	13C/12C Ratio	Conventional Radiocarbon Age(*)
Beta - 231779	3710 +/- 40 BP	-17.4 o/oo	3830 +/- 40 BP

SAMPLE : MORRO1-CH22
ANALYSIS : AMS-Standard delivery
MATERIAL/PRETREATMENT : (muscle - skin): acid/alkali/acid
2 SIGMA CALIBRATION : Cal BC 2460 to 2190 (Cal BP 4410 to 4140) AND Cal BC 2180 to 2140 (Cal BP 4120 to 4100)

Dates are reported as RCYBP (radiocarbon years before present, "present" = 1950A.D.). By International convention, the modern reference standard was 95% of the C14 content of the National Bureau of Standards' Oxalic Acid & calculated using the Libby C14 half life (5568 years). Quoted errors represent 1 standard deviation statistics (68% probability) & are based on combined measurements of the sample, background, and modern reference standards.

Measured C13/C12 ratios were calculated relative to the PDB-1 international standard and the RCYBP ages were normalized to -25 per mil. If the ratio and age are accompanied by an (*), then the C13/C12 value was estimated, based on values typical of the material type. The quoted results are NOT calibrated to calendar years. Calibration to calendar years should be calculated using the Conventional C14 age.

Figure B-1 Report of radiocarbon analysis from Beta Analytic

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CALIBRATION OF RADIOCARBON AGE TO CALENDAR YEARS

(Variables: C13/C12=-18.5:lab. mult=1)

Laboratory number: **Beta-231777**

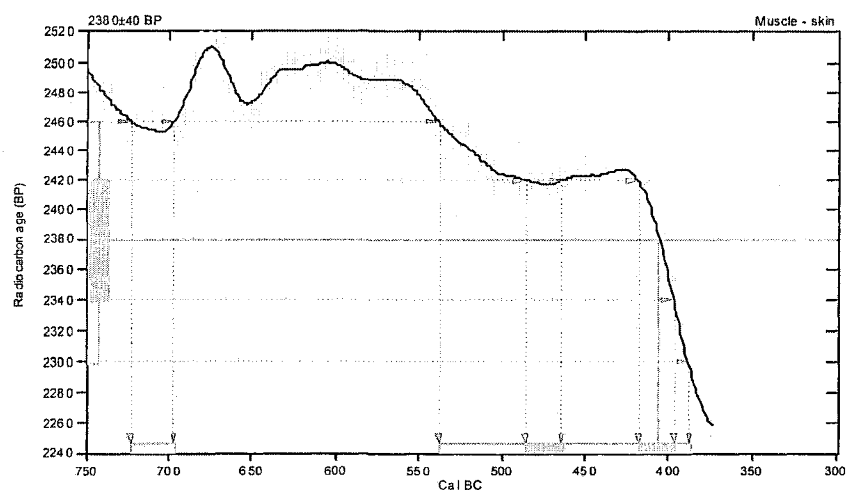
Conventional radiocarbon age: **2380±40 BP**

2 Sigma calibrated results: **Cal BC 720 to 700 (Cal BP 2670 to 2650) and**
(95% probability) **Cal BC 540 to 390 (Cal BP 2490 to 2340)**

Intercept data

Intercept of radiocarbon age
with calibration curve: Cal BC 410 (Cal BP 2360)

1 Sigma calibrated results: Cal BC 490 to 460 (Cal BP 2440 to 2410) and
(68% probability) Cal BC 420 to 400 (Cal BP 2370 to 2350)



References:
Database used
INTCAL04
Calibration Database
INTCAL04 Radiocarbon Age Calibration
IntCal04: Calibration Issue of Radiocarbon (Volume 46, nr 3, 2004).
Mathematics
A Simplified Approach to Calibrating C14 Dates
Talma, A. S., Vogel, J. C., 1993, Radiocarbon 35(2), p317-322

Beta Analytic Radiocarbon Dating Laboratory
4985 S.W. 74th Court, Miami, Florida 33155 • Tel: (305)667-5167 • Fax: (305)663-0964 • E-Mail: beta@radiocarbon.com

Figure B-2 Calibration of radiocarbon age to calendar years, AZ-70, T.7 C2

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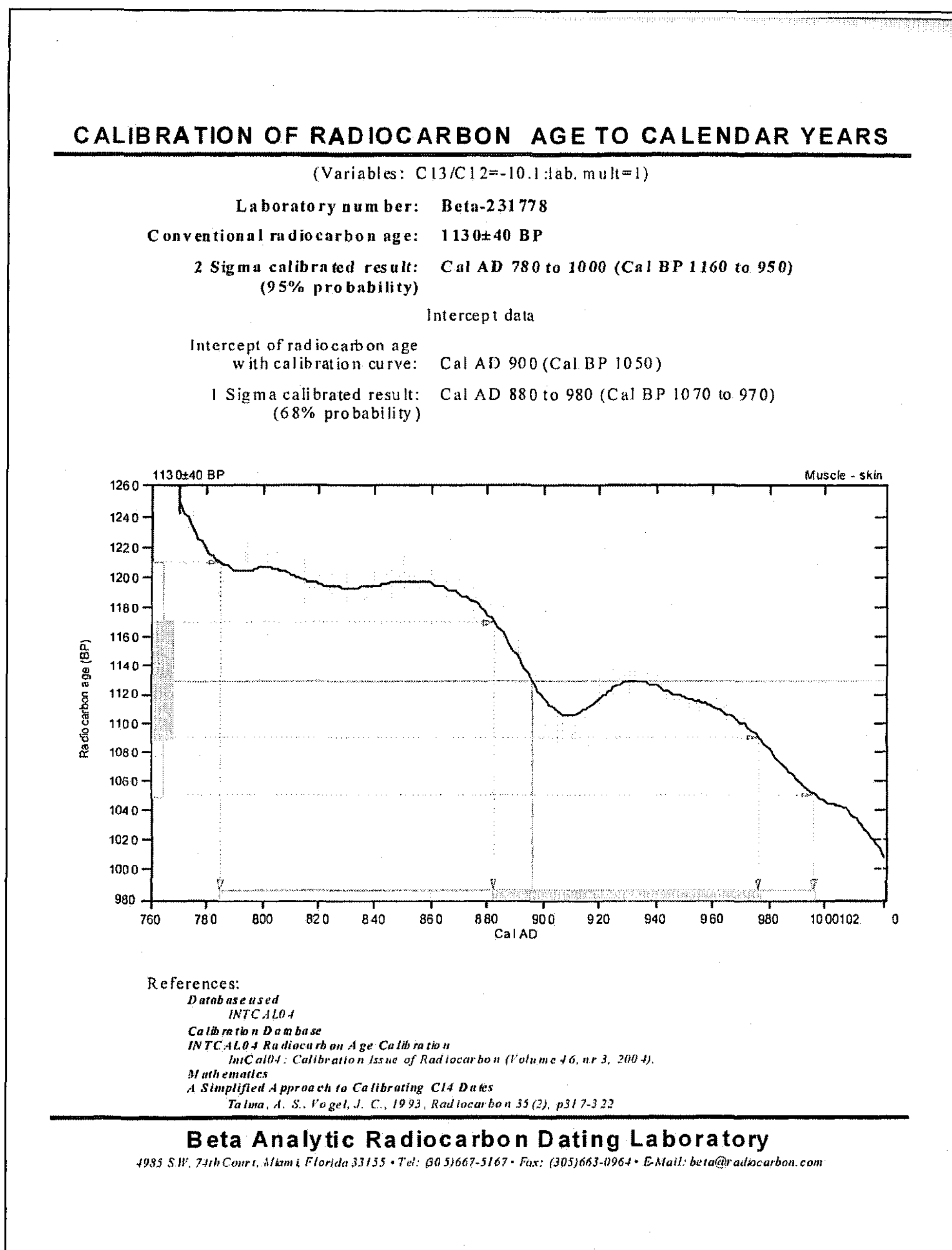

CALIBRATION OF RADIOCARBON AGE TO CALENDAR YEARS

(Variables: C13/C12=-10.1:lab. mult=1)

Laboratory number: Beta-231778

Conventional radiocarbon age: 1130±40 BP

2 Sigma calibrated result: Cal AD 780 to 1000 (Cal BP 1160 to 950)
(95% probability)

Intercept data

Intercept of radiocarbon age with calibration curve: Cal AD 900 (Cal BP 1050)

1 Sigma calibrated result: Cal AD 880 to 980 (Cal BP 1070 to 970)
(68% probability)

References:

Database used
INTCAL04
Calibration Database
INTCAL04 Radiocarbon Age Calibration
IntCal04: Calibration Issue of Radiocarbon (Volume 46, nr 3, 2004).
Mathematics
A Simplified Approach to Calibrating C14 Dates
Talma, A. S., Vogel, J. C., 1993, Radiocarbon 35(2), p317-322

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Figure B-3 Calibration of radiocarbon age to calendar years, PLM-3 T. 142

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CALIBRATION OF RADIOCARBON AGE TO CALENDAR YEARS

(Variables: C13/C12=-17.4:lab. mult=1)

Laboratory number: **Beta-231779**

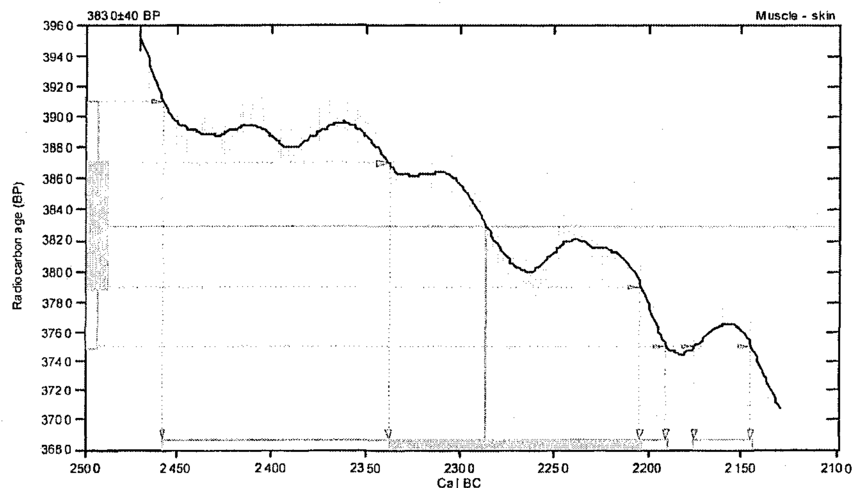
Conventional radiocarbon age: **3830±40 BP**

2 Sigma calibrated results: **Cal BC 2460 to 2190 (Cal BP 4410 to 4140) and**
(95% probability) **Cal BC 2180 to 2140 (Cal BP 4120 to 4100)**

Intercept data

Intercept of radiocarbon age with calibration curve: Cal BC 2290 (Cal BP 4240)

1 Sigma calibrated result: Cal BC 2340 to 2200 (Cal BP 4290 to 4150)
(68% probability)



References:

Database used
INTCAL04
Calibration Database
INTCAL04 Radiocarbon Age Calibration
IntCal04: Calibration Issue of Radiocarbon (Volume 46, nr 3, 2004).
Mathematics
A Simplified Approach to Calibrating C14 Dates
Talma, A. S., Vogel, J. C., 1993, Radiocarbon 35(2), p317-322

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Figure B-4 Calibration of radiocarbon age to calendar years, Morro-1, CH. 22

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CENTER FOR ACCELERATOR MASS SPECTROMETRY
Lawrence Livermore National Laboratory
^{14}C results

Submitter: Matheus DATE: July 31, 2007

CAMS #	Sample Name	Other ID	$\delta^{13}C$	fraction Modern	±	$D^{14}C$	±	^{14}C age	±
133581	1-1928-792	N80094	-13	0.8728	0.0026	-127.2	2.6	1095	25
133582	AZ 71 T601	N80095	-13	0.7662	0.0030	-233.8	3.0	2140	35

1) $\delta^{13}C$ values are the assumed values according to Stuiver and Polach (Radiocarbon, v. 19, p.355, 1977) when given without decimal places. Values measured for the material itself are given with a single decimal place.

2) The quoted age is in radiocarbon years using the Libby half life of 5568 years and following the conventions of Stuiver and Polach (ibid.).

3) Radiocarbon concentration is given as fraction Modern, $D^{14}C$, and conventional radiocarbon age.

4) Sample preparation backgrounds have been subtracted, based on measurements of samples of ^{14}C-free collagen based on historical UAK results.

5) No sample containing Thistle Creek bear bone collagen was in the package.

Figure B-5 CAMS LLNL radiocarbon report, AZ- 71, T. 601 (The first date listed is unaffiliated with this study)

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CENTER FOR ACCELERATOR MASS SPECTROMETRY
Lawrence Livermore National Laboratory
^{14}C results

Submitter: Matheus DATE: August 21, 2003

CAMS #	Sample Name	Other ID	$\delta^{13}C$	fraction Modern	±	$D^{14}C$	±	^{14}C age	±
134138	1-06-2 2:3 Morro		-15	0.6110	0.0022	-389.0	2.2	3955	30

1) $\delta^{13}C$ values are the assumed values according to Stuiver and Polach (Radiocarbon, v. 19, p.355, 1977) when given without decimal places. Values measured for the material itself are given with a single decimal place.

2) The quoted age is in radiocarbon years using the Libby half life of 5568 years and following the conventions of Stuiver and Polach (ibid.).

3) Radiocarbon concentration is given as fraction Modern, $D^{14}C$, and conventional radiocarbon age.

4) Sample preparation backgrounds have been subtracted, based on measurements of samples of ^{14}C-free coal. Backgrounds were scaled relative to sample size.

Figure B-6 CAMS LLNL radiocarbon report, Morro-1/6, T. 7

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Appendix C

Data used by Standen and Arriaza (2000b)

Note: Data is from an Excel spreadsheet used by Standen and Arriaza and given to this researcher by Arriaza

CASOS DE POSIBLE TREPONEM

POBLACIONES ARCAICAS (COSTA)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
ACHA3	C1	CHINCHORRO	25-30	M	Tibias	cuerpo completo		incipiente	
MI1	T27/C5	CHINCHORRO	25-30	M	Tibias + perone D + cubitos + clavicula I	cuerpo completo	Rx	severa	D-63
MI1	T27/C4	CHINCHORRO	>40	M	Tibias	cuerpo completo	Rx	incipiente	D-52
MI1	T15/D1	CHINCHORRO	20-22	M	Tibias + perone I + cubito D	cuerpo completo	Rx	severa	D-33
MI1	T22/C6	CHINCHORRO	15-15	M	Ext. inferiores + cubito d. + acromion	ausente solo craneo	Rx	severa	C-42
MI1	T4	CHINCHORRO	25-30	M	Tibias + perones + extr. Sup. + costillas	ausente solo craneo	Rx	incipiente	D-97
MI1	T28/C11	CHINCHORRO	>40	M	Femur + cubito + radio (derechos)	cuerpo incompleto		incipiente	D-85
MI1	T16A	CHINCHORRO	24-28	M	Tibias izquierda	cuerpo incompleto	Rx	incipiente	D-24
MI1	D.4	CHINCHORRO	A.E.L	M	Tibias	cuerpo incompleto		incipiente	D-7
MI1	D.6	CHINCHORRO	16-19	M	Clavicula d + costales + lumbares	cuerpo incompleto		moderada	D-10
MI1	T27/C8	CHINCHORRO	35-40	F	Tibias	cuerpo completo		incipiente	
MI1	T9	CHINCHORRO	35-40	F	Tibias	cuerpo completo	Rx	incipiente	D14/15
MI1	T29/C15	CHINCHORRO	35-38	F	Tibias	ausente solo craneo	Rx	minimo	D-58
MI1	14 E	CHINCHORRO	20-22	F	Tibias + perone izq.	cuerpo incompleto	Rx	severa	SC-I
MI1	T28/C9	CHINCHORRO	20-22	F	Tibias + perones + cubitos + radio y humero der.	cuerpo incompleto	Rx	severa	
MI1	C-I-18	CHINCHORRO	25-40	F	Tibias + perone D + cubitos + clavicula I	cuerpo incompleto		moderada - severo	D-92
MI1	T18/C2	CHINCHORRO	32-35	F	Costillas (2) + acromion izq.	cuerpo incompleto		incipiente	D-57
MI1	T38/C38	CHINCHORRO	18-21	F	Tibias	cuerpo incompleto		menos incipiente	D-80
MI1	T27/C5	CHINCHORRO	7.5-8.5	I	Tibias + cubitos	cuerpo completo	Rx	incipiente	D86
MI-6	T7	CHINCHORRO	>40	M	Tibias	cuerpo completo	Rx	incipiente	D131
MI-6	T21	CHINCHORRO	35-40	M	Tibias	ausente solo craneo	Rx	incipiente, menos	D146
MI-6	U7	CHINCHORRO	20-35	M	Tibias izq.	cuerpo incompleto	Rx	severa	D-115
MI-6	T17	CHINCHORRO	35-40	M	Tibias izq.	cuerpo incompleto	Rx	incipiente	D143
MI-6	T5	CHINCHORRO	20-22	F	Tibias + femures + humeros	ausente solo craneo	Rx	moderada-severa	D132
MI-6	T33	CHINCHORRO	35-40	F	Tibias + perones + cubito der.	cuerpo incompleto	Rx	moderada	D155
MI-6	U8a	CHINCHORRO	30-35	F	Cubito izq.	restos oseos	Rx	severa	D103
MI-6	U8b	CHINCHORRO	14-15	I	Tibia der.	restos oseos		incipiente	D-103
MOE	T41A	CHINCHORRO	5-6	I	Huesos largos + coxales	cuerpo incompleto	Rx	severa	D168
PLM8	T32	CHINCHORRO	35-40	F	Huesos largos + metacarpianos + craneo	cuerpo incompleto	Rx	severa	A-103
CAM17	T1/C1	CHINCHORRO	35-40	F	huesos largos + coxales + costillas	cuerpo completo		moderado	C280

POBLACIONES FORMATIVAS (COSTA)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
QUIANI	T6	QUIANI	40-45	F	Tibias	cuerpo completo		menos incipiente	B158
MI-6	T1	FALDAS MORRO	20-22	F	Huesos largos + coxal izq.	ausente solo craneo	Rx	incipiente	C117

POBLACIONES I TARDIO (COSTA)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
PLM3	T142	D. REGIONAL	58	M	Tibias + perones + radios + cubito	cuerpo completo			A103

POBLACIONES FORMATIVAS (VALLE)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
AZ71	T3 26	AZAPA	25-29	F	Tibia izq.	cuerpo completo		incipiente	
AZ70	TM7/C2	ALTO RAMIREZ	>40	F	Huesos largos + calcaneos	ausente solo craneo	Rx	severa	B181
AZ115	T1 88	ALTO RAMIREZ	35-40	M	Tibia + perone der.	ausente solo craneo	Rx	incipiente-moderada	C173

POBLACIONES PERIODO MEDIO (VALLE)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
AZ6	T108	CABUZA	14-16	M	Tibia der.	cuerpo incompleto		incipiente (-)	D200
AZ141	T53	CABUZA	20-22	M	Huesos largos+escapulas+metatarsianos	cuerpo completo	Rx	severa	C-211
AZ71	TCt	CABUZA	22-26	M	Tibia izquierda	cuerpo completo	Rx	incipiente	B-4
AZ71	T501	CABUZA	40-45	M	Tibias + clavicula iz + cubito iz.	cuerpo completo	Rx	Severo	B30
AZ71	T 69	TIWANAKU	13-15	M	Tibias - perone d.	cuerpo completo			B35
AZ71	T102	CABUZA	20-25	F	Tibias + perone iz.	cuerpo completo		severo	B16
AZ71	T230	CABUZA	8-10	M	Tibias + cubitos	cuerpo incompleto		incipiente	B60
AZ140	T91	MAITAS	35-40	M	Tibia d.	cuerpo completo		incipiente	C61
AZ140	TXP89	MAITAS	35-40	M	Tibia izq. + femur d.	cuerpo completo		incipiente	C33
AZ140	T129	MAITAS	35-40	F	Tibias	cuerpo completo		moderada	C41
AZ140	T58	MAITAS	35-40	F	Tibias izq. + cubito iz.	cuerpo completo	Rx	severa	C53
AZ140	T112A	MAITAS	48	F	Huesos largos general + craneo	cuerpo completo	Rx	severa	C112
AZ140	TXP5	MAITAS	20-25	F	Tibias + perone + cubito d.	cuerpo completo		moderada	C24
AZ140	T65	MAITAS	15-17	M	Tibias + radio + cubito d.	cuerpo completo	Rx	incipiente-moderado	C66

POBLACIONES PERIODO INTERMEDIO TARDIO (VALLE)

SITIO	TUMBA	CULTURA	EDAD	S	HUESOS AFECTADOS	CONSERVACION	Rx	OBSERVACIONES	LOCUS
AZ140	T142	SAN MIGUEL	18-20	F	Huesos largos en general + frontal	cuerpo completo	Rx	severa	C57
AZ6	T36	GENTILAR	25-30	F	Tibias	cuerpo completo	Rx	incipiente minima	C142
AZ6	T68	GENTILAR	30-35	F	Tibia + perone derecho	cuerpo incompleto	Rx	severa	C128
AZ6	T22	GENTILAR	14-15	I	Tibia d	cuerpo completo		incipiente	C124

Columns TIBIA (D, I), PERON (D, I), FEMUR (D, I), CUBIT (D, I), RADIO (D, I), HUME (D, I), CLAV (D, I), ESCA (D, I), COXAL (D, I), CR, EST, CV, COST.: [illegible]

S=	SEXO
M=	MASCULINO
F=	FEMENINO
I=	indeterminado
D=	derecha
I=	izquierda
TIBIA=	TIBIA
PERO=	PERONE
FEMU=	FEMUR
CUBI=	CUBITO
RADI=	RADIO
HUM=	HUMERO
CLAV=	CLAVICULA
ESCA=	ESCAPULA
COXA=	COXAL
CR=	CRANEO
ES=	ESTERNON
CV=	COLUMNA
COST=	COSTILLAS
1=	hueso afectado
0=	hueso normal
9=	hueso ausente
99=	hueso con tejido

Figure C-1 Standen and Arriaza data

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Appendix D

Hackett's (1976) Diagnostic Criteria

Lesions of the Crania and Calvaria

1. **Clustered Pits. (Initial Series)** This is one of the earliest changes seen and is recognized by one or several well-defined round areas (10-15mm) of clustered pits (Hackett, 1976:32). Pits usually measure 1mm in diameter and occur primarily on the frontal and parietal bones (Hackett, 1976:32). It is important to distinguish pitting related to treponematosis from porotic hyperostosis and scalp infections, which occur in the same regions and may resemble clustered pits (Wilson, 1998:107). Perforation of the calvaria is unusual. At this stage, the pits may resemble other diseases, such as carcinoma and fungal diseases such as blastomycosis and coccidioidomycosis (Wilson, 1998:106).

2. **Confluent Clustered Pits.** These pits are the result of enlarging clustered pits, which overlap with adjacent ones causing a pit with an eroded center or an eroded periphery, which leaves a central pitted plug (Stanley, 1849: Plate IV, figure 1). These conjoined pits form confluent clustered pits, which Stanley (1849) writes are unlikely to be mistaken for any other disease. These confluent clustered pits form one of two patterns according to Hackett (1976), either a discrete or a

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contiguous series. These pits affect the inner and outer table of the skull differently. In those specimens, which Hackett (1976:36) labeled "syphilis," the inner surface rarely has significant changes aside from a few pits and a small amount of new bone deposit. In those specimens labeled "neoplasm," erosion of the outer surface pitting causes a straight or concave wall perforation (Hackett, 1976:36).

3. **Focal Superficial Cavitation (Discrete Series).** These manifestations occur when the confluent clustered pits open into a superficial cavity. This cavity has concave walls; its base is comprised of the cancellous tissue of the diploë, and around the irregular and sharp-edged opening is some pitted bone (Hackett, 1976:36). Focal superficial cavitation represents the first stage of the discrete series of this sequence. Changes to the inner surface of the skull are confined to "small, slightly pitted bone deposit" (Hackett, 1976:36). This change is of short duration and is rarely seen without other symptoms (Hackett, 1976:36).

4. **Circumvellate Cavitation.** This stage occurs as the healing process progresses causing new bone to be overlaid on the opening (1-2 cm). At this stage, the rounded margin of the cavity may show fine, tiny radially extending blood vessels. The outer part of the rim may show pitting. The inner surface shows only minor changes. Hackett (1976:40) notes that several adjoining circumvellate cavities may fuse

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together; however even those fused cavities are distinct from nodular cavitations of the contiguous series of the caries sicca sequence. These fused cavities have crenulated rims and coarsely granulated bases.

5. **Radial Scars.** These radial shaped scars occur in the shallow depression that remains from the healing of the cavity with new bone. The radial pattern is recognized by thin wavy lines and in some cases, a small finely granulated center area (Hackett, 1976:40). The rim margin in this stage may be present. The margin eventually flattens. Pitting is usually not present. There may be a slight thickening from new bone deposits in the inner table of the calvarium. According to Virchow (1858), this represents the static healed stage of the discrete series. Radial scars remain, but may become shallower as time goes by. These scars must be distinguished from those left by tuberculosis, which can resemble the focal superficial cavitation and circumvellate cavitation of syphilis. Tuberculosis causes extensive changes to the inner table and erosion that extends from the sequestrum or perforation (Hackett, 1976:41).

6. **Serpiginous Cavitation (Contiguous Series).** This stage begins as an irregular confluence in the middle of a large area (3-4 cm) of clustered pits (Hackett. 1976:43). This irregular confluence opens up into confluent superficial cavities, which may extend over a large area

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(5-10 cm) (Hackett, 1976:43). The openings at this stage have sharp irregular margins and concave walls (Hackett, 1976:43).

7. **Nodular Cavitation.** This stage results from healing and is recognized by rounded margins and new bone growth that appears to "flow" from the fragments of the outer table into the cavities producing nodules of about 5 mm (Hackett, 1976). The nodules are divided by superficial cavities. Following the serpiginous cavitation, this nodular cavitation may extend approximately 5 to 10 cm in length (Hackett, 1976).

8. **Caries Sicca.** At this stage stellate depressions of caries sicca appear. These depressions have nodules 5 to 10 cm in length, with little space between them (Hackett, 1976).

9. **Sequestra. (Massive Bone Destruction. Calvarium).** These are fragments of dead bone that have separated from the tissue (Hackett, 1976). Sequestra are usually caused by pyogenic infection; however, it is thought that treponematosis may also cause sequestra (Hackett, 1976:54-55). Sequestra may be a few centimeters wide (most are around 5 cm), may be multiple, may cross sutures, and although smooth in appearance are usually crenulated (Hackett, 1976).

10. **Depressions.** This is a hollowed or lowered region, of which there are several types, only some of which are associated with treponematosis (Hackett, 1976). Those that are considered of

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diagnostic value include: depressions with protuberances on the inner surface (associated with fractures); multiple smooth surface depressions (1-3 cm); depressions with slightly raised and rounded borders (associated with treponematosis; depressions with multiple medium-sized (5 cm) depressions that are crenulated on the edges and have shallow smooth pits and rough areas; medium-sized and large (5 cm) depressions with irregular bases, a thickened inner surface and poorly defined margins; and small (1-5 cm) round depressions with raised rims and slightly thickened interiors having possibly both raised and undetermined margins (Hackett, 1976).

11. **Perforations.** Only perforations with “sloping slightly brushed margins, which often arise in the raised rolled edge of the circumvellate cavitations” are diagnostic of treponematosis (Hackett, 1976:59). These perforations may be discrete or confluent (Hackett, 1976). According to Hackett (1976:59), the confluent changes are normally not more than 6-7 cm in diameter, but may have multiple openings generally 1-2 cm in width. The inner surface may be “thickened, arabesque or smooth” (Hackett, 1976:59). Sequestra can occur.

12. **Naso-Palatine Destruction (Massive Bone Destruction. Facial Destruction).** Naso-palatine destruction results in an empty nasal cavity with smooth lateral walls and a “bored-out” appearance

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(Hackett, 1976:63). Hackett (1976:63) considered this kind of destruction to be diagnostic of treponematosis. In this form of destruction, both the palatine and ethmoid bones may show signs of perforation (Hackett, 1976). The pre-maxillary alveolus may also be affected. Naso-palatine destruction can occur alone or with calvarial lesions (Hackett, 1976).

13. **Plaques (More Bone, Localized).** These changes may occur on the inner or outer calvarium and are not considered by Hackett (1976:68-69) to be diagnostic of treponematosis. On the inner calvarium, a "thin, smooth, dull-surfaced" layer of bone represents these. They can also be represented as neoplasms, which consist of "rounded thickenings of fine cancellous tumor bone" (Hackett, 1976). These neoplasms may be discolored and erosion may be present (Hackett, 1976). In some neoplastic tumors, clustered pits and confluent clustered pits and perforations may be present (Hackett, 1976:69).

14. **General Bony Thickening (Widespread).** This change is usually found on the calvarium, can be on the inner or outer table, and is not diagnostic of syphilis unless associated with caries sicca (Hackett, 1976). According to Hackett (1976:69), on the inner surface this results in a bone build-up around new deposits of "arabesque pattered bone." On the outer table, it can accompany caries sicca. This change may also be accompanied by increased vascular grooves.

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This usually is indicative of Paget's disease and is accompanied by "grossly and uniformly thickened calvaria," which if sectioned, consist of fine cancellous bone (Hackett, 1976).

15. **Bony Excrescences.** These are prominent localized protuberances on the inner and outer table of the calvarium (Hackett, 1976:70). These changes may include osteomata, which Hackett (1976:70) describes as "smooth, shiny, white, dense, hemispherical or flat-domed benign tumors (2-20 mm) on the outer table." Osteomata are diagnostic of osteoma, but not treponematosis. Massive primary or metastatic osteogenic neoplasms are also placed in this category by Hackett (1976:70). These neoplasms begin as "pitted lenticular nodes" that result in ray-like patterned tumors on the outer surface of the calvarium (Hackett, 1976).

Lesions of the Long Bones

1. **"Normal" Bone with Plaques:** Hackett (1976:76) defined this stage as the beginning for the "node/expansion" surface changes group. He said that specimens with this type of lesion alone are rare (Hackett, 1976:76). According to Hackett (1976:76), his use of the term "normal" refers to the absence of nodes or expansions.
2. **"Normal" Bone with Fine Striation:** In this stage, fine longitudinal lines appear on the cortical surface (Hackett, 1976:77). These striae

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are usually about 1 mm apart, with a width and depth less than 0.5 mm (Hackett, 1976:77). These lines in the bone may be sharp or flat (Hackett, 1976). When the bone heals, these striations may be replaced with “rounded-edge new bone” (Hackett, 1976:77). Hackett (1976:77) has said that these changes represent a low-grade or nonspecific infection or inflammation of the periosteum.

3. **“Normal” Bone with Superficial Sequestra:** This stage is characterized by thin oval plates, about 3-5 cm of dead bone from the cortex (Hackett, 1976:79). Hackett observed these sequestra on the medial surface of the distal tibiae (Hackett, 1976:79). He defined them as being characteristic of treponematosis but not diagnostic (Hackett, 1976:79). According to Hackett (1976:77), the sequestrum may be smooth or may show changes that occurred before necrosis. These changes might include plaques and pits (Hackett, 1976:77).

4. **Nodes/Expansions with Plaques:** This stage is characterized by areas of well-defined dark colored bone that is present upon a node or expansion (Hackett, 1976:80). On the tibia, it may be seen as striated on the medial surface, but it usually shows pitting along the lateral surface of the mid-shaft (Hackett, 1976). Stratification of the plaques may occur (Hackett, 1976). Similar changes may be seen on the femora, fibulae, radii, and ulnae (Hackett, 1976). The cortex may be thickened by a new layer of bone growth on the outer surface (Hackett,

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1976). These changes may be seen in specimens with diseases other than syphilis, so Hackett (1976:80) has said these changes are not diagnostic of treponematosis.

5. **Finely Striate Nodes/Expansions:** In this stage, nodes may be single or multiple and may be widely disbursed (Hackett, 1976:81). According to Hackett (1976:81), they can be small (4-5 cm) or may involve half or more than half of the bone. The most commonly affected surface is the antero-medial surface of the tibia (Hackett, 1976:81). The posterior is the least commonly affected (Hackett, 1976:81). Single, narrow nodes are common with the surface of the nod being smooth in appearance (Hackett, 1976:81). Striations may be present on unexpanded sections of the bone (Hackett, 1976:81). A clear definition is present between the nodes and the original cortex (Hackett, 1976:81-82). The changes to the bone may be pitted and trabecular (Hackett, 1976:81-82). Hackett (1976:81-82) said that osteomyelitis, which can be similar in appearance, may be distinguished from treponemal infection by its suppurative channels.

6. **Coarsely Striate and Pitted Expansions:** In this stage, there are cylindrical expansions present on the middle two-thirds or more of the shaft (Hackett, 1976:82). These expansions show a mixture of pitting and striation with "pits that open into short narrow grooves" (Hackett, 1976:82). The bone is described by Hackett (1976:82) as looking

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"bloated" at this stage; however, he reports that the expansion of bone does not reach the articular surfaces by several centimeters. The lateral surface of the tibiae may be rugose (Hackett, 1976:82). Changes are also seen on fibulae and ulnae (Hackett, 1976:82). When the bone is sectioned, a clear boundary may be seen between the cortex and the new bone laid down on the node (Hackett, 1976:82). According to Hackett (1976:82), the coarse striated and pitted expansions may be regarded as diagnostic criteria of syphilis; however, he cautioned at the time that this criterion was still "on trial."

7. **Slight Changes -- Rugose Nodes/Expansions:** This initial stage is characterized by nodes rather than by expansions. This includes bowing of the tibia due to new bone deposits building up on the anterior surface (Hackett, 1976:83-84). On the tibia, the nodes are commonly seen on the medial surface of the proximal or middle half of the bone (Hackett, 1976:84). The change seen most frequently is "rippling" (Hackett, 1976:84). A narrow region of striation at the proximal and distal ends where the node adjoins unaffected cortex is common (Hackett, 1976). Similar changes are seen on other long bones (Hackett, 1976). These changes may also be found in Paget's disease (Hackett, 1976). The two may be distinguished by looking at where the nodes and the cortex meet (Hackett, 1976). In Paget's disease, the bone is smooth, granular, and finely pitted with no plaques

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or striations at the edge of the node. In treponemal infections, the nodes are distinct from the cortex (Hackett, 1976).

8. **Moderate Changes –Rugose Nodes/Expansions:** This stage is characterized by expansions instead of nodes (Hackett, 1976). The shaft of the bone is mostly smooth, and in some areas, the pattern of trabeculation may be hard to see because the bone is smooth and expanded. According to Hackett (1976:87), the lateral and medial surfaces of the bone usually show a patterning with the presence of vascular grooves. Pitting, striation, plaques, and nodes may also be seen, as well as rippling and trabeculation (Hackett, 1976).

9. **Gross Changes – Rugose Nodes/Expansions:** At this stage, the bones are enlarged and may be heavy (Hackett, 1976:87). The surface of the bones may be rough and granular and may show a combination of rippling and trabeculation with striations and pitting (Hackett, 1976:87). On sectioning, the cortex is thickened and is either compact or finely cancellous (Hackett, 1976:87). The medullary canal is narrowed and may be replaced with cancellous tissue (Hackett, 1976:87). According to Hackett (1976:87-88), these changes are unlikely to be mistaken for other conditions or diseases that may affect the bone.

10. **Massive Destruction – Sequestra and Expansions:** The changes seen at this stage are characterized by a single large sequestrum that

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covers anywhere from a third to the majority of the shaft of the bone (Hackett, 1976:91). Infected bones present with a "sheath" around the sequestrum (Hackett, 1976:91). This sheath may have round cloacae measuring from 1-5 cm (Hackett, 1976:91). Open pits and short striae are seen on the surface of the involcrum (Hackett, 1976:91). The surface of the involcral can also be nodular and may descend in narrow transverse folds of 5-10 mm (Hackett, 1976:91). The surface involcral is not rippled or trabeculated (Hackett, 1976:92-93). Numerous cloacae are present, especially on the medial surface (Hackett, 1976:92-93). These changes may occur in treponematosis but also occur with other conditions (Hackett, 1976).

11. **Focal Destruction – Nodes/ Expansions with Superficial Cavitation:** This change begins with a striated pitted node (Hackett, 1976). This node may develop several small flat cavities (10-20 mm) (Hackett, 1976). These initial nodes may appear stratified when sectioned (Hackett, 1976). The borders where the node and normal bone meet are usually well defined and may have striations (Hackett, 1976). When the bone begins to heal a depressed smooth surface may remain (Hackett, 1976:93-97). These cavitations in the bone usually have rough margins (Hackett, 1976). According to Hackett (1976:93-97) these changes may be considered periosteal gummata, the long bone version of caries sicca in the skull.

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12. **Metaphyseal Expansion and Cavitation:** This stage is noted by the destruction and irregular cavitation of the bone (Hackett, 1976:97-98). The enlargements and destruction that occurs looks like malignant tumors (Hackett, 1976:97-98). Changes to the bone include metaphyseal enlargement sometimes with multiple openings into cavities that may have sequestra (Hackett, 1976:97-98). The surfaces of the bone may be smooth or roughened (Hackett, 1976:97-98). According to Hackett (1976:97-98), this condition may be associated with increased rugosity and node/expansions with superficial cavitation as seen in treponematosis, but it is not diagnostic of treponemal infection. Hackett (1976:97) found that these changes were often found with tuberculosis and neoplasms. To determine if bones with these signs might have treponematosis, he looked closely at the changes occurring on the metaphyses of the bones rather than on the diaphyses (Hackett, 1976:97-98). Hackett (1976:97-98) has said that in tuberculosis these changes are seen with articular surface destruction. Changes to the bone that show irregular cavitation without articular destruction are probably neoplastic (Hackett, 1976:97-98).
13. **Expansions and Deformity-- Platforms:** These platforms are usually solitary and are seen as "well-defined, flat, oval or round areas of bone present above the cortical surface" (Hackett, 1976:98). The margins of these changes may be concave in appearance (Hackett, 1976:98).

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The changes occur primarily on the distal half of the medial surface of the tibia (Hackett, 1976:98). The surfaces of bones with these changes may be "granular, pitted or both" (Hackett, 1976:98). When sectioned the platforms may be fused to the cortex and in more extensive cases, the underlying cortex may appear finely cancellous (Hackett, 1976:98). The underlying medullary canal may be narrowed. Some platforms are seen with minor changes elsewhere on the bones; however, some are associated with large irregular platforms (Hackett, 1976:99). Hackett (1976:99) has noted that these changes with varicose ulcers. They are a diagnostic criterion of leg ulcers, but not treponematosis (Hackett, 1976:99).

14. **Bowing and Expansion:** This change is seen as bowing and expansion in the bone (Hackett, 1976). Sabre tibia is an example of this (Hackett, 1976). Fibulae may also be affected (Hackett, 1976). Deep, vascular grooves may be present on the medial surface of the tibiae and on other bones (Hackett, 1976:101). When sectioned the bone is shown to have "moderately advanced changes" to the cortex (Hackett, 1976:101). In syphilis, Hackett (1976:101) has said that the bowing is a result of new bone deposits on the anterior cortex. The cortex in bones affected by treponematosis is compact and dense and the surface is striate not granular (Hackett, 1976:101). In Paget's disease, the bone is usually cancellous (Hackett, 1976:101). Bowing

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due to treponematosis can be distinguished from Paget's disease by the fact that the changes rarely extend to the end of the bone shaft in treponematosis (Hackett, 1976:101)

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Appendix E

Examples of Data Recording Forms

INVENTORY RECORDING FORM FOR COMPLETE SKELETONS

Site Name/Number ____________ / ____________ Observer ____________

Feature/Burial Number ____________ / ____________ Date ____________

Burial/Skeleton Number ____________ / ____________

Present Location of Collection ____________

CRANIAL BONES AND JOINT SURFACES

	L(left)	R(right)		L	R
Frontal	___	___	Sphenoid	___	___
Parietal	___	___	Zygomatic	___	___
Occipital	___	___	Maxilla	___	___
Temporal	___	___	Palatine	___	___
TMJ	___	___	Mandible	___	___

POSTCRANIAL BONES AND JOINT SURFACES

	L	R		L	R
Clavicle	___	___	Os Coxae		
Scapula			Ilium	___	___
Body	___	___	Ischium	___	___
Glenoid f.	___	___	Pubis	___	___
Patella	___	___	Acetabulum	___	___
Sacrum	___	___	Auric. Surface	___	___

VERTEBRAE (individual)

	Centrum	Neural Arch
C1	___	___
C2	___	___
C7	___	___
T10	___	___
T11	___	___
T12	___	___
L1	___	___
L2	___	___
L3	___	___
L4	___	___
L5	___	___

VERTEBRAE (grouped)

#Present/# Complete

	Centra	Neural Arches
C3-6	___/___	___/___
T1-T9	___/___	___/___

Sternum: Manubrium ___ Body ___

RIBS (individual)

	L	R
1st	___	___
2nd	___	___
11th	___	___
12th	___	___

RIBS (grouped)

#Present/# Complete

	L	R	Unsided
3-10	___/___	___/___	___/___

CHAPTER 2: Attachment 1

Figure E-1-A Inventory recording form for complete skeletons, page 1 (Buikstra and Ubelaker, 1994)

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Series/Burial/Skeleton________________

Observer/Date________________

LONG BONES

	Proximal Epiphysis	Diaphysis Proximal Third	Diaphysis Middle Third	Diaphysis Distal Third	Distal Epiphysis
Left Humerus	___	___	___	___	___
Right Humerus	___	___	___	___	___
Left Radius	___	___	___	___	___
Right Radius	___	___	___	___	___
Left Ulna	___	___	___	___	___
Right Ulna	___	___	___	___	___
Left Femur	___	___	___	___	___
Right Femur	___	___	___	___	___
Left Tibia	___	___	___	___	___
Right Tibia	___	___	___	___	___
Left Fibula	___	___	___	___	___
Right Fibula	___	___	___	___	___

Left Talus____

Right Talus____

Left Calcaneus____

Right Calcaneus____

HAND (# Present/# Complete)

	L	R	Unsided
# Carpals	___/___	___/___	___/___
#Metacarpals	___/___	___/___	___/___
#Phalanges	___/___	___/___	___/___

FOOT (# Present/# Complete)

	L	R	Unsided
#Tarsals	___/___	___/___	___/___
#Metatarsals	___/___	___/___	___/___
#Phalanges	___/___	___/___	___/___

Comments:____

Attachment 1: CHAPTER 2

Figure E-1-B Inventory recording form for complete skeletons, page 2 (Buikstra and Ubelaker, 1994)

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Table 6
Skeletal Pathology Code Key

Side
1. Right
2. Left
3. Both Sides

Section
1. Proximal Epiphysis/ Articular Surface
2. Proximal 1/3 of Diaphysis
3. Middle 1/3 of Diaphysis
4. Distal 1/3 of Diaphysis
5. Distal Epiphysis/ Articular Surface
6. Proximal 2/3 Diaphysis
7. Distal 2/3 Diaphysis
8. Total Diaphysis
9. Both Prox. and Distal Articular Surfaces

Aspect
1. Superior Surface
2. Inferior Surface
3. Both Superior and Inferior Surfaces
4. Medial
5. Lateral
6. Both Medial and Lateral Surfaces
7. Posterior (Dorsal)
8. Anterior (Ventral)
9. Circumferential

Bone

2.0.0 Skull
- 2.0.1 Frontal
- 2.0.2 Parietal
- 2.0.3 Occipital
- 2.0.4 Temporal
- 2.0.5 Zygomatic
- 2.0.6 Maxilla
- 2.0.7 Mandible
- 2.0.8 Palatine
- 2.0.9 Sphenoid
- 2.1.0 Nasal
- 2.1.1 Hyoid

3.0.0 Vertebrae
- 3.1.0 Cervical vertebrae
- 3.1.1 Cl (atlas)
- 3.1.2 C2 (axis)
- 3.1.3 C3-6
- 3.1.4 C7
- 3.2.0 Thoracic vertebrae
  - 3.2.1 T1-9
  - 3.2.2 T10
  - 3.2.3 T11
  - 3.2.4 T12
- 3.3.0 Lumbar vertebrae
  - 3.3.1 L1
  - 3.3.2 L2
  - 3.3.3 L3
  - 3.3.4 L4
  - 3.3.5 L5
- 3.4.0 Sacrum
- 3.5.0 Coccyx

3.6.0 Ribs
- 3.6.1 First rib
- 3.6.2 Second Rib
- 3.6.3 Ribs 3-10
- 3.6.4 Rib11
- 3.6.5 Rib 12

3.7.0 Sternum
- 3.7.1 Manubrium
- 3.7.2 Body (gladiolus)

4.0.1 Clavicle
4.0.2 Scapula
4.0.3 Glenoid fossa
- 4.1.0 Upper limb
- 4.1.1 Humerus
- 4.1.2 Radius
- 4.1.3 Ulna
- 4.1.4 Carpals
- 4.1.5 Metacarpals
- 4.1.6 Hand phalanges

4.2.0 Os coxae
- 4.2.1 Ilium
- 4.2.2 Ischium
- 4.2.3 Pubis
- 4.2.4 Acetabulum

4.3.0 Lower Limb
- 4.3.1 Femur
- 4.3.2 Patella
- 4.3.3 Tibia
- 4.3.4 Fibula
- 4.3.5 Tarsals
- 4.3.6 Metatarsals
- 4.3.7 Foot phalanges

Pathology

1.0.0 General Abnormality of SHAPE: LONG BONE
- 1.1.0 Bowed (abnormal curvature)
- 1.2.0 Angulated
- 1.3.0 External Outline Altered
  - 1.3.1 Flaring Metaphyses
  - 1.3.2 Uniformly abnormally wide
  - 1.3.3 Fusiform (spindle) shape
  - 1.3.4 Other (see narrative)
- 1.4.0 Degree of Shape Abnormality
  - 1.4.1 Barely discernible
  - 1.4.2 Clearly discernible

1.0.0 Abnormality of SHAPE: SKULL
- 1.5.0 Craniosynostosis, suture
  - 1.5.1 Metopic
  - 1.5.2 Coronal
  - 1.5.3 Sagittal
  - 1.5.4 Lambdoid
  - 1.5.5 Other (see narrative)
- 1.6.0 Craniosynostosis, completeness
  - 1.6.1 Partial
  - 1.6.2 Complete

1.0.0 Abnormality of SHAPE: SPINAL COLUMN
- 1.7.0 Type
  - 1.7.1 Kyphosis (ant.-post)
  - 1.7.2 Scoliosis, left
  - 1.7.3 Scoliosis, right
- 1.8.0 Form
  - 1.8.1 Angular
  - 1.8.2 Gradual change in body height
- 1.9.0 Ankylosis
  - 1.9.1 Absent
  - 1.9.2 Present

2.0.0 Abnormality of SIZE
- 2.1.0 Hydrocephaly present
- 2.2.0 Size Reduction
  - 2.2.1 Achondroplastic dwarfism
  - 2.2.2 Proportional dwarfism
  - 2.2.3 Bone (or region) specific reduction
- 2.3.0 Acromegaly

3.0.0 Abnormal BONE LOSS
- 3.1.0 Location
  - 3.1.1 Periosteal or subchondral surface, external table
  - 3.1.2 Cortex, trabeculae, or diploë
  - 3.1.3 Endosteal surface, internal table
  - 3.1.4 Mixed: 3.1.1 + 3.1.2
  - 3.1.5 Mixed: 3.1.2 + 3.1.3
  - 3.1.6 Mixed: all three locations involved
- 3.2.0 Extent of involvement: % of unit affected
  - 3.2.1 <1/3
  - 3.2.2 1/3-2/3
  - 3.2.3 >2/3
- 3.3.0 Focal Bone Loss (well organized): Number of foci
  - 3.3.1 Unifocal
  - 3.3.2 Multifocal: 2 foci
  - 3.3.3 Multifocal: 3-5 foci
  - 3.3.4 Multifocal: 6-10 foci
  - 3.3.5 Multifocal: 10+ foci
- 3.4.0 Focal Bone Loss: Size
  - 3.4.1 <1 cm
  - 3.4.2 1-5 cm
  - 3.4.3 >5
  - 3.4.4 Mixed 3.4.1 + 3.4.2
  - 3.4.5 Mixed 3.4.1 +3.4.3
  - 3.4.6 Mixed 3.4.2 + 3.4.3
  - 3.4.7 Mixed 3.4.1 + 3.4.2 + 3.4.3
- 3.5.0 Focal Bone Loss: Bony Response
  - 3.5.1 Circumscription, sclerotic reaction
  - 3.5.2 Boundaries well defined, but no sclerosis
  - 3.5.3 Margins not sharply defined
  - 3.5.4 Mixed 3.5.1 +3.5.2
  - 3.5.5 Mixed 3.5.2 + 3.5.3
  - 3.5.6 Mixed 3.5.1 + 3.5.2 + 3.5.3
  - 3.5.7 Mixed 3.5.1 + 3.5.3
- 3.6.0 Diffuse (osteoporotic; irregular and poorly organized)
  - 3.6.1 With cortical thinning associated
  - 3.6.2 Without cortical thinning associated
- 3.7.0 Structural Collapse Associated

114 *RECORDING STANDARDS*

Figure E-2-A Skeletal pathology code key (Buikstra and Ubelaker, 1994: 114)

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Table 6 , continued

4.0.0 Abnormal BONE FORMATION
- 4.1.0 Periosteal Surface: Lamellar Reaction
  - 4.1.1 Reactive woven bone (fiberbone) present
  - 4.1.2 Sclerotic reaction
  - 4.1.3 Both woven and sclerotic reaction evident
- 4.2.0 Cortex Intact, spicules perpendicular to surface
  - 4.2.1 Sunburst effect
  - 4.2.2 Cauliflower appearance
- 4.3.0 Cortex Perforated
  - 4.3.1 By expansive, shell-type reaction
  - 4.3.2 Due to cloacae (sinus tracks) - from medullary cavity
- 4.4.0 Endosteal Surface
  - 4.4.1 Lamellae visible
  - 4.4.2 Medullary cavity narrowed with no visible lamellae
- 4.5.0 Abnormal Matrix
  - 4.5.1 Deposition of woven, immature bone
  - 4.5.2 Cancellous expansion
  - 4.5.3 Trabecular coarsening
- 4.6.0 Extent of Involvement; % of Unit Affected
  - 4.6.1 <1/3
  - 4.6.2 1/3-2/3
  - 4.6.3 >2/3
- 4.7.0 Ossified connective tissue
  - 4.7.1 Myositis ossificans (omit if with fracture, see 5.49)
  - 4.7.2 Enthesopathy
  - 4.7.3 Joint fusion
- 4.8.0 Specific structures
  - 4.8.1 Button osteoma(s)
  - 4.8.2 Stellate scars
  - 4.8.3 Sequestrum
  - 4.8.4 Involucrum
  - 4.8.5 Both 4.8.3 and 4.8.4

5.0.0 FRACTURES AND DISLOCATIONS
- 5.1.0 Type of fracture (indicate all)
  - 5.1.1 Complete
  - 5.1.2 Partial (greenstick)
  - 5.1.3 Simple
  - 5.1.4 Comminuted
  - 5.1.5 Spiral
  - 5.1.6 Compression
  - 5.1.7 Depressed, outer table only
  - 5.1.8 Depressed, outer and inner tables
  - 5.1.9 Pathological
- 5.2.0 Shape characteristics (indicate all)
  - 5.2.1 Blunt round
  - 5.2.2 Blunt oval
  - 5.2.3 Edged (bladed)
  - 5.2.4 Projectile entry
  - 5.2.5 Projectile exit
  - 5.2.6 Projectile embedded
  - 5.2.7 Radiating
  - 5.2.8 Amputation
- 5.3.0 Perimortem fracture (no observable remodeling)
  - 5.3.1 Clearly the result of perimortem trauma
  - 5.3.2 Ambiguous, possibly postmortem trauma
- 5.4.0 Fracture sequelae (indicate all)
  - 5.4.1 Callus formation, woven bone only
  - 5.4.2 Callus formation; sclerotic reaction
  - 5.4.3 Healing/obliteration of cranial fracture
  - 5.4.4 Nonunion
  - 5.4.5 Tissue necrosis
  - 5.4.6 infection
  - 5.4.7 Traumatic arthritis
  - 5.4.8 Joint fusion
  - 5.4.9 Traumatic myositis ossificans
- 5.5.0 Type of dislocation
  - 5.5.1 Traumatic
  - 5.5.2 Congenital
  - 5.5.3 Cause ambiguous

6.0.0 POROTIC HYPEROSTOSIS (frontal, parietal, occipital bones)
- 6.1.0 Degree
  - 6.1.1 Barely discernible
  - 6.1.2 Porosity only
  - 6.1.3 Porosity with coalescence of foramina, no thickening
  - 6.1.4 Coalescing foramina with increased thickness
- 6.2.0 Location
  - 6.2.1 Orbits
  - 6.2.2 Adjacent to sutures
  - 6.2.3 Near bosses or within squamous portion of occipital
  - 6.2.4 Both adjacent to suture and within orbits
  - 6.2.5 Both adjacent to suture and near bosses/in squamous
- 6.3.0 Activity
  - 6.3.1 Active at time of death
  - 6.3.2 Healed
  - 6.3.3 Mixed reaction: evidence of healing + active lesions

7.0.0 VERTEBRAL PATHOLOGY
- 7.1.0 Schmorl's nodes
  - 7.1.1 Barely discernible
  - 7.1.2 Moderate expression
  - 7.1.3 Marked expression
- 7.2.0 Osteophytes, degree (indicate maximum expression)
  - 7.2.1 Barely discernible
  - 7.2.2 Elevated ring
  - 7.2.3 Curved spicules
  - 7.2.4 Fusion present
- 7.3.0 Syndesmo/Enthesophytes, degree (maximum expression)
  - 7.3.1 Barely discernible
  - 7.3.2 Elevated ring
  - 7.3.3 Curved spicules
  - 7.3.4 Fusion present
- 7.4.0 Spina bifida
  - 7.4.1 Partial
  - 7.4.2 Complete
- 7.5.0 Spondylolysis
  - 7.5.1 Complete fracture, no healing
  - 7.5.2 Healing evident
  - 7.5.3 Spondylolysthesis

8.0.0 ARTHRITIS
- 8.1.0 Lipping, degree
  - 8.1.1 Barely discernible
  - 8.1.2 Sharp ridge, sometime curled with spicules
  - 8.1.3 Extensive spicule formation
  - 8.1.4 Ankylosis
- 8.2.0 Lipping, extent of circumference affected
  - 8.2.1 <1/3
  - 8.2.2 1/3-2/3
  - 8.2.3 > 2/3
- 8.3.0 Surface porosity, degree
  - 8.3.1 Pinpoint
  - 8.3.2 Coalesced
  - 8.3.3 Both pinpoint and coalesced
- 8.4.0 Porosity, extent of surface *affected*
  - 8.4.1 <1/3
  - 8.4.2 1/3-2/3
  - 8.4.3 >2/3
- 8.5.0 Eburnation, degree
  - 8.5.1 Barely discernible
  - 8.5.2 Polish only
  - 8.5.3 Polish with groove(s)
- 8.6.0 Eburnation, extent of surface affected
  - 8.6.1 <1/3
  - 8.6.2 1/3-2/3
  - 8.6.3 >2/3
- 8.7.0 Surface osteophytes, degree
  - 8.7.1 Barely discernible
  - 8.7.2 Clearly present
- 8.8.0 Periarticular resorptive foci, degree
  - 8.8.1 Barely discernible
  - 8.8.2 Clearly present
- 8.9.0 Periarticular resorptive foci extent of circumference
  - 8.9.1 <1/3
  - 8.9.2 1/3-2/3
  - 8.9.3 >2/3

Figure E-2-B Skeletal pathology code key (Buikstra and Ubelaker, 1994: 115)

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PCR Mission ______ Star Date ______

Locus ______ Buffer type ______ Taq type ______

Thermal Cycler ______

Program file ______

Initial Denature ______ ° / ______ secs

Denature ______ ° / ______ secs

Anneal ______ ° / ______ secs

Extend ______ ° / ______ secs

\# of Cycles ______ X

Final Extension ______ ° / ______ secs Final Hold 14°C/∞

expected fragment size ______ bp

	sample i.d.	dil.
1.		
2.		
3.		
4.		
5.		
6.		
7.		
8.		
9.		
10.		
11.		
12.		
13.		
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35.		
36.		
37.		
38.		
39.		
40.		

MasterMix	vol/samp (μl)	x(n+2)	= MM vol. (μl)
water			
buffer (_____X)			
dNTP (∑10mM)			
$MgCl_2$ (25mM)			
__ μm Primer______			
__ μm Primer______			
DMSO (1%)			
glycerol (1%)			
other ______			
Taq			
DNA template			
∑ volume			

Notes: ______ ladder ______

Figure E-3 PCR form (Conservation Genetics Laboratory, San Francisco, CA.)

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ExoSAP worksheet 80.xls

6/19/07

ExoSAP PCR cleanup

Date: ____________ Project: ____________________________

Thermocycler: ____________ Program: ____________________________

	sample Id.	PCR date	PCR tube #	ul DNA used	conc ng/ul		sample Id.	PCR date	PCR tube #	ul DNA used	conc. ng/ul
1						41					
2						42					
3						43					
4						44					
5						45					
6						46					
7						47					
8						48					
9						49					
10						50					
11						51					
12						52					
13						53					
14						54					
15						55					
16						56					
17						57					
18						58					
19						59					
20						60					
21						61					
22						62					
23						63					
24						64					
25						65					
26						66					
27						67					
28						68					
29						69					
30						70					
31						71					
32						72					
33						73					
34						74					
35						75					
36						76					
37						77					
38						78					
39						79					
40						80					

Notes (i.e. if using recipe rather than ExoSAP-IT):

Figure E-4 Exo-SAP-it® PCR clean-up form (Conservation Genetics Laboratory, San Francisco, CA.)

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Cadillac 800 Form | **BigDye Cycle Sequencing** | CGLab SFSU

Rxn.Date ____________________ **Expt** ____________________

Gel Date(s) ____________________ **Sample Sheet(s)** ____________________

			sample id.	purif. method	conc ng/ul	ul DNA used				sample id.	purif. method	conc ng/ul	ul DNA used
1							41						
2							42						
3							43						
4							44						
5							45						
6							46						
7							47						
8							48						
9							49						
10							50						
11							51						
12							52						
13							53						
14							54						
15							55						
16							56						
17							57						
18							58						
19							59						
20							60						
21							61						
22							62						
23							63						
24							64						
25							65						
26							66						
27							67						
28							68						
29							69						
30							70						
31							71						
32							72						
33							73						
34							74						
35							75						
36							76						
37							77						
38							78						
39							79						
40							80						

Rxn Mix Guide

	μl per sample			
	cheap	cheaper	cheapest	plasmid
5X buff	2.0	2.2	2.3	3
BigDye	0.8	0.5	0.3	2
primer(5/10um)	0.5-1.0	0.5-1.0	0.5-1.0	0.5-1.0
template	0.5-4	0.5-4	0.5-4	0.5-4
water	to 12	to 12	to 12	to 20

Thermal Cycler used: ____________

Program name: ____________

	Temp	Time
Int. denature:	___	___
Denature:	___	___
Annealing:	___	___
Extension:	___	___

X ___ cycles

Master Mix Worksheet

N = # of samps + 2 = ___

water ___(N) x ___ μl = ___

5X buffer ___(N) x ___ μl = ___

BigDye ___(N) x ___ μl = ___

primer ___(N) x ___ μl = ___

Amount of HiDye per sample: ___μl

Figure E-5 BigDye® Terminator cycle sequencing form (Conservation Genetics Laboratory, San Francisco, CA.)

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Appendix F

Reference Photos

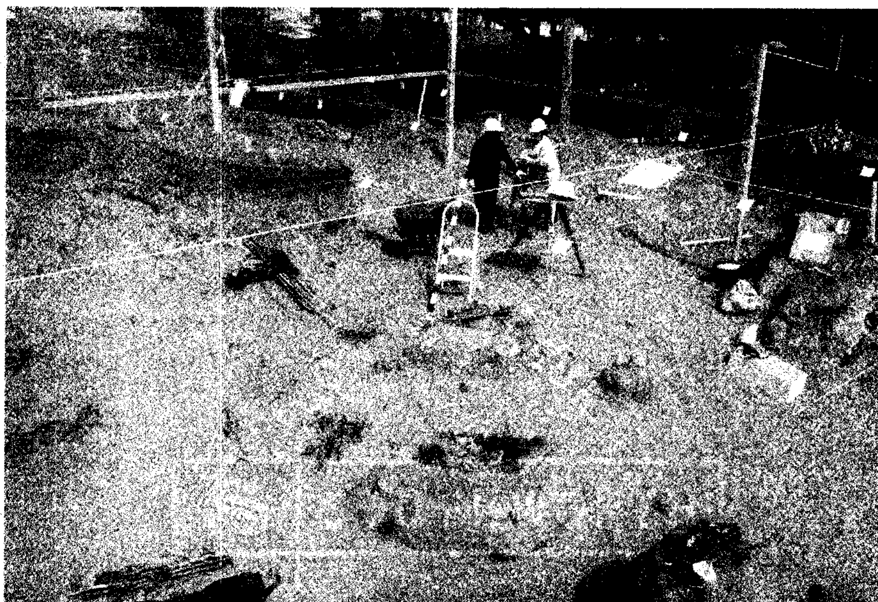

Figure F-1 An excavation site at San Miguel de Azapa

Figure F-2 A burial at San Miguel de Azapa

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Figure F-3 Right tibia PLM-8, T.3

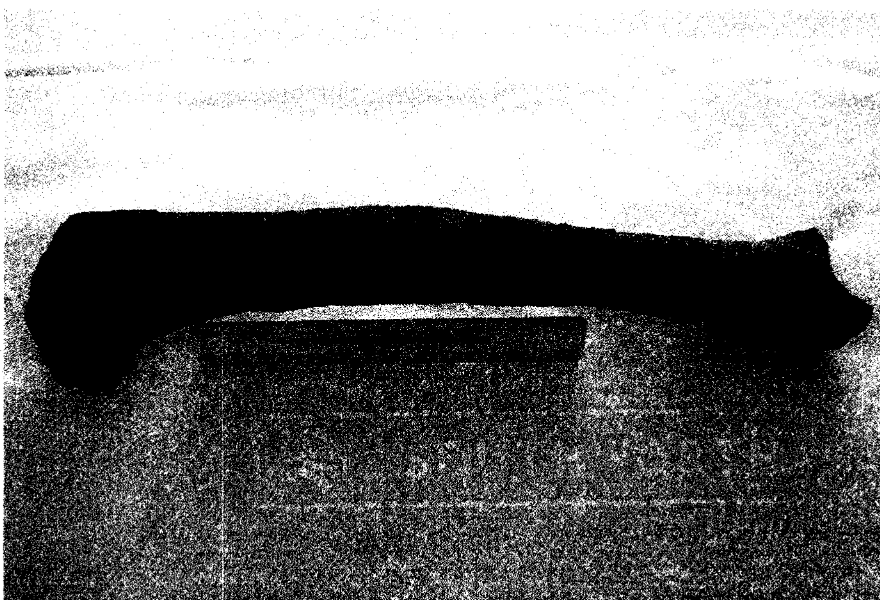

Figure F-4 Left tibia PLM-8, T.3

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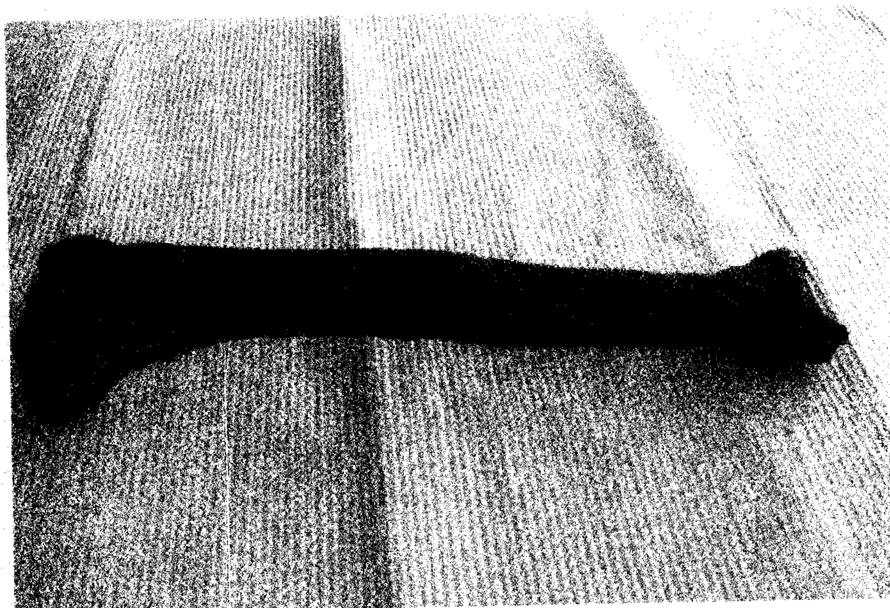

Figure F-5 Left tibia AZ-71, T.601

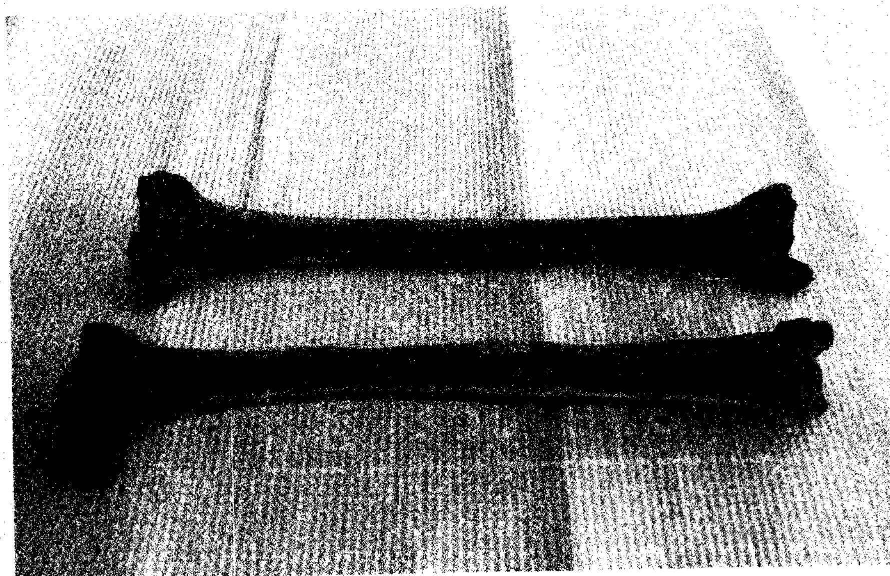

Figure F-6 Right tibia bisected AZ-71, T.601

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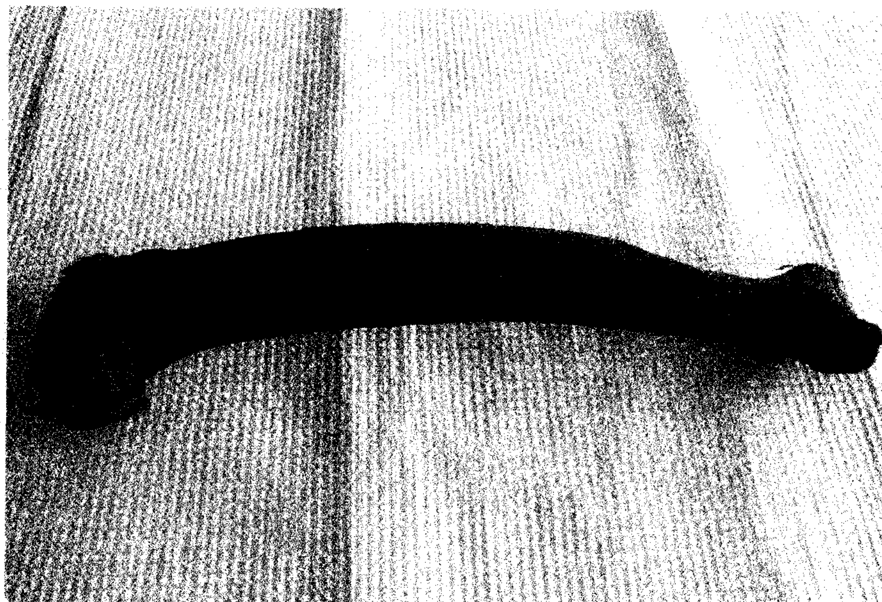

Figure F-7 Left tibia AZ-71, T.102

Figure F-8 Left tibia and left fibula Mo-1, T.22, C6

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Figure F-9 Yungay 372- A recent excavation

Figure F-10 Chinchorro mummy

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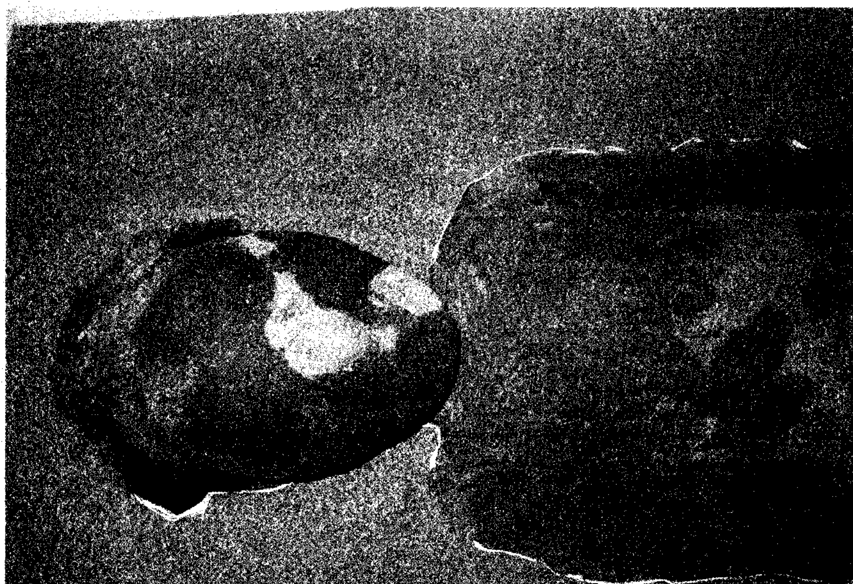

Figure F-11 Chinchorro mummy

Figure F-12 Chinchorro mummy/skeleton

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Appendix G

DNA Extraction Protocols

Note: Protocol is from the Paleo-DNA Laboratory (Thunder Bay, ON. Canada)

PK Buffer and Phenol-Chloroform Separation

» Immediately before processing samples, prepare 1X Extraction Buffer for (n+1) samples:

290 µL TNE
40 µL 20% SDS (10%)
40 µL DTT (0.39M)
2 µL Proteinase K (20 mg/mL finl. Conc. of 100 µg/mL)
28 µL of ddH_2O

400 µL

»Add 500 µL 1X Extraction Buffer above to the bone/tissue material for extraction
»Place on thermomixer at 37°C for 3 hours to overnight
»Aliquot:

1-1:1 Phenol:Chloroform/Isoamyl Alcohol, 2 tubes per sample of 1mL total
(500 µL Phenol and 500 µL Chloroform/Isoamyl Alcohol)
2-1000 µl Chloroform/Isoamyl Alcohol one tube per sample
»Add 1 mL (fill tube) of aliquoted Phenol-Chloroform/Isoamyl Alcohol mixture to
Extracted sample (do not remove supernatant from bone/tissue material)
»Shake/vortex
»Centrifuge for 5 minutes at 13,000 rpms.
»Remove top layer (aqueous phase) and place in previously aliquoted Phenol: Chloroform/Isoamyl Alcohol mixture (do not remove top layer of denatured protein at interphase) (Freeze at -86°C the left-over organic layer, bone material and interphase for later experiments)
»Shake/vortex
»Centrifuge for 5 minutes at 13,000 rpms.
»Carefully remove the top layer (aqueous phase) and place in previously aliquoted Chloroform/Isoamyl Alcohol mixture (discard organic layer)
»Shake/vortex
»Centrifuge for 5 minutes at 13,000 rpms

Continue on to ethanol precipitation

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Note: Protocol is from the Paleo-DNA Laboratory (Thunder Bay, ON. Canada)

Ethanol Precipitation

»Carefully remove top layer (aqueous phase) and place in a new 1.5 mL tube
»Add 3M Sodium Acetate (pH 5.2) to make final concentration of 0.3M sodium acetate (1/10 of aqueous phase volume)
»Shake/vortex
»Add 100% Ethanol (ice cold) in a vol:vol 2.5:1 ethanol:sample ratio
»Shake/vortex
»Place on ice for 5 minutes at max speed
»Remove and discard supernatant
»Add 500 pL to 1mL of 95% Ethanol (ice cold)
»Shake/vortex
»Centrifuge for 5 to 10 minutes at 14,000 rpms.
»Remove and discard supernatant
»Dry pellet
»Re-suspend in ddH_2O
»Put at 37°C for 10-15 minutes

Continue on to Micro Bio-Spin® P-30 Tris Chromatography Columns (Bio-Rad Laboratories, Hercules, CA.)

Micro Bio-Spin® P-30 Tris Chromatography Columns (Bio-Rad Laboratories, Hercules, CA)

Note: Directions are from Bio-Rad (Hercules, CA.).

Technical Information:

Gel Matrix: Bio-Gel P-30 polyacrylamide gel suspended in 1.0 ml of buffer

Buffer: Tris buffer (10 mM Tris-HCl, pH 7.4) with 0.02% sodium azide

Sample Application Volumes: nucleic acids, proteins, and peptides, 20-75 pL. Volumes less than 20 μL may affect recovery.

Exclusion Limits: Bio-Gel P-30 gel: 40,000 daltons (for proteins and globular peptides)

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Expected Retention and Recovery: 99% retention of unincorporated nucleotides, 95% recovery of applied DNA

Centrifuge Type: Microcentrifuge with a centrifugal force of 1,000 x (g).

Autoclavability: Micro Bio-Spin columns, Bio-Gel P-gel, and collection tubes are completely autoclavable at 121°C for 30 minutes at pH 6.0-8.0

Chemical Stability: pH 2-10, common aqueous buffers, formamide, dilute organic acids, alcohol 20% (V/V), other chaotrophic agents, detergents.

Storage: Store at 4°C. Do not freeze. Shelf life is 1 year at 4°C.

Instructions for Use

1. Invert the column sharply several times to resuspend the settled gel and remove any bubbles. Snap off the tip and place the column in a 2.0 mL microcentrifuge tube (included). Now remove the top cap.

2. Centrifuge for 2 minutes in a microcentrifuge at 1,000 x (g) to remove the packing buffer. Discard the buffer.

3. Place the column in a clean 1.5 or 2.0 mL microcentrifuge tube. Carefully apply the sample (20-75 µL) directly to the center of the column. Application of more or less than the recommended sample volume may decrease column performance.

4. After loading samples, centrifuge the column for 4 minutes at 1,000 x (g)

5. Following centrifugation, the purified sample is now in the Tris buffer

6. Properly dispose of the used column.

*Repeat (at the Paleo-DNA Laboratory the process was occasionally repeated for “dirty” samples)

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Centrifugation Notes:

To calculate the speed (rpm) required to reach a gravitational force of 1,000 x (g), use the following equation:

RCF (g) = $(1.12 \times 10^{-5})\,(\text{rpm})^2 r$

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Appendix H

Cloning Protocols

Note: Protocol is for use with Invitrogen (Carlsbad, CA.) Topo TA Cloning® Kit for Sequencing. Protocols are adapted from Instruction Manual Version K published July 31, 2003 (#25-0276). These instructions were adapted for use by Dr. Frank Cipriano in the Conservation Genetics Laboratory at San Francisco State University, San Francisco, CA.

Ligation

Table H-1 Topo Cloning® kit worksheet

Tube #	PCR date/tube	Primer 1	Primer 2	Comments
1				
2				
3				
4				

1. Fill in the blanks for “n” = # of reactions. Make a master mix for “n” reactions:

For each reaction:

Vector	x1.0 µL =	µL
Salt	x1.0 µL =	µL
Water	x2.0 µL =	µL
	(+2.0 PCR product)	
total µL		6.0 µL

Figure H-1 Ligation reaction worksheet

2. Aliquot the master mix: 4 µL each into 0.5 mL tubes; add 2.0 µL PCR product to individual tubes. Do this in cloning room/area.

3. Incubate 3 to 30 minutes at room temperature (or overnight in refrigerator), before transforming – after this time reactions can be kept in freezer (-20°C) more-or-less indefinitely.

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Transformation

1. Preliminaries: set water bath to 42°C, thaw SOC vial to room temperature, place LB/Amp (or/Kan) plates in 37°C oven for 30 minutes, spread plates with 40 µL of 40 mg/mL XGAL (Top10 cells) and let it soak in for 15 minutes. (IPTG is also needed w/TOP10F' cells.)

2. Gently thaw tubes of TOP10 cells (for "half" reactions thaw 1 tube for every two ligations, from each pipet 25 µL into another 1.5 mL tube), label tubes for corresponding sample numbers.

3. Pipet 2 µL ligation mix into tube of cells, stir gently with pipet tip, incubate on ice for 5-30 minutes.

4. Heat shock exactly 30 seconds in 42°C water bath (do not mix or shake), then ice for two minutes.

5. Add 250 µL of SOC medium (125 µL if using half tubes of cells) to each tube. Shake tilted tubes at 37°C for 1 hour at 225 rpm.

6. Spread 75/175 µL of tube contents onto two separate plates with sterile spreader. (If half tube of cells was used, spread all 125 µL onto a single plate.) Let soak 5-10 minutes. Incubate (inverted) in 37°C oven overnight (shift to 4°C for 2-3 hours for color development before screening).

Screening

1. Set up PCR reaction (12-25 µL) for each colony to be screened (usually ~6-10 colonies per plate to start with). Use m13rev and T7 [or M13(-20)] vector primers, or your PCR primers.

2. Circle and individually label white colonies to be screened on bottom of plate (e.g. plate is numbered, as per sample number, colonies are lettered, (e.g. a, b, c, d, e).

3. Using a sterile toothpick, just touch each circled colony (avoid taking too much) and twirl tip in PCR tube. To restreak colonies, touch toothpick to agar on new plate (gridded and labeled for each colony) before swirling in PCR tube.

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4. After sampling from each plate, place all back into incubator to continue growing for another 2 hours or so (then place into refrigerator to arrest growth). Seal each plate with parafilm.

PCR Profile for Screening

94°C/30s, 55°C/30s, 72°C/30s, 25-30 cycles.

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Appendix I

PCR Clean-up Protocols

Note: Protocol is adapted from ExoSAP-it® PCR Product Clean-up (USB Corporation, Cleveland, OH.). Dr. Frank Cipriano made modifications from the manufacturer's protocol for use in the Conservation Genetics Laboratory at San Francisco State University, San Francisco, CA.

ExoSAP-it® PCR Product Clean-up

In order to sequence PCR products excess nucleotides (dnTPs) and primers remaining from the PCR reaction must be removed. Exonucleases 1 (ExoI) digests single stranded DNA into free nucleotides and Shrimp Alkaline Phosphatase (SAP) dephosphorylates free nucleotides making them unavailable for polymerization. These enzymes (and a proprietary buffer) are supplied in the USB Exo-SAP-it® kit premixed and ready to use. Store kit at -20°C.

USB Exo-SAP-it® kit contains "500 reactions" (@ 2 µL/clean-up = 1000 pL) (Product # 78291)

ExoSAP-it® Protocol

1. Label a 200 pL tube (or strip tube) for each of your PCR products to be purified. Label it clearly and make sure you keep the correct orientation.

2. Transfer 5 µL of each PCR product from the PCR tube to the Exo-Sap-it® tube/strip tube.

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3. Add 0.5-1.0 µL of the Exo-Sap-it® mix to each of the Exo-Sap-it® tubes/ strip tubes and mix well. (Most products are cleaned sufficiently with only 1.0 µL of Exo-Sap-it®, some can even be cleaned with 0.5 µL of Exo-Sap-it®.

4. Place/close the lid(s) onto the tube/strip tubes and place in a thermal cycler block. Incubate with the following parameters:

   37°C 30 min to perform the digestion
   80°C 10 min to inactivate the enzymes

5. Once the Exo-Sap-it® reaction is finished, store your PCR (frozen) until ready to set-up a cycle sequencing reaction. Even with the 80°C inactivation step, your cleaned PCR product will degrade over time, so do not purify more than you need for cycle-sequencing.

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Appendix J

Cycle Sequencing Protocols

Note: These protocols have been adapted from the manufacturer's instructions for use in the Conservation Genetics Laboratory at San Francisco State University, San Francisco, CA.

ABI 3100
BigDye Cycle Sequencing Protocols
CG Lab 9/11/06

Cycle sequencing

ng DNA needed for sequencing

PCR product	
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
1000-20000 bp	10-40 ng
>2000 bp	20-50 ng
ssDNA	25-50 ng
dsDNA (cloned)	150-300 ng
Cosmid, BAC	0.5-1.0 ug
Bacterial genomic	2-3 ug

calculation from spectrophotometer*
Absorbance X Dilution X Q = ng/ul
260 Factor
Q = 50 ng/ul dsDNA Q = 40 ng/ul ssDNA
(*note: fluorometers are more accurate for PCR product)

primer needed ~ 3.2 pmoles
10 uM (umoles/liter) = 10 pmoles/ul
3.2 pmoles = 0.32 ul of 10 uM primer
(no difference observed with up to 1 ul of 10 uM primer/sample...)

Sequencing reaction recipes

"10" ul rxn mix	cheap	cheaper	cheapest	dbl-stranded (plasmid)
BigDye pre-mix	0.8	0.5	0.3	2.0
5x buffer	2.0	2.2	2.3	3.0
primer	0.5-1.0	0.5-1.0	0.5-1.0	1.0
template	0.5-4.0	0.5-4.0	0.5-4.0	1-10
water	to 12	to 12	to 12	to 20

5x* sequencing buffer: 400 mM Tris, pH 9.0; 10 mM MgCl2

Cycle sequencing reaction programs
(you may need to adjust time or ramping for different thermocyclers)
94 C/90 secs initial denature
94 C/20 sec; 50+*/30 sec; 60 C/4mins. for 25x
rapid cool to 14 degree and hold
for large (e.g. BAC) inserts
94/2 mins; 94/30 sec;50+*/30sec;60/4mins for 25x
*for better precision use higher temp., e.g. the annealing temp. used in your PCR...

Precipitation, resuspension & denaturation

1.5 mL tube with isopropanol (the usual):

Add 125 uL of 65% isopropanol to each sample
Mix well, pipet into 1.5 mL tube
Spin 30+ mins at max g
Carefully aspirate out isopropanol
Add 125 uL of 75% isopropanol, vortex
Spin 5 mins at max g; aspirate off alcohol
Dry in speedvac (5 mins – heat off)
Resuspend in 15 uL Hi-Di
Denature ~85 degrees/2 mins in big tube hot block
Snap chill 5 mins & load all into 3100 plate

Strip tube with EDTA/Ethanol/Sodium acetate:

First add 5 uL of 125 mM EDTA to each tube
Then add 50 uL of 0.11 M Sodium acetate in Ethanol to each tube
Cap and vortex tube:
Spin 45 mins at 4000 rpm (program 1)
Dump, invert spin 1 min at 700 rpm (program 2)
Add 50 uL of Sodium acetate/ethanol, vortex
Spin 5 min at 4000 rpm (program 3)
Dump, invert spin 1 min at 700 rpm (program 2)
Dry in speedvac (5 mins – no heat)
Resuspend in 15 uL Hi-Di
Denature 95 degrees/2 mins in 96-well thermocycler hot block
Snap chill 5 mins and load all into 3100 plate

96-well plate with Sephadex:

Follow the "Sephadex Spin-Column Protocol" sheet
Dry down purified cycle sequence in speedvac
Resuspend in 15 uL Hi-Di
Denature 95 degrees/2 mins in 96-well thermocycler hot block
Snap chill 5 mins and load all into 3100 plate

Figure J-1 BigDye® Terminator cycle sequencing protocols and precipitation, resuspension, and denaturation protocols

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Appendix K

Cycle Sequencing Clean-up Protocols

Note: The protocols presented here are from the Conservation Genetics Laboratory at San Francisco State University, San Francisco, CA.

12/7/07

Precipitation protocols for the 3100

1.5 mL tube with isopropanol (the usual):
Add 125 uL of 65% isopropanol to each sample
Mix well, pipet into 1.5 mL tube
Spin 30+ mins at max g
Carefully aspirate out isopropanol
Add 125 uL of 75% isopropanol, vortex
Spin 5 mins at max g; aspirate off alcohol
Dry in "Speed-Vac" (vacuum concentrator) for 5 mins – no heat
Resuspend in 15 uL Hi-Di; vortex at low speed or mix by pipetting soln up & down
Denature ~85 degrees/2 mins in big tube hot block
Immediately snap chill by placing tubes in a frozen block for 1 min-5 min
Load all samples into 3100 plate
Quick spin at a low speed (700rpm) before placing in sequencer

Strip tube with EDTA/Ethanol/Sodium acetate:
First add 5 uL of 125 mM EDTA to each tube
Then add 50 uL of 0.11 M Sodium acetate in Ethanol to each tube
Cap and vortex tubes
Spin 45 mins at 4000 rpm (program 1)
Dump (carefully) onto paper towel, invert spin 1 min/700 rpm (program 2)
Add 50 uL of Sodium acetate/ethanol, vortex
Spin 5 min at 4000 rpm (program 3)
Dump (carefully) onto paper towel, invert spin 1 min/700 rpm (program 2)
Dry in "Speed-Vac" (vacuum concentrator) for 5 mins – no heat
Resuspend in 15 uL Hi-Di
Denature 95 degrees/2 mins in 96-well thermocycler hot block
Immediately snap chill by placing tubes in a frozen block for 1 min-5 min
Load all samples into 3100 plate
Quick spin at a low speed (700rpm) before placing in sequencer

96-well plate with Sephadex:
Follow instructions on the "Sephadex Spin-Column Protocol" sheet
Dry down purified cycle sequence in a "Speed-Vac" (vacuum concentrator)
Resuspend in 15 uL Hi-Di
Denature 95 degrees/2 mins in 96-well thermocycler hot block
Immediately snap chill by placing tubes in a frozen block for 1 min-5 min
Load all samples into 3100 plate
Quick spin at a low speed (700rpm) before placing in sequencer

Figure K-1 Precipitation protocols for the 3100

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