# DNA DIAGNOSIS OF ANCIENT THALASSEMIA

# DNA DIAGNOSIS OF THALASSEMIA FROM ANCIENT ITALIAN SKELETONS

By

Dongya Yang, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of Requirements

for the Degree

Doctor of Philosophy

McMaster University

Doctor of Philosophy, 1997
(Physical Anthropology)

McMaster University
Hamilton, Ontario

TITLE: DNA Diagnosis of Thalassemia from Ancient Italian Skeletons

AUTHOR: Dongya Yang,
M.Sc. (Chinese Academy of Sciences, Beijing, China)
B.Sc. (Lanzhou University, Lanzhou, China)

SUPERVISOR: Professor Shelley R. Saunders

NUMBER OF PAGES: xiv, 211

# ABSTRACT

This thesis reports an attempt to extract DNA from the skeletal remains of five young children who died approximately 1,900 years ago and who were recovered from an Italian archaeological site, Isola Sacra. These skeletons have been tentatively diagnosed as thalassemics based on morphological observations, but alternative diagnoses are also possible. DNA diagnosis was used to attempt to identify thalassemia mutations from the human globin genes extracted from these skeletons.

Successful extraction of the human globin genes is largely dependent on two factors: retrieving sufficient amounts of ancient DNA without PCR (Polymerase Chain Reaction) inhibitors, and no contamination from modern DNA. To improve the chances of success, a more efficient and rapid method of extracting DNA from ancient skeletons was developed using a silica-based spin column technique to maximize the yield of amplifiable DNA from ancient skeletons and minimize the risk of the contamination with modern DNA. In a single step, the DNA is concentrated and separated from non-DNA substances that could inhibit PCR. This sufficiently removed PCR inhibitors but without an increased risk of contamination. A comparison test proved that this new approach is superior to current commonly used methods.

Upon application of the new method, the evidence suggests that ancient human β-globin genes were extracted from three of the five individuals from Isola Sacra. DNA diagnosis for two of the most common Italian thalassemia mutations, IVS1-110 and codon 39 (more than 50% of current Italian thalassemia mutations),

revealed that these three individuals did not have these two mutations. However, the present results cannot totally exclude the possibility of thalassemia from these specimens since five other untested mutations might occur in these specimens.

Precautions were taken to minimize the risk of contamination. Contamination was also monitored by mtDNA analysis of each individual. No systemic contamination took place in this study but a sporadic contamination was identified with one specimen. The further analysis clearly indicated that the contamination came from the author.

This thesis has shown that DNA diagnosis of diseases from ancient remains can be a new, powerful approach to the study of health and disease in past human populations. Technical improvements and revised research strategies are expected to advance DNA diagnoses of ancient diseases.

# ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor and mentor, Dr. Shelley Saunders for her unending encouragement, support and tolerance throughout my five years at McMaster. It is her vision about ancient DNA research and her faith in my abilities that have really made this thesis possible. I would also like to thank Dr. Ann Herring for her encouragement and help, which have been far beyond that warranted as a member of my supervisory committee. Her positive thinking and optimistic attitude have helped me through many difficult times. Shelley and Ann have played key roles in my graduate career, and I am honoured and privileged to have been treated by them as a colleague. Thank you, Shelley and Ann. I would also like to thank Dr. John Waye of my supervisory committee for his expert critique of ancient DNA studies and of my own project. His expertise in forensic DNA analysis has been a great help always in keeping my project on right track. I am also indebted to Dr. Edward Glanville for his time and effort taken serving as a member of my supervisory committee.

Many thanks go to Barry Eng for his help in almost all technical aspects of this project. His unmatched skill and expertise with DNA have helped me to overcome many technical problems. Thank you, Barry for being a great colleague and a good friend. I am grateful to Chris Dudar for sharing his knowledge and experience with ancient DNA with me. I also extend my thanks to Margie Patterson for her help in the DNA analysis.

I would like to thank Dr. Anne Keeleyside for her encouragement,

help and kindness during my first years at McMaster. Thanks also go to Drs. Charles FitzGerald and Rob Hoppa for their help in my preparation of the oral defence. For their friendship and camaraderie, I extend my thanks to Tina Moffat, Todd Garlie, Tracy Prowse, Clare McVeich, Sylvia Abonyi, Tosha Dupras, Debi Truscott, and Cathy Crinnion. I cannot forget to say thank you to Janis, Cookie and Rosita of the Department for their help during my years at McMaster.

I am grateful to Drs. Roberto Macchiarelli and Luca Bondioli of the National Prehistoric Ethnographic "L. Pigorini" Museum of Rome for their permission to access the Isola Sacra specimens and the use of their unpublished data on morphological observations of specimens. Thanks are also due to Alexandra Sperduti and Tracy Prowse for their efforts at gathering morphological skeletal data. I would also like to thank Drs. Wu Liu and Zhenbiao Zhang of the IVPP, Beijing for providing some ancient skeletal samples.

Special thanks go to Drs. Christopher Justice and Patricia Seymour for their invaluable friendship. Their help and encouragement in my graduate studies are greatly appreciated.

Finally, I am most grateful to my parents, brother and sisters for their faith in my ability and their support of my education. Thanks to my mother-in-law for her time taken baby-sitting my son. A very special thanks goes to my wife, Xiaobang for always encouraging me and believing in me, and to my son, Joshua, a little smart boy, who has inspired me with his unique questions.

This research was supported in part by the School of Graduate Studies, McMaster University, the Arts Research Board of McMaster University and the Social Sciences and Humanities Research Council of Canada.

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# CHAPTER 1. INTRODUCTION

## 1.1. The Study of Health and Diseases of Past Human Populations

Diseases have played an important role in the course of human history and in the development of human civilization. In the Middle Ages, the so-called "Black Death" lasted six years and killed approximately 20 million people, one-fifth of the population of western Europe (Ampel, 1992). The great influenza pandemic of 1918-1919 killed at least 20 million people worldwide (Taubenberg et al., 1997). In human history, malaria once afflicted more than two thirds of the world's populations (Wyler, 1992). The present worldwide AIDS (acquired immune deficiency syndrome) epidemic has affected more than 18 million people (data up to 1995) (Ward et al., 1997). The AIDS epidemic, like the "Black Death", is affecting human populations not only as a biological disease but also as a biological "agent" of many human social and cultural problems (Brandt, 1991; Word et al., 1997).

From the human perspective, the fight against diseases has never stopped. There was a point when it was believed that the impact of diseases on humans would decrease and would even be erased since we would be able to control and prevent them (Neu, 1992; Wilson, 1994). With the widespread use of antibiotics, vaccines and the development of new medicines, the improvement of hygienic conditions, the morbidity and mortality of most diseases that were previously major killer diseases has decreased.

Smallpox has even been wiped out from the surface of the earth (Cohen, 1992). The emergence of new infectious diseases such as AIDS (Mann, 1991) and the resurgence of old ones such as tuberculosis (TB) (Neu, 1992) clearly indicate that bacteria or viruses are more adaptable than humans had previously thought (Mims, 1987; Wright, 1990; Ampel, 1992; Wislon, 1994).

These problems are derived from complicated, interactive and dynamic relationships between diseases and humans (Fabrega, 1981, Black, 1990; Sommerfeld, 1994). Changes on either side will affect the balanced state of these dynamic relationships, promoting the emergence of new diseases (Mim, 1980; Morse, 1990; Brinkmann, 1994; Garrett, 1994). For example, urbanization brings more people together and provides opportunities for pathogens to readily spread among individuals. Humans are able to cope with new diseases biologically by developing new, resistant mutations, altered immune systems and culturally, by changing their behaviors and lifestyles (Busvine, 1980; Black, 1990; Ampel, 1992; Brinkmann, 1994). Human diseases, therefore, must be studied in terms of ecological interactions and evolution (Haldane, 1949; Lederberg, 1988; Brinkmann, 1994; Ewald, 1994). Modern medicine and epidemiology have greatly enhanced our understanding of the emergence of new diseases and their relationship with environmental factors (Kilbourne, 1991; Krause, 1992; Chen, 1994). But this relationship can also be explored historically by examining the evidence of diseases from ancient human remains (Brothwell and Sandison, 1967; Cockburn and Cockburn, 1980; Zimmerman and Kelley, 1982; Ortner and Putschar, 1985; Ortner and Aufderheide, 1991; Roberts and Manchester, 1995). Ancient remains can preserve the

marks of diseases, providing us knowledge of the origins and spread of human diseases. Records of diseases on and in ancient remains may provide invaluable insights into how humans and pathogens have interacted with each other over time and how the consequences of these interactions should be assessed. Over the course of human history, humans have experienced a number of significant events such as the transition from gathering-hunting to agriculture, from agriculture to urbanization and the contact between people of the New World and the Old World. It has been suggested that these and other events, some of which may also be inferred from the archaeological evidence, have had substantial impacts on human population health and human relationships with pathogens (Goodman et al., 1984; Cohen, 1989; Roberts and Manchester, 1995). For example, the initial contacts of Europeans with native Americans caused deaths due to diseases to which the Aboriginal peoples had no immunity (Sievers and Fisher, 1981).

One of the most important ways to search the human past for information about diseases is in the field of paleopathology, the study of diseases of ancient remains (Roberts and Manchester, 1995). Human remains have been regarded as one of the most important pieces of hard evidence regarding the health and diseases of past human populations (Cockburn and Cockburn, 1980) because diseases can leave permanent antemortem lesions on skeletons and mummified tissues. Human remains provide an opportunity for paleopathologists to directly examine these marks and infer the presence or absence of various diseases (Ortner and Aufderheide, 1991). While soft tissues can be intentionally or naturally mummified and preserved in good condition under certain circumstances, skeletons are still the most commonly preserved ancient remains, so that

most paleopathological research has been performed on skeletal remains (Brothwell and Sandison, 1967; Ortner and Aufderheide, 1991).

The study of the health and diseases of ancient remains will contribute to our understanding of the life ways and culture of past human populations (Cohen, 1990). Disease, as a harmful factor to the human health, will influence or even alter human populations culturally and biologically (Swedlund and Armelagos, 1990). In fact, pathological ancient remains are the results of long-term naturally epidemiological experiments between humans and their diseases. In summary, the study of health and diseases of past human populations is important for an understanding the past, and it is helpful to predict what will happen in the future.

## 1.2. Problems with the Diagnosis of Ancient Diseases from Skeletal Remains

One of the greatest challenges in paleopathological studies is the accurate diagnosis of individual diseases from ancient skeletons and mummies (Ortner and Aufderheide, 1991). Skeletons cannot record all of the diseases that human beings had ever experienced; only a small proportion of diseases can leave their marks on skeletons. Several different diseases can produce similar or the same skeletal lesions (Ortner, 1991). Skeletal changes cannot be solely used as diagnostic criteria for specific individual diseases. The problems seem unresolvable because skeletons are usually the only material available for investigation (Brothwell and Sandison, 1967). The difficulty is also

exacerbated by lack of standard observation criteria among paleopathologists, producing observation errors (Ortner, 1991). However, accurate diagnosis of individual diseases is essential to the study of the health and diseases of past human populations (Pfeiffer, 1991). For example, many of the following questions can be only addressed with correct diagnoses of diseases.

1. When and where did new diseases originate and spread temporally and spatially? How did new diseases originate and how were foreign diseases introduced into specific human populations? Only correct identification of these diseases can be used to locate their occurrence.

2. What impact did these new diseases have on past human populations? Accurate diagnosis of diseases is required to determine mortality and morbidity levels in specific human populations.

3. How did early human beings attempt to cope with the emergence or invasion of new diseases biologically and culturally? Identification of diseases is the first step to establishing the association between diseases and cultural and biological changes.

4. How was the interactive balance between human beings and their diseases maintained?

Although accurate diagnosis of ancient diseases cannot answer all of these questions, without convincing diagnoses, any conclusions regarding these questions can only be tentative, and in some cases, can even produce more confusion than clarification (Ascenzi et al., 1991). Accurate diagnosis of diseases, therefore, must be considered to be the first issue for any health-related study of past human populations.

Diagnosing disease is a rather complicated process even in modern clinical practice (Zimmerman and Kelley, 1982; von Lichtenberg, 1991). In most cases, correct detection and accurate diagnosis cannot be reached on the basis of one single sign or symptom. The human body is an integrated functional organism; changes or disorder in one tissue, organ or system will affect other tissues, organs or systems. A comprehensive and integrative approach is necessary in detecting and diagnosing diseases. Even though many diseases are well understood and their etiology has been revealed, mis-diagnosis still occurs. This is partly because disease symptoms might vary between different individuals and populations, and partly because diagnostic methods are not highly effective. For example, for some common infectious diseases and genetic diseases, convincing diagnosis cannot be made until successful cultures of pathogens in the laboratory or detailed pedigree analyses are available (Zimmerman and Kelley, 1982). It is expected that the difficulty with paleopathological diagnosis in paleopathology will be much greater if only limited materials such as skeletons are available.

Therefore, more effective ways must be explored for more accurate paleopathological diagnosis of ancient diseases (Ortner et al., 1992). Histochemical, immunohistochemical, protein analysis, and other biochemical analyses have been attempted to realize this goal (Montes et al., 1985; Fornacia and Marchetti, 1986; Ascenzi et al., 1991; Tuross, 1991; Nerlich et al., 1993). For example, Montes and colleagues found that elastic system fibers and collagens markedly resisted change for more than 2,000 years (Monte et al., 1985). These findings offer potential for future applications in the diagnosis of ancient diseases because the arrangement patterns of collagen are subject

to a series of diseases, such as bone tumours and osteoarthrosis (Nerlich et al., 1993). Nerlich and colleagues identified some antigens in a cartilage tissue of an infant Peruvian mummy dating between 500-1,000 A.D. and demonstrated that the antigenic determinants were still adequately preserved for immunohistochemical analysis (Nerlich et al., 1993). Fornacia and Marchetti have used immunological methods and reportedly identified human *Treponema pallidum* antigen in an Italian mummy (1,503-1,668. A.D.) (Fornacia and Marchetti, 1986). Schistosome antigens have also been reportedly identified in mummies by the ELISA (enzyme-linked immunosorbent assay) technique, a very sensitive and high specific test for the identification of pathogens (Deelder, 1990). Weisor and coworkers have even found an enzyme that still showed activity in a naturally mummified individual (1,200 B.C.) (Weisor et al., 1989). Biochemical changes are more sensitive for the detection of diseases or disorders than are pathological lesions. In some cases, a specific biochemical change can be identified where no pathological lesions can be observed. The infection can cause death before the disease can produce detectable pathological lesions. But because of chemical degradation, the amounts of preserved proteins and active enzymes are so low in the majority of ancient remains that even the most sensitive methods available with modern biotechniques cannot identify their existence (Tuross 1991).

Deoxyribonucleic acid (DNA), another type of macrobiomolecule, appears to be more useful than proteins in the biochemical analysis of ancient remains for the detection of ancient diseases (Pääbo et al., 1989, Brown and Brown, 1992). DNA is not always better preserved than proteins in ancient remains but the availability of the new

amplification technique, polymerase chain reaction (PCR), has made the detection of extremely low amounts of preserved DNA possible. With the PCR technique, DNA molecules can be retrieved and analyzed from ancient remains tens of thousands of years old (Pääbo, 1989; Hagelberg and Clegg 1991; Krings et al., 1997). Although most human ancient DNA (aDNA) studies focus on mitochondrial DNA, some nuclear genes have been amplified and identified from ancient remains (Herrmann and Hummel, 1994). Human HLA genes have been amplified from the 7,500-year-old wet brain tissues from the Windover site in Florida (Lawlor et al., 1991; Hauswirth et al, 1994).

In fact, the PCR technique and DNA analysis have brought about a revolution in clinical diagnosis of diseases in modern medical practice (Friedmann, 1990; Lee, 1993; Engleberg, 1994, Weatherall, 1996). Many protocols have been established to replace traditional diagnostic methods for the diagnosis of infectious diseases and genetic diseases. For example, it would take a week or several weeks to identify tuberculosis by culturing bacterial pathogens in a laboratory. In contrast, this disease can be diagnosed correctly in a few hours using the PCR technique (Burstain et al., 1991; Eisenach et al., 1991; Sritharan and Barker, 1991). Pathogens, such as bacteria and viruses, have their own specific DNA sequences; genetic diseases are associated with mutations - specific changes in DNA sequences. Due to the specificity of DNA sequences, the detection of their presence in samples from patients can be regarded as indicating the presence of the infectious or genetic disease (Mims, 1987; Tompkins and Falkow, 1992).

Retrieval of DNA from ancient remains and the emergence of molecular medicine or molecular pathology have provided the possibility of identifying ancient

diseases using DNA analysis. Theoretically, most techniques of modern clinical DNA diagnosis can be used directly or indirectly in the diagnosis of ancient diseases when DNA is retrieved from ancient remains. However, to date, only a few studies have been successfully carried out. DNA from *Mycobacterium tuberculosis* of tuberculosis and *Mycobacterium leprae* of leprosy reportedly has been amplified from ancient human skeletons and mummied soft tissues in the Old World and the New World (Spigelman and Lemma, 1993; Rafi et al., 1994; Salo et al., 1994; Arriaza et al., 1995). A sequence of β- thalassemia mutation reportedly has also been amplified from a child skeleton dated to the 16th to 19th centuries from Akhziv, Israel (Filon et al., 1995).

At present, DNA diagnosis of ancient diseases is fraught with difficulties due to inefficient extraction of aDNA from archaeological remains and troublesome contamination with modern DNA. Diagnosis of human genetic diseases caused by mutations in single-copy genes faces an even greater technical challenge because of low yields of aDNA. Some people are pessimistic about the possibility of DNA diagnosis of ancient human diseases. In a recent editorial of the International Journal of Osteoarchaeology, Waldron comments that "human aDNA may be able to provide some information about genetic mutations but it is not likely to be helpful for diagnostic purposes" (Waldron, 1996).

However, our accumulated knowledge about aDNA has clearly indicated that it can survive up to tens of thousands of years (Thomas and Pääbo, 1993). More recently, mitochondrial DNA (mtDNA) sequences have even been extracted from a Neanderthal fossil of 30,000 to 100,000 years old (Krings et al., 1997; Lindahl, 1997). Practical,

technical problems are major obstacles for aDNA recovery from ancient remains (Handt et al., 1994a). Nevertheless, the possibility of developing new techniques to overcome these problems will increase with the emergence of new technologies in molecular biology.

## 1.3. Diagnosis of Thalassemia from Ancient Italian Skeletons

The primary objective of this study was to explore the possibility of applying modern clinical DNA techniques to the diagnosis of diseases from ancient skeletal remains. This was attempted using DNA isolated from skeletal remains from an archaeological site, Isola Sacra in Italy, which is approximately 1,900 years old. DNA diagnosis was used in an attempt to detect thalassemia mutations of the human globin genes from the aDNA. One goal of this study was to develop and test a model for the diagnosis of ancient diseases using skeletal DNA. The model would first search morphological lesions and other archaeological evidence for primary clues for recognizing specific diseases and then use DNA diagnosis to support or refute the preliminary morphological diagnosis.

There were two reasons for choosing a genetic disease as the focus of this study as opposed to an infectious disease. First, there is a direct correlation between the presence of certain disease gene mutation and a recognizable disease phenotype. However, this is not always the case for infectious diseases. Failure to detect a specific

pathogen DNA does not necessarily mean that the pathogen is absent or the lack of disease condition. This is because technical problems can also cause negative results. Secondly, most genetic diseases are caused by mutations in single-copy nuclear genes. Low number of amplifiable aDNA templates may affect the detection, however, successful identification of genetic diseases might indicate a higher success rate for other ancient diseases.

Thalassemia is a genetic anemia caused by defects in the synthesis of the globin polypeptide chain components (Weatherall and Clegg, 1981). The identification of thalassemia was attempted in this study for a number of reasons. First, it is the most commonly inherited single-gene disorder and its pathogenesis is well understood (Friedmann, 1990). Secondly, thalassemia can leave specific bone lesions in skeletons, providing the indicators of the presence of this disorder. Thirdly, the study of ancient thalassemia can help us to better understand the effects of natural selection on human populations since the emergence of thalassemia has been attributed to the prevalence of malaria and the practice of agricultures in human history (Weatherall, 1996).

The reasons for using the Isola Sacra skeletons to identify thalassemia relate to the site location and the presence of bone lesions. These skeletons were tentatively diagnosed as thalassemics based on morphological observations and their ages at death. The Isola Sacra site is located in a lowland area and lowland populations seem to have a higher frequency of thalassemia mutations than highland populations in Italy (Weatherall and Clegg, 1981). No data are available to specifically indicate the presence or absence of malaria at the Isola Sacra site 1,900 years ago. But overwhelming evidence indicates that

malaria was highly prevalent in surrounding areas. The first observation of various malaria fevers was made in Greece by Hippocrates about 400 years B.C. (Coatney et al., 1971). The study of other early medical literature has provided further evidence that malaria was prevalent in ancient Greece and Italy (Jones, 1967). Based on these data, malaria existed in these areas at least 500 years before the Isola Sacra individuals were buried. The study of primate malaria has clearly indicated that malaria has been prevalent in the Old World primates for at least a million years (Coatney et al., 1971), indicating that human malaria might have been present in the Mediterranean long before Hippocrates first described it 2,400 years ago. If malaria is indeed a promoting factor for emergence of thalassemia, Isola Sacra people and their ancestors must have been exposed to it for a sufficient time to develop thalassemias. Therefore, it seems probably that thalassemia mutations might be present in these skeletons from the Isola Sacra site.

DNA diagnosis was therefore used to try to identify these mutations from the human globin genes and to trace the Italian history of human thalassemias, and to confirm or refute the morphological diagnostic criteria of thalassemia from archaeological skeletons. In addition, molecular diagnosis of thalassemia from ancient remains may also provide a better understanding of the interaction between thalassemia and malaria in human history.

The first part of Chapter 2 in this thesis reviews the literature on the theoretical and practical aspects of aDNA studies. The second part introduces the background knowledge on the origins and distribution, the bone manifestation and the molecular pathogenesis of thalassemia. Chapter 3-4 describe the materials and methods used in this

study, including a new DNA extraction method and method for diagnosing thalassemia mutations from aDNA. The results of the PCR amplification and the sequencing of the human β-globin gene and a mtDNA hypervariable segment, as well as the Dot Blot tests for β-thalassemia mutations are presented in Chapter 5. While focusing on the authentication of DNA sequences obtained from the Isola Sacra specimens by mtDNA sequence analysis, an analysis of the effort to identify two major thalassemia mutations, a discussion of the significance of amplification of the ancient human β-globin genes are also provided in Chapter 6. Based on the results of this study and the achievements made by others in the aDNA study and other fields, the author proposes in Chapter 7 that DNA diagnosis will be a new approach to paleopathology. The probability, possibility and applicability of DNA diagnosis of ancient diseases are critically reviewed in this Chapter. A summary of the conclusions is presented in Chapter 8.

# CHAPTER 2. BACKGROUND

## 2.1. Ancient DNA

The advent of the PCR technique in molecular biology (Saiki et al., 1985; 1988) has made it possible to amplify and analyze ancient DNA (aDNA) to reconstruct the biological history of past populations (Rogan and Salvo, 1990a; Herrmann and Hummel, 1994). "aDNA" refers to deoxyribonucleic acid (DNA) molecules which are preserved in ancient remains such as archaeological bones, mummified tissues, museum specimens of extinct organisms and even animal and plant fossils (Rogan and Salvo, 1990b; Brown and Brown, 1992). Genetic information retrieved from aDNA provides a new research perspective in anthropology, archaeology, forensic sciences, biology, paleontology and the evolutionary sciences (Pääbo, Irwin and Wilson 1989; Rogan and Salvo, 1990a; Herrmann and Hummel, 1994). Studies over the past decade have shown that aDNA research holds great potential to resolve questions that cannot be dealt with through traditional approaches.

Ancient DNA studies still face intrinsic problems with technology, methodology and interpretations. Ancient DNA is highly degraded and only minute amounts can be preserved in ancient remains. PCR, the key technique of aDNA study, is vulnerable to

contamination with modern DNA, therefore, it is difficult to verify the results of aDNA studies. However, these problems can be minimized or overcome by technical improvements and innovations in methodological strategies.

Because aDNA study is such a young discipline, I will briefly review the history, methodology, and achievements of the field in the following section.

## 2.1.1. History of aDNA Studies

The first attempt to extract DNA from ancient remains took place in China in 1980. DNA was extracted from the liver, muscle and rib cartilage of a well preserved woman from a 2,100 year old site (Hunan Medical College, 1980). However, DNA was only extracted and quantified -- no further analysis was carried out. This extraction had clearly shown that aDNA could be preserved for at least 2,100 years.

In 1984, the first clonable aDNA was extracted, cloned and sequenced from the dried muscle of a quagga, a zebra-like species (*Equus quagga*) that became extinct in 1883 (Higuchi et al., 1984). A 229 bp (base pairs) mtDNA sequence was compared with zebra, an extant member species of *Equus,* and 12 sites of base substitutions were identified. This study indicated that aDNA could be analysed by modern molecular techniques. Shortly afterward, a human DNA sequence was cloned and sequenced from a 2,400 year old Egyptian child mummy (Pääbo, 1985). This study revealed that most of the extracted DNA was fragmentary, less than 500 bp in length.

The successes of the above two studies were likely due to the fact that a high

amount of clonable DNA was obtained from these particular samples. For example, in the study of Egyptian mummies (Pääbo, 1985), among the 23 investigated mummies, only one yielded a sufficient amount of DNA for analysis (Pääbo, 1985). It was estimated that the total DNA yielded from each gram of the samples were approximately 5μg in the quagga and 20μg in the Egyptian mummy (Higuchi et al., 1984; Pääbo, 1985).

This situation was greatly improved after the PCR technique was invented in 1985 by Kary Mullis (Saiki et al., 1985; Saiki et al., 1988). The required amount of original DNA for successful analysis was then dramatically decreased. The potential of the PCR technique in aDNA studies was first demonstrated in 1988 when mtDNA was amplified from a 7,000 year old brain uncovered from Windover, Florida (Pääbo, Gifford and Wilson, 1988). Pääbo and Wilson also found that the PCR technique would produce more authentic aDNA sequences than the cloning technique does (Pääbo and Wilson, 1988).

The availability of the PCR technique has brought about a proliferation in aDNA studies in the early 1990s (Horai et al., 1989; Hagelberg and Clegg 1991) after the first extraction and analysis of ancient human bone DNA was completed in 1989 (Hagelberg, Sykes and Hedges, 1989). A 121 bp and a 205 bp mtDNA sequence were amplified from 300 and 5,500 year old human bones.

The materials available for aDNA studies were then extended to include all human or animal skeletons available from archaeological collections worldwide. The base of research was established such that aDNA could be analysed on the scale of time and space (Cherfas, 1991). The terms "molecular archaeology" and "biomolecular archaeology" were even coined to describe this new field falling between traditional

archaeology and modern molecular biological techniques (Pääbo et al., 1989; Brown and Brown, 1992).

## 2.1.2. DNA Structure and the PCR Technique

DNA is the molecule that encodes genetic information. It is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. Two polynucleotide strands are wound around each other in a helix. Each strand is a linear arrangement of nucleotides, which are each composed of one sugar, one phosphate, and a nitrogenous base. The bases are classified in two groups: the purines of adenine (A) and guanine (G), and the pyrimidines of thymine (T) and cytosine (C). The linear order of the these bases arranged along the sugar-phosphate backbone is called the DNA sequence. DNA is replicated only when a cell is reproduced. Under the presence of certain polymerase and free nucleotides, each strand can be used as a template for the synthesis of a complementary new strand. Base pairs form only between A and T and between G and C. Strict rules of base-pairing ensure that new "daughter" double-stranded DNA carries the same information as the old "parent" DNA.

The total DNA sequence in a cell is defined as a genome. The genome includes coding sequences and non-coding sequences. For coding sequences, each three nucleotides encode one specific amino acid, thus along a DNA sequence, a number of amino acids can be encoded and consequently specific proteins can be produced. When some nucleotides in a coding sequence are substituted, the sequence may produce different protein products. Consequently, their biological functions may be affected or

changed.

The determination of the nucleotide number and position in a DNA molecule is called "nucleotide sequencing", "DNA sequencing" or simply "sequencing". It is estimated that the human genome contains about 3 billion base pairs of DNA sequences (Schuler et al., 1996; Collins, 1997). Only 10% of them are coding sequences (about 100,000 genes); the rest are noncoding sequences including control sequences, intron sequences within a gene, and intergenic regions between genes. The functions of non-coding sequences are not well understood but their vast variations both within and between populations have proven to be very useful in individual identification in medicolegal situations and in the studies of biological population affinities (Lee, 1993).

DNA sequences are the result of stochastic events and a selective process in the evolutionary change of populations (Pääbo et al., 1989). The emergence of new sequence patterns can only come from random mutations. But new sequences can be passed on to subsequent generations; individuals can only receive half of their distinctive DNA sequences from their mother and half from their father. The heterozygosity in parents' sequences ensures that each child receives different combinations of sequences from their parents. DNA variations can be defined and analyzed at the level of the individual, the population, and the species. The identification of specific DNA sequences is essential for the study of aDNA. At the species level, ancient samples can be identified and classified and DNA phylogenetic analysis can be used to determine the phylogenetic positions of individual species and species groups (DeSalle et al., 1992). At the population level, sample population affinities can be analyzed and historical migration and microevolution

can be studied (Merriwether et al., 1994). At the individual level, personal identification can be performed and family kinships can be tested (Gill et al., 1994).

Successful detection of specific DNA sequences is largely dependent upon the application of certain molecular techniques. Among them, the most important one is the PCR, an *in vitro* DNA amplification system (Saiki et al., 1985). PCR is especially useful because it is highly specific, easily automated, and capable of amplifying minute amounts of a sample (Saiki et al., 1988). The PCR technique can detect an extremely small amount of DNA (even one molecule) and in the matter of a few hours, amplify a great amount of the desired DNA. To initiate a PCR amplification, four free nucleotides (A, T, G and C), two oligonucleotide primers and Taq polymerase (an enzyme for DNA synthesis) are required to mix with target DNA samples. The primers are artificially synthesized small DNA sequences (around 20 bp) that flank the target DNA sequences. When the mixture is heated, double-stranded DNA is separated into two single strands. If the template DNA samples contain the target sequence, when it is cooled, the primers find and anneal to their complementary sequences of the template DNA on the separated strands; and the polymerase extends the primers into new complementary strands. The newly synthesized DNA is then used as new DNA templates to participate in the next PCR cycle. The whole process is automatically cycled from DNA denaturation, primer annealing, to polymerase extension. This leads to the exponential accumulation of an original DNA fragment, approximately $2^n$, where n is the number of cycles. The PCR technique is an essential technique for aDNA studies because aDNA is highly degraded and the preserved DNA can only be retrieved in minute amounts (Pääbo, 1989).

## 2.1.3. Mechanisms of aDNA Degradation

The DNA structure in a living cell is always affected by destructive chemical processes: mainly spontaneous hydrolysis of bonds between base and sugar residues, breakage of the sugar backbone, and oxygen radical-induced attacks on bases and sugar residues (Handt et al., 1994a). The effects of these processes are counteracted by a cell's specific DNA repair processes (Lindahl, 1993a). This is the maintenance mechanism of DNA structure and function in a living cell.

After death, all cell repair processes stop but the destructive processes continue, and postmortem DNA degradation occurs. Two major processes, hydrolytic and oxidative damage, are thought to account for most aDNA lesions (Pääbo, 1989). Under the presence of water and/or oxygen, DNA degradation theoretically cannot be prevented (Lindahl, 1993a). Even in a dry climate, due to its hydrophilicity, DNA remains partly hydrated and hydrolytic modification still occurs (Handt et al. 1994a). Since "some of the water molecules in the grooves of the DNA double helix are structurally essential"(Lindahl, 1993a), completely "dry DNA" probably does not exist. The "drier DNA" becomes even more vulnerable to oxidative damage once it is exposed to air (Lindahl, 1993a).

Based on a degradation rate obtained from an experiment on modern DNA in a laboratory environment (Lindahl and Nyberg, 1972), Pääbo and Wilson estimate that DNA should be completely degraded after 4 million years (Pääbo and Wilson, 1991).

Lindahl believes that degradation is time-dependent on chemical damage and convincing reports of aDNA amplifications must be less than 100,000 years old (Lindahl, 1993b).

The survival ability of aDNA seems stronger than these theoretical estimates would suggest under certain circumstances (Golenburg et al., 1990; DeSalle et al., 1992; Soltis et al., 1992). Data on bacterial spores would suggest that the stability of DNA could be increased by the exclusion of oxygen and by partial dehydration. In 1990, the oldest aDNA retrieved was reportedly found in magnolia fossils from a Miocene Clarkia deposit (17-20 million years old) (Golenberg et al., 1990). An 820 bp chloroplast DNA fragment was extracted and sequenced. However, the validity of this report is questioned because it contradicts the theoretical model of aDNA degradation and an independent study did not produce positive results from samples from the same fossil sites (Lindahl, 1993b). This record of the oldest DNA was later supported by another independent report of successful reproduction of positive results, the amplification of a 1,320 bp segment of a chloroplast gene from the similar fossils from the same deposit (Soltis et al., 1992). The well-preserved DNA from this particular site is attributed to the extraordinary environmental conditions; oxygen was totally excluded from contact with materials in the deposit (Golenberg, 1994).

The record of the oldest DNA was soon rewritten when DNA was extracted and sequenced from a 120-135 million year old weevil found in Lebanese amber (Cano et al., 1993). The successful extraction was attributed to the amber resin, which could quickly withdraw the moisture from the original tissues and thus initiate the process of inert dehydration (Poinar, 1994; Poinar et al., 1994). Critics argued that it is unlikely that

aDNA could survive that long. Endogenous oxidative damage itself could degrade all DNA since amber could not provide complete protection against oxygen (Lindahl, 1993b).

The effort to search for the oldest DNA in dinosaur bones is less convincing. DNA has been reportedly extracted and sequenced from an 80 million year old bone fragment of a dinosaur-like skeleton (Morell, 1993; Gibbons, 1994; Woodward, Weyand, and Bunnel, 1994) and from a 70-80 million year old dinosaur egg (An et al., 1995; Li et al., 1995). Reanalysis of the published sequences from these studies suggests that the DNA did not come from the dinosaur (Hedge et al., 1995; Yang, 1995). The assumed dinosaur DNA was found to be closer to the DNA of humans and other mammals by phylogenetic tree analysis than to birds (Hedge et al., 1995). This strongly suggests that the DNA from these studies is more likely a result of contamination with human DNA, because birds are considered to be the closest extant relatives of dinosaurs amongst living organisms. Reanalysis of the DNA from the dinosaur egg suffered the same fate (Yang, 1995). The phylogenetic analysis reveals that the purported dinosaur egg DNA is probably from plant DNA. This was not surprising because the dinosaur DNA was extracted and amplified in a laboratory where plant DNA studies are performed daily (Yang, 1995).

The discrepancy between the theoretical predictions of DNA degradation and the reported empirical results of aDNA extraction is obvious. Mechanisms of DNA degradation appear to be more complicated than theory indicates, and laboratory studies of DNA degradation cannot reveal the whole process of aDNA degradation. Hydrolysis

is regarded as a major source of damage to DNA, but aDNA has been extracted and analyzed from a number of fully hydrated materials (Golenberg et al., 1990; Hauswirth, Dickel and Lawlor, 1994). Neutral conditions may be considered optimal for aDNA preservation because acidic conditions can depurinate the DNA and alkaline conditions can cleave it at apurinic or apyrimidinic sites (Ausubel et al., 1991). However, DNA has been extracted from 8,000 year old soft tissues uncovered from an acidic bog environment in Florida (Hauswirth, Dickel and Lawlor, 1994). Several contradictory factors may explain why. For example, chromosomal material, including DNA may be better preserved in acidic conditions. Alternatively, acidic environments can prevent microbial activity and chelate metal ions resulting in longer preservation (Eglinton and Logen, 1991).

There are many factors involved in the process of aDNA degradation. More research is needed with a focus on the mechanisms of aDNA degradation in order to understand the preservation of aDNA. It has been found that the degradation rate of aDNA is not linear over time, contradicting the theoretical model. Pääbo compared the yields of aDNA from samples ranging from 4 to 13,000 years old and found no linear correlation between the yield of the DNA and sample age. He speculated that DNA oxidative damage reaches a plateau over a short time period and then only minimal additional effects are added afterwards (Pääbo, 1989).

## 2.1.4. aDNA and the PCR Technique

According to one estimate, less than 1% of aDNA molecules from museum specimens and archaeological material can be expected to be undamaged (Pääbo et al., 1989). The results of postmortem modifications are such that the amount of DNA that can be isolated from ancient material is very small, and the size of DNA fragments that can be amplified and sequenced is also very small (only a few hundred base pairs) (Pääbo and Wilson, 1988; Herrmann and Hummel, 1994). After death, DNA molecules may break up, and some nucleotides may separate from DNA molecules, leaving a baseless region, but once the synthesis process has stopped, new DNA sequences are not produced. The DNA extracted from dead tissue (ancient material) might be very small in both amount and size, and might be heavily damaged, but, whatever the size, it can be generally regarded as comprising unchanged sequences (Pääbo et al, 1989; Rogan and Salvo, 1994).

The PCR technique has proven to be an ideal tool for amplifying small, intact or slightly damaged DNA molecules present in a vast excess of damaged molecules. Besides its ability to detect extremely small quantities of DNA, PCR has no capacity for repair or mis-repair of original DNA sequences (Pääbo and Wilson, 1988; Pääbo et al., 1989). For example, the DNA sequence from the quaaga obtained by the means of cloning showed a difference in two positions from the sequence obtained from direct PCR sequencing. Detailed comparisons with other sequences showed that the two bases were misrepaired

during the cloning process (Pääbo et al., 1989). Although little is known about the tolerance of the Taq polymerase to various types of damage in the template DNA, it can be speculated that Taq polymerase, like other DNA polymerases, will be slowed down at damaged locations such as baseless sites. Furthermore, other kinds of damage such as intermolecular and intramolecular cross-links, will completely block the enzyme activity (Pääbo and Wilson, 1988). Thus, damaged DNA molecules in the amplification reaction can be expected either not to be replicated at all, or to be at a replicative disadvantage, so that the intact DNA molecule will be amplified preferentially during PCR (Pääbo and Wilson, 1988; Pääbo et al., 1990).

There is a concern that sequences determined from PCR products may contain artefacts since the process of PCR amplification is not absolutely error-free (Pääbo and Wilson, 1988). The Taq polymerase-mediated PCR itself might cause an overall error frequency of 0.25% in a typical amplification reaction (Sambrook et al., 1989). In the case of aDNA, many chemical modifications which can cause replication errors may worsen this situation. Fortunately, this kind of replication error randomly occurs to an individual molecule, so that the effect of the random misincorporation can be averaged out when the amplification products are sequenced directly (Pääbo, 1989). If the PCR amplification products are cloned and individual clones are sequenced, a high error rate will be encountered. But if this single molecule has a base-error at the first amplification cycle, theoretically half of the amplification products will have this same error. So if the amplification is started from an extremely low number (<10 copies) of original DNA molecules, a particular site error may be expected to show up in the final sequences

(Pääbo and Wilson, 1988). If the amounts of the original DNA templates are sufficient, the PCR technique itself appears not to be a major source of errors but rather offers the possibility of avoiding cloning artefacts.

The phenomenon of "jumping PCR" in damaged DNA amplification may benefit or harm aDNA studies (Pääbo et al., 1989). When Taq polymerase encounters a lesion site of a template molecule, it sometimes inserts an adenosine residue; the prematurely terminated product then jumps to another template and polymeration continues, creating an *in vitro* recombination product (Pääbo et al., 1990). For mammalian mtDNA which is homoplasmic, the PCR products can be longer than the originally intact DNA templates. To some extent, this overcomes the disadvantage of the short length of aDNA sequences. However, a chimeric DNA molecule generated from nuclear heterozygous genes by jumping PCR may produce an incorrect sequence of an original DNA molecule. Like other PCR-generated replication errors, these errors can be averaged out by proper procedures (Pääbo et al.,1989).

## 2.1.5. Retrieval of DNA from Ancient Human Remains

It is widely accepted that DNA can be preserved in and retrieved from remains tens of thousands of years old. Fortunately, this period of time covers an important part of human history. Modern humans are believed to have originated from a single region or multiple regions between 100,000 and 200,000 years ago (Howells, 1993); peopling of the New World and the Pacific islands occurred much later (Hagelberg and Clegg, 1993;

Merriwether et al., 1994); the first crop and animal domestications took place several thousands of years ago (Brown and Brown, 1992; Rollo et al., 1994); urbanization began a few thousand years ago and continues. All of these significant events have actually changed and reshaped the course of human history and civilization. Infectious diseases are believed to be significant factors in biological and cultural history of human evolutions (Haldane, 1949; Ewald, 1993). Crop and animal domestications result in the development of agriculture which then often prompts the emergence of larger communities (Cohen, 1989). An increase in population density favors the emergence and spread of infectious diseases.

Mitochondrial DNA (mtDNA) studies of archaeological remains of Native Americans have shown that information from aDNA may add a significant dimension to studies of the peopling of the New World. So far, limited data have shown that the hypothesis of two independent migration waves from Asia to the New World cannot be confirmed by aDNA studies (Torroni et al., 1992; Merriwether et al., 1994).

Individual identification using aDNA analysis has many applications in archaeology and anthropology (Jeffeys et al., 1992; Kurosaki et al., 1993; Gill et al., 1994; Hänni et al., 1995a). Among them, sex determination and kinship determination are two common ones. Many dimorphic features of the skeletons have been identified and a high sex determination accuracy can be achieved, but the whole skeleton or certain parts such as hip bones are required for good results (Stone et al., 1996). Researchers usually have to deal with subadult or fragmentary bones. For subadults, skeletal sexual dimorphism is not well developed. For fragmentary bones, dimorphic features are usually

missing. The sex determination of these materials has proven to be very difficult. However, DNA discrimination should be a more effective way for sex determination (Gaensslen et al., 1992). Chromosome X and Y have been identified with their specific DNA sequences (Faerman et al., 1995). Theoretically, it should be very simple to find Y specific sequences only in males and X specific sequences in both males and females. DNA sex determinations have been performed on a variety of human skeletons ranging from one hundred years to less than ten thousands of years (Stone et al., 1996; Gotherstrom et al., 1997), but improvements in techniques are still necessary (Saunders and Yang, submitted).

Determination of family relationships among individuals is not as simple as sex determination (Jeffeys et al., 1992; Gill et al., 1994; Ludes et al., 1994). Parents and their children share many common specific sequences in accordance with strict inheritance rules but they are not identical because of recombination at meiosis. The determination of family relationships is a common practice in forensic identification (Hagelberg, Gray and Jeffreys, 1991; Sensabaugh, 1994; Boles et al., 1995). Prior to DNA testing, blood group tests were routine techniques for paternity testing. But now, DNA testing is the most common method. DNA studies have proven that DNA parental tests can also be used with ancient remains (Jeffeys et al., 1992; Kurosaki et al., 1993; Gill et al., 1994). Generally, if we can retrieve sufficient amounts of DNA of high quality, family identification can be readily carried out. Human mtDNA with a great number of copies, therefore, becomes an excellent DNA system for individual identification (Ginther et al., 1992; Hänni et al., 1995a). Some case studies have clearly indicated that the technique is

feasible for the determination of family relationships of skeletal populations in archaeology and anthropology.

The PCR technique and other techniques of DNA analysis have made DNA research significant for studying historical, anthropological and archaeological questions relating to human origins and evolution (Hagelberg, 1993; Hagelberg et al., 1994; Lindahl, 1997; Krings et al., 1997), population migration and mixture (Hagelburg and Clegg, 1993; Merriwether et al., 1994), diseases and health (Salo et al., 1994; Arriaza et al., 1995), social and family structure (Kurosaki et al., 1993; Shinoda and Kunisada, 1994) and crop and animal domestications (Goloubinoff et al., 1993; Brown et al., 1994; Rollo et al., 1994). In reality, the success rate of aDNA amplification is still low for a variety of reasons. To date, knowledge of DNA degradation is not sufficient for us to estimate the state of DNA preservation based on the status of morphological preservation or other criteria (Herrmann and Hummel, 1994). What is worse, PCR inhibition and contamination take place frequently, causing false negative and positive results, respectively.

PCR inhibitions occur when PCR inhibitors are introduced into PCR amplifications. PCR inhibitors include many organic and inorganic substances which can substantially decrease or extinguish the activity of Taq polymerase (Handt et al., 1994a). PCR inhibitors can result in failures of PCR amplifications, or insufficient PCR products (Niederhause et al., 1994). Human skeletons or mummified tissues have been through many chemical and physical processes and the decayed substances are accumulated within the remains. Additionally, some inhibitory substances can also diffuse into the

deposited bones from the preservation environment (Cooper, 1994). When DNA is extracted, these substances will be co-extracted and later introduced into PCR amplifications. Inhibitions caused by these substances are difficult to remove after co-extractions. However, to a certain degree, PCR inhibitions can be overcome by their removal before PCR amplifications are performed (Yang et al., 1997) or by avoiding co-extraction using better methods of extracting aDNA (Höss and Pääbo, 1993).

Contaminations have been proven to be the most troublesome problem for human aDNA studies (Cherfas, 1991; Herrmann and Hummel, 1994; Niederhause et al., 1994). In contrast to PCR inhibition, contamination will cause false positive results. Compared to other animal or plant aDNA studies, human aDNA is more likely to be subject to contamination because researchers themselves are a major contamination source. Modern human DNA contaminants can be introduced into the process of aDNA study at any time and any place from excavation to sample preparation, DNA extraction and PCR setup. Many useful suggestions have been proposed for effective contamination control and close monitoring (Handt et al., 1994a; Stoneking, 1995). Most researchers agree that the number of steps involved in aDNA extraction and PCR setup should be minimized to decrease the opportunity for human involvement (Herrmann and Hummel, 1994). It is true that contamination cannot be totally avoided in practice. This, therefore, has made contamination monitoring an absolutely necessary part of aDNA research (Hänni et al., 1995a).

PCR inhibition and contamination hinders progress in the study of aDNA. Due to the low success rate of aDNA amplification from ancient remains, we await significant

contributions in molecular techniques. Herrmann and Hummel (1994) have pointed out that the future of aDNA studies depends on (1) improvement of extraction procedures from source materials; (2) accumulated knowledge of aDNA degradation processes; (3) ability to extract and amplify longer DNA sequences and the development of repair techniques to restore longer aDNA sequences.

## 2.1.6. Summary

The study of aDNA has become feasible with the advent of the PCR technique and DNA analysis has shown its potential in many research fields. Although the mechanism of aDNA preservation and degradation is not well understood, it has been observed that aDNA can be preserved in remains that are tens of thousands of years old. At present, the broad applications of aDNA analysis are hindered by inefficient extraction of aDNA from ancient remains, PCR inhibition by some substances and contamination with modern DNA. In the future, technical improvements in DNA extractions, removal of PCR inhibitors and a better understanding of DNA preservation mechanisms would eventually make aDNA analysis more applicable and practical in many anthropological and archaeological studies.

# 2.2. Thalassemia

## 2.2.1. What Is Thalassemia?

Thalassemia is a hereditary anemia caused by defects in the synthesis of the globin part of the hemoglobin molecule (Weatherall and Clegg, 1981; Thein, 1993). The abnormal synthesis of hemoglobin in thalassemia results in the formation of erythrocytes deficient in hemoglobin content and generally smaller in volume. Clinical thalassemia varies according to the degree to which hemoglobin is synthesized. The worse the defect in hemoglobin synthesis, the more abnormal are the erythrocytes the patient manages to produce. At one extreme, such as α-thalassemia's Hb-Bart's fetal hydrop syndrome, the disease leads to death as early as *in utero*, but at the other extreme, the carrier state for some forms of β-thalassemia minor is generally harmless with few if any symptoms. Between these two extremes are some severe disorders such as Cooley's anemia which often results in death in early childhood without blood transfusions. Other forms are accompanied by anemia but are not life threatening (Dacie, 1988; Thein, 1993). Thalassemia has been a considerable health burden because frequent blood transfusions may be needed to keep patients alive (Weatherall and Clegg, 1996). With the advent of molecular biology techniques, and after the clusters of the α-globin and β-globin genes were sequenced, the pathogenesis of thalassemia became well understood at the molecular level (Lawn et al., 1980).

Hemoglobin is the oxygen-transporting protein which is composed of four

polypeptide (globin) chains. Each chain is associated with an identical iron-containing prosthetic group (heme), which is able to reversibly bind with oxygen. The tetrameric structure of hemoglobin allows it to bind with large amounts of oxygen in the lungs and then release the oxygen in the tissues. One hemoglobin molecule has two pairs of globin chains. Normal adult hemoglobin includes two α- and two β-globin chains ($\alpha_2\beta_2$) while fetal hemoglobin has two α chains and two γ chains ($\alpha_2\gamma_2$). The α-globin and β-globin chains are 141 and 146 amino acid residues long, respectively (Adams, 1986). The α-and α-like gene clusters are located on the short arm of chromosome 16, and the β-and β-like gene clusters are on the short arm of chromosome 11 (Adams, 1986). Thalassemia results from mutations which cause defective syntheses of one or more globin chains, disturbing the normal equimolar synthesis of α- and non-α-chains (Weatherall and Clegg, 1996). The clinical symptoms of thalassemias are anemia, expansion of the bone marrow with skeletal lesions, enlargement of the spleen and damage to other organs (Weatherall and Clegg, 1981).

## 2.2.2. Origins and Distribution of Thalassemia

Thalassemia was first recognized by von Jaksch a hundred years ago in Prague (Lehmann, 1982), although the first description of the disease as a distinct entity is usually attributed to Cooley and Lee for their report on thalassemia major cases in 1925 (Dacie, 1988). These authors described children with specific pathological bone changes and increased osmotic resistance of the red cells (Lehmann, 1982). In 1932, the term

"thalassemia" or "Mediterranean disease" was introduced to describe this disorder which was commonly seen in patients from Mediterranean areas (Dacie, 1988). The word "thalatta" or "thalassa" in Greek means "sea", in this sense, "thalassemia" or "thalassemia" is a good translation of "Mediterranean anemia ". Many other names have been used for the general disease or specific thalassemia variants. Today, thalassemia has been accepted as a universal term to describe the defects in the synthesis of the globin part of the hemoglobin molecule (Weatherall and Clegg, 1981). But other terms are also in use such as thalassemia major and thalassemia minor. For example, thalassemia can be mainly classified as thalassemia major and minor according to the severity of clinical symptoms. But genetically, thalassemia major can also refer to a homozygous state of thalassemia mutations and thalassemia minor to a heterozygous state (Dacie, 1988).

The distribution of thalassemia is limited to all tropical and temperate areas in the Old World and also coincides with the "malaria belt" since there is a high prevalence of malaria in these regions (Livingstone, 1986). There are almost no thalassemias or other hemoglobin variants in the areas north or south of the malaria belt. In contrast, most human populations that inhabit the malaria belt are highly polymorphic for hemoglobin and thalassemia variants (Flint et al., 1993). For example, the data on population screenings of thalassemia indicate that frequencies of heterozygotes are 15.6% in Cyprus, 12.3% in Athens, 19.4% in Sardinia, Italy (Kuliev, 1987) and 8.0% in Hongkong, respectively (Lau et al., 1997).

Many hypotheses have been proposed for the origins of thalassemia (Satinoff,

1971). In 1964, based on observations of some ancient skeletons with thalassemia-like bone lesions, Zaino proposed that thalassemia originated more than 50,000 years ago in a now inundated Mediterranean valley south of Italy and Greece (Zaino, 1964). However, it is more likely that thalassemia emerged almost simultaneously in many different places under the presence of malaria (Flint et al, 1993; Livingstone, 1986). Even in 1986, more than 400 thalassemia mutations had been identified in different human populations (Adams, 1986). The analysis of 156 β-hemoglobin variants showed that 95% of amino acid substitutions of the β-chain are functionally unacceptable and about 70% of the synonymous mutations are adaptively undesirable (Livingstone, 1986). There must have been a common selective agent behind this scenario such that so many mutations appeared in so many human populations. The heterozygotes of thalassemia also seem to provide more protection against malaria than normal homozygotes. This hypothesis regarding malaria as a selective agent was first proposed by Haldane in 1948 (Lehmann, 1982). Much research and effort has been directed to the question of how the presence of malaria could influence the occurrence of thalassemia (Wyler, 1992; Flint et al., 1993). Epidemiological and clinical evidence supports this protection hypothesis but little is known about its mechanisms (Williams et al., 1996). It has been assumed that erythrocytes in thalassemic individuals cannot provide sufficient nutritional substances such as ferrous iron for the enzymatic activity of the malaria pathogen, *Plasmodium falciparum*, resulting in impaired multiplication of the parasites (Wyler, 1992). Like sickle cell anemia, thalassemia mutations could eventually reach a balanced polymorphism state (Russell, 1986). Individuals with homozygous globin mutations

cannot survive due to their highly defective globins but people with completely normal globin genes may suffer from life-threatening malaria when there is a high prevalence of malaria (Siniscalco et al., 1961). A compromise can be found in individuals heterozygous for thalassemias who have one normal globin gene and one abnormal gene. One normal globin gene can provide normal hemoglobin to maintain minimum physiological requirements, but the abnormal hemoglobin can significantly inhibit the reproduction of *P. falciparum.* As a result, those heterozygous for thalassemia may suffer from a defective hemoglobin but obtain protection against malaria (Livingstone, 1986; Flint et al., 1993). The thalassemia mutations would then be maintained by a selective advantage of the heterozygote over either homozygote.

The practice of agriculture in human history must have played an important role in the increase of thalassemia (Flint et al., 1993). Agriculture would bring a closer association between the human host, the malaria parasite and the vector anopheline mosquito (Brinkmann, 1994). For example, the expansion of irrigation for the cultivation of rice provides an excellent breeding ground for malaria vectors. A high population density of humans and the mosquito in tropical areas could have all contributed to the emergence and maintenance of malaria (Williams et al., 1996). Agriculture, as a new human technology, could promote the dispersal of malaria throughout human populations over a short time (Livingstone, 1986). As a result, malaria would be prevalent in all areas where climates were suitable for the malaria pathogen and its vector. Under the selective pressure of malaria, thalassemias may have emerged and been maintained as a balanced polymorphism in different human populations. Theoretically, a thalassemia mutation with

a selective advantage can spread from population to population via gene flow. But compared to the dispersal of agriculture, it would take a much longer time, through human population migration and mixture, to disperse mutations throughout the whole malaria belt, especially when humans were hunters and gatherers, with a lower population density. Considering the current distribution of thalassemia and the diversity of mutation patterns, it is unlikely that thalassemia originated from a single place and time and then spread throughout the rest of the malaria belt.

This multi-regional hypothesis of thalassemia origin should not exclude exchanges of thalassemia variants as a result of gene flow among the areas within the malaria belt. Thalassemia was once thought to have originated in Mediterranean areas (Lehmann, 1982). When thalassemia was found in many other regions, a different hypothesis was proposed. In 1955, Brumpt even proposed that thalassemia might have originated in China and suggested "sinemie" as a replacement for thalassemia (Dacie, 1988). Although hemoglobin variants are maintained by malaria selection, their distribution has been determined as much by population expansion, migration and gene flow (Livingstone, 1986; Flint et al., 1993). Gene flow can be used as an explanation for an unbalanced distribution of thalassemia variants. For example, β-thalassemia is particularly frequent in Italy, Greece, Cyprus and Malta, but α-thalassemia is a major variant in Southeast Asia such as Thailand and South China.

## 2.2.3. Bone Lesions Caused by Thalassemia

Bone changes caused by thalassemia have attracted the attention of physical anthropologists since the report of the first cases in 1925. Severe bone lesions were reported with these first cases (Dacie, 1988). Subsequently, there have been many attempts to detect the presence of thalassemia from lesions on ancient skeletal remains (Angel, 1966).

Bony changes are the direct consequence of hypertrophy of the hemopoietic bone marrow as activity increases to compensate for ineffective erythropoiesis or hemolysis (Dacie, 1988). For example, in the facial bones, the maxillae enlarge, the midface broadens, the eyes become widely spaced, and the bridge of the nose is flattened. The whole face is reminiscent of facial structures often found in Asian populations (Tayles, 1996). Although the changes on some bones can be remodelled as children grow, with the shift of erythropoiesis from the bone marrow to the spleen, changes in other bones may remain with little modification.

One of the most common changes associated with thalassemia is porotic hyperostosis. This term was first introduced by Angel (1966) to describe distinctive bone changes on skulls. The compact bone is pierced by small holes of different sizes and frequencies and the diploë is increased in thickness. The lesions are symmetrical in distribution and appear mainly on the orbital roof (*cribra orbitalia*) and skull vault (Stuart-Macadam, 1985). Thalassemia was once identified as the sole etiological cause of

porotic hyperostosis, which was observed on ancient skeletons found in the Mediterranean area (Angel, 1966). More recent studies show that porotic hyperostosis can be caused either by genetic anemia s such as thalassemia or by chronic anemias such as iron-deficiency anemia (Stuart-Macadam, 1992a; 1992b). When postcranial bone changes are considered, manifestations of thalassemia seem different from chronic anemias and discrimination seems possible (Hershkovitz et al., 1991; Tayles, 1996).

The "hair on end" appearance in X-ray observations, caused by the hyperplasia of bone marrow, has been said to be one of the most distinctive features of thalassemia (Dacie, 1988). The bone trabeculae radiate out in a perpendicular arrangement to the bone tables (Stuart-Macadam, 1987a). This feature is more likely to occur in thalassemia but is also found with other hematological disorders. It has been observed in 5-10% of radiographs of patients with various anemias (Stuart-Macadam, 1987a). Outer table thinning or disappearance, and diploic thickening of the skull are two other features caused by the marrow expansion of the diploë. The pressure exerted on the bone marrow eventually results in thinning or disappearance of the outer compact bone (Stuart-Macadam, 1987a).

Postcranial bone changes reflect the same marrow hyperplasia, with expansion of the medullary cavity in long bones and coarsening of trabeculae and extreme thinning and porosity of cortices (Tayles, 1996). These postcranial skeletal changes have been found to be more common in thalassemia than chronic anemias (Stuart-Macadam, 1992b). Another more thalassemia-specific postcranial change is the premature and irregular fusion of growth plates of proximal long bones, resulting in limb deformities

(Hershkovitz et al., 1991; Williams et al., 1992). These bone changes on the postcranial skeleton, along with other criteria, have been used to diagnose thalassemia in ancient skeletons from Israel and Thailand about 8,000 years ago and 4,000 years ago respectively (Hershkovitz et al., 1991; Tayles, 1996). The authors of the former study claim to have identified the earliest case of thalassemia in the prehistoric record (Hershkovitz et al., 1991).

## 2.2.4. Pathogen Load and Porotic Hyperostosis

Stuart-Macadam has performed large scale comparisons of X-rays of individuals clinically diagnosed with anemia and X-rays of skeletons with porotic hyperostosis (Stuart-Macadam, 1987a,). Her results strongly support the interpretation that anemia is the cause of porotic hyperostosis (Stuart-Macadam, 1987b). She has further speculated that the causes of porotic hyperostosis are more likely to be produced by chronic anemia such as iron-deficiency anemia rather than thalassemia or other genetic anemias (Stuart-Macadam, 1992b) based on the following considerations. The calculated frequency of thalassemia or other genetic anemias in human populations is quite low even based on the highest gene frequencies. There are high levels of porotic hyperostosis in populations from northern Europe and North America where genetic anemias appear not have occurred in an appreciable frequency. Porotic hyperostosis is not closely correlated with the postcranial bone involvement, which is commonly seen in genetic anemias (Stuart-Macadam, 1992b).

Stuart-Macadam has introduced a new perspective on the interpretation of the high occurrence of porotic hyperostosis in some past human populations. Porotic hyperostosis should be regarded as a result of a person's active defence against diseases or "pathogen load" instead of the direct passive consequence of some specific disease (Stuart-Macadam, 1992a; 1992b). Pathogen load is defined as the total number of micro-organisms in the local environment, including fungi, viruses, bacteria and parasites (Stuart-Macadam, 1992a). Because many microorganisms need iron for their own reproduction, they will take iron from their host after they enter that host. A shortage of iron will be disadvantageous for the survival and existence of microorganisms. A human body can create a shortage of iron by binding the available iron to the transport protein, transferrin, or sending it into storage in the reticuloendothelial system, or by decreasing the absorption of dietary iron by the intestinal mucosa (Stuart-Macadam, 1992a). Stuart-Macadam argues that the increase in porotic hyperostosis during the Neolithic and early agriculture period does not derive from iron-deficient diets but resulted from greater exposure to pathogens because of increased sedentism, aggregation and population density (Stuart-Macadam, 1992a; 1992b). Although other factors such as climate, geography and culture can also contribute to the prevalence of iron deficiency, pathogen load plays a most important role. When the pathogen load is heavy and infectious diseases are prevalent, the negative effects of iron deficiency on the human body can be balanced by an effective inhibition of pathogens. Many other diseases such as chronic mycotic infectious, tuberculosis and osteomyelitis can also cause iron deficient anemias - porotic hyperostosis. Porotic hyperostosis can be considered indicative of stress responses

to diseases and pathogens.

However, this pathogen load hypothesis does not exclude the likelihood that some cases of porotic hyperostosis are really caused by genetic anemias especially when skeletons are collected in the malaria belt. If Stuart-Macadam's hypothesis is right, porotic hyperostosis reflects a physiological defensive reaction for a human body. However, in terms of evolution, this physiological reaction favours the spread of genetic anemias such as thalassemia and the sickle cell disease. On the one hand, as malaria is a severe contributor to "pathogen load", it would probably have put more pressure on human hosts than other pathogens, resulting in the emergence of thalassemia and other genetic anemias in the malaria belt. However, when, where and how this physiological reaction was converted into genetic fitness are important for understanding the evolutionary mechanism of human adaptation. One the other hand, correct identification of the types of anemia from ancient skeletons can help us to interpret the history of past human populations. For example, thalassemia can always cause porotic hyperostosis, whereas chronic anemia occurs and produces bone lesions only when the "pathogen load" is heavy or nutrients are deficient. For a particular archaeological population with a high prevalence of thalassemia but with good living conditions, the presence of porotic hyperostosis can be misinterpreted as the reflection of temporary heavy "pathogen loads" instead of genetic thalassemia.

## 2.2.5. Diagnosis of Thalassemia from Skeletons

Diagnosis of thalassemia from skeletons is largely based on the observations of porotic hyperostosis and postcranial bone lesions. However, the use of such lesions or lesion patterns in the diagnosis of thalassemia is challenging. The careful reexamination of many previously claimed thalassemic skeletons has revealed that more reasonable alternative diagnoses are possible (Ascenzi and Balistreri, 1977). This is because, in theory, any conditions that abnormally increase the rate of blood cell turnover can produce porotic hyperostosis in bones (Ascenzi et al., 1991). It is difficult to make distinctions between bone lesions caused by thalassemias or chronic anemias such as dietary iron-deficiency because they are so similar (Ascenzi et al., 1991). These difficulties are characteristic of all morphological skeletal diagnoses of ancient diseases. Although skeletal changes are important additional symptoms for some clinical diagnoses, they cannot be used alone as diagnostic features. Many other criteria have to be used for accurate diagnoses.

Additional difficulties with the diagnosis of thalassemia will be encountered when the pathogenesis of thalassemia is well understood at the molecular level. Thalassemia is a group of disorders caused by different mutations instead of a single disease with a single mutation such as sickle cell anemia (Weatherall and Clegg, 1981, Adams, 1986). When the diagnosis of thalassemia is based on bone lesions, the underlining assumption is that there is a single mutation called thalassemia; the

correlation between lesions and the mutation can be established (Hershkovitz et al., 1990, Tayle, 1996). The diversity of thalassemia mutations and the variability of clinical phenotypic symptoms have made attempts impossible, in practice, to find a single effective diagnostic method for all thalassemia variants solely based on symptoms.

## 2.2.6. Molecular Pathogenesis of Thalassemia and DNA Diagnosis

Thalassemia is the most commonly inherited single-gene disorder in the world and it was also the first human genetic disease to be characterized at the molecular level (Lau et al., 1997). A rich knowledge has accumulated about the molecular basis of thalassemia phenotypic diversity (Weatherall and Clegg, 1996). Even ten years ago, more than 400 mutations had already been identified in human globin genes (Adams, 1986). Most α-thalassemias are due to gene deletions but most β-thalassemias are the result of non-deletion mutations such as point mutations (Adams, 1986).

Based on the molecular pathogenesis of thalassemia, many effective and efficient diagnostic protocols have been developed in clinical practice to detect a variety of thalassemia mutations. The PCR technique, the Dot Blot test, the Southern Blot test and sequencing are among the commonly used molecular biology techniques. There are few genes whose structure and function are understood as well as those of the globin proteins and, in fact, few diseases whose pathogenesis is as well understood as that of the hemoglobinopathies (Friedmann, 1990). With the advent of molecular biology techniques, thalassemia can be accurately identified at the level of mutation variants. In

clinical practice, although symptoms and other evidence are still used for the preliminary diagnosis of thalassemia, more specific diagnoses come generally from DNA analysis. The DNA technique is the most definitive diagnosis of thalassemia since it directly reveals the molecular etiological cause of this genetic disorder.

It is more appropriate to raise questions about the origins and spread of specific thalassemia variants before the origins of thalassemia as a whole can be investigated. Based on prevalence and distribution, $\beta^{+}$IVS-1-110, one thalassemia variant, is proposed to have originated first in ancient Greek populations and then spread eastwards and westwards following the migration of Greek civilization in the 8th to 7th centuries B.C.(Cao et al., 1989). The $\beta^{0}$39 variant of thalassemia seems to have originated from the Phoenicians and then spread to various Western regions of the Mediterranean and Africa as far as Portugal through the migratory phase of Phoenician civilization in the 12th to 11th centuries B.C.(Cao et al., 1989).

The retrieval of DNA from ancient remains provides a potential for application of DNA diagnosis of thalassemia in ancient remains (Pääbo, 1985; Herrmann and Hummel, 1984). Theoretically, once DNA is extracted, it can be analyzed in the same way as modern DNA, making it possible to diagnose thalassemia at the molecular level in ancient remains.

## 2.2.7. Summary

Thalassemia is caused by a variety of mutations in human globin genes. As a

result of physiological compensation, thalassemia can produce some specific bone changes, such as porotic hyperostosis and postcranial bone lesions. However, other factors such as iron-deficient diets can produce similar or the same bone changes. This presents a severe difficulty to the identification of thalassemia in ancient remains based solely on skeletal lesions.

DNA has been extensively used in the diagnosis of thalassemia in clinical practice. The retrieval of DNA from ancient skeletons has made DNA diagnosis of ancient thalassemia possible.

# CHAPTER 3. MATERIALS AND METHODS

## 3.1. Materials

### 3.1.1. General Information on the Isola Sacra Site

The Isola Sacra site is a cemetery near the mouth of the Tiber River in Italy (Fig.3.1.). Located on the northern shore of the Mediterranean Sea, Italy has been continuously populated for several thousands of years by a variety of peoples. Southern Italy was colonized in the 7th and 8th centuries B.C. by the Greeks, who were the first to use the name "Italy" for this region. The Greeks and other cultures were absorbed into the Roman Empire which dominated Italy at about the time of Christ (Tentori and Marinucci, 1986).

Portus (about 23 km West of Rome) owed its origin to two political decisions taken more than half a century apart, by the Roman emperors Claudius and Trajan. At the beginning of his reign in A.D. 41, Claudius faced a dire food shortage. Seven or eight days' supply of grain was all that remained in the warehouses (Garnsey, 1988). Claudius then decided to construct a sea harbour just north of the mouth of the Tiber River to improve on Ostia, a river port, which did not permit anchorage in all weather and seasons. It took more than twenty years for Claudius to build the harbours and canals. Trajan later completed the whole project by adding an additional channel and canals in AD 100/6-112

(Sperduti et al., 1997). Portus was the port of Rome (Fig.3.2.), directing the goods into the metropolis with grains having the highest priority of the imperial government. The prosperity of Portus was tied to the imperial city.

Isola Sacra, located just off the road between Portus and Ostia, consists of a stretch of land transformed into an island in connection with construction of the port of Trajan. It was a cemetery for the city of Portus. The cemetery was in use from the first to third centuries AD, with three successive phases of use: 1) the first century AD - modest tombs along the road; 2) the second century and the first half of the third century AD - tombs built along sides the road, and covering earlier burials; 3) the third century- reuse of existing tomb structures (Sperduti, 1995). About 2,000 individuals including 248 subadults were buried in this cemetery. Most of the skeletons can be dated to 100-250 AD (Sperduti et al., 1997). The Isola Sacra necropolis, located along the via Flavia, represents generally the "middle class" people who were involved in administration, service and commerce at Portus (Sperduti et al., 1997). The other cemetery associated with the city of Portus is an elite cemetery situated along the via Portuense (Sperduti et al., 1996).

Archaeological analysis has revealed a great deal about Isola Sacra. There are no burial groups clearly associated with individual's social status, but evidence indicates that familial and social relations might be taken in account when individuals were buried. In the larger tombs individuals were not only members of an immediate family but also dependents, slaves and freemen. It cannot be clearly established that individuals buried in simple inhumations were poorer than those found in the monumental tombs. Most of the

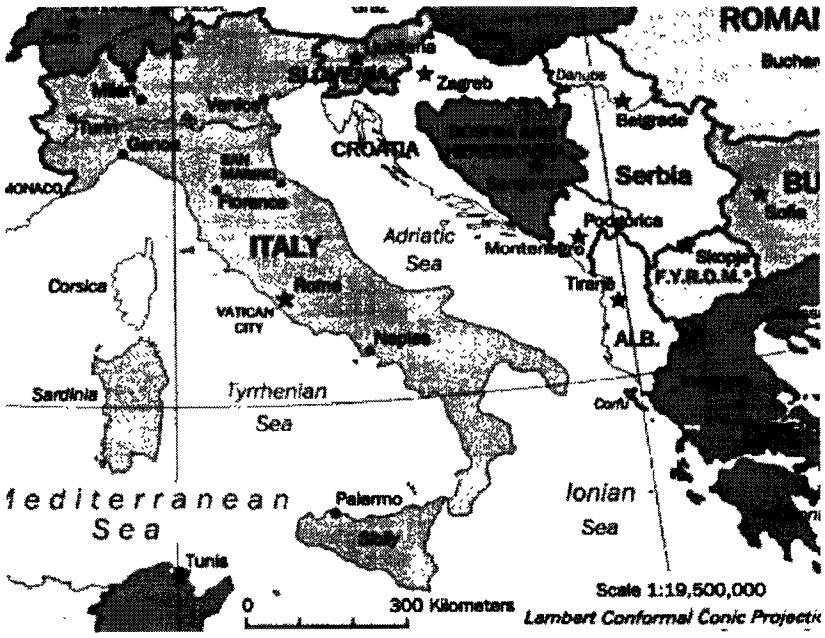



Fig.3.1. Map of Italy' s geographical position

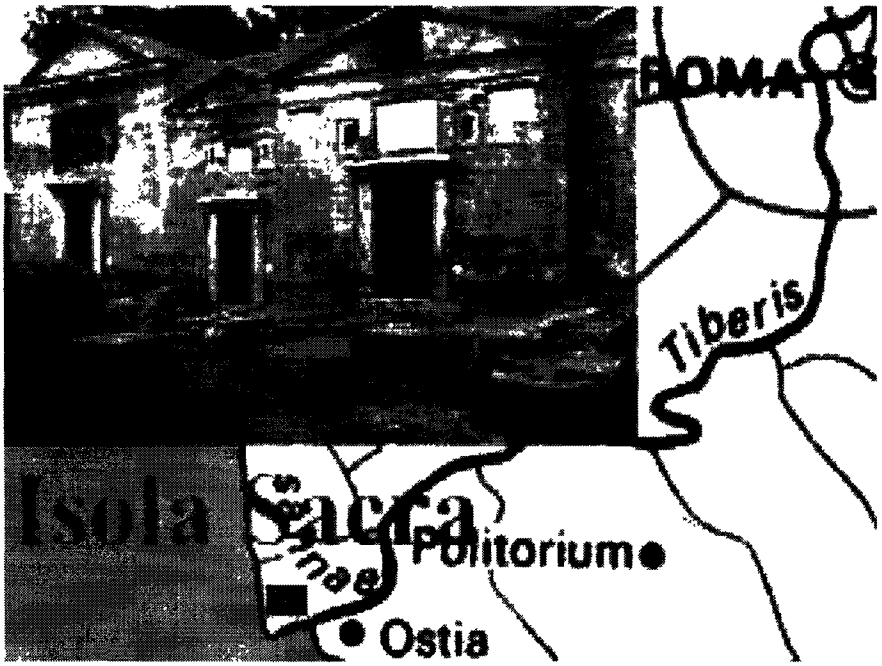



Fig.3.2. Map of location of the Isola Sacra. The inside shows the tombs.

burials did not have any associated grave goods; there were a few with some coins, lamps and "female ornaments". A significantly large number of subadults were buried in amphora inhumations (Sperduti, 1995).

The Isola Sacra site was first excavated between 1925 and 1940 (Sperduti et al., 1997). Further excavations were conducted in the 1970s and the 1980s, uncovering more than 1,000 individuals. Since 1991, a collaborative research project has been carried out between the University of Rome, la Soprintendenza Speciale del Museo 'L. Pigorini', and the Sopintedenza Archaeologica di Ostia (Sperduti et al., 1997).

The skeletons for the study presented in this thesis were chosen from the Isola Sacra skeleton collection (SCR) based on their specific thalassemia-like lesions. The morphological observations of these skeletons were originally made by Sperduti and associates in a preliminary internal report, National Museum of Prehistory and Ethnography "L. Pigorini", Rome (Sperduti et al, 1996). The author of this thesis had an opportunity to examine the bone samples sent in for aDNA extraction. But the following descriptions of the specimens are mainly derived from the preliminary internal report (Sperduti et al., 1996).

## 3.1.2. Description of the Isola Sacra Specimens

Among the skeletal sample from the Isola Sacra cemetery are individuals SCR 8, SCR 136, SCR 277, SCR 599 and SCR 1353 which have been tentatively diagnosed as possible thalassemics based on their age at death and their bone lesions. Tab.3.1.

summarizes the major bone lesions of these SCR specimens. Appendix 1 shows the preserved bones in a diagram of an infant skeleton for each of these SCR specimens (Sperduti et al., 1996).

*SCR 8*

The age at death is estimated to be 6-12 months based on dental development (Sperduti et al., 1996). The skeleton is very fragmentary. The roofs of both orbits have slight porosity. Along the metopic suture, the frontal bone is very thick. There are no bone lesions observed on the postcranial skeleton.

*SCR 136*

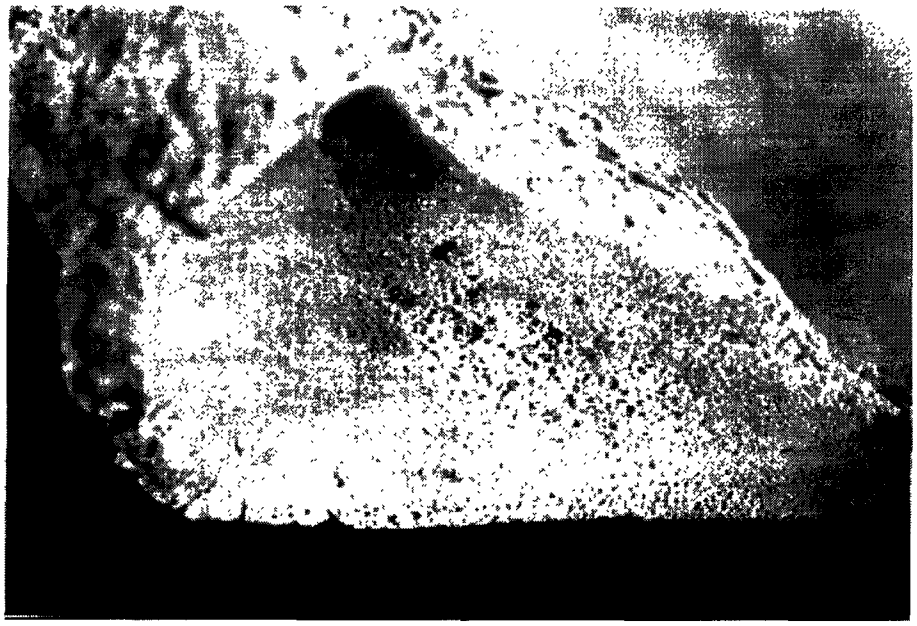
The age at death is estimated to be 9-12 months based on dental development and scapulae measurement (Sperduti et al., 1996). On the frontal bones, the external surface displays diffuse porosity. The internal surface shows new bone apposition and a porous appearance. Along the frontals the diploë is expanded, reaching a maximum thickness of 6.4 mm. There is marked thickening of the diploë in the metopic suture in the orbital region and, as a result, the roofs of the orbits appear shallow. The surface of each orbital roof shows diffuse porosity (*cribra orbitalia*) (Fig.3.3.). Along the coronal suture, the bone is extremely expanded with a spongy appearance. The parietal bones also show marked porotic hyperostosis on both the external and internal surfaces. The diploë of the parietals is considerably thick, particularly along the lateral segment of the coronal suture, and at obelion along the sagittal suture. The bones are very spongy in appearance. The

temporal bones display severe diffuse porosity. The margins of the temporomandibular fossa show trabecular-like porosity, and the petrous part of the temporal bone presents a similar porous appearance. The occipital bone shows early, anomalous fusions of both lateral portions of the squama, which usually occurs at an age of more than 3 years. The facial bones are very fragmentary; most of them are missing. But the remaining fragments are hyperostotic in appearance. The mandible is badly eroded, but the remaining segment appears porous and very light.

The radiographs of both the frontal and parietal bones reveal a granular appearance to the bones. The roof of the orbits appears thickened and the outer table is thin and tends to disappear. But in the frontal bones the typical "hair-on-end" appearance is not observed even though the diploë is clearly expanded.

In all of the preserved postcranial bones, there is a reduction in cortical bone, and an associated spongy appearance of the exposed trabecular bone. It should be noted that this involves the entire skeleton, not just one bone or fragments of a bone. All rib fragments show marked medullary expansion, cortical thinning, and osteoporosis (Fig.3.3.). In radiographs, the ribs show an osteoporotic structure and a coarsened appearance of the trabecular bone. There is also bone apposition beyond the original cortex. The scapulae are well preserved and display extensive loss of cortical bone and associated medullary expansion. Their radiographs show extreme rarefaction of the internal trabecular pattern and a very thin cortex. There are two fractures with callouses on the lateral side of the ulna. Radiographically, coarsening of the trabeculae is also evident.

A



B

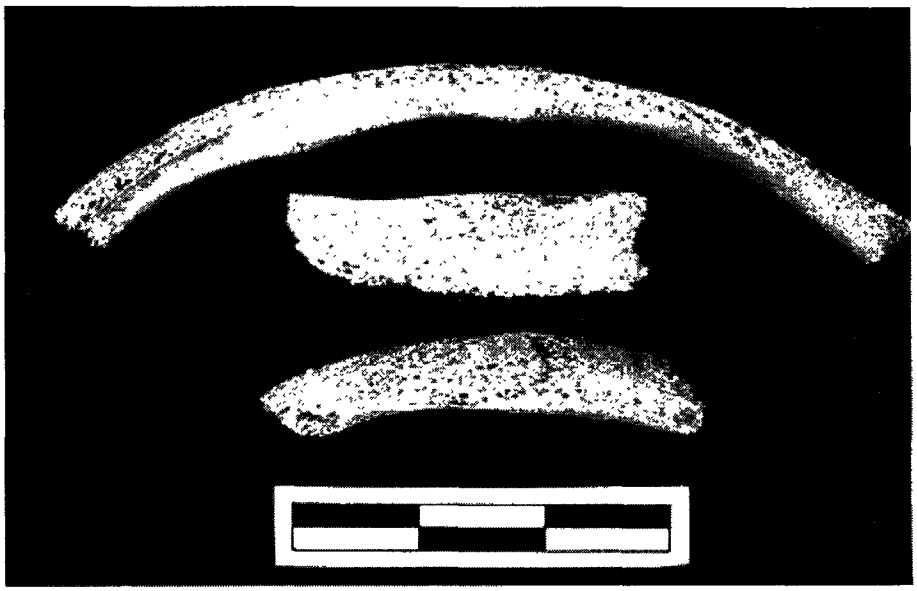


Fig.3.3. Bone lesions on SCR 136. A: the left orbit showing diffuse porosity (*cribra orbitalia*); B: the ribs showing marked medullary expansion, cortical thinning and osteoporosis

Both humeri display extensive cortical thinning and marrow hyperplasia. Radiographically, the original contour of the bones appears altered; the cortices are thinned and bone apposition is observable, the medullary cavities are enlarged, and the metaphyses are expanded. The shafts, and metaphyses in particular, show osteoporosis and coarsening of the trabeculae. At the distal end of both shafts, several lines of increased radiopacity are visible.

*SCR 277*

The age at death of this individual is estimated to be 6-12 months based on long bone length (Sperduti et al., 1996). This is a very fragmentary individual.

On the right frontal, along the metopic suture, the diploë appears expanded, and there is a clear line of demarcation between the old and new bone growth. The external surface displays porosity and new apposition of bone. The orbital roof is thickened with marked porosity. The thickness of the right frontal bone is 5.3 mm. The radiograph of the right frontal displays a thickening of the orbital roof and noticeable expansion of the diploë, but only a slightly granular texture. On the external surface of the other cranial fragments there is obvious porotic hyperostosis, however, the diploë of these fragments is not notably expanded.

There are no lesions associated with the postcranial bones.

SCR 599

The age at death of this individual is estimated to be 3-4 years based on dental

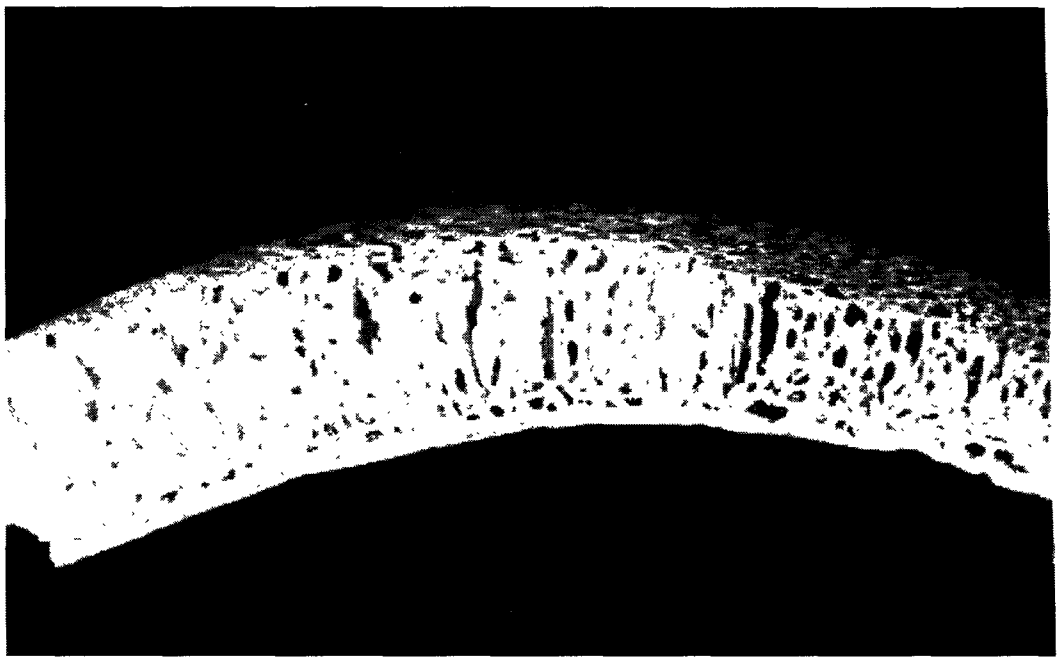


Fig.3.4. Bone lesions on SCR 599. The exposed cross section of the left parietal fragment showing characteristic honeycombed compartments of subperiosteal bone and the "hair-on-end" arrangement of trabeculae

development (Sperduti et al., 1996). Only cranial fragments are preserved for this individual.

All of the cranial fragments (mostly parietals) show pronounced thickening, and the external surfaces display severe porotic hyperostosis with a trabecular-like pattern. The exposed cross sections of these fragments clearly show characteristic honeycombed compartments of subperiosteal bone and the "hair-on-end" arrangement of trabeculae (Fig.3.4.). The temporals, maxillae and mandibular fragments display only mild porosity, the expression is not as severe as in the rest of the cranium. The orbital fragments of the frontal bone display marked *cribra orbitalia*. The roof of each orbit is extremely expanded and flattened.

Radiographically, the frontal bone shows a granular appearance, the outer table is thin, and the trabeculae show a radial arrangement. The roof of the left orbit displays anomalous thickening, such that the contour of the orbit is clearly altered. It has lost its concave shape and is straight and rather shallow. Radiographs of the frontal show two distinctive areas with diploic expansion and a characteristic "hair-on-end" pattern. The inner table is compact but the outer table is thinning. The right parietal and fragments of the left parietal show a coarsened and granular bone texture. In addition, a radial arrangement and honeycomb pattern of the trabeculae is obvious.

*SCR 1353/2*

The age at death of this infant is estimated to be 6-12 months based on the measurement of the ilium (Sperduti et al., 1996). Only the left ilium is preserved with this

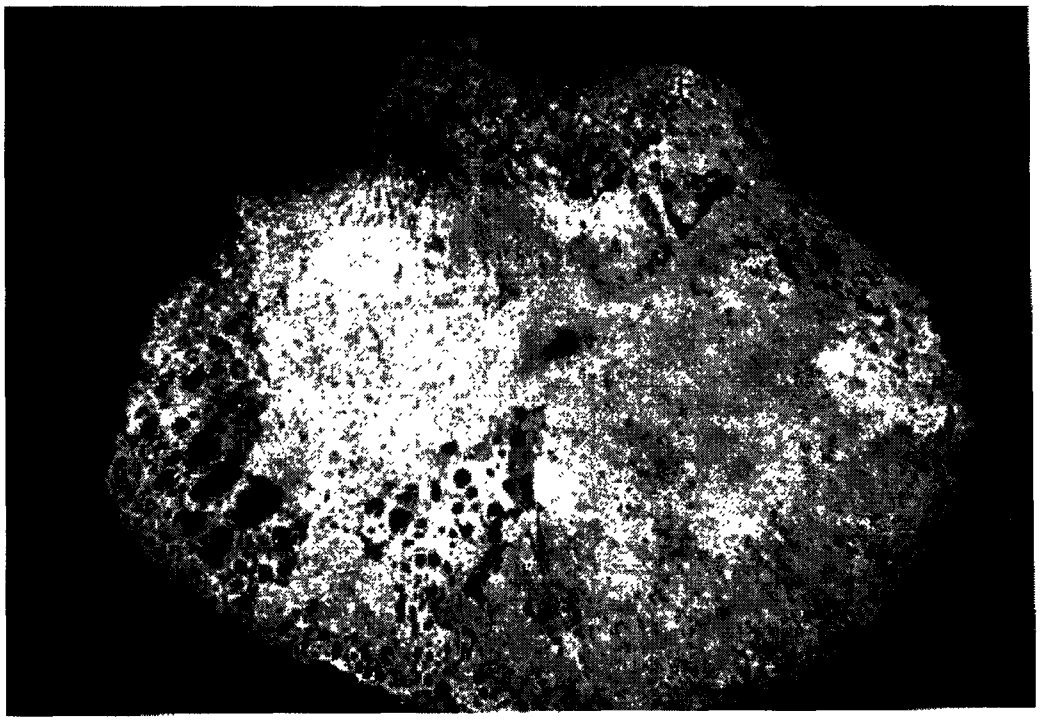


Fig.3.5. Bone lesions on SCR 1353/2. The left illium showing extremely expanded trabecular bone

Tab.3.1. The ages at death and the bone lesions of the SCR specimens (after Sperduti et al., 1996)

| SCR | No.8 | No.136 | No.277 | No.599 | No.1353/2 |
|---|---|---|---|---|---|
| Age at Death ( Method for Age Estimation) | 9-12 months (dental development) | 9-12 months (dental development and scapular measurement) | 6-12 months (long bone measurement) | 3-4 years (dental development) | 6-12 months (ilium measurement |
| State of Conservation | cranial and postcranial fragments | lacking most of the long bones | cranial and postcranial fragments | cranial fragments | left ilium |
| **Skull Vault Involvement** | yes moderate | yes, severe | yes, moderate | yes, very severe | / |
| Outer Table Thinning or Disappearance | no | yes | yes | yes | / |
| Diploic Thickening | yes, 7.1 mm at metopic | yes, 6.4 mm at metopic | yes, 5.3 mm at metopic | yes, 10.0 mm at frontal boss | / |
| Cortex/Diploë Ratio | 1.0/2.0 | 1.0/3.1 | 1.0/2.1 | 1.0/4.9 | / |
| Orbital Roof Thickening | 1.5 mm | yes 6.5 mm | yes 4.5 mm | yes 11.0 mm | / |
| Cribra Hyperostosis | yes, porotic grade | yes, porotic grade | yes, porotic grade | yes, porotic grade | / |
| Vault Porotic Hyperostosis | no | yes, diffuse porotic to trabecular grade | yes, porotic | yes, diffuse trabecula grade | / |
| X-ray 'Hair-on-End" Appearance | no | slight | no | yes | / |
| X-Ray Bone Texture | no | yes, granularity | slight granularity | yes, heavy granularity | / |

(continued)

Tab.3.1. The ages at death and the bone lesions of the SCR specimens (after Sperduti et al., 1996)

| SCR | No.8 | No.136 | No.277 | No.599 | No.1353/2 |
|---|---|---|---|---|---|
| **Facial Involvement** | / | yes, all bones are extremely light and fragile and expanded | no, but poorly preserved | yes, mild porosity | / |
| **Postcranial Involvement** | no, but poorly preserved | yes, reduction of cortical bone, medullary canal expansion, enlarged and altered contours | no, but poorly preserved | / | yes, pronounced rarefaction and disorganization of the trabecula tissue. |
| Harris Lines | / | yes, 3 on humeri | no | / | / |
| Bending Fracture | no | yes, several on ribs and ulna | no | / | / |

individual. The trabecular bone is extremely expanded and the blade has a pronounced curvature (Fig.3.5.). The internal aspect of the bone displays an expansion of the intertrabecular spaces.

The radiograph of the bone shows severe osteoporosis, and the trabeculae appear disorganized and coarsened. The bone structure is open with a widening of the marrow spaces. Areas of thickened trabeculae show a radial arrangement.

## 3.1.3. Morphological Diagnosis of Thalassemia from the Isola Sacra Skeletons

These five subadult individuals from the Isola Sacra skeletal sample present pathological skeletal lesions similar to those caused by severe anemia. Four of the five

individuals died between 6 to 12 months of age and one died at an age of about 3 to 4 years.

The pattern and severity of the pathological changes in some of these individuals seem to suggest a diagnosis of thalassemia. Because a number of genetic and chronic anemias can produce similar pathological lesions on skeletons, some specific characteristics have to be identified before we can propose that these individuals were thalassemic.

### 3.1.3.1. Age at Death

Clinical manifestation of thalassemia major usually takes place within the first few months of life (Weatherall and Clegg, 1981). However, the bone lesions of thalassemia cannot usually be observed radiographically until the afflicted infants are one year old (Dacie, 1988). This is because the fetal globins are still synthesized and persist during the first year of life.

In the SCR specimens, four individuals (SCR 8, 136, 277, 1353/2) are estimated to have died at age 6 to 12 months while for SCR 599, estimated age at death is 3-4 years. This individual showed more severe lesions of the skull. However, if these specimens were indeed homozygous for thalassemia, their ages may have been underestimated since they were assumed growing normally at the time of death. It is evident that growth retardation occurs almost invariably in homozygous β-thalassemia (Weatherall and Clegg, 1981). The growth of thalassemia children is far behind the normal growth curve.

One case study reports that the growth of a thalassemia child was below the 1st percentile from the birth to several years even with blood transfusion (Waye, 1997, personal communication). There seems to be age underestimation for SCR 136. The age at death was estimated to be 9-12 months based on dental development and scapular measurement. The earlier fusions of both lateral portions of the squama in the occipital bone suggested an age of more than 3 years (Sperduti et al., 1996).

The causes of death of these SCR specimens have not yet determined. However, it is possible that, to a certain degree, thalassemia manifestation might have contributed to the death of these individuals since thalassemia children are more prone to infection than normal children (Weatherall and Clegg, 1981).

Overall, the young age at death and the severity of the skeletal changes observed, particularly in SCR 136, are suggestive of genetic anemia but a non-genetic etiology cannot be ruled out.

### 3.1.3.2. General Pattern of Bone Manifestation

Extensive thickening of the cranial vault, diploic expansion, and the "hair-on-end" appearance are skull lesions which are usually associated with thalassemia (Resnick and Niwayama, 1981; Tayles, 1996). The pathological lesions on skulls are more common and more severe in thalassemia major than in other genetic and chronic anemias (Steinbock, 1976). Another distinctive feature associated with thalassemia is the extensive involvement of the facial bones. Although marrow hyperplasia in postcranial bones can be seen in all severe anemias, it is considered to be more severe in thalassemia

(Steinbock, 1976; Hershkovitz et al., 1991; Tayles, 1996).

Not all of these bone lesions have been observed in all five individuals. For example, SCR 136 appears to have thalassemia-like bone lesions but is lacking the "hair-on-end" appearance. Due to poor preservation conditions, many other features cannot be observed on other specimens. The presence of all thalassemia-like bone lesions on one single individual would be the ideal for identifying thalassemia, but in reality, it is very rare to observe all diagnostic lesions on a single skeleton (Tayles, 1996). This is because in clinical patients, only a portion of thalassemics develop some but not all of the diagnostic bone lesions (Stuart-Macadam, 1987a; 1987b).

### 3.1.3.3. Cranial Vault Involvement

Cranial manifestation of a severe anemia such as sickle cell disease and thalassemia tends to follow a pattern: the frontal bone is primarily affected, followed by the parietals and, to a lesser degree, the occipital bone (Steinbock, 1976; Sebes and Diggs, 1979; Stuart-Macadam, 1987). It is noted that in thalassemia there is usually little or no involvement of the occipital bone inferior to the internal occipital protuberance. In contrast, there is usually more involvement of the occipital regions in iron deficiency anemia (Zimmerman and Kelley, 1982). Radiographic analysis of thalassemics reveals radial striations from the outer table, which are usually mild, or rarely seen in individuals with chronic anemia such as iron deficiency anemia (Resnick and Niwayama, 1981).

The "hair-on-end" appearance is quite often seen in individuals with thalassemia or sickle cell disease. Although it is more likely to occur in thalassemia, it is also found

with other haematological disorders (Stuart-Macadam, 1987a). *Cribra orbitalia* is quite common with thalassemia but it cannot be used as a diagnostic feature of thalassemia on its own. In this set of specimens, the four individuals represented by cranial remains all show various degrees of *cribra orbitalia.* But this lesion is relatively common in Isola Sacra subadults less than 15 years old, with a frequency of almost 70% (Sperduti et al., 1995).

While SCR 8, 277, 599 and 1353/2 present some thalassemia-like bone lesions (see description for each individual), SCR 136 has developed more thalassemia-like changes, indicating a diagnosis of thalassemia. Bone marrow expansion and cortical thinning is widespread and severe in nearly all the cranial elements in SCR 136, while only the occipital bone is slightly less affected. Thickening of the cranium is most extreme in the frontal region, and along the sutures there is considerable marrow expansion and compression of the internal trabeculae. The radiograph reveals that there are radial striations in the frontal and parietal bones. The extensive lesions in the frontal, parietal, and temporal regions in SCR 136, combined with the relatively minimal involvement of the occipital region, suggest a diagnosis of a genetic anemia. The severity of these changes and earlier age at death also seem indicative of thalassemia.

### 3.1.3.4. Cortical Bone/Diploë Ratio

Reynolds reported that the ratio of cortical bone and diploë in genetic anemias varies from 1:2.3 to 1:7.3, with a average value of 1:4.4 (Stuart-Macadam, 1987b). The ratio is 1.37 in SCR 136 at the metopic suture and 1.49 in SCR 599 at the parietal boss.

### 3.1.3.5. Orbital Thickness

An orbital roof thickness exceeding 3 mm is often considered to be associated with severe anemias (Stuart-Macadam, 1987b; 1989). Among the five specimens, SCR 136 has a thickness of 6.5 mm; SCR 277 is 4.5 mm; and SCR 599 is 9.8 mm.

### 3.1.3.6. Facial Bone Involvement

Marrow hypertrophy of the facial bones is almost certainly indicative of thalassemia major (Moseley, 1971). In infants and children, the expansion of the nasal and temporal bones can block air flow in the paranasal sinuses, causing displacement of the orbits, and adversely affecting dental development (Resnick and Niwayama, 1981). These changes are rarely seen in other anemias and are important for a differential diagnosis of thalassemia (Resnick and Niwayama, 1981).

Generally, all five of the Isola Sacra specimens have poorly preserved facial bones. Among the five specimens, SCR 136 has zygomatic, maxillary and sphenoid fragments which reveal extensive bone changes in the facial region, characterized by fragility, trabecular expansion, and cortical resorption as well as general hypertrophy.

### 3.1.3.7. Postcranial Skeletal Changes

It has been suggested that postcranial skeletal changes must be taken into account if thalassemia is to be diagnosed as a candidate cause for skeletal changes (Ascenzi and

Balistreri 1977; Tayles, 1996). Genetic anemias such as sickle cell disease and thalassemia quite often develop severe postcranial skeletal changes, while chronic anemias such as iron deficiency anemias produce light or no postcranial bone changes.

Medullary hyperplasia is observed in the bones of the axial and appendicular skeleton of thalassemics (Resnick and Niwayama, 1981). In the long bones, the reduction of trabecular and cortical bone produces a bone reaction that maximizes mechanical stability, without encroaching on the existing marrow spaces. Thalassemics also display coarsened trabeculae in the distal and proximal ends of the long bones (Steinbock, 1976).

The extensive reduction of cortical and trabecular bone in thalassemics can also cause expansion and reinforcement on the concave surface of the ribs in response to bending stress associated with respiration (Ortner and Putschar, 1985). The expansion and rearrangement of the trabeculae can also be observed in the flat bones of the skeleton, resulting in a "fan-like" pattern in radiographs.

In addition, infarctions and fractures are often observed in thalassemics. They are mainly caused by the severe osteoporotic status of the bones. One study has shown that 33% of 75 beta-thalassemics had undergone at least one fracture (Dines et al., 1976).

Among the five specimens from the Isola Sacra, SCR 136 has the most complete postcranial skeleton with extensive cortical thinning and marrow hyperplasia indicative of thalassemia. The exposed distal portion of the left humerus as well as the radiographs reveal this characteristic thinning-out of the bone within the medullary cavity and coarsening of the remaining trabeculae, clearly arranged along stress lines. The fracture on the lateral surface of the ulna is also commonly found in thalassemics, although

usually reported for older affected individuals (Resnick and Niwayama, 1981). However, the flaring of the ends of the long bones often seen in individuals with thalassemia is not obvious in SCR 136, but other long bones of the lower limbs are missing.

The absence of infarction in the long bones of SCR 136, and the lack of any visible vertebral depressions characteristic of sickle-cell anemia, supports a differential diagnosis of thalassemia. Furthermore, the radiographs show no evidence of increased radiographic density or "bone-within-bone" appearance, also characteristic of sickle cell anemia (Resnick and Niwayama, 1981).

Because some thalassemia-like bone changes have been observed on the skulls and postcranial skeleton of these cases, thalassemia can be tentatively diagnosed as the etiological cause of the bone lesions. As discussed above, not all of these distinctive features are exclusively limited to thalassemia, namely, other anemias or even other diseases can produce all or parts of these features on bones. Furthermore, not all of these distinctive features can be found in a single thalassemic individual. Even in today's clinical practice, a particular individual who has been diagnosed with thalassemia may only show some of the above skeletal features.

In summary, a tentative diagnosis of thalassemia can be offered for these subadult specimens from Isola Sacra based on the ages at death and the presence of distinctive bone lesions.

## 3.1.4. Bone samples for Evaluating the New Extraction Method

Tab.3.2. The bone materials for evaluating the new aDNA extraction method

| Samples | Xiawanggang | Diancun | Aboriginal | Belleville | Forensic |
|---|---|---|---|---|---|
| Age | 5,000 years | 2,000 years | 300 years | 200 years | 15 years |
| Bone | mandible | rib | femur | humerus | humerus |
| Colour | light brown | light brown | brown | white | white |
| Weight | 8.0 g * | 3.0 g | 3.0 g | 1.5 g | 0.5 g |
| Preservation | good | good | poor | good | good |
| Bone Texture | fragile | fragile | fragile | hard | hard |
| Coffin | no | no | no | yes | no |
| Location | China | China | Canada | Canada | Canada |

* A semi-fossilized bone, heaver in weight.

Five human bone samples ranging in age from 15 to 5,000 years old were used to evaluate the new extraction protocol using the silica-based column (Tab.3.2.). The oldest samples, provided by Drs. Wu Liu and Zhenbiao Zhang of the IVPP, Beijing, were a 2,000 year old rib bone and a 5,000 year old mandible from two Chinese archaeological sites: Diancun and Xiawanggang. The other samples were a 15 year old humerus that had been stored in a laboratory at McMaster, a 200 year old humerus that had been buried in a pine coffin in the St. Thomas' cemetery site, Belleville, Ontario, and a 300 year old femur from an archaeological site of an Aboriginal population in Ontario where the fleshed body had been buried directly in the ground. These bone samples were provided by Dr.

Shelley Saunders.

These bone samples were not specifically chosen for their preservation states but for their ages. Morphologically, all samples seemed in a good state of preservation but their bone contexts show various degrees of degradation (Tab.3.2.). The diversity of the locations and ages will ensure that the results of the comparison are representative of a broad spectrum of ancient skeletons.

# 3.2. Methods

## 3.2.1. Protocols for DNA Extraction from Ancient Bones

Currently, there are two widely used extraction protocols for aDNA studies. The first method, designated the Centricon™ approach (Hagelberg and Clegg, 1991), involves proteinase K digestion to solubilize DNA. The DNA is then separated from the cellular debris through a series of phenol-chloroform extractions and concentrated by passage through a Centricon™ microconcentrator. The Centricon™ contains an anisotropic membrane that retains macrosolutes (including DNA) while allowing low molecular weight solutes to pass through. As the volume of the solute is reduced, the concentration of the retained DNA increases. Unfortunately, this method also concentrates any potential inhibitors of PCR that happen to fall above the molecular weight cutoff of the Centricon™ membrane.

The second method for purification involves the use of silica particles which have

a high binding capacity for DNA molecules (Höss and Pääbo, 1993). DNA extracts are treated with silica powders under conditions which allow the DNA molecules to become immobilized on the silica particles. The silica particles are then pelleted by centrifugation, washed, and treated to elute the purified DNA. This method has the advantage of being more specific for DNA, and less likely to co-purify PCR inhibitors. However, silica particles are powerful PCR inhibitors and care must be taken to ensure that the final extract is free of residual silica particles. Experience and skill are required to successfully employ this method.

These methods have been bypassed in order to identify a better DNA extraction method for retrieving aDNA from the Italian specimens. Satisfactory results could not be obtained in our laboratory using the two existing methods. We, therefore, have attempted to develop a new method of extracting DNA from ancient bones. This new method, the silica-based spin column method, is a simple extraction protocol that combines features of both the Centricon™ and silica-based approaches. The method involves the use of silica-based spin columns that simultaneously concentrate and purify DNA. Bone powders are digested with proteinase K and loaded directly onto QIAquick™ spin columns, eliminating all extraction steps involving organic solvents (phenol-chloroform).

The new DNA extraction protocol utilizes a commercially available QIAquick™ PCR Purification Kit (Yang et al., submitted). QIAquick™ spin columns were originally designed to trap PCR products that are larger than 100 bp and smaller than 10 kb, while at the same time excluding nucleotides, proteins and salts. These columns are ideally suited for aDNA samples since the DNA templates are highly degraded and the target regions

for amplification generally are quite small (*e.g.*, 150 to 250 bp); DNA molecules that are larger than 10kb associated with ancient remains are more likely to be postmortem and/or modern bacterial or fungal DNA instead of authentic aDNA.

### 3.2.1.1. New Procedures for DNA Extraction

Bone powders were generated by drilling the bone surface that had previously been polished with sandpaper. Depending on the age and state of preservation, from 0.5 to 5 g samples were taken from each bone. Larger bone samples were intentionally used in this study to obtain sufficient amounts of DNA for multiple comparisons of different protocols. Bone powders were dissolved in 8 mL extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 100 µg/mL proteinase K) and incubated in a shaking waterbath at 55°C overnight and then at 37°C overnight again. The extraction solution was centrifuged at 2,000 x g for 5 minutes and 1.75 mL aliquots of the supernatant were transferred to 2.0 mL centrifuge tubes and spun in a microcentrifuge (12,800 x g) for 5 minutes. The supernatant was then transferred to a 12 mL tube and mixed with 5 volumes of QIAquick™ PB buffer. Using a sterile disposable pipette, 750 µL was loaded directly onto a QIAquick™ column and centrifuged at 12800 x g for 1 minute. The flowthrough was discarded and the process was repeated until all of the extract had been passed through the column. The DNA was then eluted from the column by loading 100 µL TE buffer and centrifuging for 1 minute. See Fig.3.6. for reference. This figure illustrates the typical procedure protocol for this new aDNA extraction method.

Centricon™ 30 microconcentrators (Amicon Division, Danvers MA) were used to

concentrate the DNA solution after phenol-chloroform extraction (Hagelberg and Clegg, 1991). Centricon™ 30 microconcentrators were also employed to concentrate the digested bone solution to reduce the number of loadings required to pass all of the extract through the QIAquick™ column. This was accomplished by loading the extract onto a Centricon™ 30 microconcentrator and spinning at 2000 x g until the retentate was reduced to the desired volume. The retentate was then collected, mixed with 5 volumes of QIAquick™ PB buffer, and loaded directly onto the QIAquick™ column in a single loading.

### 3.2.1.2. Evaluating the New Extraction Method by PCR Amplification

The overall success of the extraction protocols was assessed based on the ability to amplify a 211 bp target sequence using PCR. The target sequence was derived from a highly repetitive alpha satellite sequence specific for human chromosome 17 (locus D17Z1) (Waye and Willard, 1986). The alpha satellite sequence is located in the centromere region with 500-1,000 copies per chromosome 17. The following primers were used for PCR amplification: 5′-CAA ATC CCC GAG TTG AAC TT-3′ and 5′-AAA ACT GCG CTC TCA AAA GG-3′. PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50 μL reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 1.5 mg/mL BSA, 0.25 U Taq polymerase (Saigon Bio, Toronto), 100 pmoles of each primer, and 5 μL of DNA template (Tab.3.4.). PCR was run for 32 cycles of 94°C

for 40 seconds, 53°C for 20 seconds, and 72°C for 20 seconds (Fig.3.7.). 5 μL of PCR product was separated by electrophoresis on a 8% non-denaturing polyacrylamide gel cast and run in 1 x TBE (90 mM Tris Borate, 2 mM EDTA). The 100 bp ladder (Life Technologies, Gaithersberg MD) was used as a size marker. After electrophoretic separation, the gel was stained with ethidium bromide and photographed under UV illumination.

## 3.2.2. DNA Extraction from the Isola Sacra Specimens

The silica-based spin column was used for extracting aDNA from the Isola Sacra specimens since the comparison test of different methods clearly indicated that the silica-spin column is advantageous over other methods in terms of PCR inhibitor removal and simplicity.

### 3.2.2.1. Bone Sample Preparation

Due to porotic hyperostosis, all specimens from the Isola Sacra site used in this study are in a poor morphological preservation state. SCR 1353/2 is in the poorest state of preservation compared to the other materials. The bone specimens provided for this study are fragments of skull and postcranial bones. All samples as well as the control samples are listed in Table 3.3. SCR 599/2 bone fragments were mixed with other Isola Sacra burials during the excavation process, but they were assumed to be from individual SCR 599. The control sample of a forensic bone which had been kept in a laboratory for 15

years was used to ensure the proper use of the extraction protocol. The control sample of SCR 35, an adult skeleton from the Isola Sacra site, was also used. If DNA could be extracted and analyzed from SCR 35, then SCR 35 could serve as a normal control for thalassemia. It is unlikely that SCR 35 was homozygous thalassemic since this person had reached adulthood and produced no skeletal lesions.

The bone samples were polished carefully using sandpaper to remove the outside surface of the bones. Due to its high fragility, this step could not be performed for SCR 1353/2. To decontaminate SCR 1353/2 and further decontaminate other samples, bone samples were wrapped in thin plastic film for UV treatment. The plastic film was previously tested to ensure it allowed the UV light through. The bone samples were then put into an Ultraviolet Crosslinker (Amersham, LIFE SCIENCE). The UV crosslinker was originally designed for the Southern and the Dot Blot tests, but it can also be used for PCR decontamination. According to the manufacturer's instructions, a few minutes are sufficient for decontaminating the surface of equipment such as pipettes at an intensity of 1500x100 μJ/cm2 . In order to sufficiently decontaminate the SCR specimens, the wrapped bones were crosslinked for 30 minutes on one side and turned over for 30 minutes on the other side. It has been demonstrated that ultraviolet irradiation can efficiently remove contaminants for PCR amplifications (Pao et al., 1993; Sarkar and Sommer, 1993). It was hoped that any external DNA on bone surfaces would be destroyed or damaged to such a degree that it would not be co-amplified with the indigenous DNA of ancient bones.

The bones were then ground with a mortar and pestle or a mini electrical drill

(Makita 10mm 6041D/DW, 450/prm) depending upon their preservation state, shape and size. All five SCR specimens were ground using a mortar and pestle. The forensic bone and SCR 35 were processed with a mini drill. Precautions were taken to decontaminate the mortar and pestle. The mortar and pestle were washed carefully using liquid detergents, and rinsed thoroughly before they were soaked in a 10% bleach solution for at least 30 minutes. They were then rinsed two times with distilled water and then two times with ultrapure water (Laboratory Supplies, Pool, UK). The clean mortar and pestle were then autoclaved. After cleaning, the mortar and pestle was used only once for each individual bone sample.

Tab.3.3. The bone specimens from the Isola Sacra site in Italy

| Specimens | Bones | Morphological Preservation | Weight | Equipment for Grinding |
|---|---|---|---|---|
| SCR 8 | Skull/Postcranial | Poor | 1.3g | Mortar & Pestle |
| SCR 136 | Skull | Poor | 0.9g | Mortar & Pestle |
| SCR 277 | Skull | Poor | 1.7g | Mortar & Pestle |
| SCR 599/1 | Skull | Poor | 1.2g | Mortar & Pestle |
| SCR 599/2 | Skull | Poor | 0.6g | Mortar & Pestle |
| SCR 1353/2 | Ilium | Very Poor | 0.9g | Mortar & Pestle |
| SCR 35 | Femur | Good | 0.4g | Mini Drill |
| Forensic bone | Femur | Very Good | 0.4g | Mini Drill |

* The 15 year old forensic bone was used as a positive control for DNA extraction. SCR 35 was used as the normal control for thalassemia. SCR 599/2 is bone fragments that may be from SCR 599/1.

The use of a mini drill is a new way to collect bone powder for aDNA extraction. It is particularly useful for larger cortical bone pieces. Although the ideal is to polish all surfaces of a cortical bone for drilling, it is convenient that only the drilling area on the bone surface be polished whereas other areas can be covered with sticky tape. The bone dust is produced by drilling holes on the polished surface of bones. The bone dust is collected as bone powder for DNA extraction. This has been proven to be a simple way to generate bone powder but without an increased risk of sample contamination (Yang et al., 1997). The drill speed used for this study was 450 rpm, an ideal speed for drilling bones because it can produce bone dust without spreading. For drill bit decontamination, two methods were compared. The first was to put drill bits into a 10% bleach solution for 20 minutes, but rust is immediately produced on the surface of the drill bits, which may interfere with later PCR amplifications. The other method was to immerse the drill bits in a 1M HCl solution for 20 minutes and rinse thoroughly two times with distilled water and two times with ultrapure water. The second method was chosen in this study since it could completely decontaminate the drill bits but without producing extra substances.

### 3.2.2.2. DNA Extraction

Approximately one half of the bone powders from each sample were used for DNA extraction. The remaining powders were reserved in case a second extraction was required owing to contamination or operator errors. The bone powders were dissolved in 2.5 mL extraction buffer [0.5 M EDTA (ethylenediaminetetraacetic acid) pH 8.0, 0.5%

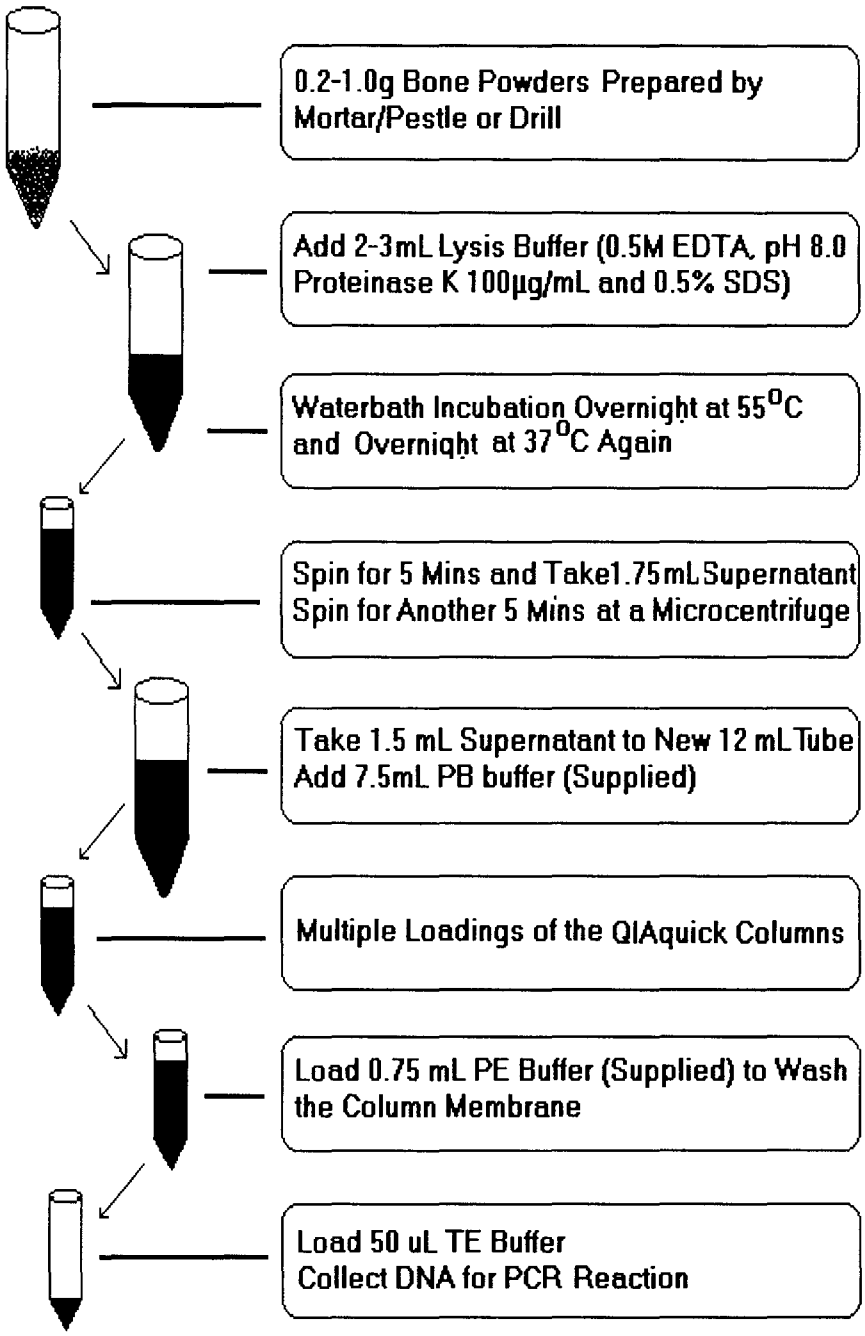



Fig.3.6. The procedure of extracting DNA from ancient human skeletons using the silica-based spin column

SDS (sodium dodecyl sulphate) and 100 μg/mL proteinase K] in a 12 mL tube and incubated in a shaking waterbath at 55°C overnight and then at 37°C overnight again. The 12 mL tubes were laid horizontally for better mixing. The shaking speed was set at 70 rpm. Since the shaking waterbath was not dedicated to aDNA extraction, in order to avoid sample contamination, all tubes were kept in a sealed microwavable container which was then put into another larger sealed microwavable container for waterbathing.

The bone extraction solution was then processed following the protocol described in Section 3.2.1.1. and illustrated in Fig.3.6. Approximately 50 μL of aDNA solution was collected and stored at -20°C in a freezer in preparation for PCR amplification.

## 3.2.3. PCR Amplification of a Highly Repetitive Human-Specific Sequence

In order to evaluate the amounts of aDNA which were extracted from each of these bone samples, a PCR amplification was performed to amplify a highly repetitive alpha satellite sequence specific for human chromosome 17 (locus D17Z1) (Waye and Willard, 1986). One advantage with this sequence is that it is a human-specific sequence. A positive result can be tentatively regarded as the extraction of human DNA from ancient skeletons. This is important since the majority of extracted DNA from ancient skeletons have been found to be DNA from bacteria and fungi (Brown and Brown, 1992).

The PCR amplification was set up and run according the parameters used in Section 3.2.1.2., listed in Tab.3.4. and illustrated in Fig. 3.7.. However, the number of

PCR cycles was reduced to 25. 5 μL of PCR product was separated by electrophoresis on a 8% non-denaturing polyacrylamide gel cast and run in 1 x TBE (90 mM Tris Borate, 2 mM EDTA). The 100 bp ladder (Life Technologies, Gaithersberg MD) was used as a size marker. After electrophoretic separation, the gel was stained with ethidium bromide and photographed under UV illumination.

Tab.3.4. The PCR setup for the human D17Z1 repeated alpha satellite sequence

| **Component** | **Concentration** |
|---|---|
| Perkin Elmer Buffer II x 10 | 5 μL |
| $MgCl_2$ | 2 mM |
| dNTP | 0.2 mM |
| Primer P1 | 100 pmoles |
| Primer P2 | 100 pmoles |
| BSA | 1.5 mg/mL |
| Taq | 0.25 U |
| DNA Sample | 5 μL |
| Total volume with $H_2O$ | 50 μL |

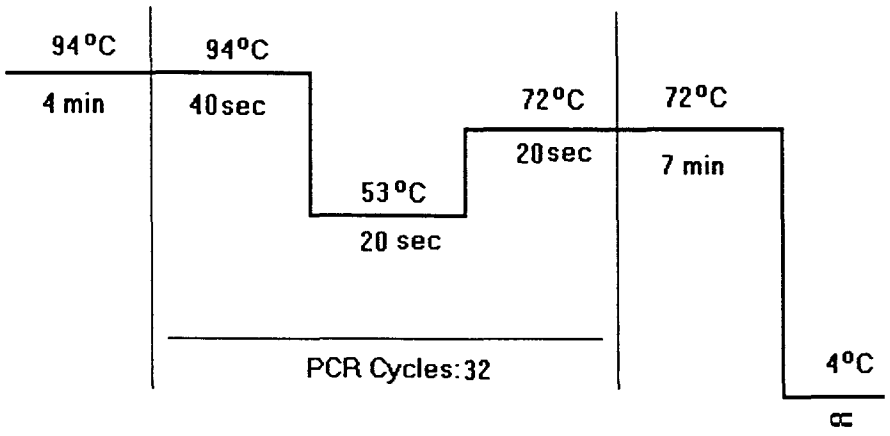



Fig. 3.7. The PCR conditions for human D17Z1 repreated alpha satellite sequence

# 3.2.4. Detection of the Human β- Globin Gene

## 3.2.4.1. PCR Amplification of the Human β-Globin Gene

Fig.3.8. shows the geographic distribution of β-thalassemia mutations in today's Italian populations (Pirastu et al., 1988). Three mutations, the G-A substitution at the position of the first intervening sequences ($\beta^+$IVS1-110), the C-T substitution at codon 39 ($\beta^o$-39) and the T-C substitution at position 6 ($\beta^+$IVS1-6) account for 80% of severe β-thalassemias in Italy (Piratsu et al., 1988). The $\beta^+$IVS1-110 and $\beta^o$-39 are the most common mutations among several common thalassemia variants (Fig.3.9.).

Information on the present distribution of β-thalassemia in Italy does not specifically indicate if the region where Isola Sacra is located has a very high frequency of β-thalassemia (Piratsu, 1988). However, if thalassemia occurs, as a general rule in Italy, the $\beta^+$IVS1-110, $\beta^o$-39 and $\beta^+$IVS1-6 mutations should be among the most common mutations in the Isola Sacra population. Namely, if the SCR specimens for this study were thalassemics, they would be more likely to be $\beta^+$IVS1-110, $\beta^o$-39 or $\beta^+$IVS1-6. This probably would be the same even if these Isola Sacra individuals were new immigrants from outside Italy because all Mediterranean areas share a similar mutation pattern with Italian populations (Cao et al., 1989).

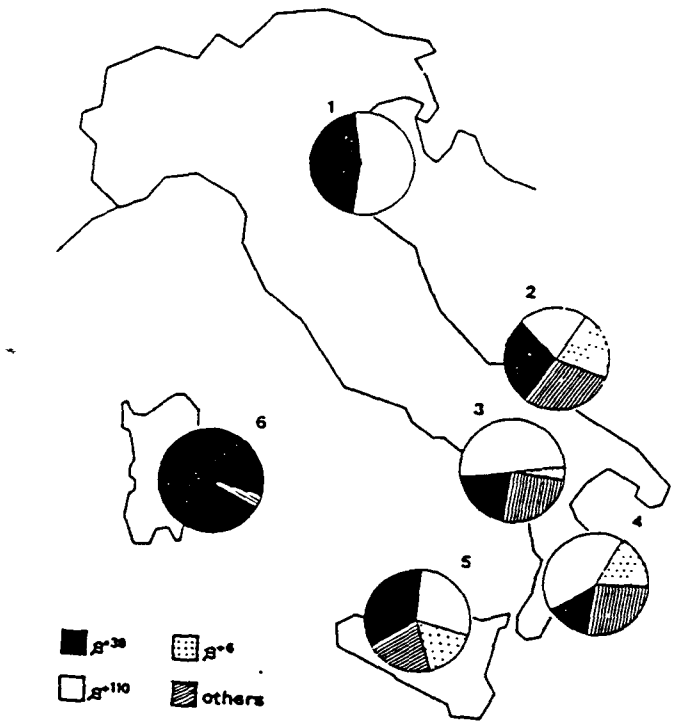



Fig.3.8. The distribution of β-thalassemia mutations in different regions in Italy. 1: Po Delta; 2: Puglie; 3: Basilicata and Campania; 4: Calabria and 5: Sicilia (after Pirastu et al., 1988).

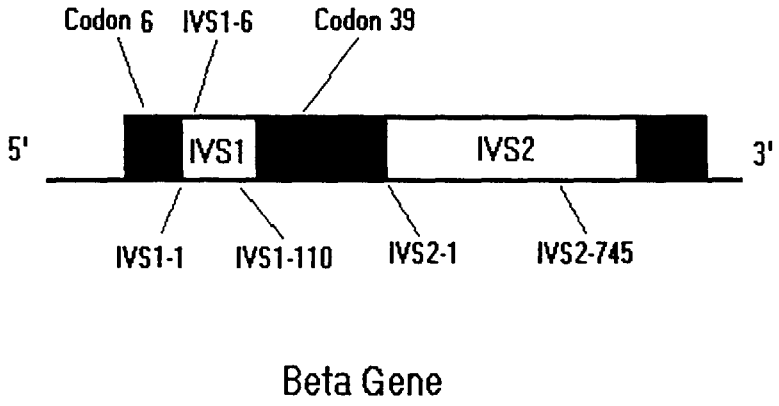



Fig. 3.9. The seven common β-thalassemia mutations and their locations on the β-globin gene in Italian populations.

### 3.2.4.1.1. Primer Design

The primary consideration for designing PCR primers for aDNA amplification is the length limitation of amplification products. aDNA has been degraded into small pieces or fragments, usually less than 300 bp in length. The PCR products of aDNA must be less than 300 bp in length. The first investigation, therefore, focussed on the most frequent types of thalassemia mutations in Italian populations: $\beta^{+}$IVS1-110, $\beta^{o}$-39 and $\beta^{+}$IVS1-6. Primer BG1 and BG2 flank the fragment of 256 bp which covers all three of the most common mutations in the Italian populations (Fig.3.10.).

However, one problem encountered when designing specific primers for the β-globin gene is due to the similarity between the β-globin gene and other β-like globin genes (Efstratidis et al., 1980). The comparison of DNA sequences reveals that there is a great similarity among the two adult β- and δ-globin genes, embryonic ϵ-globin, fetal $^{G}\gamma$- and $^{A}\gamma$-globin, and pseudogene $\psi\beta_1$ (Fig. 3.11.). Among all of the β-like globin genes, the β- and δ-globin genes are the most similar. A β-globin gene sequence (Law et al., 1980) was used for a BLAST (Basic Local Alignment Search Tool) homology comparison with all sequences in DNA databases such as the Genbank of the National Institute of Health, USA. The result clearly indicates that the human δ-globin gene (Spritz et al., 1980) shares the most similar DNA sequences with the human β-gene (Fig. 3.12.). One study estimated that both of these genes were split from the same ancestral gene 40 million years ago, whereas the β-globin gene shared the same ancestral gene with other β-like globin genes 200 million years ago (Efstratidis et al., 1980). The difference

```
ccctgtggagccacaccctagggttggccaatctactcccaggagcagggagggcaggagccagggctgg

gcataaaagtcagggcagagccatctattgcttACATTTGCTTCTGACACAACTGTGTTCACTAGCAACC

                  ValHisLeuThrProGluGluLysSerAlaValThrAlaLeuTrpGlyLysVal
TCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTG

AsnValAspGluValGlyGlyGluAlaLeuGlyArg
AACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGGTTTAAGGAGA
------BG1--->                          Ivs1-6              -----BG3---->
CCAATAGAAACTGGGCATGTGGAGACAGAGAAGACTCTTGGGTTTCTGATAGGCACTGACTCTCTCTGCC

                              LeuLeuValValTyrProTrpThrGlnArgPhePheGluSerPh
TATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTT
 ivs1-110                                              codon39
eGlyAspLeuSerThrProAspAlaValMetGlyAsnProLysValLysAlaHisGlyLysLysValLeu
TGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGAAGGCTCATGGCAAGAAAGTGCTC
                              <------BG2-------
GlyAlaPheSerAspGlyLeuAlaHisleuAspAsnLeuLysGlyThrPheAlaThrLeuSerGluLeuH
GGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGC

isCysAspLysLeuHisValAspProGluAsnPheArg
ACTGTGACAAGCTGCACGTGGATCCTGAGAACTTCAGGGTGAGTCTATGGGACCCTTGATGTTTTCTTTC

CCCTTCTTTTCTATGGTTAAGTTCATGTCATAGGAAGGGGAGAAGTAACAGGGTACAGTTTAGAATGGGA
AACAGACGAATGATTGCATCAGTGTGGAAGTCTCAGGATCGTTTTAGTTTCTTTTATTTGCTGTTCATAA
CAATTGTTTTCTTTTGTTTAATTCTTGCTTTCTTTTTTTTTCTTCTCCGCAATTTTTACTATTATACTTA
ATGCCTTAACATTGTGTATAACAAAAGGAAATATCTCTGAGATACATTAAGTAACTTAAAAAAAAACTTT
ACACAGTCTGCCTAGTACATTACTATTTGGAATATATGTGTGCTTATTTGCATATTCATAATCTCCCTAC
TTTATTTTCTTTTATTTTTAATTGATACATAATCATTATACATATTTATGGGTTAAAGTGTAATGTTTTA
ATATGTGTACACATATTGACCAAATCAGGGTAATTTTGCATTTGTAATTTTAAAAAATGCTTTCTTCTTT
TAATATACTTTTTTGTTTATCTTATTTCTAATACTTTCCCTAATCTCTTTCTTTCAGGGCAATAATGATA
CAATGTATCATGCCTCTTTGCACCATTCTAAAGAATAACAGTGATAATTTCTGGGTTAAGGCAATAGCAA
TATTTCTGCATATAAATATTTCTGCATATAAATTGTAACTGATGTAAGAGGTTTCATATTGCTAATAGCA
GCTACAATCCAGCTACCATTCTGCTTTTATTTTATGGTTGGGATAAGGCTGGATTATTCTGAGTCCAAGC

                                                    LeuleuGlyAsnValLeuValC
TAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGCAACGTGCTGGTCT

ysValLeuAlaHisHisPheGlyLysGluPheThrProProValGlnAlaAlaTyrGlnLysValValAl
GTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGC

aGlyValAlaAsnAlaLeuAlaHisLysTyrHis
TGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAG

GTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGATTCT
GCCTAATAAAAAACATTTATTTTCATTGCaatgatgtatttaaattatttctgaatattttactaaaaag
ggaatgtgggaggtcagtgcatttaaaacataaagaaatgatgagctgttcaaaccttgggaaaatacac
tatatcttaaactccatgaaagaaggtgaggctgcaaccagctaatgcacattggcaacagcccctgatg
cctatgccttattcatccctcagaaaaggattcttgtagaggcttgatttgcaggttaaagttttgctat
gctgtattttacattacttattgttttagctgtcctcatgaatgtcttttcactacccatttgcttatcc
tgcatctctctcagccttgact
```

Fig.3.10. The DNA sequence (5'-3') of human β-globin gene (Lawn et al, 1980). The uppercase and lowercase letters represent mRNA and intron sequences. The amino acid sequences are shown above the corresponding coding sequences. The bold and italic letters indicate point mutations. The bold and underlined letters represent the sequences of the primers.

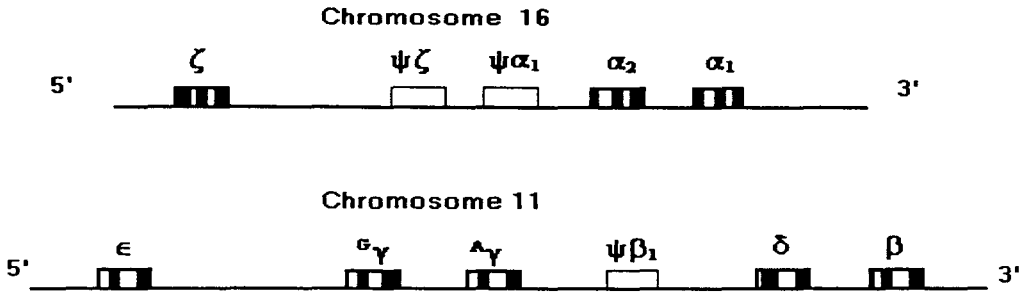



Fig.3.11.The human α-globin and β-globin gene family are shown on chromosome 16 and 11 respectively. The α-globin gene family includes the two adult ( $\alpha_1$ and $\alpha_2$) and embryonic ($\zeta$ ) gene and two pseudogenes ($\psi\alpha_1$ and $\psi\zeta$). The β-globin gene family includes the two adult ( δ and β ), embryonic ($\epsilon$ ), two fetal ($^{G}\gamma$ and $^{A}\gamma$) gene and one pseudogenes ($\psi\beta_1$).

```
Beta :    71 GCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTC 130
             ::::::::: :::::::::: :  :: ::::::::: :: ::::::::: ::: ::::::
Delta:    90 GCATAAAAGGCAGGGCAGAGTCGACTGTTGCTTACACTTTCTTCTGACATAACAGTGTTC 149

Beta :   131 ACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTA 190
             :::::::::::::::::::::::::::::: :::::::::::::::::: :::: :: :
Delta:   150 ACTAGCAACCTCAAACAGACACCATGGTGCATCTGACTCCTGAGGAGAAGACTGCTGTCA 209
                                 -------BG1------>
Beta :   191 CTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAGGTTGG 250
              ::::::::::::::: :::::::::::: :::::::::::::::::::::::::::::
Delta:   210 ATGCCCTGTGGGGCAAAGTGAACGTGGATGCAGTTGGTGGTGAGGCCCTGGGCAGGTTGG 269
                              -------BG3------>
Beta :   251 TATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCATGTGGAGACAGAG 310
             :::::::::::: :::: ::: : :::::: : ::: ::::::::::::::: ::::::::
Delta:   270 TATCAAGGTTATAAGAGAGGCTCAAGGAGGCAAATGGAAACTGGGCATGTGTAGACAGAG 329

Beta :   311 AAGACTCTTGGGTTTCTGATAGGCACTGACT 341
             ::::::::::::::::::::::::::::::
Delta:   330 AAGACTCTTGGGTTTCTGATAGGCACTGA-- 358

Beta :   342 CTCTCTGCCTATTGGTCTATTTTCCCACCCTTAGGCTGCTGGTGGTCTACCCTTGGACCC 401
             ::::::: :  :::: :: :::::: ::::: ::  : :::::::::::::::::::::
Delta:   359 CTCTCTGTCCCTTGGGCTGTTTTCCTACCCTCAGATTACTGGTGGTCTACCCTTGGACCC 418
                                                    <------BG2------
Beta :   402 AGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTA 461
             :::::::::::::::::::::::::::::::: :::::::::::::::::::::::::::
Delta:   419 AGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCTCTCCTGATGCTGTTATGGGCAACCCTA 478

Beta :   462 AGGTGAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGG 521
             :::::::::::::::::::::: ::::: ::::::::::::::::::::::::::::::
Delta:   479 AGGTGAAGGCTCATGGCAAGAAGGTGCTAGGTGCCTTTAGTGATGGCCTGGCTCACCTGG 538

Beta :   522 ACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGG 581
             ::::::::::::::: ::: :    ::::::::::::::::::::::::::::::::
Delta:   539 ACAACCTCAAGGGCACTTTTTCTCAGCTGAGTGAGCTGCACTGTGACAAGCTGCACGTGG 598

Beta :   582 ATCCTGAGAACTTCAGGGTGAGTCTATGGGACCCTTGATGTTT 624
             ::::::::::::::::::::::: : : ::  ::: :  :::
Delta:   599 ATCCTGAGAACTTCAGGGTGAGTCCAGGAGATGCTTCACTTTT 641
```

Fig. 3.12. Homology comparisons of some DNA sequences between the β-globin and δ-globin gene. BG1, BG2 and BG3 are the primers designed for the β-globin gene. The bold and underlined letters represent the sequences of the primers.

between the β- and δ-genes is greater in the non-coding sequence, but codon 39, one of the most common mutations, is located in an area where the downstream sequences (150 bp) are almost identical between the β- and δ-globin genes (Fig.3.12.). Because the sequence length for aDNA amplification should not exceed 300 bp, the primer from this area cannot be designed to be β-globin-specific.

Three sets of primers have been designed in our laboratory for the PCR amplification of the specific DNA sequence covering the major Italian thalassemia mutations, but none of them yielded satisfactory results in single-round PCR amplifications. There were either many non-specific PCR products or insufficient amounts of PCR products with the expected size. Compared to ancient mtDNA, this probably indicates that fewer DNA templates are available for PCR amplification (Lindahl, 1997).

Nested PCR amplifications have often been used in aDNA studies since they can increase amplification efficiency by increasing the number of PCR cycles (Herrmann and Hummel, 1994). The typical nested PCR techniques use two sets of primers. The first set is used to amplify a longer DNA fragment of interest for 30 or 40 cycles. The second set of primers is used to amplify internal sequences of the first-round PCR products. The internal amplification of the first-round PCR products ensures that the final PCR products are specific. Non-specific PCR products can be produced in the first-round reaction, but these products will not enter the second-round reaction since the second set of primers is only complementary to specific products from the first-round PCR. When the second-round PCR is performed for another 30 or 40 cycles, the total amount of specific PCR

products should be sufficient for post-PCR analysis even if only minute amounts of aDNA are available from ancient remains.

Semi-nested PCR amplifications were used to amplify the human β-globin gene in this study. Primers BG1 and BG2 were used for the first-round PCR amplification and primers BG3 and BG2 for the second-round PCR amplification (Tab.3.5. and Fig.3.14.). Primer BG3, 16 bp in length, has a difference at five nucleotide positions between the β-globin gene and the δ-globin gene. Primer BG1, 20 bp in length, has a difference at two nucleotide positions between the two globin genes. But primer BG2, 20 bp in length is identical between the two globin genes (Fig.3.14.). The second set of primers with more variable sequences has the power to prevent non-specific PCR products from the first-round reaction from being used as the templates for the second-round reaction. However, a complete new set of primers for the second-round amplification cannot be realized because the β-globin genes and the δ-globin genes are almost identical in this specific DNA fragment (Fig.3.14.). Therefore, primer BG2 is used twice in the semi-nested PCR amplifications.

Tab.3.5. DNA sequences of the primers for the human β-globin gene

| Primer | Sequence (5' to 3') | Tm |
|---|---|---|
| BG1 | GGT GAA CGT GGA TGA AGT TG | 55°C |
| BG2 | TTA GGG TTG CCC ATA ACA GC | 55°C |
| BG3 | CAG GTT TAA GGA GAC C | 43°C |

### 3.2.4.1.2. PCR Amplification of the Human β-Globin Gene

The semi-nested PCR method was used to obtain sufficient amounts of PCR products for DNA analysis. Semi-nested PCR uses three primers for a two-round PCR amplification. In this study, primer BG1 and BG2 would amplify a larger sequence from human β- and δ-globin genes, BG3 and BG2 would then amplify human β-globin-specific DNA sequences from the products of the first-round reaction that may include both the β- and δ-globin genes (Fig. 3.13.).

The PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50 μL reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 mg/mL BSA, 0.25 U regular Taq polymerase (Saigon Bio, Toronto) for the first-round PCR, 0.25U AmpliTaq Gold™ polymerase of Perkin Elmer for the second-round PCR amplification, 100 pmoles of each primer, and 5 μL of DNA template for the first-round PCR and 5 μL first-round PCR products for the second-round PCR (Tab.3.6.). BSA was removed from the reaction solution in the second-round PCR. PCR was run 40 cycles at 94°C for 40 seconds, 54°C for 30 seconds, and 72°C for 30 seconds for the first-round PCR, and 40 cycles at 94°C for 30 seconds, 43°C for 20 seconds, and 72°C for 20 seconds for the second-round PCR (Fig. 3.14.). 5 μL of PCR product was separated by electrophoresis on a 8% non-denaturing polyacrylamide gel cast and run in 1 x TBE (90 mM Tris Borate, 2 mM EDTA). The 100 bp ladder (Life Technologies, Gaithersberg MD) was used as a size marker. After electrophoretic separation, the gel was stained with ethidium bromide and photographed under UV illumination.

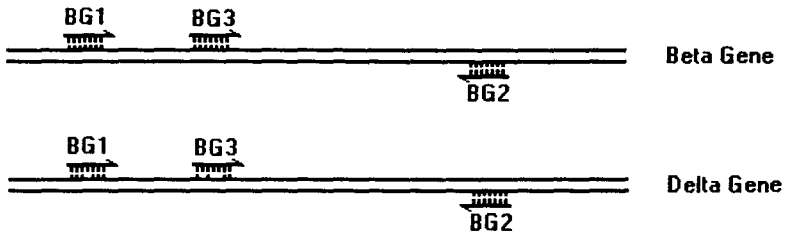



Fig. 3.13. The semi-nested PCR reaction for human β-globin gene. The PCR products of BG1 and BG2 are 256 bp in length with the β-globin gene and 254 bp in length with the δ-globin gene. The PCR products of BG3 and BG2 are 196 bp in length for the β-globin gene and 194 bp in length for the δ-globin gene.

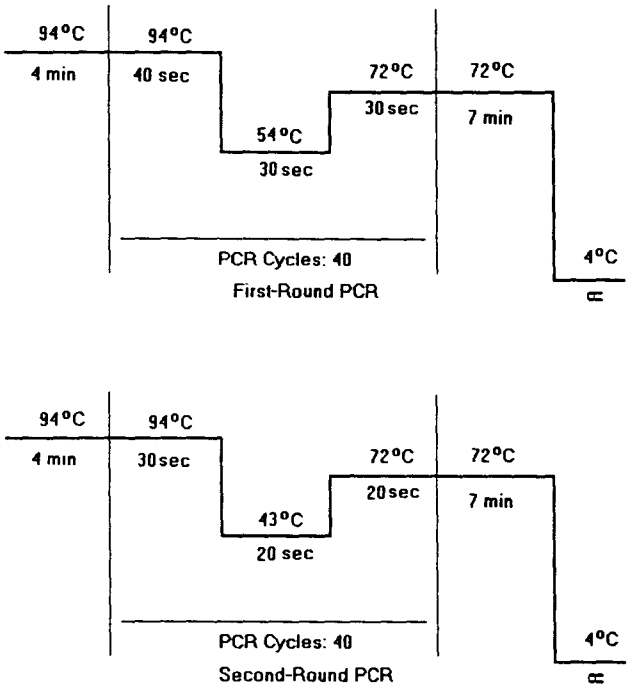



Fig.3.14. The PCR conditions for the semi-nested amplification of the human β-globin genes.

Tab.3.6. The PCR setup for the human β-globin gene amplification

| Component | 1st-Round PCR | 2nd-Round PCR |
|---|---|---|
| Perkin Elmer Buffer IIx10 | 5 µL | 5 µL |
| $MgCl_2$ | 2.5 mM | 2.5 mM |
| dNTP | 0.2 mM | 0.2 mM |
| Primer BG1 or BG3 | 100 pmoles (BG1) | 100 pmoles (BG3) |
| Primer BG2 | 100 pmoles | 100 pmoles |
| BSA | 0.75 mg/mL | |
| Taq | 0.25U (Saigon Bio) | 0.25U (AmpliTaq Gold™) |
| DNA or PCR Sample | 5 µL (DNA Sample) | 4 µL (PCR Products) |
| Total volume with $H_2O$ | 50 µL | 50 µL |

### 3.2.4.2. The Dot Blot Test for the Human β-Globin Gene

The Dot Blot test was carried out to confirm that the final PCR products amplified from primers BG1, BG2 and BG3 come from the human β-globin gene. The DNA sequence of interest for this research is located in one of the most homologous areas between the β-globin and δ-globin genes. It has not been ascertained that the set of primers is specific to human globin genes since they were designed based on the DNA sequences available to date. There is a possibility that the primers can amplify some presently unknown DNA sequences. Consequently, confirmation of amplification of the human β-globin gene is necessary. The PCR products from SCR 8, SCR 277, the forensic bone and modern DNA (1 ng of K562, Perkin Elmer) from the same PCR amplification

using primer BG1, BG2 ad BG3 were selected for the Dot Blot test. In the PCR amplification, SCR 8 did not yield any detectable PCR products on the gel, but SCR 277 and the forensic bone did. SCR 8 was used to examine whether the Dot Blot test can identify the β-globin gene from undetectable PCR products on the gel. SCR 277 was used as a control to represent the samples which had been amplified from the SCR specimens. The forensic bone was considered to be a control of bone DNA which could be confidently extracted since it is recent bone material. A blank extraction was added to the Dot Blot test to ensure that the extraction protocol was well processed for contamination. The modern DNA sample was used to confirm the success of the PCR amplification. A Dot Blot test of the human δ-globin gene was used to show whether this test was exclusive for the identification of the human β-globin gene. The human δ-globin gene was amplified using primers 5'-AAGTTAAGGGAATAGTGGAATGAAG-3' and 5'-TCATTCGTCTGTTTCCCATT-3 from modern DNA. These primers are specific for amplification of the human δ-globin gene.

Three other controls were also included to ensure that this Dot Blot test could detect three different sequences of the human β-globin gene: one with the $\beta^{+}$IVS1-110 homozygote, one with the $\beta^{+}$IVS1-110 heterozygote (carrier) and the other with no $\beta^{+}$IVS1-110 mutations. These controls were prepared in the Ontario Provincial Hemoglobinopathy DNA Diagnostic Laboratory at McMaster University using established clinical protocols. The primers for the human δ-globin gene were also obtained from the same laboratory.

The ECL™ 3'-oligolabelling and detection system (Amersham, LIFE SCIENCE)

was used for the Dot Bot test in this study. This system utilizes enhanced chemiluminescence to detect the presence of oligonucleotides tailed at the 3'-end with fluorescein-11-dUTP (FI-dUTP), hybridized to target sequences on membranes (Fig.3.15.).

The probes were prepared and chemiluminescence -labelled following the instructions from the manufacturer. The probe 5'-CTGCCTATTAGTCTATTTTC-3' is for the human β-globin gene mutation $\beta^{+}$IVS1-110 and the probe 5'-CTGCCTATTGGTCTATTTTC-3' for a normal human β-globin gene.

The PCR products were quantified before they were used for denaturing. Judging from the intensity of the bands shown on the gel, three μL of PCR products from modern DNA were used as the positive control ($\beta^{+}$IVS1-110 homozygote and heterozygote) and negative control (normal β-globin gene). Five μL of PCR products of aDNA from SCR 8, SCR 277 and the forensic bone were taken for the Dot Blot test. The PCR products were then added to 250 μL of denaturing solution (0.4 M NaOH, 25 mM EDTA, 0.00008% Bromophenol Blue).

The neutral KN biotrans membrane (Schleicher and Schuell, NH) was wetted with water for several seconds and then immersed into a 20xSSC solution for 20 seconds. The 120 μL of denatured PCR products from each sample were loaded on the duplicated membranes: one for the mutant probe and the other for the normal probe. The membranes were put into the Ultraviolet Crosslinker (Amersham, LIFE SCIENCE) at an intensity of 1500x100 μJ/cm2 for binding of the single stranded DNA to the membrane.

The membranes were placed into a 5 mL hybridization buffer (5xSSC, 0.1% w/v

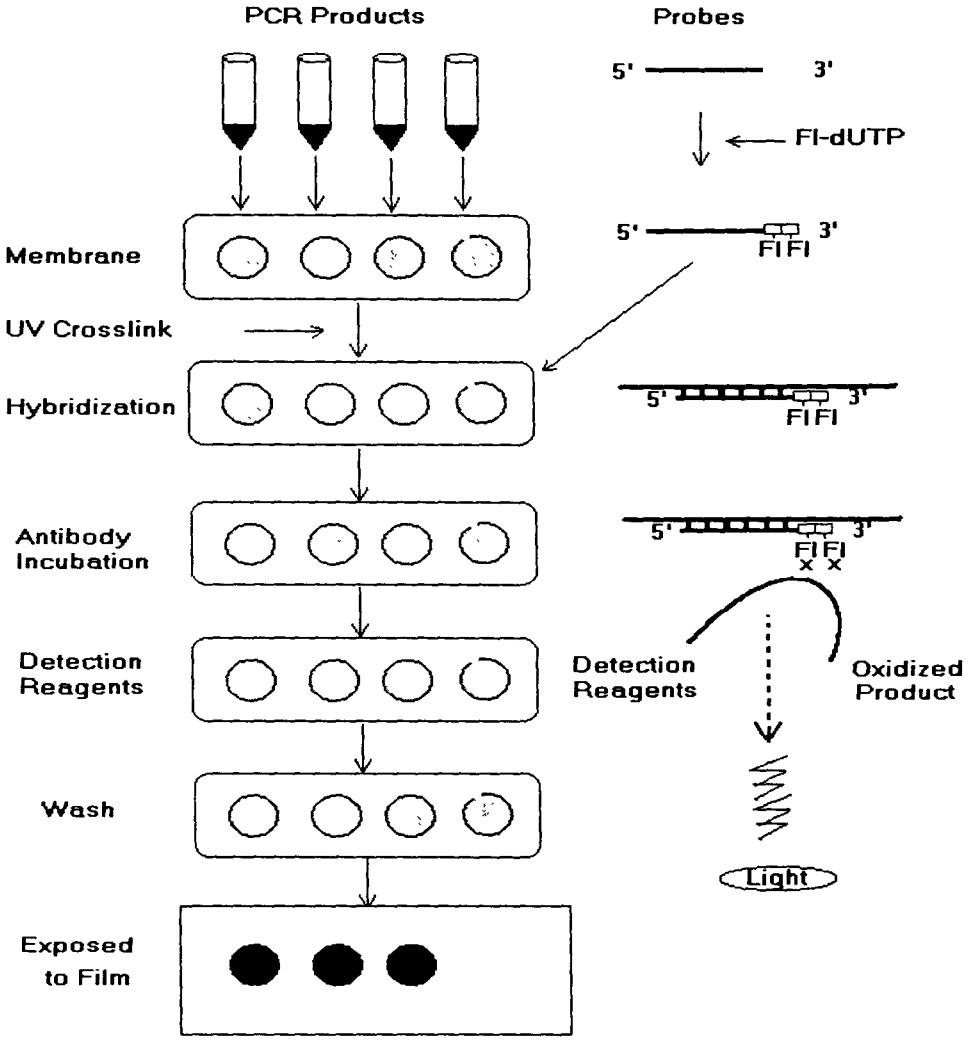



Fig.3.15. The procedures of the Dot Blot test for the human β-globin gene

hybridization buffer component, 0.02% v/w SDS, 20-fold diluted liquid block) and prehybridized at 45°C for 30 minutes in a shaking waterbath. Then, 10 μL of chemoluminescence -labelled probe was added (for the normal and the mutant, respectively) into the hybridization solution and hybridized at 50°C for one hour in a shaking water bath. The membranes were then removed from the hybridization solution and washed with 15 mL5xSSC and 0.1% (w/v) SDS and incubated at room temperature for 5 minutes under constant agitation. Next, the membranes were placed in another clean container with 15 mL pre-warmed stringency wash buffer (1xSSC, 0.1% w/v SDS) and incubated at 52°C for 10 minutes in a shaking water bath.

After this, the membranes were placed in a clean container and washed with buffer 1 (supplied with the kit) for 1 minute. Buffer 1 was replaced with block solution and incubated for 30 minutes at room temperature. The membranes were incubated in the diluted (1,000-fold) antibody conjugate solution for 30 minutes and then were placed membrane in a clean container to wash with 5 mL buffer 2 at room temperature. This step was repeated three more times.

A 2.5 mL detection solution 1 and a 2.5 mL detection solution 2 (supplied with the kit) were mixed to cover the membranes. After one minute incubation, the detection buffer was drained off and the membranes were wrapped in SaranWrap. The membrane was placed with the DNA side up and covered with a sheet of autoradiography film, Kodak XAR-5. This was exposed for 5 minutes in the film cassette. All of the above procedures are illustrated in Fig.3.15.

### 3.2.4.3. Sequencing the Human β-Globin Gene

The chain termination sequencing technique (Sanger et al., 1977) was used to sequence aDNA in this study. This technique uses specific terminators of DNA chain elongation: 2', 3'-dideoxynucleoside-5'-triphosphates (ddNTP). These deoxynucleoside triphosphate analogues can be incorporated by a DNA polymerase into a growing DNA chain through their 5'-triphosphate groups. However, since these analogues do not have hydroxyl groups at the 3'-end, they cannot form phosphodiester bonds with the next incoming deoxynucleoside-5'-triphosphate (dNTP), the chain extension will stop when an analogue is incorporated. When dNTP and ddNTP are both available, some growing chains will incorporate ddNTP and then stop, others incorporating dNTP will move to the next nucleotide position. This process generates a series of DNA chains with different lengths. To obtain a DNA sequence, each polymerase chain extension has to be run for each of four ddNTP: ddGTP, ddATP, ddCTP and ddTTP separately. The four ddNTPs corresponse to the four nucleotides: dGTP, dATP, dCTP and dTTP, respectively. Each of the chain extension reaction results in discrete length DNA chains that are specifically terminated by each ddNTP. If DNA with these different lengths is labelled with radioactives such as $P^{32}$ or chemiluminescence, then they can be read on film or special equipment. When four ddNTP chain extensions are analysed together, the complete DNA sequence will be obtained. The PCR technique has made Sanger's sequencing method more practical by providing a rapid and efficient way to prepare templates and by speeding up chain extensions by the PCR cycling reaction.

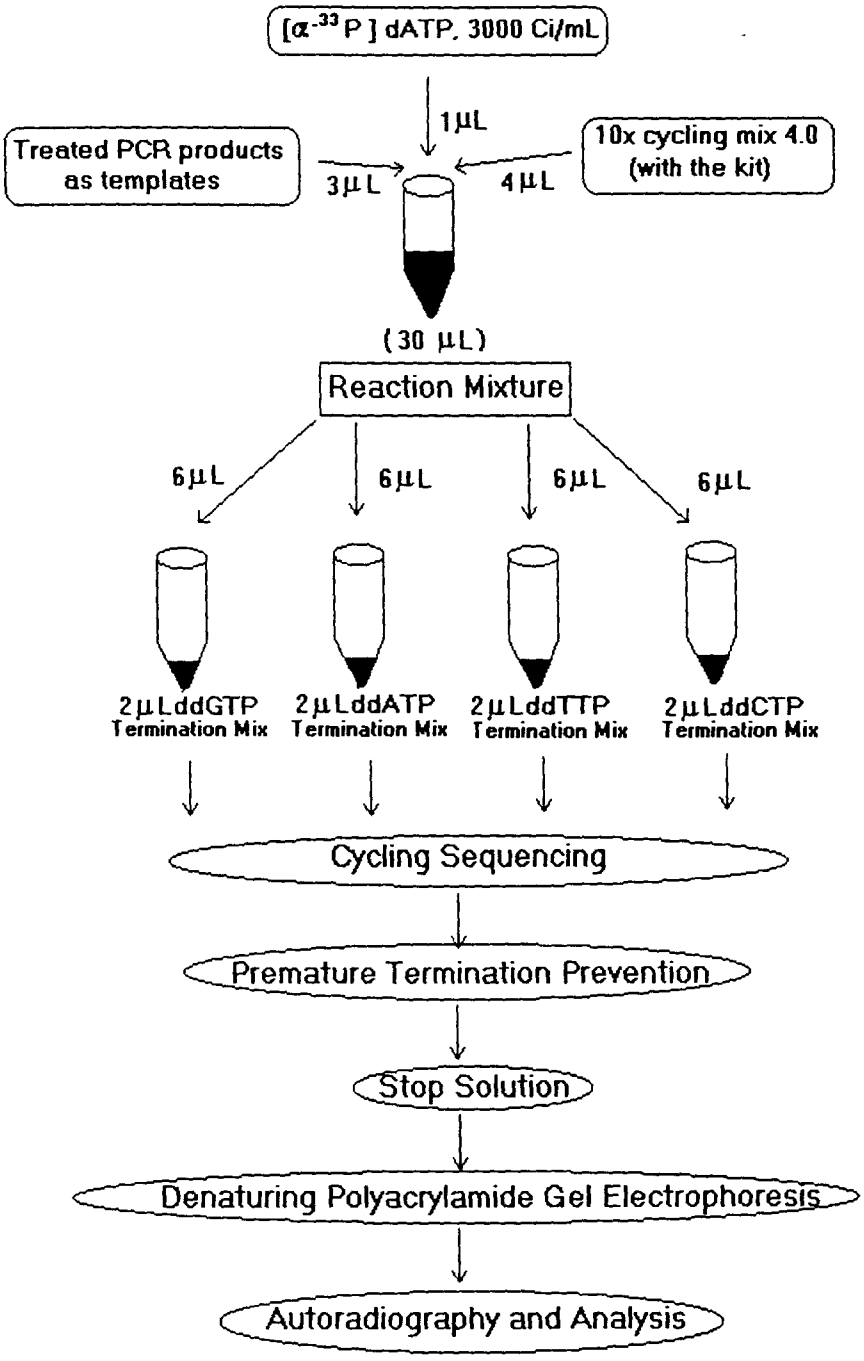



Fig.3.16. Procedures for sequencing the human β-globin gene

PCR sequencing was carried out with the PCR products from SCR 136, 277, 599/1 and 1353/2. They had yielded detectable amouts of PCR products on the gel. The AmpliCycle™ sequencing kit of Perkin Elmer was used but the recommended procedures were modified for better results (Fig.3.16.).

The PCR products were first treated with enzymes and chemicals to destroy the primers and nucleotides left from the PCR amplifications. These would interfere with the sequencing process if they were not removed. Five μL PCR products from these four samples were added to 1 μL exonuclease (1U/μL) (U. S. Biochemicals) and 2 μL alkaline phosphatase (1U/μL) (U.S. Biochemicals). They were then incubated at 37°C for 15 minutes and 80°C for another 15 minutes. Exonuclease was used to break the single stranded DNA of primers and alkaline phosphatase was used for decomposing nucleotides. After incubation, three μL of PCR products from each specimen were taken for the sequencing reaction.

The 30 μL reaction mix was prepared by mixing 4.0 μL 10xcycling mix (supplied with the Pekin Elmer kit), 1.0 μL primer BG3 (100 ng/μL), 3.0 μL treated PCR products as templates, 1 μL [ $\alpha$ $^{-33}$P]dATP (3,000 Ci/mmol), and 21 μL ultrapure water. The 2 μL G-Termination Mix (supplied with the kit) containing ddGTP and dATP, dCTP and dTTP was mixed with 6 μL of reaction mixture for detecting G positions in a given DNA sequence. Following the same rule, the 2 μL A-, T-, and C-Termination Mixes (all supplied with the kit) were prepared with 6 μL reaction mixtures.

The above mixtures were then placed in the GeneAmp™ Thermocycler (Perkin-Elmer, Norwalk CT) for cycling sequencing. The conditions were set for 25 cycles at the

following parameters: 94°C denature for 40 seconds; 55°C annealing for 40 seconds; and 72°C for 1 minute.

To avoid premature termination, 1 μL diluted terminal deoxynucleotidyl transferase (TdT) (2 U/μL) was added to each of the above cycling reaction tubes and incubated at 37°C for 30 minutes. The diluted TdT was prepared by adding TdT (15 U/μL) and dNTP to TE buffer (10 mM Tris-Cl, pH 7.5, 1mM EDTA) to make the final solution of 2 U/μL and 1mM dNTP. After the incubation, four μL of stop solution (supplied with the kit) were added to each of the tubes to stop any reactions in the tubes.

Denaturing polyacrylamide gel was prepared using Long Ranger (FMC, Bioproducts). To make 50 mL 8% gel solution, 50 gm urea, 20 mL 5xTBE, 16 mL Long Ranger Solution (50%) and 24 mL water, 1 mL 10% A.P. and 15 μL TEMED were needed.

The sequencing samples were denatured by incubating at 94°C for 3 minutes using the GeneAmp™ Thermocycler (Perkin-Elmer, Norwalk CT) and loading 3 μL of the natured samples from each of the four termination reactions of the same indiviudal templates into separate adjacent wells of the gel in 0.6% TBE. The second loading was made from the same reaction solutions when the first loading reached the bottom of the gel. Double loadings resulted in clear gel readings of the sequences. The first loading gives a clear reading on the longer terminated DNA fragments and the second can provide a clear picture of the shorter terminated fragments. The gel was dried and exposed overnight on Kodak XK-1 film.

# 3.2.5. Sequencing of Human mtDNA to Monitor Contamination

## 3.2.5.1. Human mtDNA

mtDNA has been widely used in forensic identification and anthropological studies due to its distinctive characteristics. Its maternal inheritance allows easy reconstruction of maternal history since there is no recombination (Giles et al., 1980). The entire genome (16.6 kb) has been sequenced (Anderson et al., 1981) and the structure and function of human mitochondrial DNA is therefore well understood. The mtDNA sequence evolves 6 to 17 times faster than comparable nuclear DNA sequences, providing the highest possible resolution for examining molecular evolution in populations (Cann et al., 1987; Vigilant et al., 1989) and is a very sensitive tool for distinguishing individuals in forensic situations (Stoneking et al., 1991; Ginther et al., 1992; Gill et al., 1993; Comas et al., 1996; Richards et al., 1995). Unless they are close maternal relatives, individuals have an excellent chance of having different mtDNA types. mtDNA analysis is particularly useful for degraded DNA because of its higher number of copies. The copies of mtDNA genome per cell outnumber the copies of nuclear genes, mtDNA is more likely to be preserved in old or ancient remains than nuclear DNA (Handt et al., 1994a; Lindahl, 1997). Quite often, mtDNA analysis is the only alternative to nuclear DNA analysis when DNA is highly degraded (Stoneking et al., 1991). When most DNA fingerprinting systems such as nuclear STR (short tandem repeat sequences) fail to produce detectable signals, mtDNA usually results in positive results.

The genes encoded by human mitochondrial DNA are two ribosomal RNAs (12S and 16S), 22tRNAs, and 13 polypeptides that form parts of the respiratory chain. The two strands of the circular mtDNA chromosome have an asymmetric distribution of Gs and Cs, generating heavy (H)-and light (L)-strands. The control region is the major noncoding part of the human mitochondrial DNA genome, including the origin of replication of one strand, the D (displacement)-loop region and both origins of transcription (Anderson et al., 1981). The D-loop is a triple-stranded region formed by the synthesis of a short piece of H-strand DNA, the 7S DNA. The control region (1.1kb) has been found to be the most polymorphic portion of the entire mitochondrial genome (Vigilant et al., 1989). However, variation is not randomly distributed in the control region but rather concentrated in two hypervariable segments (Stoneking et al., 1991). Each of the two segments contains a sequence approximately 400 bp long (Vigilant et al., 1989).

mtDNA fingerprinting is an ideal tool for monitoring contamination in aDNA studies (Stoneking, 1995). When DNA is extracted from ancient remains, the first question that should be asked is whether the DNA comes from ancient remains or from contaminants. The contaminants can come from the people who have handled the remains or from people who have performed the DNA analysis. DNA fingerprinting underlines the fact that each individual has his/her own distinguishing DNA patterns which can be regarded as a personal identification. In DNA analysis, contaminations from other DNA sources result in the presence of DNA patterns of more than one person in a single sample. The minute amounts of preserved aDNA make it more vulnerable to

contamination. Theoretically, aDNA work can use any DNA fingerprinting system to monitor potential contamination; mtDNA has proven to be the best (Stoneking, 1995). The mtDNA fingerprinting system usually produces good results due to sufficient amounts of ancient mtDNA templates.

In this study, mtDNA analysis was used to monitor contamination. Since the target aDNA sequences are human globin genes, the use of mtDNA will ensure that contamination can be monitored when human globin DNA is analyzed.

### 3.2.5.2. PCR Amplification of Human mtDNA

One hypervariable segment was chosen to monitor contamination in this study. Since designing the proper PCR primers plays an important role in the success of individual identification in mtDNA analysis, this study used the primers designed and used by the Armed Forces DNA Identification Laboratory (Holland, 1993) (Tab. 3.7.). The primers for the PCR amplification amplify a 261 bp DNA sequence from position 15 to 275 according to the numbering of the reference sequence (Anderson et al., 1981). (Fig.3.17.).

PCR amplification of mtDNA was carried out four times for each of the SCR specimens, the forensic bone, the modern DNA (K562) and the author.

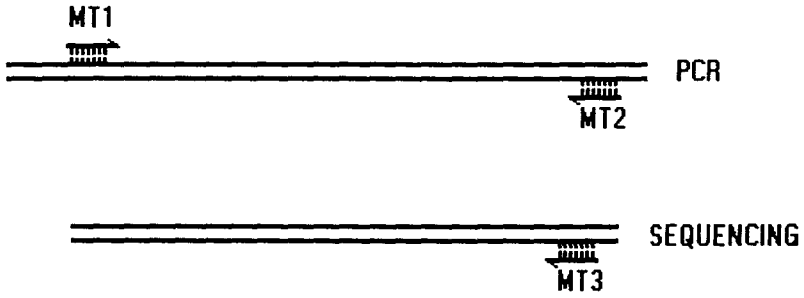



Fig.3.17. Primers MT1 and MT2 for the PCR products of 261 bp sequence from the D-loop region of human mitochondrial DNA and primer MT3 for sequencing.

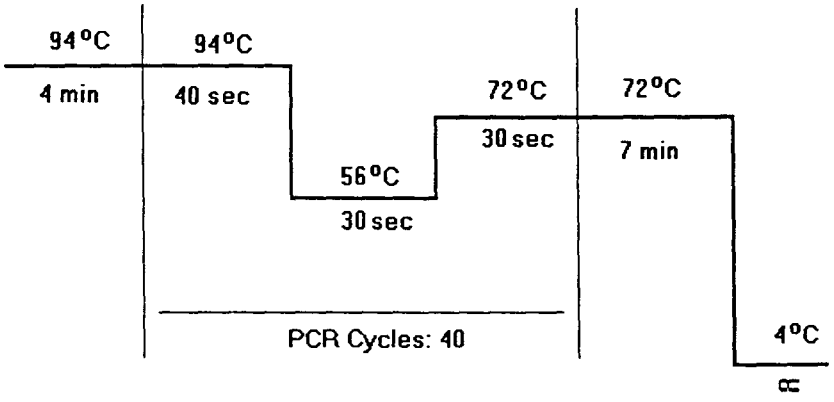



Fig.3.18. The PCR conditions for the human mtDNA sequence

Tab.3.7. The sequences of the primers for the D-loop region of human mtDNA

| Primer | Sequence | Tm |
|---|---|---|
| MT1 | 5'-CACCCTATTAACCACTCACG-3' | 55°C |
| MT2 | 5'-TGTGTGGAAAGTGGCTGTGC-3' | 53°C |
| MT3 | 5'-TGGAAAGTGGCTGTGCAGAC-3' | 57°C |

Tab.3.8. The PCR setup for the hypervariable sequence of human mtDNA

| **Component** | **Concentration** |
|---|---|
| Perkin Elmer Buffer II x10 | 5 µL |
| $MgCl_2$ | 2 mM |
| dNTP | 0.2 mM |
| Primer MT1 | 100 pmoles |
| Primer MT2 | 100 pmoles |
| BSA | 1.0 mg/mL |
| Taq AmpliTaq Gold™ | 0.25 U |
| DNA Sample | 5 µL |
| Total Volume with $H_2O$ | 50 µL |

The PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50 µL reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 1.0 mg/mL BSA, 0.25 U Taq polymerase, 100 pmoles of each primer, and 5 µL of DNA template (Tab.3.8.). PCR was

run for 40 cycles at 94°C for 40 seconds, 56°C for 30 seconds, and 72°C for 30 seconds (Fig.3.18.). 5 μL of PCR product was separated by electrophoresis on a 8% non-denaturing polyacrylamide gel cast and run in 1 x TBE (90 mM Tris Borate, 2 mM EDTA). The 100 bp ladder (Life Technologies, Gaithersberg MD) was used as a size marker. After electrophoretic separation, the gel was stained with ethidium bromide and photographed under UV illumination.

### 3.2.5.3. Sequencing Human mtDNA

Four samples, SCR 136, 277, 599/1 and 1353/2 were selected for mtDNA sequencing since only these samples had yielded detectable PCR products for the human β-globin gene. mtDNA sequencing was also performed on the author (DY) to monitor contamination.

The sequencing was carried out using the same protocol as that for sequencing the β-globin gene. The primer for cycling sequencing was MT3.

In order to examine consistence or reproducibility of the mtDNA sequencing, a second PCR sequencing was performed for the above four SCR specimens using the PCR products from a different amplification reaction. SCR 599/2 was sequenced once to compare to the sequence of SCR 599/1.

## 3.3. Contamination Controls

Contamination controls are one of the most crucial issues in the study of aDNA

since the PCR amplification of degraded aDNA is more vulnerable to modern DNA and other less degraded DNA (Handt et al., 1994a, Krings et al., 1997). Therefore, in this study, a series of controls and precautions were taken to minimize the opportunities for contamination.

## 3.3.1. Bone Decontamination

Ancient human skeletons may have been touched by many people before they are sent in for DNA analysis. This is particularly true of ancient skeletons of anthropological or archaeological interest. For this study, bone samples were usually taken from inside bones, ideally from cortical bone. This was carried out by polishing the surface of bones and drilling the polished surface to collect bone powder. This technique was applied to SCR 35 and the forensic bone. If bone materials were limited to mostly non-cortical bone, the surfaces were polished with sandpaper and then ground into a fine bone powder using a mortar and pestle. This method was used for SCR 8, 136, 277, 599/1 and 599/2. If the bone materials could not be processed by the above methods due to extremely poor preservation, the samples were UV-decontaminated by putting the materials in the UV Crosslinker (Amersham LIFE SCIENCE) for 30 minutes on each of two sides at an intensity of 1500x100 μJ/cm2. This was done for SCR 1353/2. Furthermore, bones polished for grinding by mortar and pestle were also placed into the UV Crosslinker for 60 minutes for further decontamination.

## 3.3.2. Physical Separation for aDNA Extraction

Various studies have demonstrated that one of the best ways to avoid contamination is to designate an area which is physically separated from that used for modern DNA or PCR work (Handt, 1994a; Herrmann and Hummel, 1994; Woodword et al., 1994a). In this study, specific research areas or rooms were designated for specific tasks. Bone samples or DNA samples were processed in one direction preceding from bone preparation to aDNA extraction, PCR setup, PCR amplification and post-PCR work. For example, under no circumstances were PCR products brought back to the areas of aDNA extraction or PCR setup.

One room was used exclusively for bone sample preparation and decontamination. No DNA extractions from ancient or modern sources were carried out in this area. The reasoning behind this practice is that once DNA is extracted, it remains in a relatively concentrated state. The surfaces of equipment or benches can be easily contaminated with extracted DNA, increasing the risk of contamination of new bone samples.

Another area was used specifically for extracting DNA from skeletal samples. The rule for this area was that no DNA from modern sources was extracted. For example, researchers were not allowed to extract DNA from themselves in this area. Even if the extracted DNA is spilled or contaminates the equipment, degraded aDNA of a relatively low concentration will not produce any significant threat of contamination to other aDNA samples.

The PCR setup and PCR analysis were carried out in two different laboratories in

two different buildings. The General Science Building (GSB) B/106 is a new laboratory converted from a non-biological laboratory. All PCR reagents were prepared in GSB B106. After the PCR setup was done in GSB B106, the PCR tubes were brought on ice to the Health Sciences Center (HSC) 4N76 for PCR amplifications. Modern DNA samples, such as the author's DNA were usually added into the PCR tubes in this laboratory before initiating PCR amplification.

For the second-round PCR amplification, the PCR tubes were set up in GBS B/106 with all reagents but the PCR products from the first-round reaction. When the first-round PCR amplification was done in HSC 4N76, positive disposable pipette tips were used to transfer a small amount of the PCR products from the first-round PCR amplification. This took place in an area distant from the activities related to any post-PCR analysis. The bench was always covered with new paperpads for each analysis.

## 3.3.3. Using Disposables and Commercial Kits

Using disposable products is considered to be a simple but most effective way for contamination control (Herrmann and Hummel, 1994; Handt et al., 1994a). This strategy has been followed in this study. Disposable gloves, tubes, pipette tips, transfer pipettes and commercial supplies such as buffers and ultrapure water were used whenever necessary to minimize the probability of contamination. Medical masks and hats were worn all times when handling materials or performing operations.

Ultrapure water of molecular biology grade was purchased from Laboratory

Supplies (Pool, UK) for all aDNA-related work such as setting up the PCR amplification and preparing bone samples. Using this ultrapure water, lab-made reagents such as ETDA, SDS, and TE were prepared in a microbiological laboratory where no human DNA analysis, especially the PCR amplification of human DNA, had been performed. All reagents were autoclaved before they were divided into smaller volumes.

All pre-PCR procedures were performed with commercially sterile packed aerosol-resistant, filtered pipette tips. UV light was used for routine decontamination of reagents, buffers, pipettes and other equipment.

# CHAPTER 4. A NEW METHOD FOR aDNA EXTRACTION

## 4.1. Results of Comparison

Five bone specimens from different archaeological sites were used to compare the efficiency and effectiveness of this new method with one of the two most commonly used methods - the phenol-chloroform extraction followed by Centricon™ concentration. For comparative purposes, four different procedures were used to extract DNA from the proteinase K-digested bone solutions:

A. Phenol-chloroform extraction followed by Centricon™ concentration (Fig.4.1.).

B. Phenol-chloroform extraction followed by Centricon™ concentration and QIAquick™ purification (Fig.4.2.).

C. Direct purification from proteinase K digests (without any phenol-chloroform extractions) using Centricon™ concentration followed by QIAquick™ purification (Fig.4.3.).

D. Direct purification from proteinase K digests (without any phenol-chloroform extractions) using only the QIAquick™ column (Fig.4.4.).

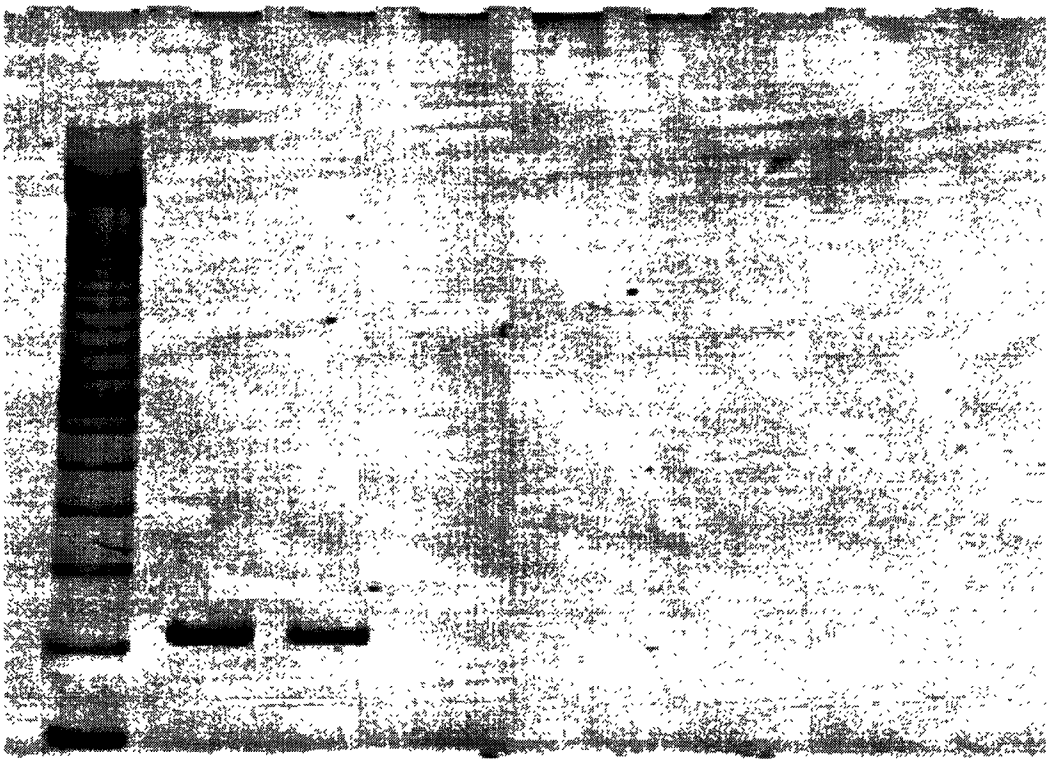


Fig.4.1. Reverse image of ethidium bromide stained gel of PCR products of DNA D17Z1 isolated from ancient bones by phenol-chloroform extraction followed by Centricon™ concentration. M: marker (100 bp); 1: 15 year old bone; 2: 200 year old bone; 3: 300 year old bone; 4: 2,000 year old bone; 5: 5,000 year old bone; B: blank extraction (no bone); and N: PCR negative control (no DNA).

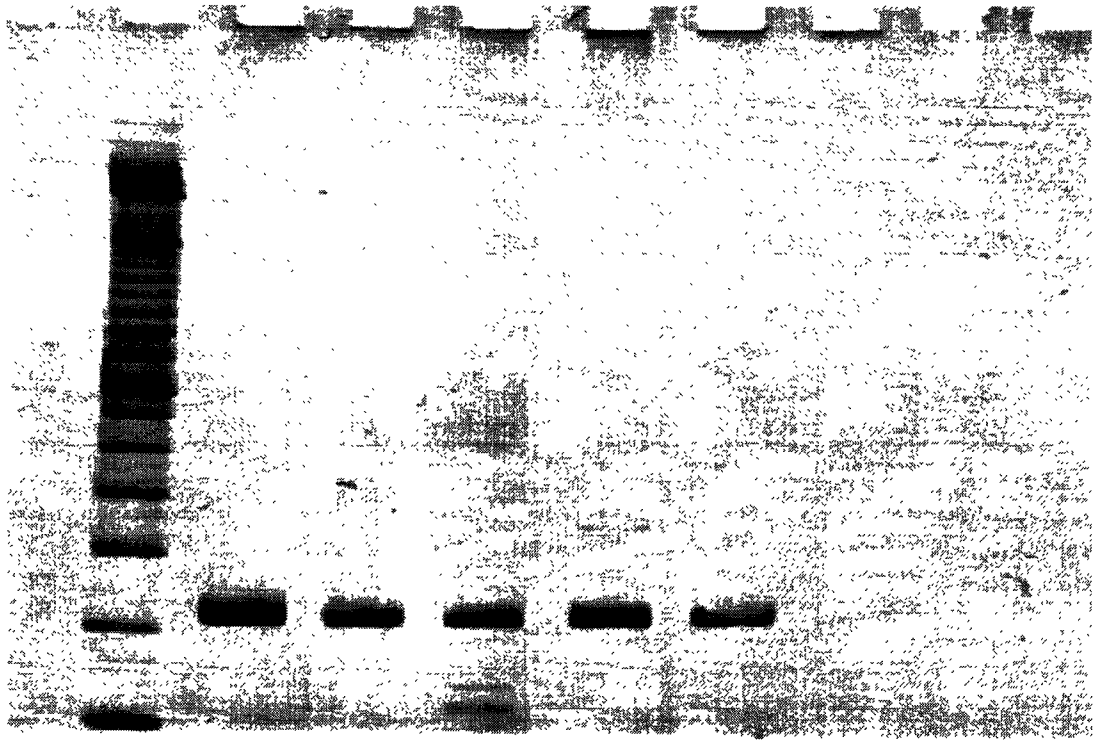


Fig.4.2. Reverse image of ethidium bromide stained gel of PCR products of DNA D17Z1 isolated from ancient bones by phenol-chloroform extraction followed by Centricon™ concentration and QIAquick™purification. M: marker (100 bp); 1: 15 year old bone; 2: 200 year old bone; 3: 300 year old bone; 4: 2,000 year old bone; 5: 5,000 year old bone; B: blank extraction (no bone); and N: PCR negative control (no DNA).

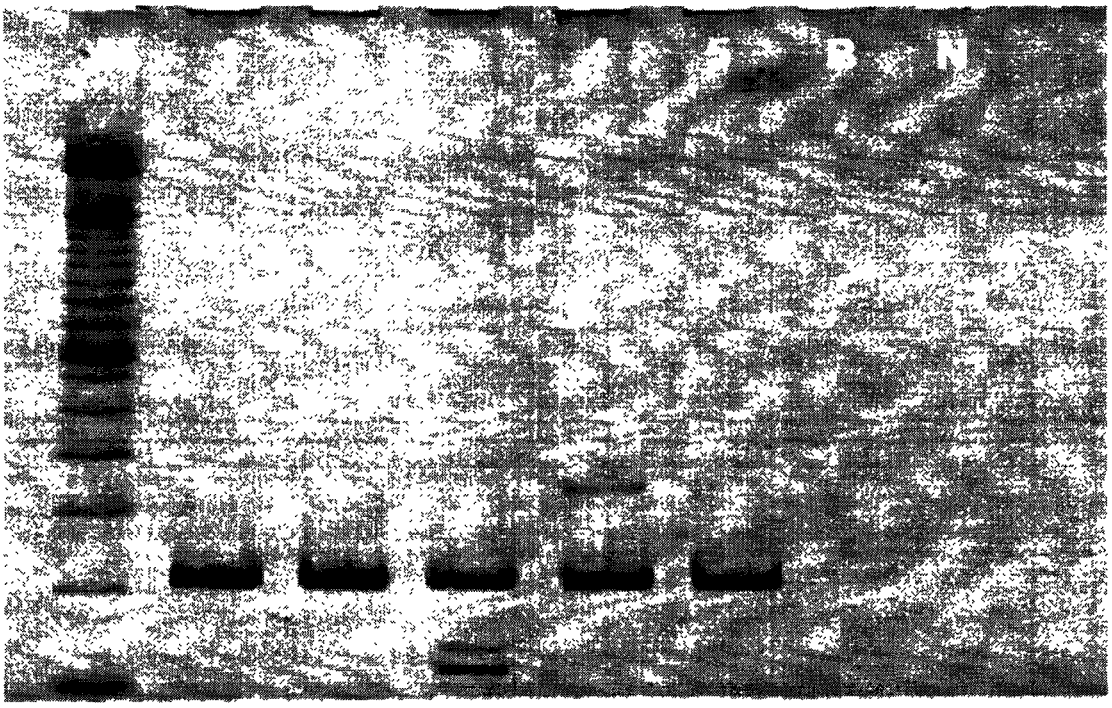



Fig. 4.3. Reverse image of ethidium bromide stained gel of PCR products of DNA D17Z1 isolated from ancient bones by direct purification from proteinase K digests (without any phenol-chloroform extractions) using Centricon™ concentration followed by QIAquick™ purification. M: marker (100 bp); 1: 15 year old bone; 2: 200 year old bone; 3: 300 year old bone; 4: 2,000 year old bone; 5: 5,000 year old bone; B: blank extraction (no bone); and N: PCR negative control (no DNA).

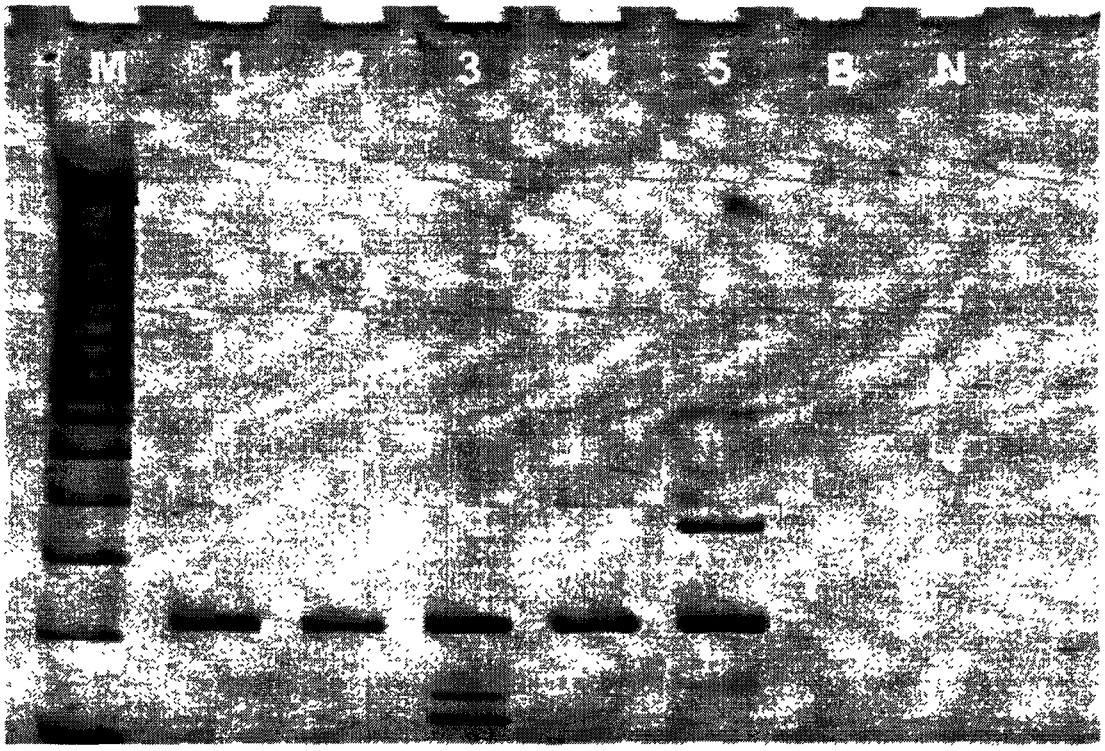



Fig.4.4. Reverse image of ethidium bromide stained gel of PCR products of DNA D17Z1 isolated from ancient bones by direct purification from proteinase K digests (without any phenol-chloroform extractions) using only the QIAquick™column. M: marker (100 bp); 1: 15 year old bone; 2: 200 year old bone; 3: 300 year old bone; 4: 2,000 year old bone; 5: 5,000 year old bone; B: blank extraction (no bone); and N: PCR negative control (no DNA).

The PCR results obtained for the five DNA samples extracted using the four protocols are shown in Fig.4.1., Fig.4.2., Fig.4.3., Fig.4.4.. With protocol A (phenol-chloroform extraction and Centricon™ concentration), only the samples from the 200 and 15 year old bones (lanes 2 and 1 respectively) showed detectable amplification (Fig.4.1.). In contrast, all of the samples extracted using protocol B (phenol-chloroform extraction, Centricon™ concentration, QIAquick™ purification) showed strong amplification (Fig.4.2.). A probable explanation for this difference is the presence of PCR inhibitors in DNA samples extracted using protocol A. This notion is supported by the observation that several of the DNA samples extracted using protocol A were pigmented, with the sample from the 300 year old bone (lane 3) appearing dark brown and the samples from the 5,000 and 2,000 year old bones (lanes 5 and 4 respectively) appearing light brown. In comparison, all five samples prepared using protocol B were free of pigmentation and readily amplified.

It was of interest to determine if the QIAquick™ columns could be applied directly to the proteinase K-digested bone solutions, without the need for phenol-chloroform extractions. The QIAquick™ columns yield PCR-amplifiable DNA from proteinase K digests that were concentrated using the Centricon™ columns (protocol C, Fig.4.3.). Moreover, the QIAquick™ purification method can be applied directly to the proteinase K digests (protocol D, Fig.4.4.).

Tab.4.1. The PCR amplification using different DNA extraction protocols

| Protocol | Protocol A | Protocol B* | Protocol C* | Protocol D* |
|---|---|---|---|---|
| 15 years | yes | yes | yes | yes |
| 200 years | yes | yes | yes | yes |
| 300 years | no | yes | yes | yes |
| 2,000 years | no | yes | yes | yes |
| 5,000 years | no | yes | yes | yes |
| Blank Extraction^ | no | no | no | no |
| PCR Negative^^ | no | no | no | no |

* Using a QIAquick™ column; ^ extraction without bone; ^^ PCR without DNA

The results (Tab.4.1.) demonstrate that QIAquick™ columns may be superior to Centricon™ microconcentrators, particularly with respect to the recovery of PCR-amplifiable DNA. This was most evident for the bone samples in lanes 3, 4 and 5. Protocol A yielded pigmented DNA preparations that could not be amplified. Subsequent purification of this sample using the QIAquick™ column (protocol B) removed all traces of pigmentation and allowed the sample to be amplified (Fig.4.5.) . Similarly, protocols C and D yielded clear DNA solutions that were readily amplified. Based on these observations, it is evident that the QIAquick™ columns are effective in removing pigments that often inhibit PCR (Yang et al., 1997).

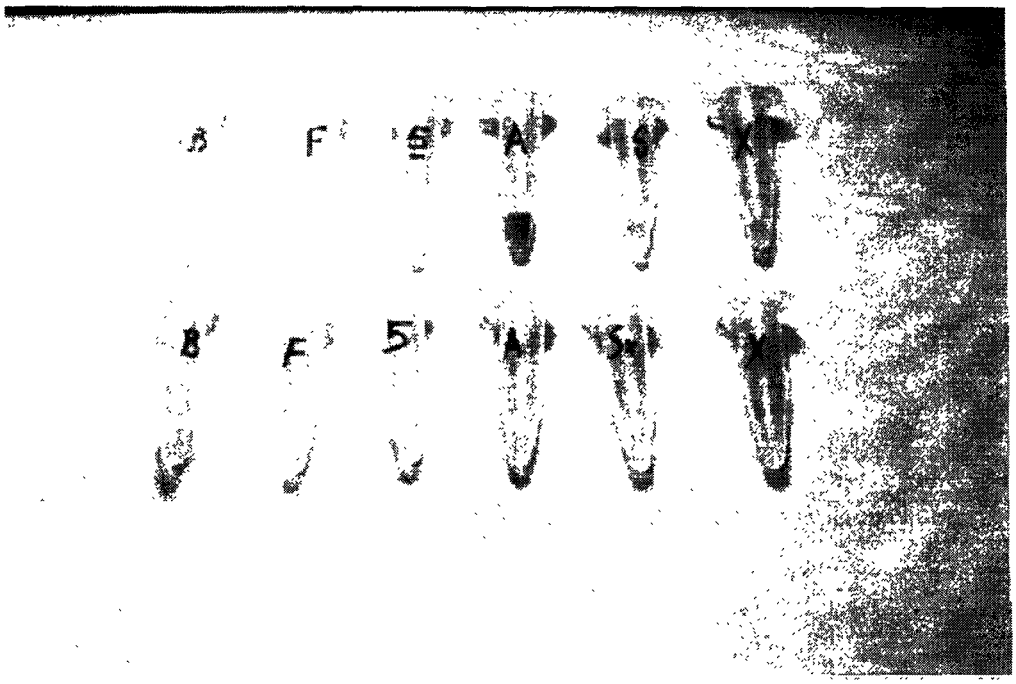


Fig.4.5. Colours of DNA samples extracted by two different protocols. DNA samples in the upper row are brownish (protocol A, without silica spin columns) but in the lower row are colourless (protocol D, with silica spin columns).

Protocol D offers several advantages over other methods. In a single step, the DNA is concentrated and separated from non-DNA substances that could inhibit PCR. A potential limitation of protocol D is that the capacity of the QIAquick™ column is only 750 μL (125 μL proteinase K digest plus 625 μL PB buffer). If the starting volume of the proteinase K digest is greater than 125 μL, multiple loadings of the QIAquick™ column are required. In this researcher's experience, as many as four or five loadings are practical. For larger volumes, it is recommended that the volume be decreased first using Centricon™ concentration (protocol C).

# 4.2. Advantages of the New Extraction Method

Protocols for aDNA extraction should optimize the recovery of DNA and minimize the impact of PCR inhibitors. The number of steps in the procedure should be minimized to lessen the possibility for contamination. Ideally, commercially available reagent kits should be employed to ensure consistency and reduce the technical requirements of the facility. The new method developed in this study has met these requirements. It purifies and concentrates aDNA in a single step with the minimum risk of contamination with modern DNA (Yang et al., submitted).

## 4.2.1. Reduced Number of Steps

The new method has greatly reduced human involvement by using commercial silica-spin columns. Obviously, human involvement cannot be totally avoided in the

procedures of aDNA extraction. But as a general rule, the involvement should be minimized to prevent contamination with modern DNA (Hermann and Hummel, 1994; Woodward et al., 1994; Richards et al., 1996).

Multiple phenol-chloroform extractions, which are necessary in many other aDNA extraction protocols (Hagelberg and Clegg, 1991), are removed from this new method. If two commercial kits, Centricon™ and Qiagen columns are used, the whole extraction procedure can be carried out with just 5 or 6 "openings and closings" of the tubes. The removal of phenol-chloroform extractions eliminates the need for a vented fume hood and makes it possible to carry out DNA extractions in conventional museums, anthropology and archaeology laboratories.

The preparation of silica particles, which involves a high risk for contamination, is no longer required in this new protocol. Silica particles are already embedded into the membranes of silica-spin columns by the manufacturers.

## 4.2.2. Removal of PCR Inhibitors

The new method developed in this study has taken a new approach to dealing with PCR inhibitors. Rather than attempting to remove or neutralize the inhibitors from aDNA samples, this method removes the DNA from the solution that contains PCR inhibitors (Yang et al., 1997). This approach is based on the principle that under high salt conditions, silica particles have a high binding capacity for DNA (Höss and Pääbo, 1993). This method employs commercially available QIAquick™ spin columns which contain a

silica-based membrane. Based on the comparison test between this new method and the phenol-chloroform extraction, passage of aDNA through the silica-based spin column effectively removes all visual traces of pigmentation from the samples (Fig. 4.5.). The silica-based spin columns appear to exclude the substances from ancient remains that give rise to the brownish pigmentation and PCR inhibition.

Other attempts have been made to remove the effects of PCR inhibitors on aDNA amplifications. The simplest approach is to dilute aDNA samples and thereby dilute the inhibitors (Hummel and Herrmann, 1994; Cooper, 1994). For aDNA samples containing limited amounts of templates, this approach is often not feasible. It has been pointed out that sufficient amounts of aDNA must be maintained for authentic aDNA sequences (Handt et al., 1996). As such, alternative strategies have been developed to remove or neutralize PCR inhibitors from aDNA samples (Yang et al., 1997). PCR inhibitors can be absorbed or neutralized by supplementing the PCR with BSA (Cooper, 1997) or compounds such as BLOTTO (DeBoer et al., 1995; Makuch et al., 1996). BSA is used as a regular PCR reagent in aDNA amplifications to prevent potential PCR inhibition. Other approaches are to remove the inhibitors by treating aDNA samples with Chelex (Sensabaugh, 1994), or thiopropyl Sepharose 6B beads (Williamson et al., 1996) or to precipitate aDNA using isopropanol rather than ethanol (Hänni et al., 1995). These methods may be only effective in removing or inactivating certain kinds of the inhibitors depending on specific interactions between added reagents such as BSA and inhibitory substances. As a result, the inhibition can be effectively eliminated from some ancient remains but cannot be removed from others.

## 4.2.3. Efficient Retrieval of Amplifiable aDNA

The new method has shown that an effective recovery of amplifiable DNA is possible from many archaeological bone samples. Almost all SCR specimens used in this study have yielded sufficient amounts of aDNA for PCR amplifications of human mtDNA and highly repetitive sequences. In the comparison test, only two of five samples were amplified with phenol-chloroform extractions whereas five of five samples were well amplified with this new method.

Improvement of PCR amplifications may be also due to the removal of PCR inhibitors from aDNA samples, but it is difficult to distinguish the effects of increased amounts of aDNA templates from the results of the removal of PCR inhibitors. The quantitation of absolute amounts of aDNA is very difficult since aDNA may be always mixed with PCR inhibitors. Chemical quantitation methods are generally not sensitive or specific enough to detect minute amounts of DNA from ancient remains (Handt et al., 1996). Therefore, amounts of amplifiable aDNA are measured as an indicator of the efficiency of aDNA retrieval (Handt et al., 1994).

The amounts of aDNA can be easily doubled or tripled by this new method. In this study, 1.5 mL of bone lysis solution was used for aDNA extraction, yielding sufficient amounts of aDNA for some PCR amplifications. However, up to 5-6 mL of bone lysis solution can be used for retrieving larger amounts of aDNA when a Centricon™ column is used first to concentrate bone solutions.

The silica spin column method is particularly well-suited for aDNA research

because it can be applied directly to proteinase K digests, without the need for phenol-chloroform extractions. Not only does this eliminate the need for a vented fume hood, but it reduces the number of manipulations and the potential for contamination. Moreover, the commercial availability of the QIAquick™ kits makes it possible to carry out DNA extractions in conventional museum, anthropology and archaeology laboratories (Yang et al., submitted).

# CHAPTER 5. RESULTS

## 5.1. PCR Amplification of a Highly Repetitive Human-Specific Sequence for the SCR Skeletons

Fig. 5.1. shows the PCR amplification results of the highly repetitive sequence. SCR 35, 136, 277, 599/1, 1353/2, forensic bone and 100 pg modern DNA (K562) were well amplified. SCR 8 and SCR 599/2 were amplified with very weak bands on the gel. The results indicate that problems will be encountered with amplification of the human β-globin gene for SCR 8 and 599/2.

Controls for blank extraction and PCR setup did not yield detectable PCR products on the gel, indicating no systematic contamination with the SCR specimens during DNA extraction and PCR setup. Therefore, the results of the SCR specimens in Fig.5.1. provide a general indication of aDNA extraction from each of these SCR specimens.

These results are listed in Tab.5.1.

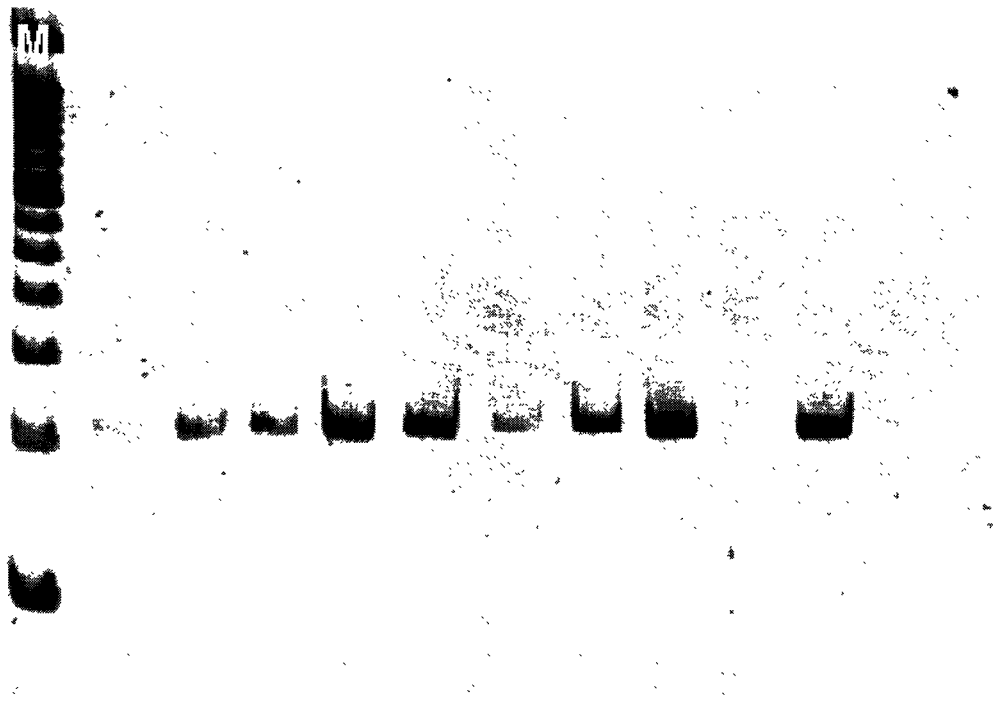



Fig.5.1. Reverse image of ethidium bromide stained gel of PCR products of DNA D17Z1 isolated from the SCR specimens. M: marker (100 bp); 1: SCR 8; 2: SCR 35; 3: SCR 136: 4: SCR 277; 5: SCR 599/1, 6: SCR 599/2; 7: SCR 1353/2; 8: forensic bone; 9: 100 pg modern DNA (K562); 10: 10 pg modern DNA (K562); B: blank extraction (no bone); and N: PCR negative control (no DNA).

Tab.5.1. PCR amplification and mtDNA sequencing of the SCR specimens

| Tests | D17Z1 PCR | mtDNA PCR | β-Globin PCR | mtDNA Sequencing ^ |
|---|---|---|---|---|
| SCR 8 | +/_ | +/_ | # | / |
| SCR 35 | + | + | # | / |
| SCR 136 | + | ++ | ++ | T->C (195) |
| SCR 277 | ++ | ++ | ++ | T->C (152) |
| SCR 599/1 | ++ | ++ | ++ | A->G ( 73) |
| SCR 599/2 | + | + | # | C->T (150), T->C (152) T->C (195) |
| SCR 1353/2 | ++ | ++ | + | A->G (73) and/or A (73) |
| Forensic | ++ | ++ | ++ | / |
| Blank Extraction* | # | # | # | / |
| 100pg K562 | ++ | ++ | # | / |
| 10pg K562 | # | # | # | / |
| Author DY | / | ++ | / | A->G (73), T->C (131) T->C (146), T->C (199) T->C (204) |
| PCR Negative** | # | # | # | / |

# No amplification; +/_ very weak amplification; + strong amplification; ++ very strong amplification; *extraction without bone; **PCR without DNA; / N/A; ^ difference from the reference sequence (Anderson et al., 1981)

# 5.2. DNA Diagnosis of Thalassemia from the SCR Specimens

## 5.2.1. PCR Amplification of the Human β-Globin Genes

DNA from samples SCR 136, 277, 599/1 and 1353/2 were well amplified using the semi-nested PCR amplification, but SCR 8, 35 and 599/2 failed to yield detectable PCR products on the gel. The first-round PCR amplification using primers BG1 and BG2 was used to amplify a 256 bp fragment of the β-globin gene and probably a 254 bp fragment from the δ-globin gene. The second-round PCR amplification used primers BG3 and BG2 to amplify a 196 bp fragment specifically from the β-globin gene.

It was not expected that SCR 35 would amplify well. The D17Z1 and mtDNA amplifications suggested that the amounts of DNA extracted from SCR 35 were less compared with SCR 136, 277 and 599/1 and 1353/2 (Fig.5.1. and 5.7.). This was probably because insufficient amounts of bone powder (0.2 g) were used for DNA extraction (Tab.3.3.). Low amount of bone powder in SCR 599/2 (0.3 g) might also contribute to the failure of its amplification of the β-globin gene.

No systematic contamination was observed with amplification of the β-globin gene. The blank extraction (with all extraction reagents but no bone samples) and the PCR negative control (with all PCR reagents but no DNA samples) did not produce detectable PCR products on the gel. The forensic bone used as a positive control for extraction and amplification produced a strong PCR band on the gel (Fig.5.2.). Therefore, the results were tentatively regarded as the indication of successfully amplification of the β-globin gene from SCR 136, 277, 599/1 and 1353/2.

These results are also listed in Tab.5.1.

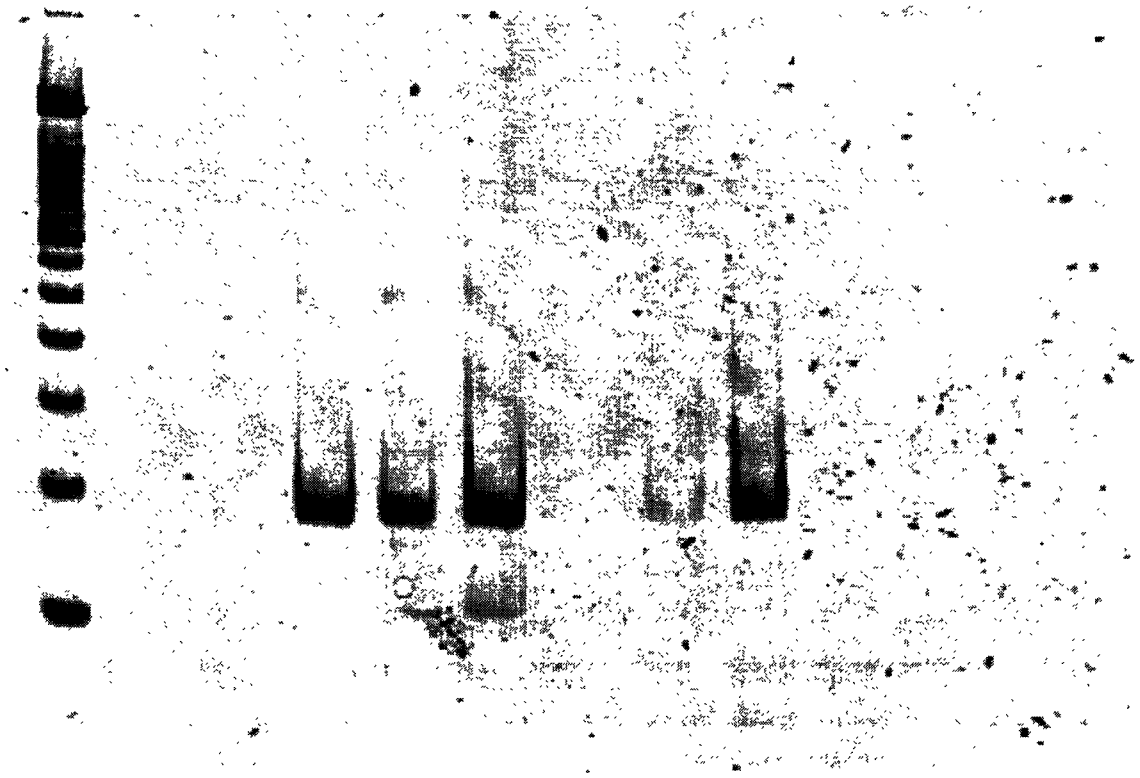


Fig.5.2. Reverse image of ethidium bromide stained gel of the semi-nested PCR products of the human β-globin gene from the SCR specimens. M: marker (100 bp); 1: SCR 8; 2: SCR 35; 3: SCR 136: 4: SCR 277; 5: SCR 599/1, 6: SCR 599/2; 7: SCR 1353/2; 8: forensic bone; 9: 100 pg modern DNA (K562); 10: 10 pg modern DNA (K562); B: blank extraction (no bone); and N: PCR negative control (no DNA).

The successful amplification of the human β-globin genes is largely due to the use of two different Taq polymerases in the semi-nested PCR amplification. Saigon Taq polymerase (Saigon Bio, Toronto), a regular Taq with a moderate specificity and efficiency, was used in the first-round PCR amplification, while AmpliTaq Gold™ (Perkin Elmer), a more efficient Taq polymerase for amplifying DNA, was used in the second-round PCR amplification. In previous PCR amplifications, AmpliTaq Gold™ was used in both the first-round and second-round PCR amplifications. Amplifications from the first-round PCR amplification showed up but also with non-specific bands; the results from the second-round reaction produced even more non-specific bands on the gel (Fig.5.3., Fig.5.4.). This result was not expected because the second-round PCR used BG3 and BG2 which should be more specific to the human β-globin gene. A further analysis found that a high annealing temperature and too many PCR products carried over from the first-round reaction could be responsible for the results. Theoretically, the annealing temperature should be increased to raise the specificity of the second-round reaction. The increase of annealing temperature may increase the specificity of the BG3 but would definitely decrease the efficiency of the second-round PCR amplification since the Tm (melt temperature) for primer BG3 is only 48°C. Fig.5.4. shows the results of the second-round PCR amplification with the annealing temperature at 55°C. The amplification of the specific products (196 bp) was much weaker compared to non-specific products because 55°C was too high for the BG3 to bind its target sequences. However, the decrease of annealing temperature increased the amplification of the specific products but still produced strong non-specific products (data not shown). One

possible explanation for this is that the primers used in the first-round PCR amplification might continue to amplify with fresh Taq polymerase in the second PCR amplification. In PCR amplification, more than sufficient amounts of primers are added into the PCR solution. In this study, primer BG1 and BG2 might be carried over from the first-round PCR solution, which was supposed to be used as a template for the second-round PCR amplification, and continued to amplify. The primers BG1 and BG2 would be able to compete with primers BG3 and BG2 since the annealing temperature suitable to BG3 and BG2 (lower) would be far more suitable to the BG1 and BG2. As a result, non-specific bands on the gel appeared again after the second-round PCR amplification.

One way to fix this problem is to redesign primer BG3 by increasing its length so as to increase the BG3 annealing temperature. BG3 was not originally designed to be used with BG2. Another approach would be to increase the specificity in the first-round PCR amplification and to increase the efficiency in the second-round PCR amplification, or to increase the efficiency in the first-round PCR amplification and to increase the specificity in the second-round PCR amplification. After many attempts were made to modify the times and temperatures of the PCR amplifications, it was found that the use of different Taq polymerases helped in reaching the desired goal.

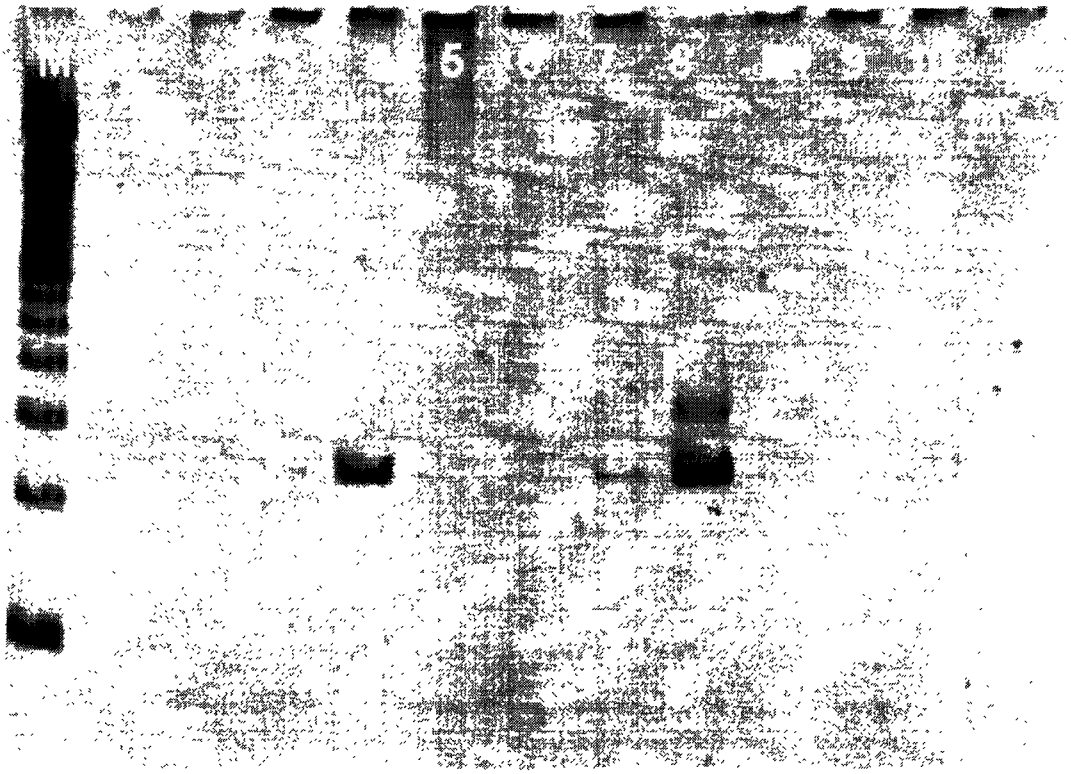



Fig.5.3. Reverse image of ethidium bromide stained gel of the first-round PCR reaction of the human β-globin gene from the SCR specimens using AmpliTaq Gold™. M: marker (100 bp); 1: SCR 8; 2: SCR 35; 3: SCR 136: 4: SCR 277; 5: SCR 599/1, 6: SCR 599/2; 7: SCR 1353/2; 8: forensic bone; 9: 100 pg modern DNA (K562); 10: 10 pg modern DNA (K562); B: blank extraction (no bone); and N: PCR negative control (no DNA).

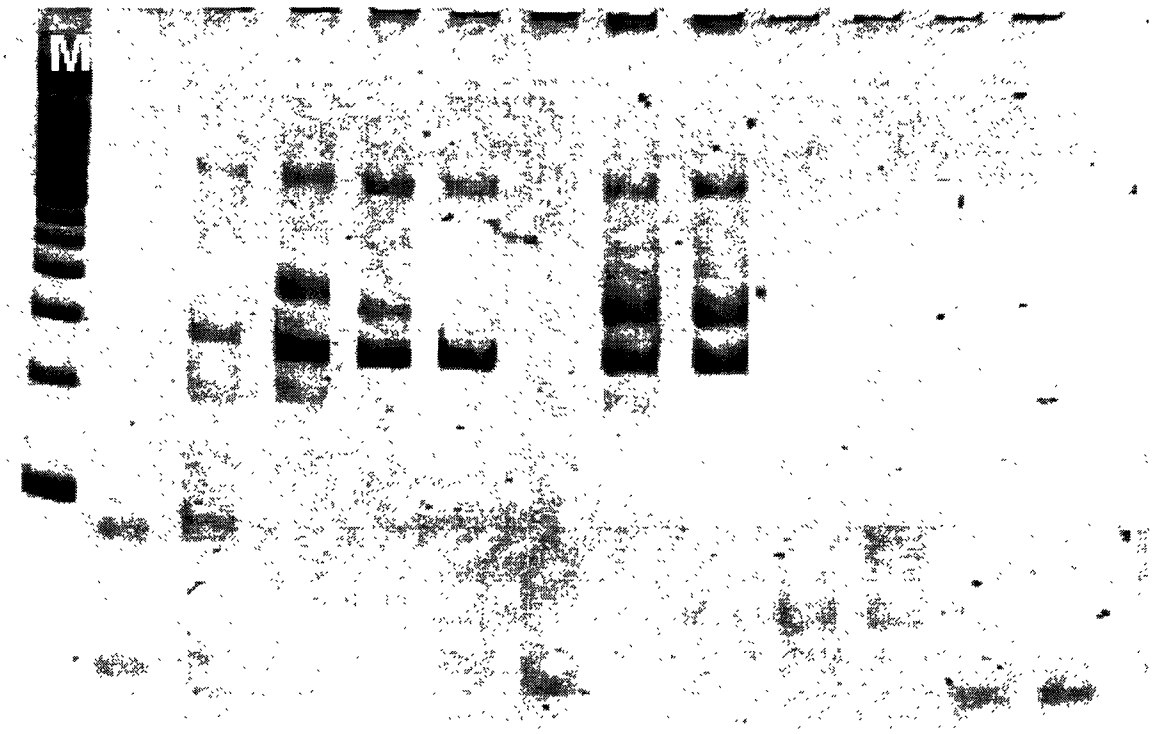



Fig.5.4. Reverse image of ethidium bromide stained gel of the second-round PCR reaction of the human β-globin gene from the SCR specimens using AmpliTaq Gold™ M: marker (100 bp); 1: SCR 8; 2: SCR 35; 3: SCR 136: 4: SCR 277; 5: SCR 599/1, 6: SCR 599/2; 7: SCR 1353/2; 8: forensic bone; 9: 100 pg modern DNA (K562); 10: 10 pg modern DNA (K562); B: blank extraction (no bone); and N: PCR negative control (no DNA).

Saigon Taq polymerase (Saigon Bio, Toronto) was used in the first-round PCR amplification but only very weak amplification bands or no bands could be seen on the gel (Data not shown). Weak bands or no bands were also observed on the gel in the second-round PCR amplification (data not shown). But when AmpliTaq Gold™ was used in the second-round PCR amplification, strong bands with a correct length were shown on the gel (Fig.5.2.). The PCR amplifications with these conditions and these Taq polymerases were repeated three times and the same results were always obtained.

Modifications of the PCR conditions and the concentrations of PCR reagents can affect the outcome of PCR amplifications. When the first PCR amplification was carried out on the SCR specimens, no PCR products could be detected on the gel. In this study, more than 15 semi-nested PCR amplifications with different conditions were run to amplify the human β-globin gene from the SCR specimens. It was interesting to note that SCR 8, SCR 599/2, 10 pg DNA (K562), blank extraction and the PCR negative control never yielded any detectable PCR products on the gels. SCR 136, 277, 599/1 and 1353/2 could produce more or less detectable PCR products on the gels (except the very first two PCR amplifications). On the other hand, 100 pg modern DNA (K562) could produce detectable amounts of PCR products with certain conditions. SCR 35 could be occasionally amplified to produce specific and non-specific bands under different PCR conditions (data not shown), suggesting that only a limited amount of DNA was extracted from the specimen. Based on these observations, it can be assumed that the human nuclear genomic DNA had not been extracted from SCR 8 and 599/2 at the level of more than 100 pg for each PCR amplification. This is consistent with the results from the

highly repetitive human specific sequences (Fig.5.1.).

It is obvious that the optimal PCR amplification for aDNA is more difficult than for modern DNA. The attitude of "try once and give up" is not appropriate for aDNA studies. In this study, optimization of the PCR amplifications for aDNA was finally achieved without sacrificing the specificity of the reaction.

## 5.2.2. Results of the Dot Blot Test

The results from the Dot Blot test showed that mutation $\beta^+$IVS1-110 G->A was not identified from the PCR products of the SCR specimens. The probe for the normal β-globin gene was bound to the PCR products of SCR 277, the forensic bone, and modern DNA (Fig.5.5). However, neither probe was bound to the PCR products from SCR 8, the blank extraction or the δ-globin gene. The failure to detect either the normal or the mutant β-genes for SCR 8 was probably due to the fact that there were not sufficient amounts of PCR products extracted from SCR 8. Fig.5.6. indicates that no detectable PCR products were amplified. The results of the blank extraction indicated that no system contamination occurred to these SCR specimens.

The three controls were successfully detected by this Dot Blot test (Fig.5.5.). The probe for the mutant allele was bound to the $\beta^+$IVS1-110 homozygote and the $\beta^+$IVS1-110 heterozygote (carrier) but not to the normal individual. The other probe was bound to the $\beta^+$IVS1-110 heterozygote and the normal individual. The two probes could be bound to the $\beta^+$IVS1-110 heterozygote. The results of these controls showed that the probes

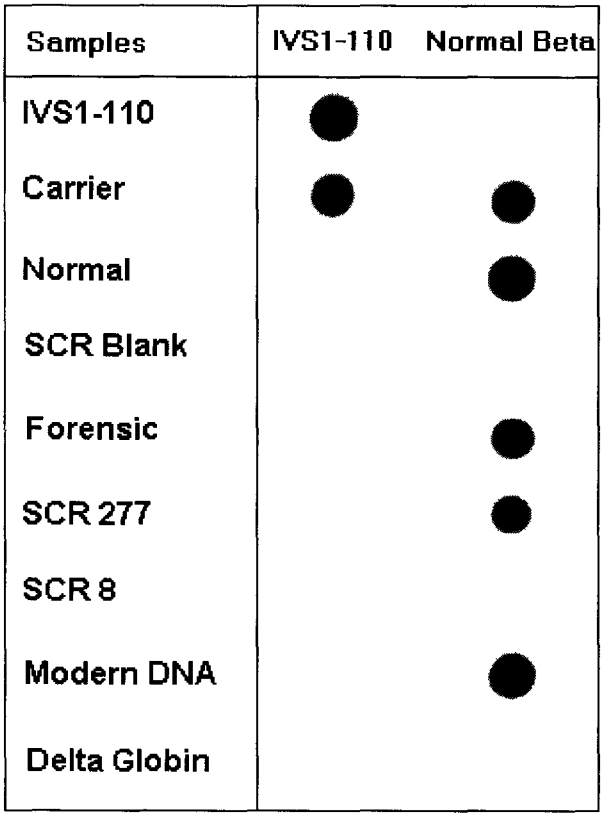



Fig. 5.5. The results of the Dot Bot test for the β-thalassaemia mutation $\beta^{+}$IVS1-110 on the PCR products of the SCR specimens.

were specific to the human β-globin gene.

Both probes failed to bind to the PCR products from human δ-globin gene, demonstrating that the probes of this Dot Blot test were exclusive for the human β-globin genes, not for the human δ-globin genes - the closest gene to the human β-globin genes. Therefore, positive results can be regarded as proof of the presence of the human β-globin genes.

As shown in this test, probes for mutations can definitely be used for detecting thalassemia from PCR products. The probe for $\beta^{+}$IVS1-110 mutants has successfully identified the control samples, $\beta^{+}$IVS1-110 homozygote and the $\beta^{+}$IVS1-110 heterozygote (carrier) in this study. But one disadvantage associated with the Dot Blot test is that each probe can only identify one mutation. In this study, seven β-thalassemia mutations are commonly seen in Italian populations (Fig.3.6). Direct sequencing would be a more efficient way for diagnosing thalassemia mutations for this study. Obviously, the Dot Blot test can be regarded as an alternative way for crosschecking results from direct sequencing.

However, the Dot Blot test has proven that the primers BG1, GB2 and BG3 and the PCR conditions optimized by a combination of different strategies are specific for amplification of the human β-globin genes from ancient remains.

## 5.2.3. Sequences of the Human β-Globin Gene

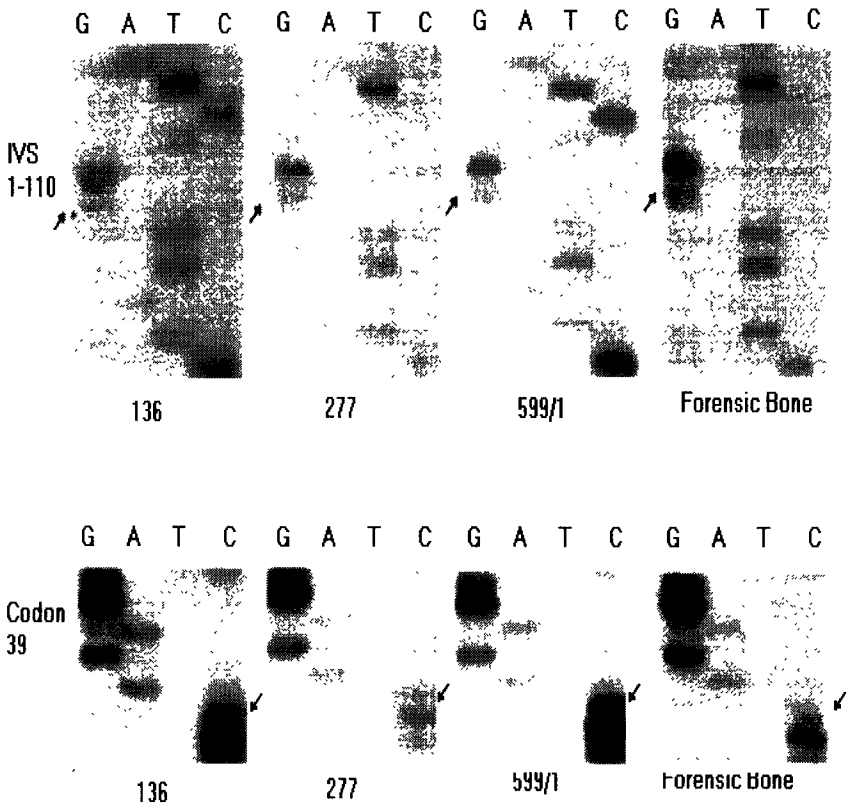



Fig.5.6. The sequencing gel of the human β-globin genes from SCR 136, 277 and 599/1 (SCR 1353/2 not shown). The mutation points of IVS1-110 and codon 39 are normal on all of these samples.

All four samples for sequencing in this test produced quite clear sequencing results (Fig.5.6.). The sequenced fragments cover two of the most common mutations in Italian populations. The sequences from SCR 136, 277, 599/1 and 1353/2 show that they do not have the $\beta^{+}$IVS1-110 or the codon 39 mutation.

These results are also listed in Tab.5.1.

# 5.3. Sequences of Human mtDNA from the SCR Specimens

The results of the PCR amplification of human mtDNA showed that except for SCR8, all samples from Isola Sacra were well amplified (Fig.5.7.). The previously quantified DNA sample (K562, Perkin Elmer) was well amplified at the level of 100 pg for each PCR reaction but failed at the level of 10 pg for each PCR amplification. The mtDNA result from SCR 8 is consistent with the PCR result for the highly repetitive DNA sequence, indicating that SCR 8 either lacks sufficient mtDNA templates or contains too many PCR inhibitors. Since all other samples were well amplified with mtDNA or alpha satellite DNA or both, failure of PCR amplification in SCR 8 is more likely due to the possibility that DNA templates were badly degraded and no significant amounts of DNA were preserved in this specimen.

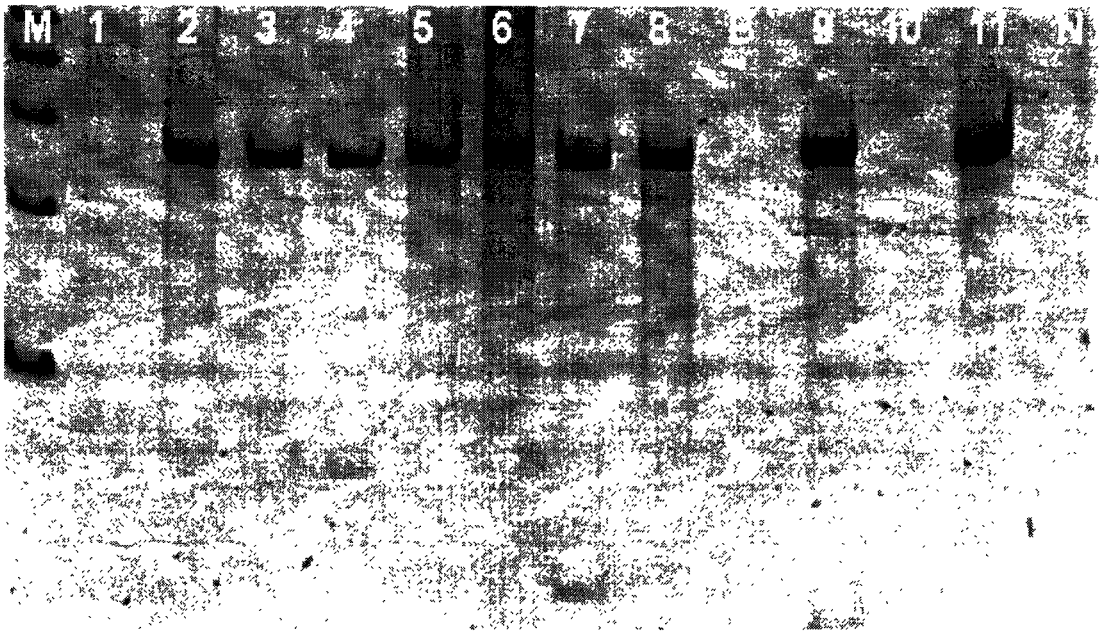



Fig. 5.7. The reverse image of ethidium bromide stained gel of the PCR products of human mtDNA. M: marker (100 bp); 1: SCR 8; 2: SCR 35; 3: SCR 136: 4: SCR 277; 5: SCR 599/1, 6: SCR 599/2; 7: SCR 1353/2; 8: forensic bone; 9: 100 pg modern DNA (K562); 10: 10 pg modern DNA (K562); 11: researcher DNA sample (DY); B: blank extraction (no bone); and N: PCR negative control (no DNA).

Only SCR 136, 277, 599/1 and 1353/2 were selected for direct sequencing of human mitochondrial DNA because they were the only samples from which a fragment of the human β-globin gene was sequenced. A DNA sample from the author was also included to monitor contamination. The sequences of all five samples were obtained and compared with the reference sequence from Anderson et al. (1981) (Fig.5.9.). In an approximate 200 bp readable sequence, six different nucleotides were observed in SCR 136 from the reference sequence, one in SCR 277, one in SCR 599/1, one in SCR 1353/2 and five in DY (author, Chinese). But it was observed that there are two nucleotides present in SCR 136 where only one is supposed to appear. These results are listed in Tab.5.1.

Theoretically, heteroplasmy is possible, but it is unlikely that a 200 bp sequence contains six heteroplasmic positions. Contamination seems to be a more reasonable cause of the observation. A comparison with the DY sequence supported the interpretation that the observed sequence dimorphism in SCR 136 was due to contamination with the DY sequence (Fig.5.8.A.; Fig.5.9.). Compared to the reference sequence, wherever a different nucleotide occurs in the DY sequence, the same nucleotide is also present in SCR 136 at the same position. But the reverse is not necessarily true. At position 195, both T and C were observed in the SCR 136 sequence but the DY sequence has only T. Obviously, the C at position 195 is not from the DY sequence. If it is assumed that SCR 136 was mixed with just one contaminant sequence from DY, then 195 C must be from SCR 136 itself instead of someone else. The removal of DY's sequence from the SCR 136 sequence results in a corrected sequence, c136. This new sequence only has one nucleotide

difference from the reference sequence at position 195. It is also possible that SCR 136 was contaminated by more than one person, but this second person must share an identical sequence with c136. This probability cannot be considered high. The 195 C is not common in European-based populations (17.6 %) or in Asian populations (4.0%) (Stoneking et al., 1991).

SCR 277 only has one nucleotide that is different from the reference sequence at position 152 (T to C). This change occurs more frequently in European-based populations than in Asian populations (Stoneking et al., 1991). The frequency is 15.5% in European-based populations, almost double the frequency of 8.1% in Asian populations (Stoneking et al., 1991). Among all five individuals, only 152 C occurs in SCR 277. This can be regarded as evidence that SCR 277 was not contaminated by other samples or by the author (DY).

SCR 599/1 is another sample with one nucleotide difference from the reference sequence at position 73 (A to G). This change is commonly seen in European-based populations (59.9%) although it is more frequent in Asian populations (97.3%) (Stoneking et al., 1991). It is unlikely that the G at position 73 was from the author (DY) because if the contamination takes place, the six changes in DY's mtDNA difference should be observed in the SCR 599/1 sequence.

SCR 1353/2 was problematic due to the presence of two nucleotides at position 73 (G and A) but it was difficult to figure out the reason. Both contamination and heteroplasmy are possible. With contamination, the presence of G and A at position 73 could occur only if SCR 1353/2 had the 73 G originally but was contaminated with an

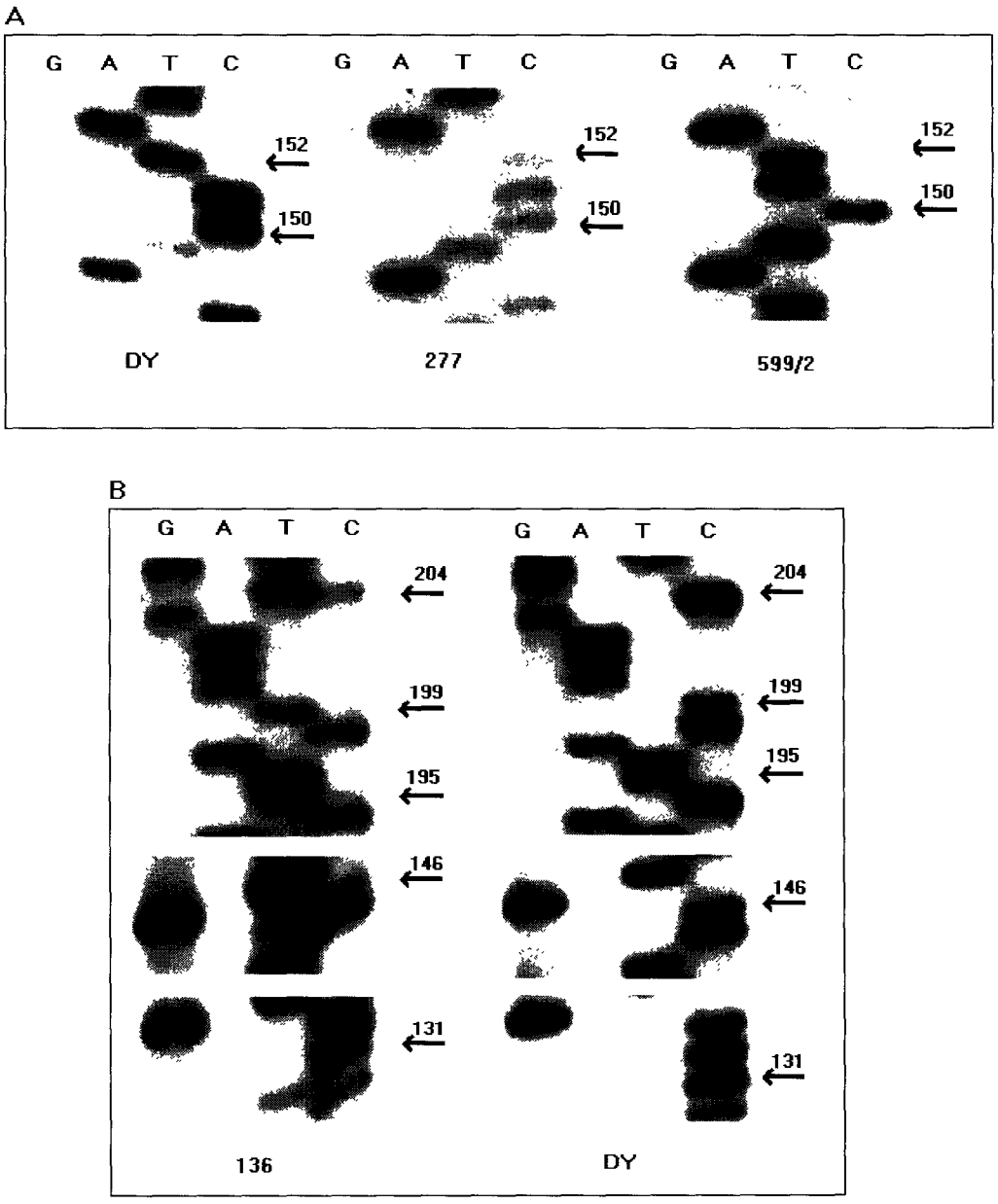
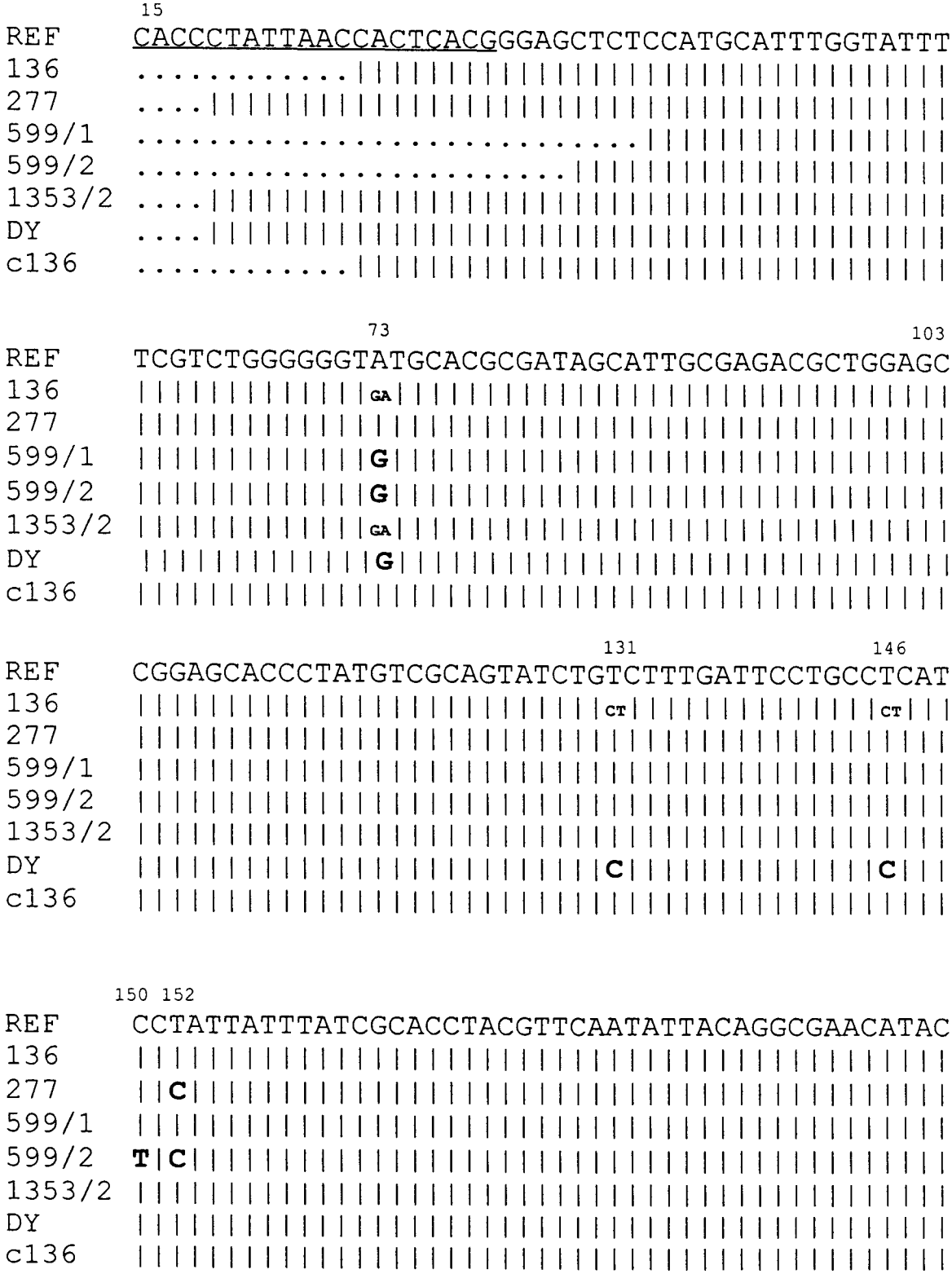



Fig.5.8. The sequencing gels of human mtDNA from SCR 136, 277, 599/2 and DY. A: different patterns of human mtDNA sequence at position 147-154 from DY, SCR 277 and SCR 599/2; B: SCR 136 contamination with mtDNA from DY (researcher).


15
REF CACCCTATTAACCACTCACGGGAGCTCTCCATGCATTTGGTATTT
136
277
599/1
599/2
1353/2
DY
c136
73
103
REF TCGTCTGGGGGGTATGCACGCGATAGCATTGCGAGACGCTGGAGC
136 GA
277
599/1 G
599/2 G
1353/2 GA
DY G
c136
131
146
REF CGGAGCACCCTATGTCGCAGTATCTGTCTTTGATTCCTGCCTCAT
136 CT CT
277
599/1
599/2
1353/2
DY C C
c136
150 152
REF CCTATTATTTATCGCACCTACGTTCAATATTACAGGCGAACATAC
136
277 C
599/1
599/2 T C
1353/2
DY
c136

```
          195  199     204
REF       TTACTAAAGTGTGTTAATTAATTAATGCTTGTAGGACATAATAAT
136       CT|||CT||||CT|||||||||||||||||||||.............
277       |||||||||||||||||||||||||||||||||..............
599/1     |||||||||||||||||||||||||||||||||..............
599/2     C|||||||||||||||||||||||||||||||||.............
1353/2    |||||||||||||||||||||||||||||||||..............
DY        ||||C||||C|||||||||||||||||||||||||||||||||||||
c136      C|||||||||||||||||||||||||||||||||.............
```

```
                                                    275
REF       AACAATTGAATGTCTGCACAGCCACTTTCCACACA
136       ...................................
277       ...................................
599/1     ...................................
599/2     ...................................
1353/2    ...................................
DY        ...................................
c136      ...................................
```

Fig. 5.9. The sequences of the D-loop fragment of human mitochondrial DNA from the Isola Sacra specimens. The " | " represents the same nucleotide as the reference sequence (Anderson et al, 1981). The "." indicates that this nucleotide cannot be read on sequencing results. The DY represents the researcher (D. Yang). The c136 is the corrected sequence for specimen 136. The underlined nucleotides in the reference sequence are the primers for the PCR amplification, whereas the bold nucleotides are the primers for sequencing. The single bold letters show nucleotide substitution at these specific positions. The two smaller bold letters mean that two nucleotides have been observed at this single position. The whole sequence is numbered from 15 to 275 according to the reference sequence (Anderson et al, 1981).

individual who had the 73 A, or originally if SCR 1353/2 carried the 73 A but was contaminated with the 73 G. Heteroplasmy is a possibility since it has been observed in some cases (Gill et al., 1994).

The DY (author, Chinese) sequence was expected to have more nucleotide differences from the reference sequence which was obtained from a European (Anderson et al., 1981). The differences had been observed at five positions between the DY sequence and the reference sequence, whereas only one nucleotide difference was found with all SCR specimens. These Italian SCR specimens were expected to have fewer differences from the reference sequence.

BLAST homology searches were carried out through the Internet to compare the sequences of the SCR specimens and the researcher to the published sequences in the DNA databases such as Genbank (National Institute of Health, 1997). For each individual sequence, the search listed 100 most homologous DNA sequences from the DNA databases. The results revealed that the sequence from DY was clearly closer to those from Asian populations, whereas the sequences of the SCR specimens were closer to those from European populations. A detailed phylogenetic analysis will be conducted in future for further confirmation.

The DY sequence is a rich source of contamination since it is modern DNA without degradation. Fortunately this contamination can be easily identified because the author and the study specimens have large differences in their mtDNA sequences.

The mtDNA sequences obtained for SCR 136, 277, 599/1 and 1353/2 from the

first sequencing were also observed in the results from the second sequencing of different PCR products. The DY sequence was still seen mixed with the SCR 136 sequence and 73 A/G was observed in the SCR 1353/2 sequence. This indicates that the sequences from SCR 277, 599/1 and probably 1353/2 were not amplified from contaminants or caused by PCR amplification errors. Contaminants were probably introduced into SCR 136 before or during DNA extraction. It is unlikely that the contamination took place during the PCR setup or PCR amplification, otherwise, different mtDNA sequences might have been observed in two separate sequencings using different PCR products.

Four positions in the SCR 599/2 were observed to be different from the reference sequence, A -> G at 73, C -> T at 150, T->C at 152 and T->C at 195 (Fig.5.8; 5.9.). The 150 T is found only in SCR 599/2 (Fig.5.8B). The SCR 599/2 sequence was different at three positions from the SCR 599/1 sequence. The mtDNA discrepancy between SCR 599/1 and 599/2 clearly indicates that the bone samples did not come from the same individual.

# CHAPTER 6. DISCUSSION

## 6.1. Authentication of aDNA from the SCR Specimens

Authentication of aDNA is an extremely important part of aDNA studies due to the possibility of contamination with modern DNA (Richards et al., 1996; Stoneking, 1995; Handt et al., 1996). The human β-globin genes were amplified from the SCR specimens but their sequences must be carefully examined before they can be considered to be authentic. Human mtDNA analysis and quantitation of aDNA templates can be helpful in verifying the outcome of PCR amplifications and DNA sequencings.

Contamination controls should be carried out during all procedures involved with aDNA extractions and analyses. Strict precautions have been suggested to avoid contamination with modern DNA (Handt et al., 1994a; Stoneking et al., 1995; Richards et al., 1996). In this study, these guidelines were followed while handling ancient remains and analyzing aDNA. The bone samples were prepared in a room where no DNA extraction was previously carried out; ancient DNA was extracted in an area where no modern DNA was ever extracted; pre-PCR and post-PCR procedures were performed in two different laboratories; all buffers and PCR reagents were prepared and autoclaved in a laboratory where no human DNA or PCR analysis was ever carried out; different sets of pipettes were dedicated specifically to aDNA extraction and aDNA setup; aerosol-

resistant, filtered pipette tips were used in all pre-PCR procedures; whenever possible, bone samples were decontaminated by removing the outer surface and/or placing them under intensive UV light; blank extractions (no bone samples) and PCR negative setup controls (no DNA) were performed at the same time with aDNA. These measures are very basic requirements for aDNA studies. Obviously, failure to meet these requirements definitely makes contamination more likely. But adherence to these rules can ensure that aDNA may be obtained but does not necessarily guarantee that aDNA must be obtained.

There is a difficulty associated with the authentication of aDNA derived from the aDNA sequence itself. When DNA is extracted from ancient remains, the recovery of a DNA sequence does not prove whether it is from ancient remains or from modern contaminants. Other criteria must be used to help to authenticate aDNA such as DNA fingerprinting identification.

In the following sections, quantitation of aDNA templates and analysis of ancient mtDNA will be discussed to illustrate the monitoring of contamination and the authentication of aDNA sequences.

## 6.1.1. Quantity of aDNA from the SCR Specimens

Quantitation of aDNA is of importance since it helps not only to predict the efficiency of PCR amplifications but also to authenticate aDNA sequences and to estimate the risk of contamination with modern DNA (Handt et al., 1994a; 1996). It is also noted that sufficient amounts of aDNA templates are needed for obtaining

reproducible, authentic aDNA sequences. PCR amplifications from insufficient amounts of aDNA templates are more vulnerable to contamination and more likely to produce artificial aDNA sequences (Handt, 1994a; 1996).

A competitive PCR assay has been developed by other researchers for aDNA quantitation (Handt et al., 1994a; Handt et al., 1996; Krings et al., 1997). The assay uses a synthesized DNA template which is almost identical to the target sequence but has an insertion or deletion of a few bases between the two primer locations. When a series of diluted and quantitated synthesized templates are added with a constant amount of aDNA for PCR amplification, equal amounts of PCR products can be obtained from the target templates and synthesized templates when the two types of templates are present in equal amounts in a single PCR tube. When two bands appear on the gel with an equal intensity, a quantity of correspondent synthesized templates can be read as the amount of aDNA templates. The quantity of aDNA measured by this assay reflects equivalent amounts of aDNA for a specific PCR amplification. Namely, it can determine how many aDNA molecules can be actually amplified with the same set of PCR primers under the same PCR conditions, even with the presence of the same amount of PCR inhibitors. However, this assay is very expensive since templates of hundreds of nucleotides must be synthesized for each set of primers.

In this study, quantified modern DNA was amplified along with aDNA from the SCR specimens to determine the amounts of aDNA that could be obtained from these ancient remains. Fig.5.1. shows the results of a highly repetitive human-specific DNA sequences of D17Z1, indicating that except for SCR 8, more than 10 pg of DNA in each

of the 5 μL DNA samples was extracted from all SCR specimens. The comparatively less sensitive PCR amplification of human mtDNA confirms the above conclusion (Fig.5.7.). PCR amplifications of the human β-globin genes shows that both 100 pg and 10 pg of modern DNA (K562, Perkin Elmer) did not yield any detectable PCR products but SCR 136, 277 and 599/1 produce strong amplification bands while SCR 1353/2 produced a weak band (Fig.5.2.), indicating these four specimens have more than 100 pg DNA for each 5 μL DNA samples. One control sample, SCR 35, did not yield any amplification under this PCR condition. However, under highly efficient PCR conditions, SCR 35 yielded strong PCR bands on the gel but 100 pg of modern DNA still failed to produce any detectable PCR products (Fig.5.3. and Fig.5.4.). It seems that there is more than 10 pg of each PCR amplification of SCR 35. Comparatively, a conservative estimate of aDNA templates for SCR 136, 277, 599/1 and 1353/2 should be more than 200 -300 pg for each PCR amplification.

In fact, the amount of aDNA from the SCR specimens may have been underestimated by the method used in this study. In all of the above PCR amplifications, quantitated modern DNA was not mixed with the aDNA solution, avoiding potential PCR inhibition of the aDNA extract. If the modern DNA had been mixed with aDNA solution, similar to the above competitive assay, it is likely that more than 100 pg of modern DNA would be needed for each PCR amplification to produce detectable PCR amplifications of mtDNA in the same PCR amplification as the SCR specimens.

In addition to the above considerations, the intensity of PCR amplifications from SCR 136, 277, 599/1 and 1353/2 (Fig.5.1.) indicates that these specimens can probably

yield DNA templates for SCR 136, 277, 599/1 at the level of approximately 300 pg for each 5 μL aDNA solution and SCR 1353/2 at the level of more than 200 pg for each 5 μL aDNA solution.

## 6.1.2. Authentication of Human mtDNA from the SCR Specimens

Hypervariability of mtDNA and multiple copies of the mitochondrial genome in each cell make mtDNA ideal for monitoring contamination in aDNA studies (Stoneking, 1995). Unless they are closely maternally related, individuals are more likely to present with a different DNA sequence. Generally, if no contamination takes place, each individual should have a unique mtDNA pattern. However, when sporadic contamination occurs, more than one pattern can be detected in the affected individuals. If buffers or reagents are contaminated with modern DNA (systematic contamination), the same mtDNA pattern can be seen in all or most of the individuals.

A series of experiments were carried out to determine whether the amounts of aDNA templates had any influence on the outcome of mtDNA sequencings (Handt et al., 1996). It has been noted that when PCR amplifications start from less than 40 molecules, several different sequences can be obtained. In contrast, when starting amounts of templates are a few thousand molecules, unambiguous and reproducible results can be achieved (Handt et al., 1996). These results clearly indicate that sufficient amounts of original aDNA are crucial for obtaining authentic aDNA.

The number of mtDNA templates from the SCR specimens can be calculated

roughly based on the results of PCR amplification of highly repetitive sequences of D17Z1 (Fig.5.1) and the human β-globin genes (Fig.5.2.). Almost all SCR specimens have at least 10-50 pg of aDNA templates for each PCR amplification. If 10 pg genomic DNA is equivalent to the DNA from one cell (Sensabaugh, 1994), the amounts of aDNA equivalent to one to five "cells" were used in PCR amplifications from these SCR specimens. Ancient bone DNA is mainly preserved in three types of cells: osteocytes, osteoblasts, and osteoclasts (Hummel and Herrmann, 1994). Although we do not know the exact copy number of mtDNA for each of these cells, it can be assumed that each of them has at least a few hundred copies of mtDNA. It can then be estimated that PCR amplifications for each SCR sample started from at least a couple of thousand mtDNA copies.

Amplifications of mtDNA were not used to calculate the number of aDNA templates since the control sample of modern DNA (K562) is not derived from bone cells. Otherwise, calculation errors will result from inaccurate calibration because different tissues have a substantially different copy number of mtDNA for each cell (Goto et al., 1992).

Artificial mtDNA changes due to PCR errors can then be excluded from the SCR specimens. Ancient DNA is often extensively damaged and may have chemical modifications to the nucleotide bases which can misincorporate wrong complementary bases in PCR amplifications. If PCR amplification starts from insufficient amounts of DNA templates with chemical modification, and misincorporation takes place in the first few PCR cycles, a large proportion of the PCR product with incorrect DNA sequences

would be expected (Handt, 1984a; Handt et al., 1996). However, if more DNA templates are available for PCR amplifications, misincorporation can be averaged out in the final PCR products, presenting no significant amounts of incorrect PCR products (Pääbo, 1989). In this study, PCR artifacts can be ruled out since the DNA sequences obtained from the first sequencing were also found in the second sequencing. PCR amplifications do cause artificial misincorporation in some circumstances, but these PCR errors are usually not reproducible (Krings et al., 1997).

Contamination was then a primary concern for detecting non-authentic aDNA sequences in this study. Since the blank extractions and the PCR negative controls were negative and no identical mtDNA pattern was seen in the mtDNA sequences from all SCR specimens, therefore, systematic contamination can be ruled out in this study. However, sporadic contamination was more troublesome since at least one of four specimens clearly showed this type of contamination (SCR 136). The contamination was easily detected in this study since the contaminant DNA was identified as coming from the researcher (DY). Contamination may also have taken place in another specimen (SCR1353/2) but cannot be confirmed.

For the other specimens, even if there are no signs of systematic or sporadic contamination, the authentication of aDNA must go further to examine all possibilities for causing non-authentic DNA sequences. In this study, a detailed analysis was carried out to estimate the authenticity of aDNA sequences from the SCR specimens.

One theoretical possibility is that no DNA was preserved and retrieved from SCR 136, 277 and 599/1. Instead, the sequences of these specimens were all contaminations

from people who handled these specimens. However, unlike SCR 1353/2, only one pattern was identified with each of these individuals. The only theoretical explanation for this observation would be that, among people who handled all of these specimens, one of them would leave his/her DNA with only one of all of the specimens that he/she handled. But at the same time, one specimen would only acquire DNA from one person although it might be handled by many people. Otherwise, there would be the presence of two nucleotides in the same position, as with SCR 1353/2. Since these specimens had been handled by at least several people due to their morphological importance to physical anthropologists, if contamination takes place, it is more likely that more than one person would leave their DNA with each of these specimens.

Commenting on a study using many skeletal samples from different archaeological sites, Cooper argued that there is a plausible potential that each bone can be uniquely contaminated by different individuals (Beraud-Colomb et al., 1995; Cooper, 1997). However, the possibility that each specimen was uniquely contaminated by different individuals in this study should not be considered high since all SCR specimens are from the same archaeological site and were handled by the same people.

Another scenario of contamination is that aDNA was retrieved from these specimens but the contaminants had identical mtDNA sequences to these specimens. However, statistically, the probability of this scenario is extremely unlikely. In European-based populations, the probability of two persons having an identical nucleotide G at position 73 (SCR 599/1) is 35.88% (59.9% x 59.9%) (Stoneking et al., 1991). The probability of an identical C at position 152 (SCR 277) is 2.40% (15.5% x

15.5%) (Stoneking et al., 1991). The probability of an identical C at position 195 (SCR 136) is 3.10% (17.6% x 17.6%) (Stoneking et al., 1991). Based on these numbers, the probability of this type of contamination of all three specimens would be 0.027% (35.88% x 2.40% x 3.10%). According to Stoneking et al (1991), there are at least 30 mutation hot spots in this 261 bp sequence. Although there might be many links among these mutations, the probability that three contaminating individuals would have identical 200 bp sequences at the same time would be very low when more mutation positions are taken into account.

The results also suggest that contamination took place with SCR 1353/2 and the UV light could not sufficiently decontaminate the specimen. SCR 136 was arbitrarily contaminated by the researcher since no other samples have been found with contamination from the researcher. The other SCR specimens might have been contaminated by other people but the polishing process sufficiently removed DNA contaminants from these specimens during the bone preparation in the laboratory.

Therefore, a more likely explanation would be that the mtDNA sequences for SCR 136, 277 and 599/1 are authentic. However, more evidence is required for a thoroughly convincing authentication. One type of evidence is whether all sequences from the SCR specimens can be repeated. In this study, the mtDNA sequences of these specimens were confirmed by several PCR amplifications and two DNA sequencings. However, this seems insufficient for ancient DNA authentication unless the whole experiment can be completely repeated from a new bone preparation to the stage of DNA sequencing in an independent laboratory (Handt, 1994a; Cooper, 1997). One concern is

that DNA can deeply infiltrate ancient bone because of handling, washing or preservative treatments (Cooper, 1997). However, this can only be detected by extracting DNA from different bone samples. Due to extensive porosity of these young child skeletons used in this study, the extensive handling and treatments could not be performed on them. However, a new bone extraction is being planned for future work.

There are two possible explanations for the sequence of SCR 1353/2: contamination and heteroplasmy. Recent studies show that heteroplasmy of human mtDNA is more common than previously thought (Howell et al., 1996; Parsons et al., 1997). A more accurate mutation rate is estimated based on familial analyses which generate a mutation rate roughly twenty-fold higher than estimates derived from phylogenetic analyses (Parsons et al., 1997). However, the author considers contamination to be more probable for SCR 1353/2. When SCR 1353/2 was prepared for DNA extraction, its bone surface could not be removed because of extensive porosity. Only UV decontamination was applied to this specimen. It is unlikely that specimens that have had bone surfaces removed coincidently produce a single mtDNA type whereas specimens that have not had bone surfaces removed happen to have mixed mtDNA types. If the two nucleotides persist at the same position in the DNA from the new extraction, then this is more likely to be due to heteroplasmy.

In summary, the mtDNA sequences of SCR 277, SCR 599/1 appear to be from ancient remains, as does that of SCR 136 after the DY sequence is removed. It is more likely that dual bases at one single position in SCR 1353/2 are due to contamination rather then heteroplasmy.

## 6.1.3. Authentication of the Human β-Globin Genes from the SCR Specimens

The human β-globin genes at mutation points IVS1-110 and codon 39 are normal from SCR 136, 277, 599/1 and 1353/2. Normal sequences of these specimens make it impossible to discriminate individuals by the β-globin sequences themselves. Since almost all possible contaminant sources (researchers) have normal β-globin genes, the possibility of contamination with modern DNA should be carefully examined.

The mtDNA analysis reveals sporadic contamination (SCR 136) but also indicates that authentic DNA was probably obtained in other specimens (SCR 277 and 599/1). The results can also be used to monitor possible contamination of the human β-globin genes from the SCR specimens. If a contamination occurs and yields a non-authentic DNA sequence for the human β-globin gene, contaminant mtDNA sequences can also be detectable since much more copies of contaminant mtDNA are also available for PCR amplifications. It has also been noted that modern, intact contaminant DNA will be preferentially amplified over ancient, damaged DNA (Pääbo, 1989). This makes the mtDNA analysis a very sensitive approach for monitoring contamination not just for mtDNA itself but also other nuclear DNA sequences.

The quantity of aDNA templates for the human β-globin genes seems sufficient for obtaining authentic DNA sequences from ancient SCR specimens. When mtDNA analysis indicates there is no contamination with ancient mtDNA, the human β-globin gene sequences from the SCR specimens cannot be automatically granted authenticity. Quantitation of aDNA templates must be carried out to estimate the efficiency of the

amplifications and the risk of the contamination (Handt et al., 1996). The previous estimates of aDNA templates are 200-300 pg (equivalent to 20-30 cells) for SCR 136, 277, 599/1 and 1353/2. The 40-60 molecules of the human β-globin genes would be expected for the five μL DNA samples. If the conclusion that more than 40 molecules should be used for authentic mtDNA sequencing is applicable to nuclear DNA or the human β-globin genes (Handt et al., 1996), aDNA amounts from the SCR specimens seem sufficient for yielding authentic aDNA β-globin sequences.

The evidence so far indicates that the human β-globin genes have been extracted from SCR 277, 599/1 and probably from SCR 1353/2. But the reproducibility of these results need to be examined to ensure that they are indeed from the original ancient remains.

# 6.2. Thalassemia

## 6.2.1. Diagnosis of Thalassemia

### 6.2.1.1. Strategy for Diagnosing Ancient Thalassemia

#### 6.2.1.1.1. The Akhziv Skeleton

To date, there has been one attempt to diagnose thalassemia mutations from ancient human remains using aDNA analysis (Filon et al., 1995). Filon and associates identified a β-thalassemia mutation in the DNA of an eight year old child skeleton from an Israeli archaeological site, Akhziv, dated to the Ottoman period, sometime between the 16th and 19th centuries (Filon et al., 1995). The skull was observed to have specific

bone lesions including porotic hyperostosis and honeycombed compartments of subperiosteal bone. Sequencing and the Southern analysis of PCR products both showed that the β-thalassemia of this skeleton was caused by a homozygous frameshift mutation in codon 8 due to a TT deletion.

Among the three skull fragments studied in this case only one produced good PCR amplifications. This may indicate that different parts of the skull in question had different preservation states (Filon et al., 1995), but it may also mean that the amounts of amplifiable DNA templates extracted from the bones were not sufficient for efficient PCR amplification. Unfortunately, quantitation of aDNA templates was not carried out in this analysis (Filon et al., 1995). As discussed earlier, quantitation of aDNA should be carried out in aDNA studies to estimate the authenticity of aDNA sequences and the risk of contamination with modern DNA (Handt et al., 1996). If amounts of the aDNA templates are insufficient, then it is possible that PCR amplifications may have preferentially amplified one specific allele (mutant codon 8) over another (normal codon 8), resulting in homozygous sequences.

The issue of contamination needs to be further addressed in the Akhziv case Filon and associates did not mention if codon 8 was chosen as their first target or if it was successfully identified after many different mutations were attempted. Contamination should be suspected if the first target mutation such as codon 8 is identified as positive in a hematology laboratory. Theoretically, the possibility of identifying the codon 8 is not very high since it is a rare mutation.

Contamination was not carefully monitored during the aDNA analysis of the

Akhziv child since only blank extractions were performed (Filon et al., 1995). Like systematic contamination, sporadic contamination can also take place frequently at any step of aDNA analysis. For example, in our study, SCR 136 was found to have been contaminated from one researcher while other samples remained uncontaminated. The presence of a thalassemia mutation itself can be regarded as a good monitor for contamination since researchers are generally not homozygous or even heterozygous for thalassemia. If the work was performed in a haematology laboratory where thalassemia mutations were analysed frequently, DNA fingerprinting systems such as mtDNA analysis should have been used to monitor the contamination routes. For example, if the unique mtDNA patterns of laboratory workers are found in aDNA amplifications, thalassemia mutations previously analysed in the laboratory might also find their way into aDNA amplifications.

#### 6.2.1.1.2. The Isola Sacra Skeletons

The study presented here has been carefully designed to overcome some of the above problems. Several SCR specimens were chosen based on many specific thalassemia-like bone lesions including postcranial bone changes. The postcranial bone lesions were considered to be more thalassemia-specific than the skull lesions (Tayles, 1996). The Akhziv skeleton seems to be the only one that was identified as a possible thalassemic from the archaeological site.

The study of the SCR specimens used an optimal strategy for aDNA analysis. A more efficient DNA extraction method was used to remove PCR inhibition; a mtDNA

fingerprinting system was set up to monitor contamination; quantitation of aDNA templates was performed; pre-PCR work was carried out in a laboratory where no human DNA analysis was ever performed. It is worth mentioning that the failures of PCR amplifications in the two other skull fragments from the Akhziv skeleton might be due to the presence of PCR inhibitors since only successful amplifications were achieved through diluted aDNA samples (Filon et al., 1995).

The study of the SCR specimens is significant in terms of evolution of thalassemia since the Isola Sacra site dates to 1,900 years ago and is an undisturbed cemetery population with about 2,000 excavated skeletons (Sperduti et al., 1996). Theoretically, a plan for population screening can be prepared for determining the frequencies of thalassemia DNA mutations in ancient skeletal populations. Different frequencies of thalassemia mutations can be expected from the SCR population. The Akhziv child is one sporadic case of possible thalassemia from a site 100 to 400 years old from disturbed graves.

Therefore, a good strategy for successful diagnosis of thalassemia from ancient skeletons using the DNA analysis should include the following considerations. Skeletons must come from a geographic area where malaria was prevalent; individuals should be of an appropriate young age; skeletons should present bone lesions which are similar to those caused by thalassemia; bone preparation and DNA extraction should be carried out in a laboratory where no work on human globin genes has previously been performed; aDNA should be efficiently extracted with PCR inhibitors removed; quantitation of aDNA should be performed to estimate the efficiency of PCR amplifications and the risk

of contamination; mtDNA analysis should be used to monitor contamination from modern DNA; and finally more than one independent extraction from more than one bone sample should be used to examine the reproducibility of the results.

### 6.2.1.2. No Thalassemia mutation from the SCR Specimens?

The mutations $\beta^{+}$IVS1-110 and $\beta^{o}$ codon 39 have not been identified from SCR 136, 277, 599/1 and 1353/2 which are identified as thalassemic individuals based on specific bone lesions. However, it is too early to conclude that these specimens do not have a thalassemia mutation.

First of all, only the two most common β-globin mutations in contemporary Italian populations have been searched in these samples. There are at least five other common mutations which could be investigated (Fig.3.9.).

Secondly, failures to detect β-globin mutations may also be due to false negative results caused by contamination with modern β-globin genes. Based on morphological diagnoses of these SCR specimens, SCR 136 is more likely thalassemic than the other samples. But the result of mitochondrial sequence of SCR 136 indicates that this sample was contaminated. Therefore, it is more likely that the sequence of the β-globin gene of SCR 136 may come from contaminants rather than SCR 136. For those specimens with a single mtDNA pattern, contamination is not automatically excluded. Further, reproducibility experiments could be conducted.

Finally, there is also the possibility that none of these samples were thalassemic. Although bone changes are strongly indicative of thalassemia, alternatives are always

possible.

The next step in this work is to concentrate on re-extraction of aDNA from SCR 136 and SCR 1353/2 skeletal samples to remove contaminants. When all SCR samples are free of contamination, which can be judged from the results of mtDNA fingerprinting, pursuit of a diagnosis of thalassemia will target more β-thalassemia mutations which are common in Italian populations.

However, larger questions have to be considered. Information on the timing and emergence of detectable bone lesions in the first year of growth is necessary for interpreting and developing a differential diagnosis of observed bone changes on infant skeletal remains. Theoretically, bone changes associated with thalassemia can develop only after birth when the synthesis of fetal globins stops and the site of hemoglobin synthesis switches to the bone marrow (Russell, 1986). Although clinical symptoms of thalassemia can be observed as early as a few months after birth (Weatherall and Clegg, 1981), there are few data about the time and severity of development of morphological bone changes in infants.

High infant mortality, especially in the 6-12 month age range, is associated with many other causes, including the weanling diarrhea syndrome (Katzenberg et al., 1996). Can the weanling diarrhea syndrome cause bone changes that are similar to those of thalassemia? At present, no data are available to address this critical issue. The ability to exclude non-genetic diseases based on morphological observations, however, would increase the chance of DNA diagnosis of thalassemia and other genetic anemias. Clearly, this is an important area of future research.

## 6.2.2. Significance of Amplification of the Ancient Human β-Globin Genes

Compared with human mitochondrial DNA, it is more difficult to amplify nuclear DNA due to the low number of copies (Krings et al., 1997, Lindhal, 1997). In this study, the human β-globin genes appear to have been extracted and sequenced from 1,900 year old bones. A polymorphic fragment of the human β-globin gene has also reportedly been amplified from archaeological human skeletons up to 12,000 years old (Beraud-Colomb et al., 1995) although this work has been criticized for not being able to further exclude the possibility of contamination with modern DNA (Cooper, 1997; Handt et al., 1996).

If aDNA analysis can be further optimized and applied to larger skeletal populations, the significance of the amplification of the human β-globin genes also lies in the potential that in the future, it can be used to test the protection-against-malaria hypothesis of thalassemia and the defence-against-pathogen load hypothesis of porotic hyperostosis. A number of skeletal samples have been studied from different prehistoric and historic periods in many places in the malaria belt (Stuart-Macadam, 1992). The emergence of agriculture in human history is less then ten thousand years old (Cohen, 1989), the optimal time period for aDNA extraction and amplification based on our knowledge of DNA preservation in ancient remains (Herrmann and Hummel, 1994). The natural selection hypothesis suggests that for thalassemia, alleles have gradually accumulated over time in the presence of malaria. If porotic hyperostosis is common in one particular skeletal population from the malaria belt, and/or archaeological evidence

reveals the practice of agriculture, and if malaria can be regarded as an important component of pathogen loads therefore, the population should be under selective pressure from malaria. Population screening could be performed to see how many skeletal individuals were carrying thalassemia alleles. An increase in the frequency of thalassemic alleles in specific areas over time is expected.

# CHAPTER 7. DNA DIAGNOSIS - A NEW APPROACH TO PALEOPATHOLOGY

As demonstrated in this study, the DNA diagnosis technique can be applied to detection of diseases from ancient remains. The significance of aDNA diagnoses lies in the potential of this approach to produce highly accurate diagnoses of ancient diseases and detect many more diseases that could not be diagnosed by morphological methods. However, few attempts have been made to comprehensively assess this potential. While examining the availability of necessary techniques and knowledge for DNA diagnosis of ancient diseases at present and in the future, in this chapter, the author will propose some strategies and methodology for this new approach to paleopathology. DNA diagnosis of ancient diseases is particularly useful in the identification of infectious diseases and genetic disorders from ancient remains.

## 7.1. Genetic Diseases

More than 5,000 human genetic disorders have been clinically characterized (Lee, 1993). More than 3,000 of them are caused by a defect in a single gene, such as thalassemia, Huntington's disease, cystic fibrosis, and Duchenne muscular dystrophy. The numbers are expected to grow rapidly as more genes responsible for genetic disorders are

identified (Savill, 1997). In addition, many diseases that were previously thought to be non-genetic disorders have been found to be closely associated with genetic factors (Friedmann, 1990). The diseases of this category include heart disease, stroke, diabetes, and several kinds of cancer. Compared with genetic disorders caused by a single gene defect, understanding of the mechanism of polygenic disorders is limited. Pathogenesis of polygenic disorders is complicated by more genes, as well as the environmental factors involved (Weatherall, 1996). Obviously, much research and effort is required to uncover the etiology of these common but complicated human disorders.

Molecular biology technology has created an efficient and effective way to detect many diseases at the molecular level (Collins, 1997). The study of molecular pathogenesis has enabled us to detect these genetic disorders with high accuracy. In fact, the PCR technique has become a primary tool in DNA diagnosis of diseases in today's clinical practices (Lovell and Grody, 1996). But, the PCR technique has also played an extremely important role in the study of aDNA.

Shorter degraded DNA sequences and low amounts of aDNA templates require the modification of clinical diagnostic protocols for aDNA. Currently, many problems and difficulties are associated with transferring clinical molecular diagnostics to aDNA. For example, new primers must be redesigned to amplify shorter DNA sequences of interest from aDNA. The nested PCR amplification, in most cases, must be carried out to increase PCR products. However, as it has been shown in this study, these problems can be overcome with the advent of new biotechnology and accumulated understanding of mechanisms of aDNA preservation and degradation.

DNA diagnosis is generally more suitable to genetic diseases caused by single gene defects, particularly point mutations. However, it is expected that this application can be extended to other genetic diseases after their etiology is defined at the molecular level. For example, chromosome changes can cause genetic diseases. If these chromosome changes could be detected by PCR amplifications, then identifying the diseases caused by chromosome changes from ancient skeletons would be possible.

Besides the direct detection of mutant sequences themselves, the markers of polymorphism can also be used to identify genetic disorders (Weatherall, 1996). Polymorphisms have been found using many different systems, such as restriction fragment length polymorphism (RFLP), variable number of tandem repeats (VNTR) and short tandem repeats (STR) (Stoneking, 1997). These polymorphism are closely linked with some genetic diseases (Li et al., 1996; Mizuki et al., 1997). For example, a triplet repeat polymorphism in the transmembrane region of a gene has been found with a strong association of six GCT repetitions with Behçet disease (Mizuki et al., 1997). The CAG repeat number with specific DNA sequences of Huntington disease are strongly associated with variation in the age of onset of the disorder (Rubinsztein et al., 1997). STRs seem more suitable for DNA diagnosis of ancient diseases since the sequences of the STRs are usually within the range of aDNA sequences. For example, the locus VWA31/A has been amplified from early medieval skeletons in Germany (Zierdt et al., 1996). Thus, STR analysis from aDNA samples could provide preliminary indication for certain genetic disorders.

mtDNA is another source for the diagnosis of ancient diseases since it is easily

preserved and retrieved from ancient remains. The high variability of mtDNA has been extensively used in the study of human evolution and variability (Cann et al., 1987; Comas et al., 1996). However, mitochondria are also responsible for encoding 13 proteins involved in respiratory chains required for oxidative phosphorylation (Anderson et al., 1981). It has recently been found that mutations or defects in mtDNA can also cause genetic diseases (Weatherall, 1996). A number of degenerative diseases involving the central nervous system, heart, muscle, endocrine system, kidney and liver have been associated with systemic mtDNA mutations, either base substitutions or insertion/deletions (Holt et al., 1988; Goto et al., 1990; Goto et al., 1992; Ballinger, 1994). Leber's Hereditary Optic Neuropathy (LHON) was the first genetic disease identified as associated with a mitochondrial mutation (Lee, 1993). LHON, caused by a single point mutation, involves mid-life acute or subacute central vision loss resulting in blindness. Eighteen mutations in mtDNA electron transport genes have been associated with this phenotype, four of which are generally found to play a significant role in the etiology of LHON (Wallace et al., 1988; Shoffner et al., 1995). Mounting evidence suggests that defects in energy metabolism contribute to the pathogenesis of Alzheimer disease. A new study indicates that Alzheimer disease results from specific mutations in mitochondrial genes with maternal inheritance patterns (Davis, 1997).

To date, there have not been any attempts to diagnose ancient mitochondrial diseases, but the potential is great since mtDNA is well preserved in ancient remains. The low frequency of mtDNA diseases as well as the small number of diseases associated with mitochondrial DNA may limit this application. But this situation may change as

more and more diseases are found to be associated with mitochondrial DNA mutations (Shoffner et al., 1995).

Identification of genetic diseases from ancient remains has puzzled paleopathologists for a long time. No distinctive diagnostic bone lesions can be linked to individual genetic diseases (Roberts and Manchester, 1995). In some circumstance, preliminary diagnosis of one or other individual genetic diseases can be obtained from morphological observations; however, quite often, this diagnosis cannot be confirmed in skeletal samples by pedigree analysis which is necessary to verify the diagnosis of genetic diseases. It is evident that kinship cannot be determined solely based on morphological comparisons of skeletons because many environmental factors such as nutrition and disease could produce morphological similarities. Sometimes, archaeological evidence does indicate close relationships from burial practices at archaeological sites. Nevertheless, this relationship may not necessarily mean biological kinship between individual skeletons. This problem may be easily overcome by aDNA analysis - parentage tests using the DNA fingerprinting technique (Alford et al., 1994; Hammond et al., 1994; Olaisen et al., 1997). The maternal inheritance pattern of mtDNA has been used to identify mother-child or sibling relationships among the related skeletal individuals (Giles et al., 1980; Gill et al., 1993; Handt et al., 1994b; Hänni et al., 1995a). The STRs of nuclear DNA, which are recombined between paternal and maternal lines, have been employed in aDNA analysis to determine biological relationships between individuals (Kurosaki, 1993; Epplen, 1994; Zierdt et al; 1996). HLA-DQA1 has been successfully used on DNA from ancient Egyptian bones and teeth (Ludes, et al., 1994; Woodward et

al.,1994). HLA DQA1 is a powerful identification tool which has been widely used in forensic cases (Hochmeister et al., 1995; Martinelli et al., 1996). Sex determination is another dimension which can be added to reveal biological kinships among individuals (Gaensslen et al., 1992; Sullivan, 1993; Lassen et al., 1997). The discrimination power of sex determination of skeletons is not high since there are only two categories: male and female. But one advantage, which is absent in other DNA fingerprinting systems, is that the result of sex typing can be checked by sexual dimorphism of skeletal morphology (Stone et al., 1996). One of the examples of successful DNA determination of kinship is the identification of the remains of the Romanov family (Gill et al., 1994). Based on skeletal observations and other evidence, the five individuals of nine skeletons from a grave in Russia were tentatively assumed to be the last Tsar, Tsarina and three of their five children. DNA Sex determination, STR analysis and mtDNA sequence all confirmed that these five individuals were from the same family and matched the tentative conclusion of identification. In forensic DNA analysis, more research and effort are currently being focussed on developing more sensitive, effective and efficient DNA fingerprinting systems for forensic identification. No doubt, these forensic DNA fingerprinting systems can be applied to reconstruct the kinship of ancient skeletal individuals for DNA diagnosis of ancient genetic diseases.

When the ongoing Human Genome Project completes in 2005, approximately 100,000 human genes of three billion nucleotides will be determined (Schuler et al., 1996; Collins, 1997). Then, we will enter the so-called post genome era. It is expected that more diseases will be found associated with gene mutations and more functions of

individual genes will be defined at a more detailed molecular level. This trend can be illustrated by the human mitochondrial genome sample (Pääbo, 1996). Before the human mitochondrial genome project was completed in 1981 (Anderson et al.), few diseases were found to be associated with mtDNA mutations. Now, a search through the Human Mitochondrial Genome Database at Emory University, Atlanta, Georgia reveals 28 groups of diseases that are caused by or associated with mtDNA mutations (Emory University, 1997). Compared to the human mtDNA genome (approximately 16.6 kb in length), the human nuclear genome contains more genetic information (approximately 3,000,000 kb in length). The pathogenesis of many diseases is expected to be revealed at the molecular level and eventually, effective, efficient and accurate diagnoses of most genetic or genetic-related diseases will be developed in the future (Friedmann, 1990; Collins, 1997; Savill, 1997).

## 7.2. Infectious Diseases

This study does not specifically deal with the diagnosis of ancient infectious diseases, but they should be included to understand the larger scenario of DNA diagnosis of ancient diseases.

The assumptions for DNA (RNA for some viruses) diagnosis of infectious diseases are that pathogens infect and enter a human body, cause illness and attack human physiological systems. The pathogens will remain inside the human body and duplicate themselves unless they are removed by medication or by human immune systems. As

with DNA fingerprinting, pathogen-specific DNA sequences can be identified. The detection of these DNA sequences can be regarded as indicating the presence of pathogens. The method has been widely used in clinical practice for diagnosing tuberculosis, syphilis, AIDS and a number of other bacterial and viral infectious diseases (Burstain, 1991; Eisenach, 1991; Sritharan and Barker, 1991).

Like DNA diagnosis of genetic diseases, DNA identification of infectious diseases can also overcome some intrinsic problems associated with paleopathological diagnoses and interpretations. The paradox of interpretations of skeletal lesions lies in the fact that two opposite interpretations can be derived from the same set of paleopathological data (Wood et al., 1992). For example, the higher frequency of skeletal lesions in specific populations was once explained as due to high pathogen loads or poor nutrition, suggesting poor health status. However, these lesions can be explained in a more positive way. Bone lesions may no longer be regarded as indications of poor health status or heavy pathogen loads. Instead, they can be considered a sign of stronger immunity or defence system with a generally good health for those skeletal populations (Wood et al., 1992). Populations with weaker immunity and defence systems, and poor health have difficulty withstanding the attacks from bacterial and viral pathogens. Populations with stronger defence systems and good health can probably survive the first attacks and allow pathogens to produce morphological lesions on their bones (Ortner, 1991). This makes more sense than the first interpretation which says that more lesions mean more diseases and poor health status. But, it is not always true that skeletal populations with lesions should be considered to be healthier than populations without lesions. The latter

populations may live without these pathogens or diseases and therefore have no chance to be tested by these specific pathogens if these populations are stronger or weaker. Therefore, a more reasonable statement would be that populations with more skeletal lesions are not necessarily of poorer health than populations without bone changes. But the question of health and diseases of past populations remains unanswered.

Furthermore, the scenario becomes even more complicated when the evolution or changes in pathogens over time are taken into account. There is a theory regarding the evolutionary relationship between hosts and their parasites. Parasites should evolve toward a benign state to coexist with their hosts to maintain their mini microenvironment inside the hosts. Newly mutated pathogens, especially those of zoonotic origins, would cause high mortality and morbidity when they attack populations no matter how healthy these populations might be (Ewald, 1994). Nevertheless, it would also be expected that populations with poorer health might have a lower mortality and morbidity if pathogens had become more benign.

Obviously, these problems cannot be resolved by further skeletal morphological examinations. DNA analysis, however, can be used to overcome some obstacles in detecting diseases. DNA diagnosis looks for pathogens themselves instead of effects that pathogens cause. Although morphological bone changes are indicative of possible presence of specific pathogens, theoretically, DNA diagnosis can be verified independently without further verification from other sources. If pathogens invade a human body, they may kill the body instantly, destroy the body slowly, or may not affect the body at all. The process will be determined by the virulence of the pathogens and the

nature of effectiveness of the body's immune system. The useful information from aDNA research is whether pathogens actually enter the body. If pathogens reach bone tissues and remain within the tissues, pathogens can probably be identified from ancient skeletons. Skeletons with bone lesions may indicate the invasion of pathogens; however, the presence of bone changes does not necessarily imply the presence of pathogens in lesion sites. Therefore, pathogenesis of infectious diseases must indicate that the pathogens will actually invade bone tissue, whether there is a bone lesion or not. This is because it is theoretically possible that bone changes can be the result of secondary effects of pathogen invasions, rather than caused by the pathogens themselves. For specific pathogens, spread routes of bones should be well confirmed before we attempt to detect their DNA or RNA sequences in ancient human skeletal remains. This is the most efficient way of diagnosing diseases from a limited preserved resource - human ancient skeletons.

To date, all DNA diagnoses of ancient diseases have been conducted on skeletal materials showing specific bone lesions (Spigelman and Lemma, 1993; Rafi et al., 1994; Salo et al., 1994; Arriaza et al., 1995). The specific patterns of morphological bone lesions are useful indicators of the presence of specific diseases. But many other sources can also be explored. For example, paleodemographic analysis may reveal a sudden increase of morbidity in certain age groups in skeletal populations. It may be that an epidemiological pattern can be defined and compared to the known patterns of some specific diseases. If this is proven to be practical, many diseases which have not been mentioned in the paleopathological literatures can be identified by DNA diagnosis. DNA diagnosis of ancient diseases therefore need not be confined to specimens with bone

changes. Many diseases such as viral infections can not be morphologically diagnosed.

The pathogen itself is an important aspect of paleopathology that has been ignored for a long time. As mentioned earlier, pathogen virulence may change and this may greatly affect outcomes of population mortality and morbidity (Ewald, 1994). A recent study of ancient RNA reveals that no RNA sequences of 1918-1919 influenza viruses are like the sequences from avian influenza viruses but rather are similar to swine influenza viruses (Taubenberg et al., 1997). This unexpected discovery contradicts the current theory about the outbreak of 1918-1919 influenza pandemic. The current theory proposes that this influenza virus was a new one to which human populations had no immunity because it came from one or more genes from avian influenza viruses via swine influenza viruses (Oxford, 1987). Pigs played a pivotal role in the exchange of genes between human influenza viruses and avian influenza viruses. Pigs could share common influenza viruses with humans and with birds respectively. Agricultural practices in some areas in the world provided the opportunity to bring human, swine and avian viruses together to allow the exchange to take place (Scholtissek, 1992). If this ancient RNA study is confirmed by further studies, the current interpretation regarding the 1918-1919 outbreak of the influenza needs to be rethought. This example illustrates the importance of understanding pathogens in the study of health and diseases of past human populations.

# 7.3. More Advantages of aDNA Diagnosis

High accuracy is expected for DNA diagnosis of ancient diseases because it is directly aimed at the causes of diseases instead of the effects of diseases. The relationship between cause and effect is not simple, especially between pathogens and their morphological effects. However, the task of DNA diagnosis is quite simple - examining the presence or absence of the DNA sequences of interest. Positive results can mean the presence of pathogens, the existence of mutation sequences or false positive results. It is possible that technical improvements will significantly decrease the occurrence of false positives. Negative results would be caused by the absence of pathogens, nonexistence of mutation sequences or by false negative results. False negative results can be confirmed or refuted by a series of tests. As used in this study, mtDNA is amplified first to estimate the state of the DNA preservation. If mtDNA cannot be amplified, it is less likely that nuclear DNA sequences would be amplified and analyzed. One can also employ another approach to quantify the amount of the DNA preserved in ancient remains by using a series of diluted synthesized oligonucleotides (Handt et al., 1994a; Krings et al., 1997). The latter is very sensitive and can quantify aDNA as low as a few molecules (Krings et al., 1997).

Clinical DNA diagnostics can be applied to aDNA as they are to clinical DNA. Paleopathology has not been closely linked with clinical practices because the nature of skeletal tissue prevents application of clinical diagnostics which are often more suitable

for soft tissues. Bone is seldom involved in the diagnostic process of most diseases unless the diseases are bone disorders. Frequently, it is paleopathologists who have to establish the diagnostic criteria specific to bone changes (Stuart-Macadam, 1987a; 1987b; 1989). Usually, the sizes of skeletal populations are small and statistical errors, therefore, are difficult to control. Criteria established from one skeletal population or one small group of "patients" is probably not applicable to all skeletal populations, especially when large temporal and geographic differences are taken into account. Abnormality in one population may constitute the normal form in other populations. Thalassemia can cause facial expansion but similar characteristics can be often found in Asian populations. To a certain degree, DNA diagnosis of ancient diseases can overcome these problems since it follows from DNA diagnostic criteria which have been clinically proven. Molecular diagnostics is a rapidly growing field, with more effective, efficient and sensitive methods becoming available (Lovell and Wayne, 1996). Here, the advantage of DNA diagnosis of ancient diseases is self-evident.

Mummified tissues have been favoured by paleopathologists since they can provide more morphological information (Zimmerman and Kelley, 1982). The paucity of mummies has made it impossible to use mummified tissues as common materials to study ancient diseases. The reality is that skeletons remain the largest portion of ancient human remains. Several studies have shown that DNA seems preserved better in bones and teeth than in mummified soft tissues because bones seem able to provide more protection against damage and produce fewer PCR inhibitors (Lassen et al., 1994; Woodward et al., 1994). Therefore, skeletal samples may be more suitable for DNA diagnosis of ancient

diseases.

DNA diagnosis of ancient diseases is superior to protein diagnosis of ancient diseases. The presence of specific proteins can be attributed to the occurrence of some specific diseases (Cattaneo et al., 1994; Waldron, 1996). This approach has been used extensively in modern clinical practice. For example, infectious diseases can be diagnosed by the presence of specific antibodies and genetic diseases can be detected by the presence of abnormal enzymes. However, this approach will encounter great problems in the diagnosis of ancient diseases. First, most of these non-collagenous proteins have probably lost all of their activity, which is one of most important things in protein diagnosis of diseases. On the other hand, there is no technique so sensitive for protein identification as the PCR technique is for DNA detection.

## 7.4. Summary

The probability, possibility and applicability of DNA diagnosis of ancient diseases have been discussed in this section. Obviously, DNA diagnosis of ancient diseases is still in its early developmental stage. More research and effort are needed to understand many questions such as infection patterns of pathogens in the human body and the molecular pathogenesis of more genetic diseases.

The success of DNA diagnosis of ancient diseases is largely determined by the availability of aDNA from human remains or from pathogens. As demonstrated in this study, with improved DNA extraction methods and better strategies, DNA can be

extracted and analyzed with a higher success rate.

DNA diagnosis of diseases is emerging as a powerful approach to the diagnosis of infectious diseases and genetic disorders from ancient human skeletons as many technical and methodological problems are solved. The preliminary results of this study as well as work done by others in this field have indicated that ancient DNA diagnosis of diseases can be a practical approach for the study of diseases and health of past human populations.

# CHAPTER 8. CONCLUSIONS

Accurate diagnosis of diseases from ancient skeletons cannot be achieved from morphological observations of bone lesions alone. Study of health and disease in past human populations is therefore hindered by inaccurate diagnosis (Wood et al., 1992). An alternative approach, DNA diagnosis of ancient disease, has become possible due to recent progress in aDNA studies and clinical DNA diagnosis of diseases. DNA has been extracted from many human remains from different archaeological periods, and techniques for DNA diagnoses have been developed in clinical practice. DNA diagnosis can provide a quick and accurate identification of infectious and genetic diseases.

In this study, we have developed a new method for extracting DNA from ancient skeletons and obtained human β-globin genes from Roman skeletons uncovered at the Isola Sacra site in Italy. While the search for thalassemia goes on in the aDNA from these skeletons, at present, some preliminary conclusions can be drawn from this study.

1.) DNA diagnosis of diseases from ancient human remains is a feasible approach not only for human infectious diseases but also for human genetic diseases. When DNA is extracted from ancient remains, current clinical DNA diagnostic techniques can be used for aDNA diagnosis.

2.) The aDNA extraction technique using silica-spin columns developed in this study is superior over other aDNA extraction methods. Its strength lies in the fact that it can concentrate and purify aDNA in a single step with a minimal risk of contamination.

The new method produces a higher success rate of amplification than other aDNA extractions, suggesting that failures of aDNA extractions may be due to improper extraction protocols rather than the preservation states of DNA in ancient remains.

Sufficient amounts of amplifiable aDNA are crucial for a successful diagnosis of diseases. Highly efficient aDNA extraction methods are needed to maximize the yield of aDNA extract and minimize the presence of PCR inhibitors and the risk of contamination with modern DNA.

3.) The amounts of aDNA can be roughly quantitated by parallel amplifications of modern quantitated DNA with aDNA samples. Quantitation is a necessary step for diagnosis of diseases since it can help to predict the efficiency of PCR amplifications and to estimate the risk of contamination with modern DNA (Handt et al., 1996).

4.) Successful amplification of ancient human β-globin genes in this study has shown that the optimization of PCR amplification for aDNA requires a great deal of time and effort. The same PCR amplifications can be easily carried out with modern DNA but will encounter problems with damaged aDNA.

5.) Successful amplification of the human β-globin genes from the Roman skeletons of Italy are attributed to the use of efficient DNA extraction methods and the optimization of PCR conditions for ancient β-globin genes.

6.) Precautions for contamination controls should be taken to minimize the risk of contamination; DNA fingerprinting systems such as mtDNA analysis should be used to monitor contamination. Contamination cannot be totally avoided in aDNA amplifications but it can be minimized; more importantly, it can be monitored. Contamination control is

the most important issue in DNA diagnosis of ancient diseases, especially for human genetic diseases.

7.) DNA diagnosis of ancient diseases may bring a new perspective to the study of diseases and health of past human populations. In this study, a careful search for thalassemia mutants has provided an opportunity to study the interaction between human genes and their environments at the molecular level, and to test the protection-against-malaria hypothesis of thalassemia and the defence-against-pathogen load hypothesis of porotic hyperostosis on the scale of time.

8.) Paleopathology as a science which studies diseases in the past has a new approach to identification of diseases from ancient remains. For DNA analysis, skeletons are considered to be more useful than mummified tissues since bones or teeth can provide better protection for aDNA. Additionally, paleopathology can take advantages of the progress in clinical DNA diagnosis. Molecular pathogenesis is being understood for more and more genetic and infectious diseases; efficient techniques of using DNA analysis to diagnose these diseases are being developed.

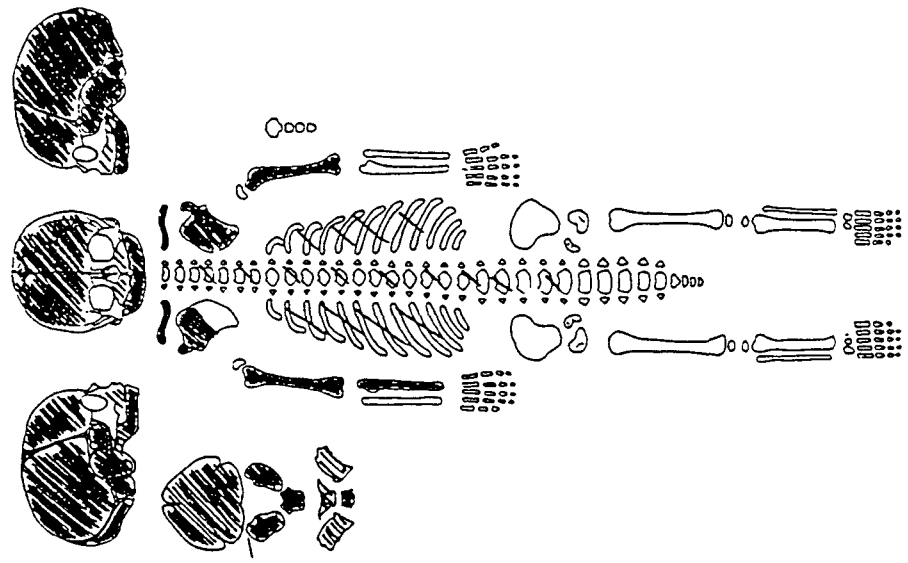
DNA diagnosis of diseases from ancient remains is still at its very early stage, but this study and the results from others in DNA diagnosis of ancient diseases clearly indicate that technical improvements and strategy innovations will help it to become a practical approach to the study of health and diseases of past human populations.

# LIST OF ABBREVIATIONS

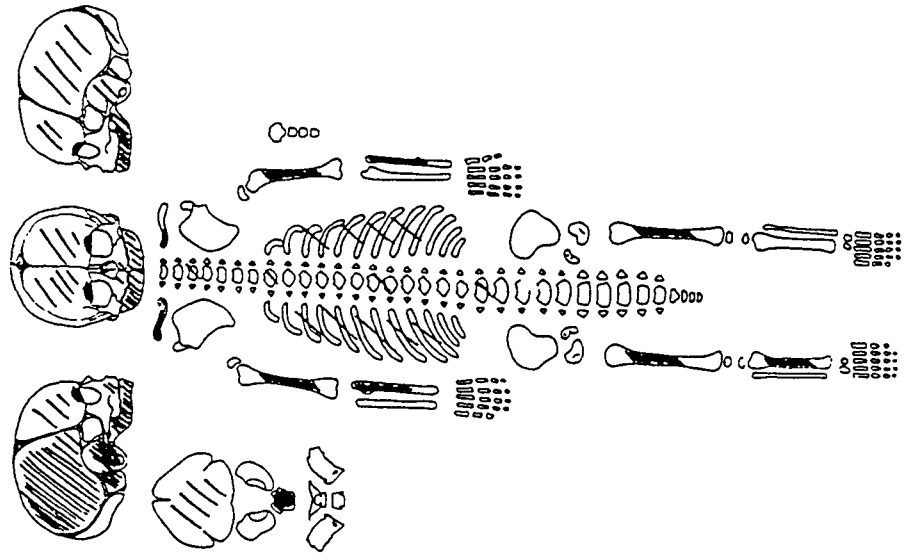
| | |
|---|---|
| aDNA | ancient DNA |
| AIDS | acquired immune deficiency syndrome |
| BLAST | basic local alignment search tool |
| BLOTTO | bovine lacto transfer technique optimizer |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| $dH_2O$ | distilled water |
| ddNTP | 3'-dideoxynucleoside-5'-triphosphates |
| DNA | deoxyribonucleic acid(s) |
| dNTP | deoxynucleoside-5'-triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| FI-dUTP | fluorescein-11-dUTP |
| HLA | human leucocyte antigen |
| kb | kilobase(s) |
| LHON | Leber's Hereditary Optic Neuropathy |
| M | molar |
| mg | milligram |
| mL | millilitre(s) |

| | |
|---|---|
| mM | millimolar |
| mtDNA | mitochondrial DNA |
| PCR | polymerase chain reaction(s) |
| pg | picogram(s) |
| pmole | picomole |
| RFLP | restriction fragment length polymorphism(s) |
| RNA | ribonucleic acid (s) |
| SCR | bone collection from the Isola Sacra site in Italy |
| SSC | sodium chloride citrate |
| STR | short tandem repeat (sequence) |
| TBE | Tris-Boric acid-EDTA buffer |
| TE | Tris-EDTA buffer |
| TEMED | N,N,N',N'-tetra-methylenediamine |
| TdT | terminal deoxynucleotidyl transferase |
| Tm | melting temperature |
| Tris | tris(hydroxymethyl)aminomethane |
| UV | ultraviolet |
| VNTR | variable number of tandem repeats |
| $\mu$Ci | microcurie(s) |
| $\mu$g | microgram(s) |
| $\mu$L | microlitre(s) |

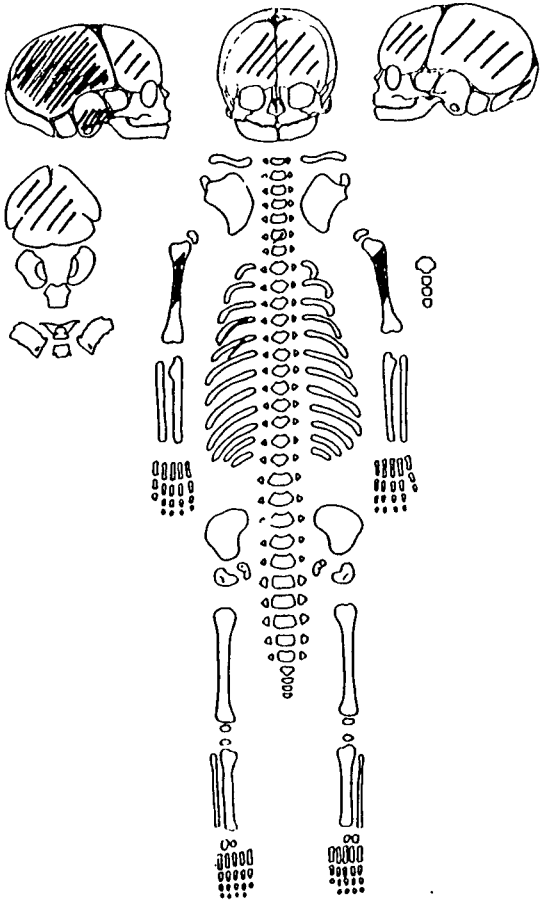
# APPENDIX 1
# Preserved Bones for Each SCR Specimen



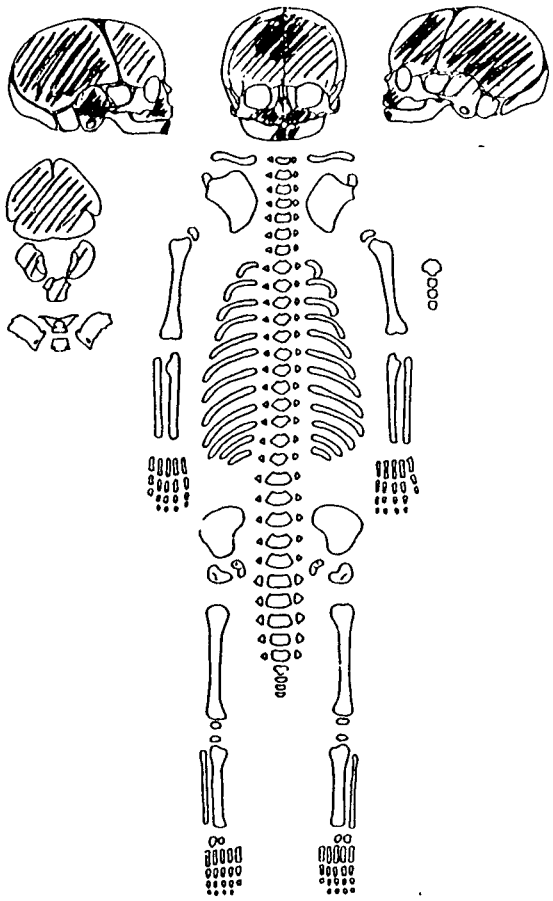
SCR 136 (the shadow areas represent preserved bones)



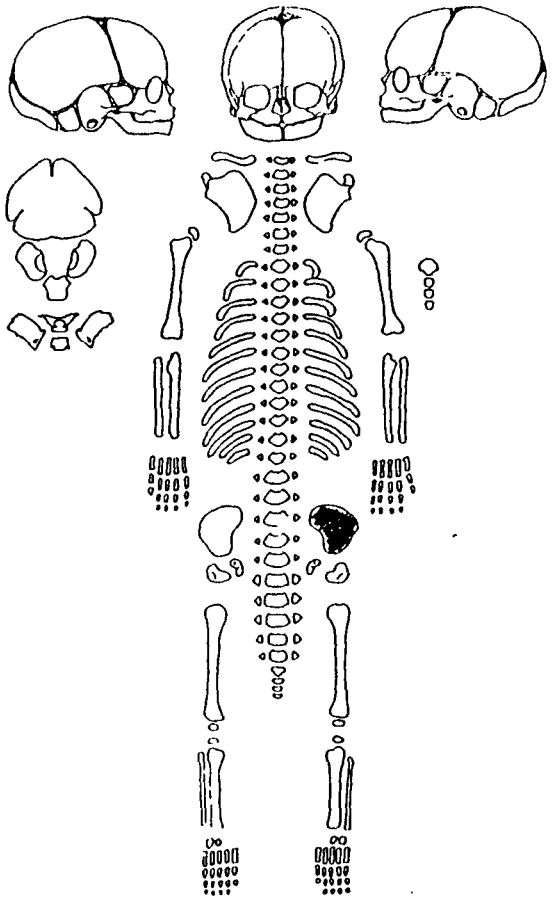
SCR 8 (the shadow areas represent preserved bones)



SCR 277 (the shadow areas represent preserved bones)



SCR 599 (the shadow areas represent preserved bones)



SCR 1353/2 (the shadow areas represent preserved bones)

# REFERENCES


Adams, J. G. 1986. The genetics of human hemoglobin. In: *Hemoglobin Variation in Human Populations* edited by William P. Winter. CRC Press, Inc. Boca Raton, Florida.

Alford, R., H.A. Hammond, I. Coto and C. T. Caskey. 1994. Rapid and efficient resolution of parentage by amplification of short tandem repeats. *American Journal of Human Genetics.* Vol.55:190-195.

Ample, N. M. 1992. Plagues -- what's the past is present: thoughts on the origin and history of new infectious diseases. *Reviews of Infectious Diseases.* Vol.13:658-665.

An, Y., Y. Li, Y. Zhu, X. Shen, Y. Zhang, L. You, X. Liang, X. Li, S. Wu, P. Wu, H. Gu, Z. Zhou and Z. Chen. 1995. Molecular cloning and sequencing the 18S rDNA from specialized dinosaur egg fossil found in Xixia, Henan, China. *Acta Scientiarum Naturalium Universitatis Pekinensis.* Vol. 31:1140-147.

Anderson, S., A.T. Bankier, B.G.Barrell, M.H.L. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D. P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H.Smith, R. Staden, and I.G. Young. 1981. Sequence and organization of the human mitochondrial genome. *Nature.* 290:457-465.

Angel, J. L. 1966. Porotic hyperostosis, anemias, malarias, and marshes in the prehistoric Eastern Mediterranean. *Science.* Vol.153:760-763.

Arriaza, B. T., W. Salo, A. C. Aufderheide and T. A. Holcomb. 1995. Pre-Columbian tuberculosis in Northern Chile: molecular and skeletal evidence. *American Journal of Physical Anthropology.* Vol 98:37-45.

Ascenzi, A., and P. Balistreri. 1977. Porotic Hyperostosis and the Problem of Origin of Thalassemia in Italy. *Journal of Human Evolution.* Vol.6:595-604.

Ascenzi, A., A. Bellelli, M. Brunori, G. Citro, R. Ippoliti, E. Lendaro, and R. Zito 1991 Diagnosis of Thalassemia in Ancient Bones: Problems and Prospects in Pathology. In: *Human Paleopathology: Current Syntheses and Future Options* edited by Donald J. Ortner, and Arthur C. Aufderheide. Smithsonian Institution Press. Washington, D.C..

Ausubel, F.M., R. Brent, R. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, K. Struhl. 1991. *Current Protocols in Molecular Biology.* Greener Publishing Associates and Wiley-Intersciences.

Ballinger, S.W., J. M. Shoffner, S. Gebhart, D.A. Koontz, D. C. Wallace. 1994. Mitochondrial diabetes revisited. *Nature Genetics* Vol.7: 458-459.

Beraud-Colomb, E., R. Roubin, J. Martin, N. Maroc, A. Gardeisen, G. Trabuchet and M. Goossens. 1995. Human β-globin gene polymorphisms characterized in DNA extracted from ancient bones 12,000 years old. *American Journal of Human Genetics.* Vol.57:1267-1274.

Black, Francis L. 1990. Infectious disease and evolution of human populations: the example of South Forest Tribes. In *Disease in Populations in Transition, Anthropological and Epidemiological Perspectives. Bergins and Garvery.* New York.

Boles, T. C., C. C. Snow, E. Stover. 1995. Forensic DNA testing on skeletal remains from mass graves: a pilot project in Guatemala. *Journal of Forensic Sciences.* Vol.40:349-355.

Brandt, A. M. 1991. AIDS and metaphor: toward the social meaning of epidemic disease. In: *Time of Plague, the History and Social Consequence of Lethal Epidemic Disease,* edited by Arien Mack. New York university Press. New York.

Brinkmann, U. K. 1994. Economic development and tropical disease. *Annals of New York Academy of Sciences.* Vol. 740:303-313.

Brothwell, D. and A. T. Sandison (editors). 1967. *Diseases in antiquity, A survey of the Diseases, Injuries and Surgery of Early Populations.* Charles C Thomas. Publisher. Springfield.

Brown, T. A., R. G. Allaby, K .A.Brown, K. O'Donoghue and R. Sallares. 1994. DNA in wheat seeds from European archaeological sites. *Experientia.* Vol.50:571-575.

Brown, T. A. and K. A. Brown. 1992. Ancient DNA and the archaeologist. *Antiquity.* Vol.66:10-23.

Burstain, J. M., E. Grimprel, S. A. Lukehart, M. V. Norgard and J. D. Radolf. 1991. Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. *Journal of Clinical Microbiology.* Vol.20:62-69.

Busvine, J. R. 1980. The evolution and mutual adaptation of insects, microorganism and man. In *Changing Disease Patterns and Human Behaviour* edited by the N. F. Stanley and R. S. Jokse. Academic Press.

Cann, R. L., M. Stoneking, and A. C. Wilson. 1987. Mitochondrial DNA and human evolution. *Nature*. Vol.325:31-36.

Cano, R. J., H. N. Poinar, J. J. Pieniazek, A. Acra and G. O. Poinar, Jr. 1993. Amplification and sequencing of DNA from a 120-135 million year old weevil. *Nature* 363:536-538.

Cao, A., M. Gossens and M. Pirastu. 1989. β-thalassemia mutations in Mediterranean populations. *British Journal of Haematology.* Vol.71:309-312.

Cattaneo, C., K. Gelsthorpe, P. Philips, T. Wadron, J. R. Booth and R. J. Sokol. 1994. Immunological diagnosis of multiple myeloma in medieval bone. *International Journal of Osteoarchaeology.* Vol.4:1-2.

Chen, L. C. 1994. New disease, the human factors. *Annals of New York Academy of Sciences.* Vol. 740:319-324.

Cherfas, J. 1991. Ancient DNAs: still busy after death. *Science*. 253:1354-1356.

Coatney, G. R., W. E. Collins, M. Warren and P. G. Contacos. 1971. *The Primate Malaria.* U.S. Department of Health, Education, and Welfare. Washington D.C..

Cockburn, A and E. Cockburn (editors). 1980. *Mummies, Disease and Ancient Culture.* Cambridge University Press. New York.

Cohen, M. L. 1992. Epidemiology of drug resistance: implication for a post-antimicrobial era. *Science.* Vol.257:1055-1057.

Cohen, M. N. 1989. *Health and the Rise of Civilization.* Yale University Press. New Haven

Collins, F. S. 1997. Sequencing the human genome. *Hospital Practice.* Jan:35-53.

Comas, D., F. Calafell, E. Mateu, A. Pérez-Lezaun, and J. Bertranpetit. 1996. Geographic variation in human mitochondrial DNA control region sequence: the population history of Turkey and its relationship to the European populations. *Molecular Biology and Evolution.* Vol.13:1067-1077.

Cooper, A. 1994. DNA from museum specimens. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Cooper, A. 1997. Reply to Stoneking: ancient DNA - how do you really know when you have it?. *American Journal of Human Genetics.* Vol.60:1002-1003.

Dacie, J. 1988. *The Haemolytic Anaemias Vol.2. The Hereditary Haemolytic Anaemias Part 2.* Third Edition. Churchhill Livingstone. Edinburgh.

Davis, R. E., S. Miller, C. Herrnstadt, S. S. Ghosh, E. Fahy, L. A. Shinobu, D. Galasko, L. J. Thal, M. F. Beal, N. Howell and W. D. Parker Jr. 1997. Mutations in mitochondrial cytochrome c oxidase genes segregate with late-onset Alzheimer disease. *Proceedings of the National Academy of Sciences, USA.* Vol. 94:4526-4531.

DeBoer, S. H., L. J. Ward, X. Li and S. Chittaranjan. 1995. Attenuation of PCR inhibition in the presence of plant compounds by the addition of BLOTTO. *Nucleic Acid Research.* Vol.23:2567-2568.

Deelder, A.M. et al. 1980. Detection of schistosome antigen in mummies. *The Lancet.* Vol. 335:159.

DeSalle, R., J. Gatesy, W. Wheeler, D. Grimaldi. 1992. DNA sequences from a fossil termite in oligo-miocene amber and their phylogenetic implications. *Science.* 257:1936.

Dines, D. M., V.C. Canale and W.D. Arnold. 1976. Fractures in thalassemia. *Journal of Bone and Joint Surgery.* 58A: 662.

Dunn, F. L. 1965. On the Antiquity of Malaria in the Western Hemisphere. *Human Biology.* Vol.37:385-393.

Eisenach, K. D., M. D. Cave, J. H. Bates and J. T. Crawford. 1991. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis. The Journal of Infectious Diseases.* Vol. 161:977-981.

Efstratidis, A., J. et al. 1980. The structure and evolution of the human β-globin gene family. *Cell.* Vol.21:653-668.

Eglinton, G. and G. A. Logen. 1991. Molecular preservation. *Philosophical Transactions of the Royal Society of London (series B).* 333:315-328.

Emory University. 1997. Human Mitochondrial Genome Database. [ *http://www.gen. emory.edu/cgi-bin/mitomap*].

Engleberg, N. Cary. 1994. Molecular methods: applications for clinical infectious diseases. *Annals of Emergence Medicine.* Vol.24:490-502.

Epplen, J. T. 1994. Simple repeat loci as tools for genetic identification. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Ewald, P. W. 1994. *Evolution of Infectious Disease.* Oxford University Press. New York.

Fabrega, H. Jr. 1981. Culture, biology, and the study of disease. In: *Biocultural Aspects of Disease,* edited by Henry R. Rothschild. Academic Press. Toronto.

Faerman, M., D. Filon, G. Kahila, C. L. Greenblatt, P. Smith and A. Oppenhim. 1995. Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles. *Gene.* Vo.167:327-332.

Filon, D., M. Faerman, P. Smith and A. Oppenheim. 1995. Sequence analysis reveals a β-thalassemia mutation in the DNA of skeletal remains from the archaeological site of Akhziv, Israel. *Nature Genetics.* Vol.9:365-368.

Flint, J. R., M. Harding, A. J. Boyce and J.B. Clegg. 1993. The population genetics of the haemoglobinpathies. *Balliere's Clinical Haematology.* Vol.6:215-261.

Fornacia, G. and A. Marchetti. 1986. Intact smallpox virus particles in an Italian mummy of sixteenth century. *The Lancet,* September:625.

Friedmann, T., 1990. Opinion: the human genome project-some implications of extensive "reverse genetic" medicine. *American Journal of Human Genetics.* Vol.46:407-414.

Gaensslen, R. E., K. M. Berka, D. A. Grosso, G. Ruano, E. M. Pagliaro, D. Messina and H. C. Lee. 1992. A polymerase chain reaction (PCR) method for sex and species determination with novel control for deoxyribonucleic acid (DNA) template length. *Journal of Forensic Sciences.* Vol.37:6-20.

Garnsey, P. 1988. *Famine and food-Supply in the Graeco-Roman World: Responses to Risk and Crisis.* Cambridge University Press. Cambridge.

Garrett, L. 1994. Human movements and behavioural factors in the emergence of diseases. *Annals of New York Academy of Sciences.* Vol. 740:312-318.

Gibbons, A. 1994. Possible dino DNA find is greeted with skepticism. *Science.* Vol. 266:1159.

Giles, R.E., H. Blanc, H. M. Cann, D. C. Wallace. 1980. Maternal inheritance of human

mitochondrial DNA. *Proceedings of the National Academy of Sciences, USA.* Vol. 77: 6715-6719.

Gill, P., P. L. Iranov, C. Kimpton, R. Piercy, N. Benson, G. Tully, I. Evett, E. Hagelberg and K. Sullivan. 1994. Identification of the remains of the Romanov family by DNA analysis. *Nature Genetics.* Vol.6:130-135.

Ginther, C. , L. Issel-Tarver and M. King. 1992. Identifying individuals by sequencing mitochondrial DNA from teeth. *Nature Genetics.* 2:135-138.

Golenberg, E. M. 1991. Amplification and analysis of Miocene plant fossil DNA. *Philosophical Transactions of the Royal Society of London (series B).* Vol.333: 419-427.

Golenburg, E. M. 1994. DNA from plant compression fossils. In *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Golenberg, E. M., D. E. Giannasi, M. T. Clegg, C. J. Smiley, M. Durbin, D. Henderson and G. Zurawski. 1990. Chloroplast DNA sequence from ancient a Miocene Magolia species. *Nature.* Vol.344:656-658.

Goloubinoff, P., S. Pääbo, and A. C. Wilson. Evolution of maize inferred from sequence diversity of an *Adh2* gene segment from archaeological specimens. *Proceedings of the National Academy of Sciences, USA.* Vol.90:1997-2001.

Goodman, A. H., D.L. Martin, G. J. Armelagos and G. Clark. 1984. Health changes at Dickson Mounds, Illinois (A.D.950-1300). In: *Paleopathology at the Origins of Agriculture*. edited by M.N.Cohen and G.J.Armelagos. Academic Press. Orlando.

Gotherstrom, A., K. Liden, T. Ahlstrom, M. Kallersjo and T. A. Brown. 1997. Osteology, DNA and sex identification: morphological and molecular sex identifications of five neolithic individuals from Ajvide, Gotland. *International Journal of Osteoarchaeology.* Vol. 7: 71-81.

Goto, Y., S. Horai, T. Matsuoka, Y. Koga, K. Nihei, M. Kobayashi and I. Nonaka. 1992. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. *Neurology.* Vol.42: 545-550.

Goto, Y., I. Nonaka, S. Horai. 1990. A mutation in the tRNA Leu (UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. *Nature.* Vol.348: 651-653.

Hagelberg, E, L. 1993. Ancient DNA studies. *Evolutionary Anthropology.* Vol.2:199-207.

Hagelberg, E, L. S. Bell, T. Allen, A. Boyde, S. J. Jones and J. B. Clegg. 1991. Analysis of Ancient DNA: Techniques and Applications. *Philosophical Transactions of the Royal Society of London (series B).* 333:399-407.

Hagelberg, E. and J. B. Clegg. 1991. Isolation and characterization of DNA from archaeological bone. *Proceedings of the Royal Society of London (series B).* Vol. 244:45-52.

Hagelberg, E. and J. B. Clegg. 1993. Genetic polymorphisms in prehistoric pacific islanders determined by analysis of ancient bone DNA. *Proceedings of the Royal Society of London (series B).* Vol.252:163-170.

Hagelberg, E., I. C. Gray, A. C. Jeffreys. 1991. Identification of the skeletal remains of a murder victim by DNA analysis. *Nature.* Vol.352: 427-429.

Hagelberg, E., S. Quevedo, D. Turbon and J. B. Clegg. 1994. DNA from anceint Easter Islanders. *Nature.* Vol 369:25-26.

Hagelberg, E., B. Sykes and R. Hedges. 1989. Ancient bone DNA amplified. *Nature.* Vol.342:485.

Haldane, J.B.S. 1949. Disease and evolution. Supplement to *La Ricerca Scientifica* Vol.19:68-76.

Handt, O., M. Höss, M. Krings and S. Pääbo. 1994a. Ancient DNA: methodological challenges. *Experientia.* Vol.50:524-529.

Handt, O., M. Krings, R. H. Ward and S. Pääbo. 1996. The retrieval of ancient DNA sequences. *American Journal of Human Genetics.* Vol.59:368-376.

Handt, O., M. Richards, M.Trommsdorff, C. Kilger, J. Simanainen, O. Georgiev, K. Bauer, A. Stone, R. Hedges, W. Schaffner, G. Utermann, B. Sykes, S. Pääbo. 1994b. Molecular genetic analysis of the Tyrolean Ice Man. *Science.* Vol. 264:1175-1178.

Hänni, C., A. Begue, V. Laudet and D. Stéhelin. 1995a. Molecular typing of Neolithic human bones. *Journal of Archaeological Sciences.* Vol.22: 649-658.

Hänni, C., T. Brousseau, V. Laudet and D. Stehelin. 1995b. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic Acids Research.*

Vol.23:881-882.

Hammond, H. A., L. Jin, Y. Zhong, C. T. Caskey and R. Chakraborty. 1994. Evaluation of 13 short tandem repeat loci for use in personal identification application. *American Journal of Human Genetics.* Vol.55:175-189.

Hauswirth, W. W., C. D. Dickel and D. A. Lawlor. 1994. DNA analysis of the Windover population. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Hedges, S. B. et al., 1995. Detecting Dinosaur DNA. *Science*. Vol.268:1191-1194.

Herrmann, B. and S. Hummel. 1994. *Ancient DNA.* Springer-Verlag, New York.

Hershkovitz, I., B. Ring, M. Speirs, E. Galili, M. Kislev, G. Edelson, and A. Hershkovitz. 1991. Possible Congenital Hemolytic Anemia in Prehistoric Coastal Inhabitants of Israel. *American Journal of Physical Anthropology.* Vol. 85:7-13.

Higuchi, R., B. Bowman, M. Freiberger, O.A. Ryder and A. C. Wilson, 1984. DNA sequences from the Quagga, an extinct member of the horse family. *Nature,* Vol.312: 282-284.

Hochmeister, M. N., B. Budowle, U. V. Borer, O. Rudin, M. Bohnert and R. Birnhofer. 1995. Confirmation of the identity of human skeletal remains using multiplex PCR amplification and typing kits. *Journal of Forensic Sciences.* Vol.40:701-705.

Holland, M. M. 1993. Automated DNA sequence analysis: sequencing mitochondrial DNA. *The Fourth International Symposium on Human Identification, Promega Meeting.*

Holt, I.J., A. E. Harding, J. A. Morgan-Hughes. 1988. Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature.* Vol. 331: 717-719.

Horai, S., K. Hayasaka, K. Murayama, N. Wate, H. Koike and N. Nakai. 1989. DNA amplification from ancient human skeletal remains and their sequence analysis. *Proceeding of the Japanese Academy.* Vol.65:229-233.

Höss, M. and S. Pääbo. 1993. DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic Acids Res.* Vol.21:3913-3914.

Howell, N., I. Kubacka and D. A. Mackey. 1996. How rapidly does the human

mitochondrial genome evolve? *American Journal of Human Genetics.* Vol.59:501-509.

Howells, W. 1993. *Getting here, the Story of Human Evolution.* The Compass Press. Washington. D.C.

Hummel, S. and B. Herrmann. 1994. Y-chromosome DNA from ancient bones. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag, New York.

Hunan Medical College, 1980. *Study of an ancient cadaver in Mawangtui Tomb 1 of Han Dynasty in Changsha.* Relics Press, Beijing, China.

Jeffeys, A. J., M.J. Allen, E. Hagelberg and A. Sonnberg. 1992. Identification of the Skeletal remains of Josef Mengele by DNA analysis. *Forensic Science International.* Vol.56: 65-76.

Jones, W. H. S. 1967. The Prevalence of Malaria in Ancient Greece. In: *Diseases in Antiquity, A Survey of the Diseases, Injuries and Surgery of Early Populations,* edited by Don R. Brothwell and A. T. Sandison. Charles C Thomas, Springfield, Illinois.

Katzenberg, M. A., D. A. Herring and S. R. Saunders. 1996. Weaning and infant mortality: evaluating the skeletal evidence. *Yearbook of Physical Anthropology.* Vol.39:177-199.

Kilbourne, E.D. 1991. New viruses and new disease: mutation, evolution and ecology. *Current Opinion in Immunology.* Vol.3:518-524.

Krause, R. M. 1992. The origin of plagues: old and new. *Science.* Vol.257: 1073-1078.

Krings, M., A. Stone, R. W. Schmitz, H. Krainitzki, M. Stoneking, and S. Pääbo. 1997. Neanderthal DNA sequences and the origin of modern humans. *Cell.* Vol. 90:19-30

Kuliev, A. M. 1987. The WHO control program for hereditary anemia. *Birth Defects: Original Articles Series.* Vol.23(5B):283-394.

Kurosaki, K., T. Matsubita and S. Ueda. 1993. Individual DNA identification from ancient human remains. *American Journal of Human Genetics.* Vol.53:638-643.

Lassen, C., S. Hummel and B. Herrmann, 1994. Comparison of DNA extraction and amplification from ancient human bone and mummified soft tissue. *International*

*Journal of Legal Medicine.* Vol.107:152-155.

Lassen, C., S. Hummel and B. Herrmann, 1997. PCR based sex identification of ancient human bones by amplification of X- and Y-chromosomal sequences: a comparison. *Ancient Biomolecules.* Vol.1: 25-35.

Lau, Y-L, L-C. Chan, Y-Y. A. Chan, S-Y. Ha, C-Y. Yeung, J. S. Waye, D. H.K. Chui. 1997. Prevalence and genotypes of α-thalassemia and β -thalassemia carriers in Hong Kong - implications for population screening. *The New England Journal of Medicine.* Vol.336:1298-1301.

Lawlor, D. A., C. D. Dickel, W. W. Hauswirth and P. Parham. 1991. Ancient HLA genes from 7,500-year-old archaeological remains. *Nature.* Vol.349:785-788.

Lawn, R. M., Efsreatiadis, C. O'Connell and T. Maniatis. 1980. The nucleotide sequence of the human β-globin gene. *Cell.* Vol.21:647-651.

Lederberg, J. 1988. Pandemic as a natural evolutionary phenomenon. *Social Research.* Vol.55: 343-259.

Lee, T. F. 1993. *Gene Future, the Promise and Perils of the New Biology.* Plenum Press. New York and London.

Lehmann, H. 1982. The History of Thalassemia. *Birth Defects Original Article Series.* Vol.18:1-11.

Li, J.Y., K. W. Kong, M. H. Chang, S. C. Cheung, H. C. Lee, C. Y. Pang, Y. H. Wei. 1996. MELAS syndrome associated with a tandem duplication in the D-loop of mitochondrial DNA. *Acta Neurologica Scandinavica.* Vol.93:450-455.

Li, Y., C. An, Y. Zhu, Y. Zhang, Y. Liu, L. Qu, L. You, X. Liang, X. Li, L. Qu, Z. Zhou and Z. Chen. 1995. DNA isolation and sequence analysis of dinosaur DNA from Cretaceous dinosaur egg in Xixia, Henan, China. *Acta Scientiarum Naturalium Universitatis Pekinensis.* Vol. 31:148-152.

Lindahl, T. 1993a. Instability and decay of the primary structure of DNA. *Nature.* Vol.362:709-715.

Lindahl, T. 1993b. Antediluvian DNA research. *Nature.* Vol.367:700.

Lindahl, T. 1997. Facts and Artifacts of Ancient DNA. *Cell.* Vol: 90, 1-3.

Lindahl, T. and B. Nyberg. 1972. Rate of depurination of native deoxyribonucleic acid.

*Biochemistry*. Vol.11:3610-3618.

Lindahl, T. and O. G. Poinar. 1993. Recovery of antediluvian DNA. *Nature*. Vol.365:700.

Livingstone, F. B. 1986. Anthropological aspects of the distributions of the human hemoglobin variants. In: *Hemoglobin Variation in Human Populations,* edited by William P. Winter. CRC Press, Inc. Boca Raton, Florida.

Lovell, M. A. and W. G. Wayne. 1996. Molecular pathology 1995. *Diagnostic Molecular Pathology.* Vol. 5:1-2.

Ludes, B., H. Pfitzinger, E. Crubézy, P. Mangin, T. Janin and B. Midant-Reynes. 1994. Polymerase chain reaction (PCR) amplification and human leukocyte antigen (HLA)-DQA1 oligonucleotide typing on bones of Egyptian Predynastic times (3400 to 3200 BC). *C. R.. Acad. Sci. Paris. série II*: t318:1571-1575.

Mann, J. 1991. AIDS and the next pandemic. *Scientific American*. March:126.

Makuch, J., E. Brucker, R. Schmid, P. Mynahan and T. Owen. 1996. Removal of PCR inhibitors from forensic samples and enhanced sensitivity for D1S80 using fluorescent scanning. *Canadian Society of Forensic Science Journal.* Vol.29:29-32.

Martinelli, G., E. Trabetti, P. Farabegoli, M. Buzzi. A. Zaccaria, N. Testoni, M. Amebile, A. Casartelli, A. De Vivo, P. F. Pignatti and S. Tura. 1996. 'Fingerprinting' of HLA-DQA by polymerase chain reaction and heteroduplex analysis. *Molecular and Cellular Probes.* Vol.10:123-127.

Merriwether, D. A., F. Rothhammer and R. E. Ferrell, 1994. Genetic variation in the New World: ancient teeth, bone, and tissue as sources of DNA. *Experientia* Vol.50: 592-601.

Mim, C. 1980. The emergence of new infectious diseases. In: *Changing Disease Patterns and Human Behaviors* edited by the N. F. Stanley and R. S. Jokse. Academic Press.

Mims, C. A. 1987. *The Pathogenesis of Infectious Diseases.* 3rd Edition. Academic Press. New York.

Mizuki, N., M. Ota, M. Kimura, S. Ohno, H. Ando, Y. Katsuyama, M. Yamazaki, K. Watanabe, K. Goto, S. Nakamura, S. Bahram and H. Inoko. 1997. Triplet repeat polymorphism in the transmembrane region of the MICA gene: A strong

association of six GCT repetitions with Behçet disease. *Proceedings of the National Academy of Sciences, USA*. Vol. 94:1298-1303.

Montes, G. S. et al, 1985. Preservation of elastic system fibers and of collagen molecular arrangement and stainability in an Egyptian mummy. *Histochemistry*. Vol.83:117-119.

Morell, V. 1993. Dino DNA: the hunt and the hype. *Science*. Vol: 261: 160-162.

Morse, S. S. 1990. Stirring up trouble, environmental disruption can divert animal viruses into people. *Science*. Sept:16-21.

Moseley, J. 1971. Hematologic disorders. *In: Radiology of the Skull and Brain, Volume 1.* Book 1. edited by T.H. Newman and D.G. Potts. Mosby. St. Louis.

Nakaghori, Y., O.Takenaka and Y. Nakagome. 1991. A human X-Y homologous region encodes "amelogenin". *Genomics*. Vol.9:264-269.

National Institute of Health. 1997. "The BLAST Search" [*http://www.ncbi.nlm.nih.gov/cgi-bin /BLAST*].

Neeser, D., and S. Liechti-Gallati, 1995. Sex determination of forensic samples by simultaneous PCR amplification of α-satellite DNA from both the X and Y chromosomes. *Journal of Forensic Sciences.* Vol.40:239-241.

Nerlich, A. G. et al. 1993. Immunohistochemical detection of interstitial collagens in bone and cartilage tissues remainants in an infant Peruvian mummy. *American Journal of Physical Anthropology.* Vol.91:279-285.

Neu, H. C. 1992. The crisis in antibiotic resistance. *Science*. Vol.257:1064-1073.

Niederhause, C., C. Höfelein, B. Wegmüller, J. Lüthy and U. Candrian. 1994. Reliability of PCR decontamination systems. *PCR Methods and Applications.* Vol.4:117-123.

Olaisen, B., M. Stenersen and B. Mevåg. 1997. Identification by DNA analysis of the victims of the August 1996 Spitsbergen civil aircraft disaster. *Nature genetics.* Vol.15:402-405.

Ortner, D. J. 1991. Theoretical and methodological issues in paleopathology. In: *Human Paleopathology.* edited by D.J. Ortner and A.C.Aufederheide. Smithsonian Institution Press. Washington.

Ortner, D. J. and A.C. Aufderheide (editors). 1991. *Human Paleopathology.* Smithsonian Institution Press. Washington.

Ortner, D.J., and W. G. J. Putschar. 1985. *Identification of Pathological Conditions in Human Skeletal Remains.* Smithsonian Contributions to Anthropology, No. 28.

Ortner, D. J., N. Tuross and A. Stix. 1992. New approaches to the study of disease in archaeological New World populations. *Human Biology.* Vol.64:337-360.

Oxford, J. S. 1987. What is the true nature of epidemic influenza virus and how do new epidemic viruses spread? *Epidem. Inf.* Vol.99:1-3.

Pao, C. C., J. J. Hor, P. L. Tsai, and M. Y. Horng. 1993. Inhibition of *in vitro* enzymatic DNA amplification reaction by ultra-violet light irradiation. *Molecular and Cellular Probes.* Vol.7:217-219.

Pääbo, Svante. 1985. Molecular cloning of ancient Egyptian mummy DNA. *Nature.* Vol.314:644-645.

Pääbo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning , and enzymatic amplification. *Proceedings of the National Academy of Sciences, USA.* Vol.86:1993-1943.

Pääbo, S. 1996. Mutational hot spots in the mitochondrial microcosm. *American Journal of Human Genetics.* Vol.59:493-496.

Pääbo, S, J. A. Gifford and A. Wilson. 1988. Mitochondrial DNA sequences from a 7,000-year-old brain. *Nucleic Acids Research.* Vol.16:9775-9787.

Pääbo, S, R., G. Higuchi and A. C. Wilson. 1990. DNA damage promoters jumping between templates during enzymatic amplification. *Journal of Biological Chemistry.* Vol.265:4718-4721.

Pääbo, S, D. M. Irwin and A. C. Wilson. 1989. Ancient DNA and the polymerase chain reaction, the emerging field of molecular archaeology. *Journal of Biological Chemistry.* Vol.264:9709-9712.

Pääbo, S. and A. Wilson. 1988. Polymerase chain reaction reveals cloning artefacts. *Nature.* Vol.334:387-388.

Pääbo, S. and A. Wilson. 1991. Miocene DNA sequences - a dream comes true ? *Current Biology.* Vol.1:45-46.

Parsons, J. T., D. S. Muniec, K. Sullivan, N. Woodyatt, R. Alliston-Greiner, M. R. Wilson, D. L. Berry, K. A. Holland, V. W. Weedn, P. Gill and M. M. Holland. 1997. A high observed substitution rate in the human mitochondrial DNA control region. *Nature Genetic.* Vol.15:363-368.

Pfeiffer, S. 1991. Is paleopathology a relevant predictor of contemporary health patterns? In: *Human Paleopathology.* edited by D.J. Ortner and A.C.Aufederheide. Smithsonian Institution Press. Washington.

Pirastu, M., G. Saglio, C. Camaschella, A. Loi, A. Serra, T. Bertero, W. Gabutti, and A. Cao. 1988. Delineation of specific β-thalassemia mutations in high risk areas of Italy: a prerequisite for prenatal diagnosis. *Blood.* Vol.71:983-988.

Poinar, G. O. Jr. 1994. The range of life in amber: significance and implications in DNA studies. *Experientia* Vol.50: 536-542.

Poinar, G. O. Jr., H. N. Poinar and R. J. Cano. 1994. DNA from amber inclusions. In *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Rafi, A., M. Spigelman, J. Stanford, E. Lemma, H. Donoghue and J. Zias. 1994. DNA of *Mycobacterium leprae. International Journal of Osteoarchaeology.* Vol. 4:287-290.

Resnick, D., and G. J. Niwayama. 1981. *Diagnosis of Bone and Joint Disorders.* Philadephia: W.B. Saunders. Co.

Richards, M., H. Corte-Real, P. Forster, V. Macaulay, H. Wilkinson-Herbots, A. Demaine, S. Papiha, R. Hedges, H. Bandelt, and B. Sykes. 1996. Paleolithic and Neolithic lineages in the European mitochondrial gene pool. *American Journal of Human Genetics.* Vol.59:185-203.

Rechards, M. B., B. C. Sykes and R. E. M. Hedges. 1995. Authenticating DNA extracted from ancient skeletal remains. *Journal of Archaeological Sciences.* Vol.22:291-299.

Roberts, C. and K. Manchester. 1995. *The Archaeology of Disease.* Second Edition. Cornell University Press. Ithaca.

Rogan, P. R. and J. J. Salvo. 1990a. Study of nucleic acids isolated from ancient remains. *Yearbook of Physical Anthropology.* Vol.33:195-214.

Rogan, P. R. and J. J. Salvo. 1990b. Molecular genetics of pre-Columbian South

American mummies. UCLA Symposia on Molecular and Cellular Biology, New Series. Vol.122:223-234.

Rogan, P. R. and J. J. Salvo. 1994. High-fidelity amplification of ribosomal gene sequences from South American mummies. In *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Rollo, F., F. M. Venanzi and A. Amici. 1994. DNA and RNA from ancient plant seeds. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Rubinsztein, D.C., J. Leggo, M. Chiano, A. Dodge, G. Norbury, E. Rosser, and D. Craufurd. 1997. Genotypes at the GluR6 kainate receptor locus are associated with variation in the age of onset of Huntington *disease. Proceedings of the National Academy of Sciences, USA.* Vol.94:3872-3876.

Russell, P. J. 1986. *Genetics.* Little, Brown and Company. Toronto.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G.T. Horn, K. B. Mullis and H. A. Erlich. 1988. Primer-directed enzymatic application of DNA with a thermostable DNA polymerase. *Science.* Vol.239:487-491.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim. 1985. Enzymatic amplification of β-globin genomic sequences and restriction sites analysis for diagnosis of sickle cell anemia. *Science.* Vol.230:1350-1354..

Salo, W. L., A. C. Aufderheide, J. Buikstra and T. A. Hocomb. 1994. Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. *Proceedings of the National Academy of Sciences, USA.* Vol.91:2091-2094.

Sambrook, J., E. F. Gritsch and T. Maniatis. 1989. *Molecular Cloning - A laboratory Manual.* 2nd. Cold Spring Harbour Laboratory Press.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA.* Vol.74:5463-5467.

Sarkar G. and S. S. Sommer. 1993. Removal of DNA contamination in polymerase chain reaction reagents by ultraviolet irradiation. *Methods in Enzymology.* Vol.218:381-389.

Satinoff, M. I. 1971. The origins and geographical spread of the Thalassaemias and

abnormal haemoglobins. *Journal of Human Evolution.* Vol. 1:79-82.

Saunders, S. R. and D.Y. Yang. (In press). *Sex isn't easy!: XX or XY from the human skeleton.* In: Chilled to the Bone: Case Studies in Forensic Anthropology, edited by Scott Fairgrieve. Cambridge University Press. Cambridge.

Savill, J. 1997. Prospecting for gold in the human genome. *BMJ.* Vol.314:43-45.

Scholtissek, C. 1992. Cultivating a killer virus. *Natural History.* No.1:16.

Schuler, G. D. et al., 1996. A gene map of the human genome. *Science.* Vol.274:540-549.

Sebes, J. and Diggs, L. W. 1979. Radiographic changes of the skull in sickle cell anemia. *American Journal of Radiology.* Vol.132:373-377.

Sensabaugh, G. F. 1994. DNA typing of biological evidence material. In: *Ancient DNA* edited by Bernd Herrmann and Susanne Hummel. Springer-Verlag. New York.

Shinoda, K. and T. Kunidada. 1994. Analysis of ancient Japanese Society through mitochondrial DNA sequencing. *International Journal of Osteoarchaeology.* Vol.4:291-297.

Shoffner, J.M, M. D. Brown, C. Stugard, A. S. Jun, S. Pollok, R. H. Haas, A. Kaufman, D. Koontz, Y. Kim, J. Graham, E. Smith, J. Dixon, D. C. Wallace. 1995. Leber's hereditary optic neuropathy plus dystonia is caused by a mitochondrial DNA point mutation in a complex I subunit. *Annals of Neurology.* Vol.38:163-169.

Sievers, M. L., J. R. Fisher. Diseases of North American Indians. In: *Biocultural Aspects of Diseases*, edited by Henry R. Rothschild. Academic Press. Toronto.

Silvestroni, E. And I. Bianco. 1975. Screening for microcythemia in Italy: analysis of data collected in the past 30 years. *American Journal of Human Genetics.* Vol.27:198.

Siniscalco, M., L. Bernini, B. Latte and A. G. Motulsky. 1961. Favism and thalassemia in Sardinia and their relationship to malaria. *Nature.* Vol.190:1179-1180.

Soltis, P.S., D. E. Soltis and C. J. Smiley, 1992. An rbcL sequence from a Miocene Taxodium (bald cypress). *Proceedings of the National Academy of Sciences, USA.* Vol.89: 449-451.

Sommerfeld J. 1994. Emergencing epidemic diseases, anthropological perspectives. *Annals of New York Academy of Sciences.* Vol. 740:276-284..

Sperduti, A. 1995. *I Resti Scheletrici Umani della Necropoli di Età Romano Imperiale di Isola Sacra (I-III sex. d.C.).* Ph.D. Thesis. Department of Animal and Human Biology, Univ. 'La Sapienza', Rome.

Sperduti, A., L. Bondioli, T. L. Prowse, F. Salomone, D. Yang, R. D. Hopper, S. R. Saunders and R. Macchiarelli, 1997. The juvenile skeletal samples of the imperial roman site of Portus Romae. *Annual meeting of Canadian Association of Physical Anthropologists.* London, Ontario.

Sperduti, A. et al. 1996. Thalassemia in the Imperial Roman period: evidence from the Isola Sacra necropolis. *Preliminary Internal Report*, National Museum of Prehistory and Ethnography "L. Pigorini", Rome, Italy.

Spigelman, M. and E. Lemma. 1993. The use of the polymerase chain reaction (PCR) to detect *Mycobacterium tuberculosis* in ancient skeletons. *International Journal of Osteoarchaeology*. Vol.3:137-143.

Spritz, R. A. , J.K. DeRiel B.G. Forget, S. M. Weissman. 1980. Complete nucleotide sequence of the human delta-globin gene. Cell. Vol.21:639-646.

Sritharan, V. and R. H. Barker Jr. 1991. A simple method for diagnosing *M. Tuberculosis* infection in clinical samples using PCR. *Molecular and Cellular Probes.* Vol.5:385-395.

Steinbock, T. 1976. *Paleopathological Diagnosis and Interpretation.* C.C. Thomas. Springfield, Illinois.

Stone, A.C., G. R. Milner, S. Pääbo and M.Stoneking. 1996. Sex determination of ancient human skeletons using DNA. *American Journal of Physical Anthropology.* Vol.99:231-238.

Stoneking, M. 1995. Ancient DNA: how do you know when you have it and what can you do with it. *American Journal of Human Genetics.* Vol.57:1259-1262.

Stoneking, M. 1997. The human genome project and molecular anthropology. Genome Research. Vol.7:87-91.

Stoneking, M, D. Hedgecock, R. G. Higuchi, L. Vigilant, and H. A. Erlich. 1991. Population variation of human mtDNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. *American*

*Journal of Human Genetics.* Vol.48:370-382.

Stuart-Macadam, P. 1985. Porotic hyperostosis: representative of a childhood condition. *American Journal of Physical Anthropology.* Vol.66:391-398.

Stuart-Macadam, P. 1987a. A radiographic study of porotic hyperostosis. *American Journal of Physical Anthropology.* Vol.74:511-520.

Stuart-Macadam, P. 1987b. Porotic hyperostosis: new evidence to support the anemia theory. *American Journal of Physical Anthropology.* Vol.74:521-526.

Stuart-Macadam, P. 1989. Porotic hyperostosis: relationship between orbital and vault lesions. *American Journal of Physical Anthropology.* Vol.80:187-193.

Stuart-Macadam, P. 1992a. Porotic hyperostosis: a new perspective. *American Journal of Physical Anthropology.* Vol.87:39-47.

Stuart-Macadam, P. 1992b. Anemia in past human populations. *In: Diet, Demography and Disease.* Edited by P. Sturt-Macadam and S. Kent. Aldine de Gruyter. New York.

Sullivan, K. M., A. Mannucci, C. P. Kimpton and P. Gill (1993) A rapid and quantitative DNA sex test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin. *Biotechniques* Vol.15:635-641.

Swedlund, A. C. and G. J. Armelagos. 1990. *Disease in Populations In Transition, Anthropological and Epidemiological Perspectives*. Bergins and Garvey. New York.

Taubenberg, J. K., A.H. Reid, A. E. Kraft, K. E. Bijwaard, T. G. Fanning. 1997 Initial genetic characterization of the 1918 "Spanish" influenza virus. *Science*. Vol.275:1793-796.

Tayles, N. 1996. Anemia, genetic diseases, and malaria in prehistoric mainland southeast Asia. *American Journal of Physical Anthropology.* Vol. 101:11-27.

Tentori, L. and M.Marinucci. 1986. Hemoglobin variants and thalassemas in Italy. In: *Hemoglobin Variation in Human Populations* edited by William P. Winter. CRC Press, Inc. Boca Raton, Florida.

Thein, S. L. 1993. β-thalassemia. *Bailliere's Clinicla Hematology.* Vol.6:151-262.

Thomas, K. W. and S. Pääbo. 1993. DNA sequences from old tissue remains. *Methods*

*in Enzymology.* Vol.224:406-419.

Tompkins, L. S. and S. Falkow. 1992. Molecular biology of virulence and epidemiology. In: *Infectious Diseases*, edited by S. L. Gorbach, J. G. Bartlett and N. R. Blacklow. W. B. Saunders Company. Toronto.

Tuross, N. 1991. Recovery of bone and serum proteins from human skeletal tissue: IgG, osteonectin, and albumin. In: *Human Paleopathology*, edited by D.J. Ortner and A.C.Aufederheide. Smithsonian Institution Press. Washington.

Torroni, A. et al, 1992. Native American mitochondrial DNA analysis indicates that the Amerind and the NaDene populations were founded by two independent migrations. *Genetics* Vol.130:153-162.

Vigilant, L., R. Pennington, H. Harpending, T. D. Kocher and A. C. Wilson.1989. Mitochondrial DNA sequences in single hairs from a southern African population. *Proceedings of the National Academy of Sciences, USA.* Vol.86:9350-9354.

von Lichtenberg F. 1991. *Pathology of Infectious Diseases.* Raven Press. New York.

Waldron, T. Editorial: biomarkers of diseases. *International Journal of Osteoarchaeology.* Vol.6:324-325.

Wallace, D.C., G. Singh, M.T. Lott, J. A. Hodge, T. G. Schurr, A. M. Lezza, L. J. Elsas, E. K. Nikoskelainen. 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science.* Vol.242:1427-1430.

Waye, J. S. and H. F. Willard. 1986. Structure, organization, and sequence of alpha satellite DNA from human chromosome 17: evidence for evolution by unequal crossing-over and an ancestral pentamer repeat shared with the human X chromosome. *Molecular and Cellular Biology.* Vol.6:3156-2165.

Weatherall, D. J. 1996. Molecular biology and medicine. In: *Oxford Textbook of Medicine*. Third Edition. Vol. 1. edited by D.J.Weatherall, J.G.G.Ledingham and D.A. Warrell. Oxford University Press. Oxford.

Weatherall, D. J. and J. B. Clegg. 1981. *The Thalassemia Syndromes.* Blackwell Scientific Publication. Oxford.

Weatherall, D. J. and J. B. Clegg. 1996. Thalassemia - a global public health problem. *Nature Medicine.* Vol.2:847-849.

Weiser, U. et al, 1989. Mummified enzymes. *Nature.* Vol.341:696.

Williams, B. A., L. L. Morris, I. R. G. Toogood, J. L. Penfold and B. K. Foster. 1992. Limb deformity and metaphyseal abnormalities in thalassemia major. *The American Journal of Pediatric Hematology/Oncology.* Vol.14:197-201.

Williams, T. N., K. Maitland, S. Bennett, M. Ganczakowski, T. E.A. Peto, C. I. Newbold, D. K. Bowden, D. J. Weatherall, and J. B. Clegg. 1996. High incidence of malaria in α-thalassaemic children. *Nature.* Vol.383:522-525.

Williamson, J. M., J. S. Waye, P., Newall, D.H. Bing and E. Black. 1996. The use of a comprehensive approach for neutralization of PCR inhibitors found in forensic samples and its use in a homicide/sexual assault case. In: *Proceedings from the Sixth International Symposium on Human Identification 1995.* Promega Corporation, Madison, WI.

Wilson, Mary E. 1994. Disease in evolution, introduction. *Annals of New York Academy of Sciences.* Vol. 740:1-12.

Wood, J. W., G. R. Milner, H. C. Harpending, and K. M. Weiss. 1992. The Osteological Paradox: Problems of Inferring Prehistoric Health from Skeletal Samples. *Current Anthropology.* Vol.33:343-370.

Woodward, S. R., M. J. King, N. M. Chui, M. J. Kuchar and C. W. Griggs. 1994. Amplification of ancient nuclear DNA from teeth and soft tissues. *PCR Methods and Applications.* Vol.3:244-247.

Woodward, S. R., N.J. Weyand, M. Bunnell. 1994. DNA sequence from Creteceous period bone fragments. *Science.* Vol.266:1229-1232.

Word, J. W., L. R. Petersen and H. W. Jaffe. 1997. Current trends in the epidemiology of HIV/AIDS. In: *the Medical Management of AIDS*, edited by Merle A. Sande and Paul A. Volberding. Fifth Edition. W. B. Saunders Company. Toronto.

Wright, K. 1990. Bad news bacteria. *Science.* Vol.249:22-29.

Wyler, D. 1992. Palsmodium and babesia. In: *Infectious Diseases*, edited by S. L. Gorbach, J. G. Bartlett and N. R. Blacklow. W. B. Saunders Company. Toronto.

Yang D.Y., B. Eng, J. C. Dudar, S. R. Saunders and J. S. Waye. 1997. Removal of PCR inhibitors using silica-based spin columns: application to ancient bones. *Canadian. Society of Forensic Sciences Journal.* Vol.30:1-5.

Yang D.Y., B. Eng, J. S. Waye, J. C. Dudar, S. R. Saunders. In Press. Improved DNA Extraction from Ancient Bones using Silica-Based Spin Columns. *American Journal of Physical Anthropology.*

Yang, H., 1995. Authentication of ancient DNA sequence - a reassessment of 18S rDNA sequence from a fossil dinosaur egg. *Acta Palaeontologica Sinica.* Vol.34:657-673.

Zaino, E. C. 1964. Paleontologic Thalassemia. *Annals of the New York Academy of Sciences.* Vol.119:402-412.

Zierdt, H., S. Hummel and B. Herrmann. 1996. Amplification of human short tandem repeats from Medieval teeth and bone samples. *Human Biology.* Vol.68:185-199.

Zimmerman, M. R. And M. A. Kelley. 1982. *Atlas of Human Paleopathology.* Praeger Publishers. New York.