Development Of Molecular Sex Determination Methods And Their Application To Archaeological Material Sourced From The Raymond Dart Collection Of Human Skeletons

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Declaration

I declare that this thesis is my own, unaided work, except where otherwise acknowledged. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

Signed this ___ day of _____ 20___

Victoria Elaine Gibbon.

Abstract

Within biological anthropology there is a movement toward the use of molecular methods and their application to anthropological questions. In South Africa there is great potential for research in anthropological genetics as this country is a vast repository of skeletal remains. This study aims to develop novel methods of molecular sex determination for skeletal material and apply these to an archaeological collection of historical importance.

The bone extraction method developed here is minimally destructive to skeletal material and does not interfere with any anthropometric landmarks. The extracted bone powder is subjected to DNA extraction procedures adapted for bone. In addition, two novel systems of molecular sex determination ideal for skeletal material are developed on the amelogenin gene, beginning in intron 2-3, spanning exon 3 and ending in intron 3-4. This area is optimal for sexing, as it includes 14 sex specific polymorphic regions in addition to an indel (insertion or deletion of nucleotides). Once these procedures of molecular sex determination are optimised and working with 100% efficiency on the controls, they will be applied to a collection of miscellaneous archaeological skeletons (*ex-situ*) sourced from the Raymond Dart Collection of Human Skeletons (Dart Collection). This collection is used to optimise these techniques for specimens derived from an archaeological context. These methods yielded 46.66% sex results for the *ex-situ* sample, which is within the normal range for ancient DNA studies. These novel methods are then applied to an archaeological sample with good provenance, this being 36 skeletons of Chinese indentured miners sourced from the Dart Collection. While a previous morphometric study showed that this collection was represented by equal male to female ratios, the historical

records suggest however that very few women accompanied these Chinese labourers. In using these procedures, 41.93% of this sample produced results all of which were male, correlating well with the historical records. The value of molecular approaches to investigate sex determination is that they do not rely on intact specimens as morphmetric methods do. Therefore in cases where the use of morphometrics is complicated, molecular approaches offer an accurate solution for diagnosing sex.

The techniques of bone and DNA extraction are applicable for both human and animal skeletal tissue, and the methods of molecular sex determination are optimal for archaeological or forensic derived human skeletal material.

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Abbreviations

aDNA	Ancient deoxyribonucleic acid
AMEL	Amelogenin gene
AMELX	Amelogenin X gene
AMELY	Amelogenin Y gene
archDNA	Archaeological deoxyribonucleic acid
BP	Before present
bp	Base pair
DNA	Deoxyribonucleic acid
EDTA	Tetrasodium ethylenediaminetetra-acetate dihydrate
PCR	Polymerase chain reaction
mtDNA	Mitochrondial deoxyribonucleic acid
SRY	Sex determining region Y gene
ZF	Zinc finger protein

Chapter 1

Introduction

Anthropology is a scientific discipline that uses a holistic approach to the study of humans, encompassing both biological and socio-cultural perspectives. A sub-discipline within anthropology is biological anthropology, which focuses on the biological aspects of human adaptation, evolution, variation, as well as growth and development.

For biological anthropologists skeletal remains are the most abundant remnants from the past that retain information regarding the individual, including factors such as age, sex, stature and population affinity. The analysis and knowledge gained from these variables assists in understanding past populations through sex orientated patterns of burial, marriage, differential mortality, diseases, diet, societal status and material possessions (Kaestle & Horsburgh 2002). It allows the biological profile to be reconstructed and to understand the demographics of the individual and the population represented.

The determination of sex is important as it is the first stage in the biological reconstruction process and assists in assessing sex specific demographic variables such as age and stature (France 1998). Sexing the individual is of particular importance as it excludes half the population. It also allows the researcher to better understand the gender and sex-specific roles in a society. Therefore, having accurate methods of sex determination are valuable in biological anthropology.

A problem with sex determination in humans is that the level of sexual dimorphism is relatively low, being greater in soft than in skeletal tissue. Biological anthropologists commonly use anthropometry and gross morphology (morphometrics) to determine sex, using the subtle sex-related differences in the skeleton (White 2000).

1.1 Traditional Morphometric Sex Determination

Traditional morphometric methods are based on the examination of bones and teeth that contain indicators of the demographic features of a person (Byers 2002). The underlying assumption of these conventional methods are that females are small and gracile, while males are robust, with heavy muscle attachments. Morphometric methods of sex determination also include the interpretation of size and architecture (Byers 2002). On average, females are roughly 92% the size of males, size being intrepreted as the level of robusticity, height and weight. With regards to skeletal architecture, women are structually adapted in the pelvic area for pregnancy and childbirth, so this is a distinguishing feature for sex determination.

Morphometric methods require the use of population specific standards for the skeletal remains of the population represented in a specific time period and geographic location (Buikstra & Ubelaker 1994; Byers 2002). Two commonly used regions of the skeleton for sex determination are the cranium and pelvis, as the greatest number of characteristics for sexing are seen on these structures (Byers 2002). There is variation within the structures used for sex determination among different human population groups (Byers 2002). The accuracy of conventional anthropometric methods for sex determination in adults with the entire skeleton is 90-100% (Byers 2002). With the pelvis alone these methods are 90-95% accurate, with only the skull the accuracy is 80-90%, and in using only the long bones the accuracy is 80% (Byers 2002).

Morphological sexing is diagnosed through the observational analysis of the overall shape and structure of specific skeletal elements where sex differences are discernible. This must be done in combination with the appropriate population-specific chart or table. Each of the morphological characteristics are normally scored on a standard of one to five, one being gracile (female) and five being robust (male) (Buikstra & Ubelaker 1994; White 2000; Byers 2002). Within this scoring system, a score of three is an ambiguous option, which results in non-identifiable sex. As inexperience affects the accuracy of the observations, morphological methods are reliant on the experience of the researcher.

Metrical sexing is diagnosed through a number of measurements using specialised instruments. These are based on the assumption that males when compared to females can on average be up to 20% larger in some skeletal measurements (White 2000). In order to determine sex, the measurements acquired from these methods are used with a mathematical formula and the result is evaluated with a population specific standard table of ratios. As with all metrical methods measurement error is an issue, arising either through the lack of a repeatability standard, or the use of uncalibrated tools thus resulting in incorrect measurements.

The limitations of morphometric methods are known. As stated previously morphometric methods are based on the assumption that females are more gracile in structure compared to males. There are many cases in modern populations with robust women and gracile men who fall outside of this generalisation, which result in an incorrect diagnosis of sex. Another example are male adolescent individuals who are typically gracile in appearance and could be erroneously diagnosed as female (Buikstra & Ubelaker 1994).

Conventional anthropometric methods of sex identification are considered to be unreliable on fragmented skeletons as well as foetuses, neonates and pre-adolescents (Saunders 1992; Buikstra & Ubelaker 1994; Weaver 1998). The features on the skeleton used for sexing are located on fragile aspects that are often damaged or even missing in archaeological and forensic contexts.

Another drawback is that most archaeological graves do not contain the entire skeleton and this clearly limits the application of morphometric methods (Byers 2002). In addition, the standards used in morphometric methods for sex determination vary among different human groups, which is problematic when applying the methods to a population from a remote time or under-researched geographical area (White 2000; Buikstra & Ubelaker 1994).

For all of the above reasons there has been a trend in biological anthropology to increase the use of other methods of sex determination, one method being to diagnose sex using molecular biological techniques, through the analysis of DNA obtained from ancient tissues.

1.2 Anthropological Genetics

Anthropological genetics is a complex, ever-expanding discipline and it eludes any simple definition. The Cambridge Dictionary of Human Biology and Evolution defines it as: "the synthesis of anthropology and population genetics in order to study the interactions between culture and allele frequencies; the use of genetic techniques to reconstruct the past, including the use of DNA sequences to reconstruct likely phylogenetic or genealogical relationships among populations; and the use of archaeological samples, including ancient DNA, to achieve these goals" (Mai *et al.* 2005).

In 1962 Emile Zuckerkandl a biochemist, introduced anthropological genetics as the study of human evolution at the molecular level (Goodman *et al.* 1976). The field was transformed and improved with the invention of polymerase chain reaction in 1985 by Kary Mullis, and in the same decade with the development of a thermal cycling machine (Mullis *et al.* 1989). These new developments in molecular biology were followed by interest and experimentation in DNA extraction from skeletal material (Capelli *et al.* 2003).

The first archDNA sequence was derived from the skin of a 140 year-old Quagga, an extinct member of the Zebra (*Equidae*) family (Higuchi *et al.* 1984).

Shortly thereafter, Pääbo (1989) published the first archDNA human sequence from an artificially preserved 2400 year-old Egyptian mummy, and Hagelberg and Sykes (1989) amplified DNA from 5500 year-old ancient human skeletal remains.

These initial publications were riddled with technical difficulties, as the integrity of the DNA was severely limited by physical and chemical degradation. There was scepticism and criticism towards the efficacy of the technique of DNA analyses from ancient tissue,s due to reports that were proven to be incorrect. Consequently, intensive research has led to the improvement of extraction methods and PCR amplification for authentic ancient DNA results (Pääbo 1991; Kaestle & Horsburgh 2002). This has led to a wider acceptance and a more progressive use of the available technology. This was followed by a sudden increase of interest in DNA extraction when it was discovered that DNA could be extracted from bones and teeth (Brown & Brown 1994).

Due to the potential contribution of anthropological genetics to science, this field is rapidly expanding and is being increasingly used within the field of biological anthropology. The genetic information retrieved from the past provides the opportunity to examine anthropological issues regarding pre-history in novel ways. Genealogical information is often used in forensic cases (criminal, mass graves, accidents etc.) for personal identification where DNA is frequently the only method of identification. The extraction and isolation of DNA from skeletal tissue can also be applied to investigate issues related to human variation and evolution over both long and short periods of time (Cooper & Wayne 1998). Anthropological genetics is a way of directly measuring genetic traits without phenotypic involvement (Williams *et al.* 2002).

Studies have clearly demonstrated that the extraction of DNA from ancient soft-tissues is possible (Kaestle & Horsburgh 2002). However, the application of these techniques to the more abundant remains of hard tissues such as bones and teeth provides the opportunity for large-scale population sampling (Hagelberg & Sykes 1989).

Although DNA is often degraded, under favourable conditions skeletal remains retain sufficient DNA for extraction and analysis (White 2000). Amplification of DNA molecules older than one million years is overly optimistic, as after 100 000 years due to hydrolytic damage all DNA that could reasonably be retrieved would be destroyed (Hofreiter *et al.* 2001). This is assuming physiological salt concentrations, neutral pH and a temperature of 15 °C, and certain environmental conditions will extend or reduce this time limit (Hofreiter *et al.* 2001).

Historically the laboratory methods in this field were extremely time consuming and difficult, but as the method of DNA extraction from ancient tissues improves, its use is more widely accepted. In addition, much research has been conducted to develop and perfect authentic DNA sequences (Machugh *et al.* 2000). In particular, forensic organisations involved in the discovery of missing people have led to major improvements in molecular identification techniques. One such organisation, the International Commission on Missing Persons developed in 1996, locates and identifies people that have disappeared during armed conflict or human rights violations (International Commision on Missing Persons 2008). In the past seven years, this and organisations like it have led the way for major improvements in DNA extraction and amplification methods from bone (Holland *et al.* 2003; Bille *et al.* 2004; International Commision on Missing Persons 2008). Due to the technological advancement within anthropological genetics, DNA can also be analysed from ancient tissues and can be applied to anthropological questions.

1.2.1 Anthropological Genetics in South Africa

Although South Africa, due to the work of scientists such as H. Soodyall and T. Jenkins, are in the forefront of research in the area of population genetics, arch/aDNA research is still in its infancy. Apart from some forensic studies that focused on the degradative resistance of DNA to different temperatures (Pillay & Kramer 1997; Urbani *et al.* 1999), very little work has been carried out in this field using skeletal material. As skeletal materials are particularly

resistant to the ravages of the physical environment, they would obviously provide good sources of intact DNA. Bearing in mind that South Africa is a vast repository of skeletal remains, there exists great potential for research in this area of anthropological genetics. One such repository is the Raymond Dart Collection of Human Skeletons (Dart Collection) in the School of Anatomical Sciences at the University of the Witwatersrand, Johannesburg. This collection provides a wealth of information on various South African populations, and has preserved skeletal material from the many migrations of people to and from South Africa. One sample stored within the Dart Collection of significant historical importance is that of the Chinese indentured miners, who were part of the initial migration of people to the Witwatersrand area in the gold mining era, early in the twentieth century. This is a valuable source of information regarding an important period in South African history that is characterised by the use of indentured labour.

1.2.2 Molecular Sex Determination

As is well known, the sex chromosomes can be used to genetically sex individuals. In order to determine sex one must isolate a gene/s on the sex chromosomes (X and Y). Molecular sex determination has certain advantages over morphometric methods as they are not affected by individual variation in the size and architecture of skeletal material. It can be used to determine sex from foetal and juvenile remains; and it is not restricted by physical fragmentation in the same way, requiring only one complete bone or a tooth to use for sex determination. Anthropological genetics does have its disadvantages related to molecular preservation. This is relevant to molecular sex determination because if the DNA cannot be extracted, then sex cannot be determined by molecular methods.

Each of the traditional morphometric and the molecular methods of sex determination have known limitations and it is important to be aware of these. However, these methods complement one another, where one fails the other may succeed. Therefore, when it is viable, in an attempt to reconstruct as

much of the biological profile as possible, all of the available methods for sex determination should be used.

1.3 Research Aims

The present thesis is divided into two parts that employ the technological advancement of anthropological genetics to add new information to the archaeological record in South Africa.

Aim 1 The first aim is to devise novel methods of molecular sex determination suitable for skeletal material and to optimise their use on a collection of miscellaneous archaeological skeletal material sourced from the Dart Collection. It is necessary to first devise an acceptable bone extraction method that is minimally destructive to skeletal material. The DNA extraction procedures will then be adapted for bone and finally a novel method of molecular sex determination appropriate for skeletal material is developed.

Aim 2 The second aim is to apply the developed methods of molecular sex determination for skeletal tissue to an archaeological sample of Chinese indentured miners sourced from the Dart Collection. In a previous study, morphometric sex determination was conducted on this collection wherein the sex ratio obtained was almost half male and half female (Edwards 2004). This is surprising as only a few women accompanied the imported Chinese miners to the gold mines in South Africa (Reeves 1954; Sacks 1967). This collection is suitable for the application of these methods of molecular sex determination to examine questions regarding the sex profile of this sample, and to compare the results produced from the molecular methods to results gained from a previous study conducted on this collection using traditional morphometrical methods.

Chapter 2

DNA extraction and Analysis from ancient tissues

2.1 Introduction

Research in anthropological genetics requires that a clear distinction is made between ancient DNA (aDNA), archaeological DNA (archDNA) and modern DNA. Although there are disagreements on the precise definition of these terms, it is generally accepted that modern DNA is DNA extracted from living populations; that archaeological DNA derives from any *post mortem* tissue taken immediately after death right up until to 10 000 years-old; and that ancient DNA is DNA extracted from tissues that are at least 10 000 years-old (Mai *et al.* 2005). The definitions outlined above are used throughout the present research.

2.2 The Challenges Associated with Anthropological Genetics

Even though DNA has been successfully extracted from ancient tissues of various ages and of differing states of preservation, several challenges still

face molecular anthropologists. Three main concerns have been identified surrounding DNA extraction from ancient tissues. These include the destructive nature of the extraction procedure, degraded molecular mass/molecular preservation of the DNA molecule and contamination from modern DNA (Höss 2000; O'Rourke *et al.* 2000; Gilbert *et al.* 2005). While contamination can be successfully avoided through the implementation of strict laboratory measures, the first two problems are related to the physical and molecular preservation of the specimen that continue to pose a problem for molecular biologists.

2.2.1 Destructive Nature of the Method

The methods of bone and tooth extraction have traditionally been destructive to skeletal remains, which has made it difficult to gain access to important collections of human skeletal remains. Obtaining DNA often represents a compromise between the level of destruction to the tissue and the amount of bone required for a successful extraction.

2.2.2 Molecular Preservation

While the survival of DNA for purposes of aDNA analysis is of central importance, it is unclear what proportion of skeletal remains will yield sufficient human DNA for genetic analysis (Hagelberg & Sykes 1989). It does not matter how stringent and accurate the methodology is; should the quality of the starting material be poor there will not be any DNA to amplify (Machugh *et al.* 2000). It follows that survival of DNA is positively correlated with the quality of the starting material, which includes factors related to preservation, morphology and handling (Richards & Sykes 1995). In view of these considerations, it can be said that preservation of DNA depends less on the age of the specimen and more on the environmental conditions surrounding the burial (Hagelberg & Sykes 1989). The likelihood of good preservation can be assessed by analysing the gross morphology of the specimen, in which favourable indicators include weight, robustness and completeness of the specimen.

2.2.3 Molecular Degradation

Molecular degradation is a normal feature of all living cells that is counter-balanced by mechanisms of cellular repair. However, post mortem degradation of DNA leads to the physical destruction of the DNA molecule (Pääbo *et al.* 2004; Pruvost *et al.* 2007). It first occurs by bacterial and fungal damage, followed by chemical degradation reacting to hydrolysis and oxidation. Degradation of DNA includes the physical and chemical breakdown of DNA, which can destroy most of the DNA molecules contained in ancient remains. The DNA remaining in ancient tissues is often poorly preserved and fragmented, causing problems with the molecular analysis (Yang & Watt 2005).

Various categories of DNA damage are found in samples from ancient tissues; these are dependent on many factors including, the age of the specimen, the environment, its storage and extraction conditions (Mitchell *et al.* 2005). The accumulation of environmental heat and chemical damage can cause gradual degradation of DNA. The DNA molecule degrades over time in a relatively predictable manner and consequently after thousands of years, at moderate temperatures, only short fragments of DNA may survive (Brown & Brown 1994; Cina 1994).

Pääbo *et al.* (2004) describe four types of molecular damage: DNA strand breaks, crosslinks, hydrolysis and oxidation. DNA strand breaks are the most common form of damage, being caused primarily by micro-organisms, nucleases and other chemical processes, all of which serve to reduce DNA to the small average size of 100-500 base pairs. DNA when exposed to heat may be altered by cross-links between DNA and other biomolecules, causing modification of the phosphate sugar and amino acid moieties that result in malliard products (condensation of the amino group). Strand breaks and cross-links are typical of bacterial and fungal damage arising during the *post mortem* degradation of DNA. During hydrolytic damage the bonds between the phosphate sugar and the nucleic acid base break down in the presence of water, causing base loss and single strand DNA breaks (Richards & Sykes 1995; Hofreiter *et al.* 2001; Poinar

2003). Oxidative damage is caused by radiation effects on DNA, which results in ring fragmentation and strand scission (Höss *et al.* 1996; Poinar 2003).

It is largely attributed to hydrolytic and oxidative damage that the retrieval of authentic DNA sequences older than 100 000 years is expected to be difficult to obtain, if not impossible (Pääbo 1991; Poinar 2002). These degradative processes may be overcome in proficient laboratory settings (Pääbo *et al.* 2004).

2.2.4 Environmental Conditions

In order to predict the level of molecular preservation or degradation of the specimen being examined, both favourable and unfavourable burial conditions must be taken into consideration (Höss 2000; Wurmb-Schwark *et al.* 2004*a*; Gilbert *et al.* 2005). Environmental conditions are important in the preservation of DNA in animal and human remains, which ultimately impinge both on the quantity and quality of the isolated DNA.

Taphonomy is a discipline that studies the processes that affect an organism after death, including both biological and environmental factors, through its burial process, fossilisation and collection (Mai *et al.* 2005). These incorporate decomposition, temperature, the type of ecosystem, geography, insect and carnivore activity (Hunter 2002). In this regard, a good understanding of the general ecosystem is important; for example, decomposition in water ecosystems is entirely different to that of terrestrial ecosystems.

Some of the relevant factors within the environment that cause DNA degradation include long exposure to UV light, drastic temperature gradients, high salt concentrations, burial conditions, low/high pH and humidity conditions (Hofreiter *et al.* 2001; Capelli *et al.* 2003; Wurmb-Schwark *et al.* 2004*a*). Cold and dry conditions are more favourable for molecular preservation.

The collections used in the present study were found in and around the greater Johannesburg area. Johannesburg is naturally a grassland ecosystem (Environment Affairs and Tourism, South Africa n.d), although in the last few hundred years

in particular areas of Johannesburg numerous exotic trees/plants have been introduced, which have changed the ecosystem to an urban forest. According to the World Meteorological Organization based on monthly averages for the period of 1961-1990 for Johannesburg, the annual rainfall is 713 mm, with an average maximum temperature of 22 °C and a average minimum temperature of 10 °C (South African Weather Service n.d). This amount of precipitation is within the world average and is a good level for DNA preservation. The deserts in comparison receive 250 mm or less, while the tropics receive over 2000 mm annually. Johannesburg does not normally reach temperatures above 30 °C or below 0 °C, and it is not humid (South African Weather Service n.d). These conditions are fair for DNA preservation as the temperatures do not fluctuate excessively, nor is it extremely hot with excessive precipitation.

There is little to no information on the excavations for the collections used in the present study. Therefore information regarding the pH and salt concentrations in the soil, along with the depth of burial (access to UV radiation) are unknown. Damage inflicted by insect and rodent activity can be assessed from the condition of the bones. Scavengers are known to drag limbs or bony elements away. However, these are not the only reasons for skeletal elements to be missing. Therefore, it cannot be assumed that missing elements are due to scavenger activity.

2.2.5 Contamination

A particular limitation of working with human derived arch/aDNA is the contamination of specimens by exogenous DNA, including DNA derived from micro-organisms, carnivore activity, laboratory derived DNA and during the excavation of material by archaeologists. These raise questions of the authenticity of amplified sequences. It is only possible to recover material of high molecular mass if contamination from modern DNA is avoided (Hagelberg & Sykes 1989). In addition, DNA extracted from ancient tissues is often contaminated with microbial DNA (Mitchell *et al.* 2005). A contaminant of microbial

or fungal DNA is relatively easy to exclude if the target DNA is human. Contaminant DNA is most often from modern human DNA, which is identical to the target template, therefore it can be amplified by PCR and is difficult, if not impossible, to exclude from the target. Contamination must be avoided, which can be achieved through extensive decontamination methods and proper handling of the specimens.

Kemp and Smith (2005) describe three common types of contamination related to research in anthropological genetics. The first is surface contamination, the result of contact with excavators and laboratory personnel. The second type pertains to labware, including reagents, glassware, and lab disposables. The third type relates to the carry over of DNA between tubes during PCR amplification. Contamination can be monitored by checking the repeatability and reproducibility of the results (Hagelberg *et al.* 1991).

2.2.6 Decontamination and Authenticating Criteria

The field of anthropological genetics has attracted much criticism and scepticism, as a number of unauthenticated DNA extraction claims have been discredited (Austin *et al.* 1997). This has led to investigation into techniques to limit contamination and methods to ensure the authenticity of the target sequence. A good example relates to the claim of DNA extraction from a 120-135 million year-old weevil *Nemonychidae, Coleoptera* preserved in amber (Cano *et al.* 1993) that was discredited after further research (Machugh *et al.* 2000). Strict authenticating criteria have been developed to ensure a generation of validated arch/aDNA data (Cooper & Poinar 2000).

Cooper and Poinar (2000) published a list of the criteria for DNA authentication. Their list consists of nine authenticating controls: a physically isolated work environment, control PCR amplifications (negative controls), appropriate molecular behaviour (concentration of PCR amplification should be related to product size), reproducibility (repeatable results), cloning, independent replication, biochemical preservation (DNA compared to amino acids), quantitation

(DNA copy number) and associated remains (faunal remains as negative controls).

While it is important to follow guidelines such as those listed above, there is no absolute set of criteria that excludes all possibilities of contamination. The best policy is to attempt to reduce the number of false reports (Lindahl 1993; Hebsgaard *et al.* 2005). It is recommended that separate work areas exist for DNA extraction and PCR amplification and there should be dedicated materials and instruments available for exclusive use in each area (Capelli *et al.* 2003). Results should ideally be reproducible and verifiable by independent laboratories.

2.3 Applications of DNA Analysis in Anthropology

From an anthropological perspective, DNA sourced from skeletal remains has many applications. It can address questions that offer a historical perspective to molecular investigations, such as biological relationships in pre-history (Williams *et al.* 2002).

In recent times, DNA has been extracted from ancient tissues of differing ages ranging from current forensic cases through to fossilised Neandertal DNA. Authentic or non-contaminant DNA has been extracted from many animal and human remains, so over the years the diversity and versatility of the technique has been well authenticated.

2.3.1 Phylogenetic Relationships and Evolutionary Analyses

Studies of extinct taxa allow the phylogenetic relationship of species to be traced and compared to contemporary species, while tracking population changes

over both time and space (Capelli *et al.* 2003; Pääbo *et al.* 2004). In this area, there is an astonishing amount of attention with regards to human evolution as this is of scientific as well as general interest. This subject has been extensively researched and produced significant results. For example, DNA extracted from skeletal remains of fossil *Homo sapiens* and *Homo neanderthalensis* proved invaluable in understanding modern human origins.

Neandertals lived in Europe and Asia from 300 000 to 30 000 years ago (Klein 1989). Krings *et al.* (1997) were the first to successfully extract mitochondrial DNA (mtDNA) from the Neandertal species. A similar study by Ovchinnikov *et al.* (2000) extracted mtDNA from a Neandertal individual with a radiocarbon date of 29 000 BP. The work on the Neandertal DNA reflects the problems of aDNA work, as some sequence results have proved to be authentic while others have not (Cooper *et al.* 2004). However, with the perseverance of researchers in the field, the partial DNA sequences of Neandertal genome were recently published (Noonan *et al.* 2006; Green *et al.* 2006). This represents a significant advance in DNA analysis and evolutionary studies, since it was initially thought impossible to extract DNA from Neandertals due to their age.

The study of phylogenetic relationships has also been conducted on other animal species. DNA was extracted from a 25 000 year-old ancient Alaskan horse and identified it as *Equus hemionus* (Höss & Pääbo 1993). These investigators demonstrated that the Pleistocene horse is closely related but not identical to the modern domestic horse and is more distantly related to the modern cow. In another study mtDNA was extracted from the bones of a frozen woolly mammoth (*Mammuthus primigenius*) that have a radiocarbon date of 47 000 BP (Hagelberg *et al.* 1994). Cooper (2006) used ancient mammoth DNA to show a phylogenetic relationship to living African and Asian elephants; that of the mammoth being more closely related to the Asian elephant.

2.3.2 Genealogical Relationships and Identifications

In modern identifications of recently missing or found persons, where the identification is complicated beyond the conventional means of a coroner, a forensic scientist is employed to assist in the identification of these victims. In many of these cases an identification can only be made through molecular methods. Some common examples of such cases could relate to criminal activity (serial killers, war crimes with mass graves), acts of god (earthquake, tsunami) or accidents (plane crash). Molecular identification of the victim/s allows closure for families and relatives. The international commission on missing persons has been involved in identifying the remains of missing people of conflict, having typed over 25 000 bones (International Commision on Missing Persons 2008). Notably, from mass graves in the former Yugoslavia they have been able to identify 13 455 missing people (International Commision on Missing Persons 2008). Another instance occurred during the disaster at the World Trade Centre in New York 2001, an identification project was set-up and through this approximately 1500 victims were identified (Holland *et al.* 2003; Bille *et al.* 2004).

2.3.3 Interpretation of Mummified Remains

Mummies are human or animal remains with preserved non-bony tissue, including epidermis, hair and organs (Lynnerup 2007). These tissues are highly sought-after for DNA research as the preservation appears good with the assumption being that the DNA is also well preserved (Lynnerup 2007). While these tissues are well preserved, the integrity of nucleic acids for molecular research remains a problem. This is especially dependent on the type of mummification. Natural mummification can occur due to both extremes of hot or cold. In artificial mummification there is the risk of inhibitors and chemical modification of the DNA which affects PCR amplification. There are cases such as the famous "Ice Man" where DNA has been successfully extracted. Eight DNA extractions were prepared from muscle and connective tissue and

these results were compared to living populations in search of the closest living population, these were present-day Europeans living north of the Alps (Handt *et al.* 1994). Since this report on the "Ice Man", Konomi *et al.* (2002), in a bid to improve the methodology and to obtain better yields of DNA, have reviewed the DNA extraction methods previously used on mummified tissues and found that methods containing Guanidium thiocyanate are more efficient than commercial kits. With mummified remains it is essential to understand the taphonomic conditions responsible for mummification as these directly affect the preservation of the DNA molecule.

2.3.4 Environmental Reconstruction

DNA studies can be used to discover the origins of plant/animal domestication and the subsistence practices of previous populations (Pääbo *et al.* 2004; Yang & Watt 2005). DNA taken from permafrost ice cores in Siberia dated at 2000-4000 BP revealed a broad range of DNA diversity from fungi, plants, algae and protists, which aided in the reconstruction of the local palaeobiology (Mitchell *et al.* 2005). Diet and the behaviour of prehistoric populations can be reconstructed by extracting mammalian DNA from deposits in residues found on middle palaeolithic stone tools (Hardy & Raff 1997).

2.3.5 Palaeodemography

Palaeodemography is the study of a population that existed in the past by assessing population size, rate of fertility, mortality and to gauge the health of a population (Mai *et al.* 2005). DNA studies can be applied to different levels of society, ranging from the local population to the family (lineage reconstruction) and also to the species level (Kaestle & Horsburgh 2002). A study by Jones (2003) provides a good example of palaeodemography. He reviewed DNA information on the local kinship and the movements of people in Pre-Columbian times. More recently, Yue *et al.* (2006) used DNA analysis to map the genetic relationship of Chinese Khitan nobles from 1043-825 BP in the Liao Dynasty

to their closest living relatives, those of present North Asian populations.

Closely linked to palaeodemography is palaeopathology, a discipline that assists in understanding the origins of diseases in human populations and how they have changed over both time and space (Mitchell *et al.* 2005; Papagrigorakis *et al.* 2006). In order to identify the pathogen, DNA is extracted from bacterial, protozoal and viral pathological skeletal lesions. There has been a specific focus on *Mycobacterium tuberculosis* and sickle cell anaemia DNA derived from archaeological human bone samples (Faerman *et al.* 2000; Haas *et al.* 2000; Taylor *et al.* 2001; Mays *et al.* 2002; Fletcher *et al.* 2003).

Part I

Development of Methods

Chapter 3

Introduction

The aim here is to devise novel methods of molecular sex determination suitable for skeletal material, and to optimise their use on a collection of miscellaneous archaeological skeletons (*ex-situ*) sourced from the Dart Collection. As mentioned previously, in South Africa DNA extraction has been conducted on fresh teeth and blood from living populations. However, molecular sex determination from archaeological skeletal tissue has not previously been accomplished on material from the Dart Collection, nor in this country.

In order to develop a novel molecular sex determination system from bone, it was first necessary to devise an acceptable bone extraction method that is minimally destructive to skeletal material. For a successful outcome the objectives of bone extraction, DNA extraction and molecular sex determination outlined below are reliant on each other.

Bone Extraction: An appropriate location on the skeleton is selected that does not interfere with any morphometric points, and meets specific criteria needed for a positive bone powder extraction. The rationale here is to choose an optimal method of bone extraction which minimises contamination, but maximises the yield of bone powder.

DNA Extraction: This entails selecting the most appropriate DNA extraction method for bone and modifying the protocol in order to increase the amount and integrity of the extracted DNA. DNA is extracted from

human control samples of blood and bone, and archaeological bone from the *ex-situ* collection.

Molecular Sex Determination: Two systems of conventional PCR are used with specific primers to amplify the amelogenin gene, which is suitable for sex determination from skeletal material. In the first system, sex is diagnosed through sequencing to identify 10 sex specific polymorphisms. The second system uses automated microfluidic gel electrophoresis to identify a sex specific indel and the product is subsequently confirmed with sequencing. In addition, 10 sex specific polymorphic regions are localised. The efficacy of both systems is first tested using blood controls from male and female subjects, and then applied to male and female bone controls. Once the procedures have been tested and are performing with 100% efficiency on the controls, they are optimised on pooled DNA from the *ex-situ* sample. Finally these methods are applied to determine sex from DNA extracted from the *ex-situ* specimens sourced from the Dart Collection.

The methods developed here are discussed and incorporated into each of the following chapters.

Chapter 4

Materials

4.1 Introduction

When conducting research on skeletal tissue of any age, the recommendations for aDNA research should be used, as they provide strict parameters that ensure an authentic result. Therefore, for the present research even though the skeletal material falls under the category of archDNA the materials and methods are designed using the guidelines for aDNA research.

4.2 Control Samples

Throughout the optimisation of this research, different specimens were used. With regard to the bone extraction method miscellaneous skeletal remains of cadaver origin were accessed from the so-called X collection housed in the School of Anatomical Sciences. This collection consists of incomplete or damaged skeletal specimens that were de-accessioned from the Dart Collection.

For the optimisation of the PCR and DNA extraction methods, 6 human blood controls were used. One female and one male were used from three populations from different geographical regions (African, Asian and European). In addition, one male and one female sample of control bone were used (donated by Dr. N Duneas, Centre for Tissue Engineering, Tshwane University of Technology).

Ethical clearance was obtained to use these specimens through the University of the Witwatersrand Human Research Ethics Committee, under the ethics number M050465.

4.3 Dart Collection

The School of Anatomical Sciences at the University of the Witwatersrand contains a large skeletal collection known as the "Raymond Dart Collection of Human Skeletons". This collection was built up by an eminent anthropologist Raymond Arthur Dart (1893-1988), the second head of the Anatomy Department (now the School of Anatomical Sciences) at the University of the Witwatersrand. While Dart was in America he was exposed to two cadaver-derived collections, at Western Reserve University in Cleveland, Ohio and at Washington University in St. Louis, Missouri. These influenced him to institute a similar collection at the University of the Witwatersrand (Tobias 1987; Tobias 1991).

Dart was appointed head of the Anatomy Department in 1923 by the time he retired in 1958, he and his staff had accumulated roughly 2000 skeletons. When P.V. Tobias assumed headship, he ensured that this collection became a legacy within the Anatomy Department and continued to collect skeletons for the duration of his tenureship. Today, this is a world renowned collection, to which additions continue to be made (Tobias 1987; Tobias 1991; Tobias 1998).

The Dart Collection currently comprises of more than 3000 skeletons. Two types of skeletons are represented: those derived from cadavers and those derived from an archaeological context. Cadaver-derived skeletons are unclaimed bodies or bodies donated by relatives to science through the auspices of the University for anatomy dissection. After dissection the cadavers are chemically and physically de-fleshed and the skeletons are stored for research purposes. While some 2605 skeletons in this collection are cadaver-based, the remainder are comprised of skeletal remains derived from archaeological context, the latter being used in the present research.

Figure 4.1: Professor Raymond Arthur Dart (Courtesy of the School of Anatomical Sciences, University of the Witwatersrand).

The archaeologically-derived skeletons housed within the Dart collection have either been excavated, found with no provenance, or are donated to the University. Both donated skeletons and those found without provenance are known as '*ex-situ* material', because they are not associated with any archaeological records. It is essential to point out that they still fall under the category of archaeological material since they decomposed and de-fleshed naturally, as opposed to the remainder of the collection that is cadaver derived. The excavated materials in the collection were retrieved by archaeologists from the University of the Witwatersrand, which are also stored under the archaeological category. Much of this material in the Dart Collection is carefully protected from potentially destructive methods of research in order to preserve the skeletal material. With this in mind, it was important to optimise the methods used in this research first on the *ex-situ* material to ensure their efficacy.

The *ex-situ* material of the Dart Collection represents skeletal elements found mainly in the surrounding areas of Johannesburg, that were not excavated by archaeologists; rather they decomposed naturally and upon exposure to the surface were subsequently brought to the University. While the exact age of these skeletons is unknown, it can be estimated that they are at least fifty years old, as most of them were accessioned into the Dart Collection in the 1950's.

These skeletons serve as an important control for the present study, as they are chemically untreated. Ethical clearance for this collection falls under the human tissues act of 1983, therefore permission was obtained from the School of Anatomical Sciences Collections Committee.

There is a database associated with the Dart Collection that contains information regarding the completeness of the skeleton including the presence or absence of the crania, mandibles and post-cranial skeletons. This database was used to select 30 *ex-situ* specimens from the Dart Collection. Only complete skeletons were used and 180 specimens in the *ex-situ* collection fit this description. The assumption behind the limiting criteria was that the more complete the skeleton, the higher the probability of good DNA preservation. A simple random sample (SRS) was then applied on the 180 specimens to select a sample of 30. Where the specimen was unusable, due to fragmentation or missing the femur, then the next specimen number drawn by the SRS was used, until 30 specimens suitable for use were drawn. DNA extracted from the *ex-situ* specimens was also pooled and used for the final stages of optimisation to be representative of the sample being studied.

4.4 Assessment of Collection

The colour and condition of each bone was assessed using the investigator's criteria based on observation found in appendix A, (tables A.1 & A.2) and were recorded in table B.1. An understanding of the condition of the bone is important as an indicator of molecular preservation. For example, specimens 173, 312, and 1096 were juvenile with unfused proximal or distal ends of the femur (figure 4.2), specimens 294 and 302 were partially mummified (figure 4.3), while other specimens were fragmented.

Figure 4.2: *Ex-situ* specimen 312, demonstrating an unfused metaphysis (arrow) on the proximal end of the left femur. This is indicative of the individual still growing and as a result the specimen was considered to be fragmented. Scale=1 cm.

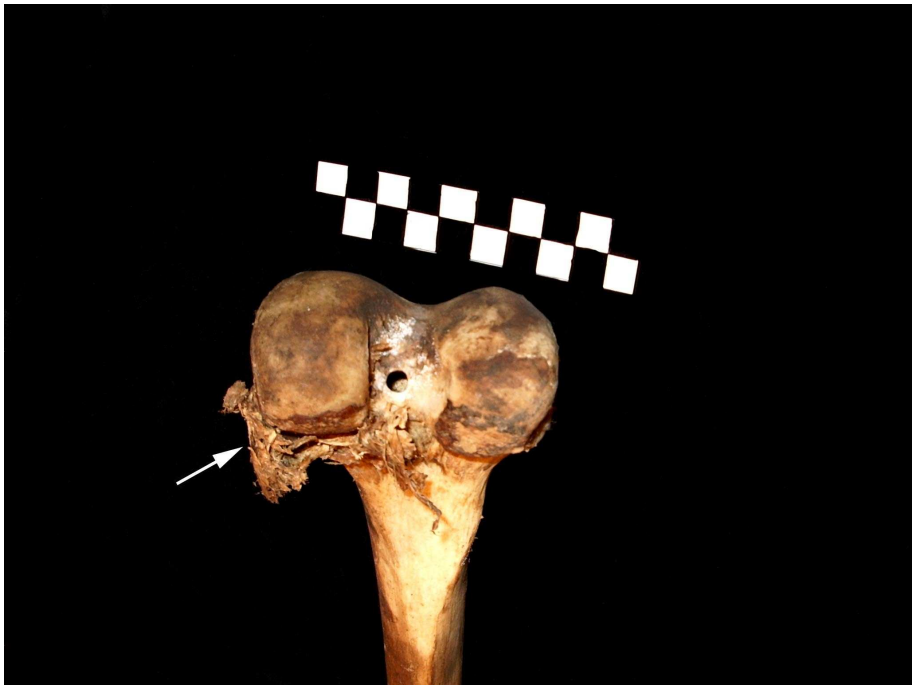

Figure 4.3: *Ex-situ* specimen 302, demonstrating partial mummification (arrow) on the distal end of the left femur. Scale=1 cm.

Chapter 5

Bone Extraction Method

5.1 Introduction

Human skeletal material found in archaeological localities provides a wealth of bio-cultural data on the individuals and populations represented. These data were until recently collected exclusively through the traditional morphometric methods of biological anthropology. In the 1980s, the amount and variety of information that could be gathered dramatically increased, due to the new techniques in molecular biology that made the analysis of genetic material (DNA) from deceased organisms possible (Kaestle & Horsburgh 2002). Bone is often considered an optimal source of DNA, due to the binding of DNA to a calcium phosphate hydroxyapatite, which stabilises and delays DNA degradation (O'Rourke *et al.* 2000). Due to the compact structure of bones, DNA is generally less degraded with time in comparison to soft tissue, which undergoes rapid degeneration (Wurmb-Schwark *et al.* 2003). Skeletal material is also relatively abundant in museums and university collections, providing an ample source of material for sampling.

Historically, DNA extraction from skeletal remains has been detrimental to the holistic value of the specimen, as large sections of bone were removed and destroyed, often leaving behind a mutilated skeleton with missing elements or sections (O'Rourke *et al.* 2000; Kaestle & Horsburgh 2002). This

obviously limits the subsequent use of the specimen for anthropological analysis, which relies on the detailed examination of the skeleton in order to reconstruct the life history of an individual. The skeletal elements commonly used for DNA extraction are the femur (Hagelberg *et al.* 1991; Cattaneo *et al.* 1995; Kolman & Tuross 2000; Wurmb-Schwark *et al.* 2003; Gilbert *et al.* 2005), ribs (Kemp & Smith 2005), vertebrae (Cattaneo *et al.* 1995) and teeth (Meyer *et al.* 2000*a*; Cobb 2002; Gilbert *et al.* 2005). Vertebrae, ribs and teeth are used due to their number; the rationale being that even if one is destroyed (e.g. one rib or a vertebra), the morphometric data can be gained from other such elements. Teeth are valuable as they are more robust than bones and hence a good element for preserving DNA. However, as teeth are extremely valuable for stable isotope and morphometric analysis, it is often difficult to obtain permission to access them for DNA studies. The most commonly used bone for DNA extraction is the femur. Usually, a femoral head, a section of the mid-shaft, or a femoral fragment is removed or an unspecified femoral part is used.

To ensure that the final DNA extraction is uncontaminated, it is vital to use decontamination standards from this point on. There are a number of criteria relating to the bone's physical condition that must be considered for the bone extraction method. For the present study, it was necessary to devise a relatively non-destructive method of bone removal.

5.2 Bone Biology

The adult human skeleton is comprised of approximately 206 individual bones, constituting less than 20% of the body weight (White 2000). Bones are an imperative mechanical component of the musculoskeletal system and in locomotion. They have important functions for protecting and supporting soft tissues. Bones also produce blood cells as well as store fat and minerals (White 2000). They are a living tissue that can repair and reshape itself in response to external stresses.

There are four types of bones according to shape: long (femur, humerus, tibia), short (metacarpals, metatarsals, phalanges), flat (scapulae, cranial plates, innominates) and irregular (vertebra, carpals, tarsals, cranial bones) (Bass 1995). Compact bone or cortical bone is the solid, dense layer that is found in the walls of bone shafts and on the external surfaces. In comparison cancellous or trabecular bone is spongy or porous with a honeycomb structure. It occurs inside the compact layer, particularly under protuberances (where tendons attach), in vertebral bodies and at the ends of the long bones (Bass 1995; White 2000). The molecular and cellular compositions of compact and cancellous bone are identical, differing only in porosity. Bone is a composite material formed of protein (collagen) and mineral (hydroxyapatite) (White 2000). Long bones have the same structural components, and these are illustrated in figure 5.1.

Bone is comprised of a connective tissue composed of osteocytes with the same molecular and cellular composition in all mammals. It is made up of both organic and inorganic material. The organic matrix attests for bone's tough and elastic nature, while the inorganic material provides hardness and rigidity. The majority of the material in the bone matrix consists of inorganic minerals. This section of bone is made up of hydroxyapatite crystals, 85% of which is calcium phosphate, 10% calcium carbonate, with the remainder being an admixture of calcium fluoride, magnesium phosphate and sodium chloride (Bass 1995; White 2000). The mineral component fills and mixes with the organic collagen matrix creating a weave of protein and minerals, which gives bone its structural properties. The organic matrix consisting of collagenous fibres, mucopolysaccharides (chondroitin sulphate and hyaluronic acid) and proteins enables nutrients and blood to flow through the bones (Coetzee 1987). Collagen intertwines to form flexible and slightly elastic fibres in bone and 90% of the bone's organic matrix is comprised of collagen (White 2000). Cells and DNA are found within the organic matrix; post mortem these fuse to the bone's inorganic structure, specifically to the hydroxyapatite crystals.

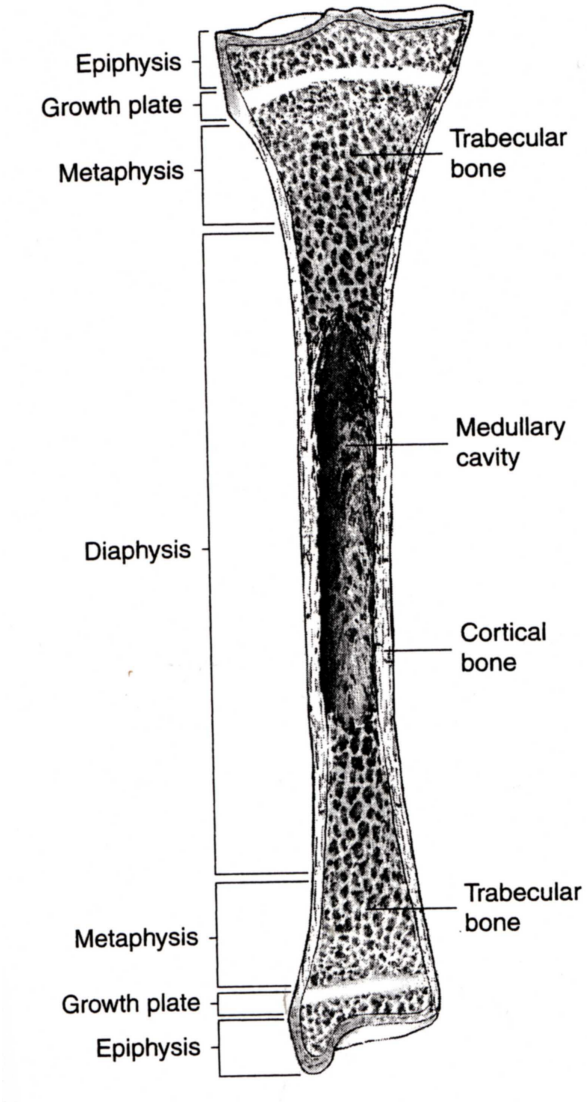

Figure 5.1: The structural components of a human long bone (tibia). Note the main structures: the deposits of trabecular/cancellous bone in both ends and the thick outer layer of cortical bone (From figure 2.33, Byers, 2005).

5.3 Bone Extraction Method

Specific criteria were developed in the present research to determine the optimal area on the skeleton to harvest a bone sample suitable for DNA analyses. The bone extraction method should firstly, not interfere with any known morphometric sites used for individual reconstruction. Bone with a significant amount of the cancellous component should be used. The skeletal element must not be destroyed. Lastly, bone preservation should be considered as it is

associated with DNA integrity (Haynes & Searle 2002). This preservation was assessed by examining the physical appearance of the bone based on softness, cracking and flaking. Damaged surfaces and lesions provide avenues for contamination from the environment into the bone. It follows that no bone with any damage or lesions on the external surface, or at the distal and proximal ends should be used.

Cancellous bones can yield 10 to 20 fold more DNA compared to compact bone (Lee *et al.* 1991). The compact outer layer of bones provides a layer of protection for the cancellous bone, therefore the DNA is generally less degraded with time (Machugh *et al.* 2000; Wurmb-Schwark *et al.* 2003). The chosen element must have a good yield of cancellous bone, as it is much easier to pulverise and is typically protected by a layer of robust compact bone. This eliminates the ribs and smaller bones from the selection as they do not contain ample cancellous bone. Taking into consideration the above criteria, the distal and proximal ends of long bones were considered the most suitable for bone extraction.

Unlike the proximal end of the femur, the distal end is superior as the lateral and medial condyles have no definitive features of identification. In addition, there is a good store of cancellous bone at the distal end that is protected from environmental contamination by a hard layer of compact bone. Bearing this in mind, in addition to the criteria listed above, it appears that the best area on the skeleton for the bone extraction was the distal end of the femur in the region of the intercondylar fossa. The left side was chosen for all specimens as the right side is more commonly used in metrical and morphological analysis.

It has been found that 40 to 210 mg of bone powder is sufficient for DNA extraction (Mays *et al.* 2002). This range was used as a guideline for the amount of bone necessary for a successful DNA extraction.

To avoid contamination the sample must be carefully prepared prior to extraction, using strict decontamination measures to minimise the risk of a false positive result in the PCR (Yang & Watt 2005). Hypochlorite (household bleach)

was the most cost-effective means for decontaminating the surface of bones (Kemp & Smith 2005). The surface was washed with hypochlorite and ethanol (Saarchem) and the first layers of bone physically removed: if possible the material was extracted from the internal surface (Kemp & Smith 2005).

Identifying the most suitable method for bone extraction involved numerous procedures. The sample must be mechanically broken down to a powder form. Extra manipulation however, increases the surface area for contaminant DNA molecules to bind to (O'Rourke *et al.* 2000). The most commonly used method for powdering the bone uses liquid nitrogen with a pestle and mortar (Hagelberg *et al.* 1991; Höss & Pääbo 1993; Cattaneo *et al.* 1995; Kolman & Tuross 2000). In a pilot study for the present research this method was found to be inadequate, as the bone dispersed and the end product was not a fine powder. In addition, the pestle and mortar were difficult to clean due to the fine grain of the composite material.

Figure 5.2: Method of bone extraction from a human femur. A sterile titanium bit attached to a power drill was used to make a small hole and extract bone tissue from the inner table of the long bone.

Other researchers have had positive results using a commercially available bone mill or freezer mill (Hagelberg & Clegg 1991; Meyer *et al.* 2000*a*), however, this

method was not financially viable.

A simple effective method of producing bone powder was accomplished using a sterile titanium bit attached to a power drill, illustrated in figure 5.2. After drilling, the bone powder was poured directly into a sterile container. This method decreases the amount of contamination. It only forms a small hole on the surface and does not interfere with any known anthropometric landmarks, as shown in figure 5.3. While the use of this region ensures minimal destruction to the bone, it also provides enough bony tissue for DNA analysis. This technique was only marginally invasive, and does not physically compromise the femur's use in future studies.

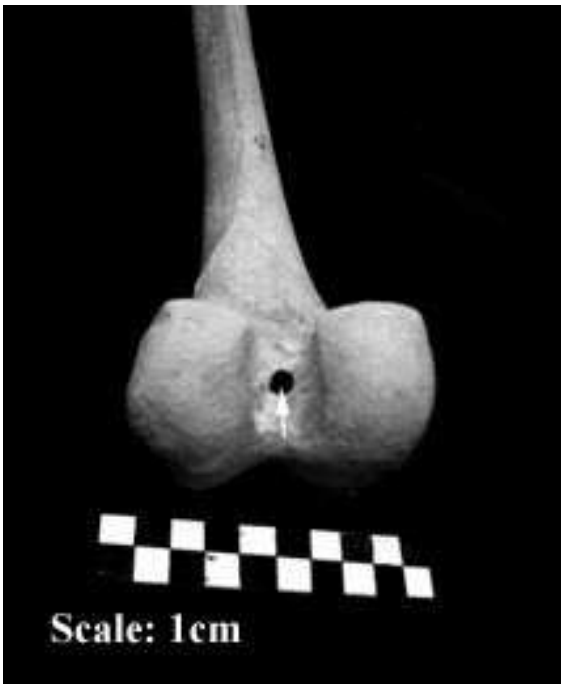

Figure 5.3: Demonstration of the hole created in the femur following the bone extraction process.

5.4 Methods

Strict contamination precautions were taken as prescribed for aDNA research (Hagelberg & Sykes 1989; Haas *et al.* 2000; Machugh *et al.* 2000; O'Rourke *et al.* 2000; Brown 2001; Mays *et al.* 2002). Protective clothing was worn, consisting

of doctor's scrubs, latex gloves, hairnet, face mask and booties (Hagelberg *et al.* 1991; Kolman & Tuross 2000; Cobb 2002). Drill bits and 50 ml centrifuge tubes were autoclaved (Stericlav-28), in addition a new bit and pre-weighed tube were used for each extraction.

The bone extraction was performed under a sterilised laminar flow hood to protect the working surfaces. The extraction area was sterilised with 0.6% sodium hypochlorite followed by 70% ethanol (Hebsgaard *et al.* 2005; Kolman & Tuross 2000; Wurmb-Schwark *et al.* 2003; Wurmb-Schwark *et al.* 2004*a*). Under the flow form hood, paper towelling was placed over the cleaned working surfaces. The entire surface of the femur was first wiped down with 0.6% sodium hypochlorite followed by 70% ethanol (Kemp & Smith 2005). The surface in the intercondylar fossa was scraped with a scalpel to remove contaminant DNA from the surface, then wiped down with 70% ethanol (Hagelberg & Clegg 1991; Zoledziewska *et al.* 2002; Holland *et al.* 2003). The femur was held by the neck in the left hand, in a vertical position, while a power drill (Bosch PSB 650RE) with a sterile 4.5 mm titanium masonry drill bit, was used to create a hole between the lateral and medial condyle in the intercondylar fossa. Once through the cortical surface, a rotating action was used to pulverise the cancellous bone from the inner table. The drill bit was withdrawn and the femur inverted over the open mouth of a 50 ml tube. The bone powder was gently tapped into the tube to collect approximately 1 g of bone powder. The extracted bone powder was stored for a short period of time at 4 °C. In between each extraction gloves and surfaces were washed and wiped down with 0.6% sodium hypochlorite, followed by 70% ethanol.

5.5 Results

Once devised, this optimised method was applied to 30 *ex-situ* skeletons sourced from the Dart Collection. This was a good sample to establish the method on and to demonstrate its use for more valuable material. Access to these skeletons was only granted after it was shown that the method was minimally

invasive to the skeletal material from the X-collection.

The results from the 30 *ex-situ* samples was a fine to medium bone powder with an average yield of 0.85 g (table B.1). There was individual variation in the amount of the bone yielded, which could be attributed to the fact that bone mass decreases with the ageing process.

5.6 Discussion

It has been suggested that the use of an electronic drill could reach very high temperatures degrading the DNA within the bone. Two experiments were set-up to test the temperature emitted from the drill for this newly developed method. The materials and methods for these experiments are available in appendix B. To measure the temperature a copper-constantan thermocouple probe connected to an laboratory digital thermometer (Physitemp, Model BAT-12; Sensortek Inc., Clifton, NJ, USA) was used. This was tested against a mercury thermometer. On average a mercury thermometer took up to 15 seconds to take a measurement, while the superior thermocouple probe took only 3-5 seconds. The accuracy of the measurements according to the Sensortek Inc. company "The BAT-12 has 0.1 °C resolution and super accuracy of 0.1 °C 1 digit between 0-50 °C, 0.1 °C 1 digit over full range. The temperature range is -100 to +200 °C. The BAT- 12 provides fast readings, usually within 2-5 seconds depending on the temperature probe in use."

For the first experiment the change in temperature was measured for initial breakthrough and during the pulverisation procedure for both the drill bit and the bone. The results are recorded in tables B.2 and B.3. The average increase in temperature for initial breakthrough of the cortical bone on the drill bit was 0.62 °C and for the hole in the bone was 0.36 °C. For the pulverising action the average increase in temperature of the drill bit was 0.58 °C and for the hole in the bone it was 0.42 °C. During this experiment it was noted that the drill bit had the greatest increase in temperature during the initial breakthrough. On the other hand the bone experienced the greatest temperature change during

the pulverisation procedure.

In the second experiment the temperature was recorded from within the bone during the entire drilling process. The temperature probe was placed inside an existing hole and the temperature was recorded while drilling and the measured temperatures are recorded in table B.4. The change in average temperature recorded both while and after drilling was 0.84 °C. One notable feature of the change in temperature seen in both experiments was that the temperature increase being dependent on the quality of the starting material. For younger specimens with thicker cortical bone and a high density of cancellous bone the extraction process takes slightly longer, thus increasing the temperature. The opposite result was found for specimens with low bone density and thin cortical bone, there was virtually no change in temperature.

The maximum change from ambient temperature was 0.84 °C, therefore it can be stated with confidence that the temperature for this drilling process does not reach excessively hot temperatures that would destroy the DNA. Especially in comparison to studies that have extracted DNA from incinerated remains that have endured temperatures upwards of 150 °C (Urbani *et al.* 1999; Wurmb-Schwark *et al.* 2004*b*).

5.7 Conclusion

The method developed here is suitable for analysis from human skeletal remains, using a novel area on the human skeleton within the intercondylar fossa of the left femur. Further, following the bone extraction the specimen can still be used for future morphometric studies. This method has been published (Woodward *et al.* 2006) (appendix J).

Chapter 6

DNA Extraction Method

6.1 Introduction

The technique involved in extracting DNA from skeletal tissue is sensitive and innovative. In extracting DNA, irrespective of the source, there are three main objectives to be considered: the DNA should not be contaminant DNA, the DNA should not be degraded and it should be as pure as possible.

Mitochondrial DNA has been commonly used in arch/aDNA studies due to its high rate of mutation, matrilineal descent and because it consists solely of exons, all of which are excellent characteristics to study human variation and evolution. However, it is necessary to have both sex chromosomes to distinguish between a male and a female. Nuclear DNA was used in this research as it carries both parental genetic information.

6.2 DNA Extraction

6.2.1 Blood

DNA was extracted from blood and used as a control to develop the methodologies to determine sex that would ultimately be applied to DNA derived from

bone; the rationale being that if the system works on blood it only requires optimisation to be applied to bone. DNA was extracted from the 6 blood controls using an ethanol based precipitation method (Miller *et al.* 1988) as it allowed a large volume of blood (10 ml) to be processed at once, resulting in enough control DNA for the entire study. The silica membrane method is limited for blood extractions, as it can only process 100 μl of blood at a time.

6.2.2 Bone

Over the relatively short history of anthropological genetics, many DNA extraction methods have been used to assist in the identification of human remains. The two most commonly used techniques are phenol chloroform and silica column extractions. The phenol chloroform method requires a fumehood as it is hazardous. It is time-consuming and contains PCR inhibitors (Yang *et al.* 1998). This method is not commonly used in forensic cases as this system usually fails due to PCR inhibitors. Therefore, it is not suitable for anthropological genetics (Cattaneo *et al.* 1995). The silica column extraction methods or variations thereof are preferred, as the result is high quality DNA. This method is simple, fast, effective and results in a product with fewer PCR inhibitors (Höss & Pääbo 1993; Yang *et al.* 1998; Brown 2001; Mays *et al.* 2002; Fletcher *et al.* 2003; Holland *et al.* 2003; Bille *et al.* 2004; Kemp *et al.* 2006; International Commision on Missing Persons 2008). Based on the acceptance and reliability of the silica-based DNA extraction method in the literature, the use of this approach was explored. The South African Police Services was contacted to enquire about the method of DNA extraction they use and prefer for processing forensic samples. Their approach uses the silica-column based kit (Qiagen Micro kit 50) that was also employed in the present research.

Prior to the DNA extraction the sample was prepared using strict decontamination measures. Failure to follow these directly affects the amount and quality of the DNA retrieved (Holland *et al.* 2003). It is suggested that the laboratory where the DNA extraction is conducted should be physically separated from

both the initial bone extraction area and subsequent PCR amplification laboratories (Faerman *et al.* 2000). A blank control extraction without bone was carried out in parallel with the bone extractions to monitor potential contamination.

As bone is understandably a difficult tissue from which to extract DNA, it is logical that chemically reducing the bone as much as possible will result in an increased yield of DNA (O'Rourke *et al.* 2000). An important step in this process is the enzymatic lysis of the sample. Hagelberg and Clegg (1991) compared three different DNA extraction methods from bone, each of these involved changing the lysis step during the extraction, followed by a phenol chloroform extraction method. The method containing the calcium chelating agent tetrasodium ethylenediaminetetra-acetate dihydrate (EDTA) and the enzyme proteinase K provided the highest yields of DNA.

EDTA reduces the concentration of calcium in bone, while the proteinase K purifies the nucleotides by digesting the associated proteins. The advantages of using proteinase K and EDTA together is that they assist in the breakdown of the mineral aspect of bone, releasing the organic components which contains the DNA. Having reviewed many different DNA methodologies (for example Hagelberg & Clegg 1991; Höss & Pääbo 1993; Yang *et al.* 1998; Kolman & Tuross 2000; Zoledziewska *et al.* 2002; Fletcher *et al.* 2003; Holland *et al.* 2003; Bille *et al.* 2004), it was decided to include EDTA in the lysis step.

Based on the ratio of the organic content (35%) relative to the mineral content (65%) of bone 0.5 M EDTA was used (Duneas 2005). The efficacy of EDTA on DNA extraction from human control bone was tested at three different stages in the protocol, prior to lysis, during lysis together with proteinase K, post lysis and with and without a "QIA-shredder" (Qiagen). The shredder assists in the breakdown of any remaining tissue in the tube. The addition of EDTA to the lysis buffer, without the use of the QIA-shredder gave the highest yield of DNA. Therefore, the addition of 0.5 M EDTA (pH 8.0) was included in the lysis buffer with the proteinese K (Qiagen) and this was incubated overnight at 56 °C. The DNA extraction protocol from bone was used with 40 mg of

bone powder from each of the 30 *ex-situ* specimens.

6.3 Methods

6.3.1 From Blood:

The recipes for the reagents required for this protocol are detailed in appendix C.

DNA Extraction Protocol For Blood (Miller *et al.* 1988)

Prepare solutions: Sucrose Triton X lysing buffer, T20E5, Saturated NaCl, 10% SDS, 0.5 M EDTA, 10 mg/ml Proteinase K, Proteinase K mix, ice cold 70% ethanol and room temperature absolute ethanol. Autoclave 3 x 50 ml tubes per sample, and 1.5 ml eppendorfs.
Blood must be collected in EDTA tubes.
Work on ice. Two ice buckets, one for solutions and one for samples.

1. Use 10 ml of blood, place into a 50 ml sterile tube and mix well.
2. Add 45 ml cold lysis solution, **Mix well**.
3. Spin for 10 minutes at 2300 rpm at 4 °C.
4. Discard supernatant and re-suspend pellet in 20 ml lysing buffer.
5. **Put on ice for 5 minutes or at -20 °C for 5 minutes.**
6. Spin for a further 10 minutes at 2300 rpm 4 °C.
7. **A white pellet should be observed; if red blood cells are present then lyse again.**
8. Discard supernatant and add 3 ml T2OE5 to each tube, and **mix well**.
9. Add 20 μl 10% SDS to each tube and **mix well**.

10. Add 500 μl Proteinase K Mix and place samples at 42 °C to 50 °C.

11. Vortex after 1 hour and put back at 42 °C overnight.

DAY 2

Pellet should be dissolved, if not then vortex until dissolved.

Work on ice.

1. Add 1 ml saturated NaCl solution to each tube. Mix well.

2. **Place on ice for 5 minutes.**

3. Spin at 2300 rpm 4 °C for 30 minutes.

4. Supernatant should be clear with a pellet at the bottom.

5. If supernatant is cloudy: decant into a new tube and spin for another 15-20 minutes at 2300 rpm, then follow for clear supernatant.

6. If supernatant is clear: the pellet **(IS NOT DNA)** consists of precipated proteins and the DNA should be present in the supernatant. **Keep Supernatant.**

7. Transfer supernatant to a clean 50 ml tube and add 20 ml absolute ethanol at room temperature.

8. DNA should be present as a white aggregate.

9. **If not present:** Place at -70 °C for 30 minutes then spin for 20 minutes at 2300 rpm 4 °C. **OR** at -20 °C overnight and spin for 20 minutes at 2300 rpm 4 °C.

10. **If present:** Fish or spool DNA out using a pipette. Wash pellet gently with ice cold 70% ethanol, add just enough to cover centrifuge at 2000 rpm for 5 minutes then take as much ethanol off as possible with pipette. **Do not spin genomic DNA faster than 2000 rpm or it will redissolve.**

11. Air Dry pellet for 30 minutes to 1 hour at room temperature.

12. Dissolve DNA in appropriate amount of water to a maximum of 1 ml.

13. Stand tube at 37 °C, or place on a mixing wheel for 1-2 hours.

14. Measure concentration of DNA on a spectrophotometer.

6.3.2 From Bone:

The fine bone powder extracted from the femur was processed using a silica-based DNA extraction kit (QIAamp DNA Micro Kit 50). Additional items to supplement the DNA extraction kit include 100% ethanol, 1.5 ml plastic eppendorf tubes, a vortex (MT19 Delus Vortex Mixer), a mini centrifuge (Stratagene Picofuge), thermometer, heating block (Häger Designs Dry Block Heater) and a bench-top centrifuge (Sigma 302).

Prior to the DNA extraction the counter tops in the DNA extraction area were sterilised with 0.6% sodium hypochlorite followed with 70% ethanol. Then paper towelling was placed over all the countertops. All the materials used were sterilised in an autoclave (Sterilclav-28). The pipettes were wiped down with 0.6% sodium hypochlorite and 70% ethanol before use. Gloves and protective clothing were worn at all times, and hair held in a hair net.

From each specimen, 40 mg of the powdered bone sample was weighed out on a digital scale (Ohaus GA200D) and then placed in a 1.5 ml micro-centrifuge tube. The protocol followed was modified from the "Isolation of Genomic DNA Tissues protocol" of the Qiagen DNA handbook. The modification involved the addition of 100 μl 0.5 M EDTA (pH 8.0) in the lysis step to assist with the digestion of the bone prior to the incubation. During the lysis period of 24 hours, the samples were vortexed three times, followed by the remainder of the protocol. A negative tube without bone was run in parallel with the samples to ensure no contamination during this procedure.

DNA Extraction Protocol For Bone: Modified from QIAamp DNA Micro Kit Handbook (pg 35)

1. Transfer 40 mg of bone powder into a 1.5 ml microcentrifuge tube.
2. Add 180 μl of buffer ATL.
3. Add 20 μl of Proteinase K.
4. Add 100 μl of Na_4EDTA and mix by pulse-vortexing for 15 seconds.
5. **Place the 1.5 ml microcentrifuge tube in an incubator for 24 hours at 56** °C**.**
6. Add 200 μl buffer AL and 1 μl of carrier RNA then mix by pulse-vortexing for 15 seconds.
7. Add 200 μl of 100% ethanol and mix by pulse-vortexing for 15 seconds.
8. **Incubate at room temperature for 5 minutes.**
9. Briefly centrifuge to remove drops from inside the lid.
10. Transfer entire lysate to QIAamp MiniElute column without wetting the rim, centrifuge at 8000 rpm for 1 minute.
11. Place column into a clean 2 ml collection tube and discard the collection tube containing the flow-through.
12. Add 500 μl of buffer AW1, centrifuge at 8000 rpm for 1 minute.
13. Place column into a clean 2 ml collection tube and discard the collection tube containing the flow-through.
14. Add 500 μl of buffer AW2, centrifuge at 8000 rpm for 1 minute.
15. Place column into a clean 2 ml collection tube and discard the collection tube containing the flow-through.
16. **Centrifuge at 10 000 rpm for 3 minutes to dry the membrane completely.**
17. Place column into a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the flow-through.

18. Add 40 μl of H_2O, **incubate at room temperature for 5 minutes**.

19. Centrifuge at 10 000 rpm for 1 minute.

After the elution in 40 μl of H_2O, the concentration and purity of the extracted DNA was assessed spectrophotometrically (Nanodrop-1000). The DNA samples were then stored at -20 °C until the PCR amplification.

6.4 Results

Instead of a conventional spectrophotometer the Nano-Drop (ND-1000, Thermo Fisher Scientific) was used here, due to its accuracy and its sensitive detection of DNA concentrations from 2-3700 ng/μl. In addition, it uses only 1 μl of sample and this is important as the volume of extracted archDNA from each sample in the *ex-situ* collection is minute.

Table C.2 shows the results of the DNA extracted from the *ex-situ* specimens obtained from 1 μl of the DNA solution. Based on the A260/A280 ratio, which measures the purity of the DNA sample. In these results the purity of the DNA varied considerably. A value of 1.8 is pure and has no contamination. However, a value below 1.8 indicates contamination with proteins and aromatic substances and above 2 is indicative of RNA contamination. For example, sample A75 has a ratio of 1.82, which means that this DNA sample is relatively pure without contamination from RNA, proteins or aromatic substances. Sample A173 has a purity ratio of 1.53 which is indicative of contamination from aromatic substances and proteins. Sample A26 with a purity ratio of 2.02 is indicative of RNA contamination.

Chapter 7

Molecular Sex Determination Method

7.1 Introduction

The sex chromosomes can be used to determine the sex of individuals sourced from skeletal remains. In order to determine sex, the DNA must be isolated and amplified using specific primers for sex linked genes.

There are a number of systems of sex identification available that use skeletal tissue. Four types of systems are mainly used: real-time PCR with fluorescent labelled probes specific to each the X and Y chromosomes (Alonso *et al.* 2004); a common forward primer and specific reverse primer to each of the X and Y chromosomes (Faerman *et al.* 1995; Faerman & Bar-Gal 1998; Sivagami *et al.* 2000); STR typing with multiplex PCR that is designed to isolate multiple loci for personal identification including the determination of sex (Hummel & Schultes 2000; Holland *et al.* 2003; Bille *et al.* 2004; Wurmb-Schwark *et al.* 2004*a*; Reuter *et al.* 2005; International Commision on Missing Persons 2008); and a common forward and reverse primer are used to isolate both the X and Y fragment in one PCR reaction that span an indel, and sex is determined based on band separation on a 10% polyacrylamide gel (Mannucci *et al.* 1994; Haas-Rochholz & Weiler 1997; Cattaneo *et al.* 1997; Meyer *et al.* 2000*a*; Zoledziewska *et al.* 2004; Arnay-de-le-Rosa *et al.* 2007; Fontanesi *et al.* 2008).

The use of real-time PCR with fluorescently labelled probes was extensively researched over the course of a year. However while a product was obtained and the correct amelogenin amplicon was amplified, the specific isolated polymorphism failed to distinguish sex. This method failed even after extensive reanalysis and expert redesign of the probe by the supplier.

A common forward primer with two sex specific reverse primers was also attempted using real-time PCR to determine sex based on melting curve analysis. As this method produced inconsistent sex results even on the blood controls, it could not be used to accurately determine sex.

STR typing kits are designed for forensic cases to isolate numerous loci for comparison to existing DNA and to search for a positive match. This is important in cases involving the recently deceased, as the purpose of forensic study is to identify DNA from a specimen to a specific person or a living relative. These kits are very expensive and in arch/aDNA cases the use of these kits is often not feasible for studies focused only on the diagnosis of sex. one such sex identification kit (Promega) was tested; it spans an indel and distinguishes sex based on band separation on a 10% polyacrylamide gel. When the system failed it was difficult to troubleshoot, as amplification in kit form is pre-designed and the results cannot be sequenced. The primers are also patented, which makes it difficult to determine whether or not the correct fragment has amplified. Further, it is now published knowledge that this specific kit produces problematic results (Michael & Brauner 2004). Sex identification in a kit form also has the problem of optimisation, these kits are designed for optimal conditions, which are specific to different forensic related circumstances, and these conditions are often not applicable for arch/aDNA samples.

After attempting these existing systems of molecular sex determination, it was found that the only feasible method was to use a common forward and reverse primer, to isolate both the X and Y fragment in a single PCR reaction. These primers need to span identifying features such as polymorphic sites or an indel where sex is determined based on electrophoretic separation of PCR specific products and on sequencing. Electrophoresis using a 10% polyacryalmide gel

was assessed with the Promega kit and did not prove to be a reliable method of band separation. Therefore, another method to distinguish two bands on a indel system was deemed necessary. Sequencing is a superior option, not only to determine sex, but also to confirm that the correct area on the genome has been amplified. Through the examination of these methods it was found that isolating only one polymorphic site or an indel runs the risk of producing a lower number of results, as sometimes one area will not amplify in arch/aDNA samples. A novel method of sex determination was deemed necessary to improve on existing techniques by designing an efficient and more effective method. In particular the rationale was to perfect a technique that isolates more than one definitive sex feature, using a unique area on the genome. In addition, the intent is to devise a suitable method for distinguishing sex on an indel not previously used.

7.2 Sex Determining Genes

The analysis of DNA sequences specific to the X and Y chromosomes provides an expedient solution for sex determination (Faerman *et al.* 1995). Many sex specific (X-Y) genes have been discovered in the human genome (Lahn & Page 1999). The most commonly used sex determining genes are the sex determining region Y gene (SRY locus), zinc finger protein (ZF) and the amelogenin (AMEL) gene.

The SRY gene is located on the Y chromosome and controls the development of mammalian testes (National Center for Biotechnological Information n.d). The major disadvantage with the SRY locus is that it only amplifies the Y chromosome. This is particularly significant when using ancient samples where there is a high rate of failure in PCR amplification. Further, a negative amplification cannot be assumed to be a female specimen. Therefore, in order to determine sex using SRY, it must be used in conjunction with another gene that is indicative of the female sex, either a gene on the X chromosome or with the use of mtDNA.

As the ZFX/ZFY gene is located on both sex chromosomes, it can be co-amplified in one PCR reaction. There are, however, limitations that relate to the structural variations between these genes. The number of exons vary between the two genes and the introns are very short, which make it difficult to design primers that co-amplify both genes (Ensembl Genome Browser n.d; Fredsted & Villesen 2004).

In the present research, the amelogenin gene was used for sex determination. As this gene does not have the same limitations as the two described above, it appears to be the best option of the three sex determining genes.

7.2.1 Amelogenin

As already mentioned amelogenin is the most commonly used human sex determining gene, and it has been used as a molecular sex-typing system in numerous studies involving the use of skeletal material (Faerman *et al.* 1995; Cattaneo *et al.* 1997; Faerman & Bar-Gal 1998; Cipollaro *et al.* 1998; Meyer *et al.* 2000*b*; Sivagami *et al.* 2000; Matheson & Loy 2001; Alonso *et al.* 2004; Fredsted & Villesen 2004; Arnay-de-le-Rosa *et al.* 2007).

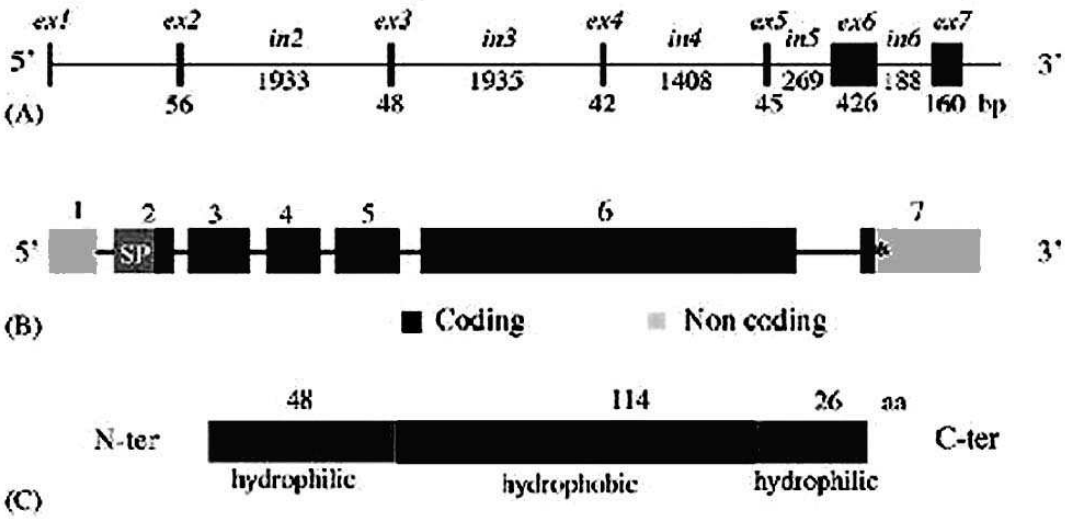

Figure 7.1: Amelogenin gene (A) Gene structure (B) Coding and uncoding exons (C) Linear representation of the native protein (From figure 1, Sire *et al.*, 2005).

AMEL was present in the common ancestor of vertebrates, at least 450-500 million years-ago and was generated by a gene duplication (Sire *et al.* 2005). It is the major protein secreted by ameloblast cells of the inner enamel epithelium of the tooth (Sivagami *et al.* 2000; Moradian-Oldak 2001; Habelitz *et al.* 2004), being responsible for the development and the unique properties of enamel in dental tissues (Snead 2003). Enamel is the hardest tissue in the vertebrate body, and the only place AMEL proteins are found (Chen *et al.* 1998; Snead 2003). These are essential for normal enamel structure and mineralisation in mammals (Moradian-Oldak 2001). If AMEL proteins do not develop properly, this results in amelogenesis imperfecta, an inherited disease affecting the formation of dental enamel in primary and permanent teeth (Sasaki & Shimokawa 1995).

During meiosis a sex chromosome is gained from each parent resulting in either XX (female) or XY (male) genotype. In humans the AMEL gene is present on both the X and Y chromosomes. The level of expression is different for the AMELX and AMELY genes, the X chromosomal gene being expressed at four to ten times higher levels compared to the Y chromosomal gene (Haas-Rochholz & Weiler 1997; Chen *et al.* 1998; Matheson & Loy 2001). The AMELY gene is significantly less active compared to the AMELX genes and could be considered a pseudo gene (Iwase *et al.* 2007).

However, as AMEL varies in size between the two chromosomes, it can be used to differentiate males from females. The major advantage of using this gene is that it can be amplified using one set of primers that target the same region on both of the X and Y AMEL genes (Buel *et al.* 1995; Holland *et al.* 2003). Another advantage is that it can be used to determine sex using short fragments of DNA, which is ideal for aDNA studies. Large portions of this gene are also highly conserved. AMEL is located on both sex chromosomes on the Y at p11.2 and on the X at p22.2. The genome location in base pairs of the X is 11.22 m and the Y is at 6.79 m. The two alleles are similar for the exonic sequences as they both have 7 exons, although they differ in the intronic sequences that vary in length (figure 7.1).

7.3 Polymerase Chain Reaction

In 1986 the polymerase chain reaction (PCR) was developed. This technique mimics DNA replication of living cells, allowing fragments of DNA to be replicated to create multiple copies of a gene (Klug & Cummings 1997). Two primer molecules are essential for the amplification process. These are short chains of nucleotides that duplicate the nucleotide sequences on either side of the desired strand of DNA.

PCR is performed by incubating the sample at three different temperatures corresponding to three steps, denaturation, annealing and extension in a thermal cycler (Saiki 1990). Denaturing the DNA forces the double strand to unwind and separate, while the primers bind at the annealing stage. In the extension phase, the specific area the primer codes for is replicated. This is repeated 30 to 40 times resulting in millions of strands of the desired gene being produced (Watson *et al.* 1998).

While PCR can be used for biological samples containing very little or even degraded DNA (Hagelberg & Clegg 1991) there are various difficulties working with arch/aDNA including, no detectable product; a low yield of the desired product, the presence of non-specific background products due to mispriming or mis-extension of the primers; the formation of primer-dimers that compete for amplification with the desired product; and mutations or heterogeneity due to mis-incorporation of nucleotides (Innis & Gelfand 1990). Some of these problems can be overcome by thorough analysis of the appropriate gene sequences and careful primer design to amplify the gene of interest.

Conventional PCR amplifies the desired product and at the end of the cycle its products are analysed based on band size discrimination using a gel electrophoresis. In contrast, real-time PCR demonstrates the progression of the sample amplification from cycle to cycle, by measuring kinetics of the reaction early on during the PCR, so allowing the researcher to observe the results in real-time. While both conventional and real-time PCR are tested here, conventional PCR generated better and more reliable results.

7.4 Molecular Sex Determination Design on the Amelogenin Gene

A unique design of primers based on the amelogenin gene was necessary to isolate and amplify the desired fragment on the AMEL gene. The initial steps toward this, were to first find the entire gene sequence for the human amelogenin gene on both the X and Y chromosomes. Two on-line databases that store current genetic information were used for this purpose: the National Centre for Biotechnology Information (NCBI) and the Ensembl Genome Browser. The correct sequences were verified through, BLAST and BLAT to ensure the sequences were that of the human amelogenin X and Y genes. The entire gene sequence was entered, and the subsequent output matched the sequence to the corresponding gene. The human amelogenin Y sequence was identified and verified with the Ensembl identification number ENSG00000125363 and the human amelogenin X sequence with the Ensembl identification number ENSG00000099721 (Ensembl Genome Browser).

The next step in the primer designing process was to align the two genes to provide a visual comparison of the differences and similarities between them. The gene alignment was conducted using the on-line program ClustalW. This program allows both genes to be entered with an output of the gene alignment. This alignment was then examined for sex specific polymorphisms and indels (insertion or deletion of nucleotides). Primers were designed around these features on the gene using the following rules of design:

Rules for primer design (Dieffenbach *et al.* 1995)

1. Primers should be 17-28 bp long.
2. GC content should be 50-60%.
3. The 3' end should end in a G or a C, or CG GC to increase the efficiency of the primer.
4. Melting temperature (Tm) should be between 55-80 °C.

5. At the 3' end of the sequence of 3 base repeats should be avoided, as this promotes mis-priming.

6. The 3' ends should not be complementary or this could lead to primer dimers.

7. Primer self-complementary, and primer complementary must be tested as this leads to hairpins, where the primer binds to itself or to each other.

The area on the gene that is focused on begins in intron 2-3, spans exon 3 and ends in intron 3-4. This area is ideal for sex determination as it includes 14 highly conserved sex specific polymorphic regions (table 7.1), in addition to an indel of 6 bp (table 7.2). These noticeable differences in the two forms of the gene allow a classification of sex to be determined.

These primers were designed by the investigator and synthesised by the Synthetic DNA laboratory, University of Cape Town, Department of Molecular and Cellular Biology, South Africa. Prior to synthesis these primers were analysed for primer compatibility, hairpins, primer dimers etc. using two programs, PRIMER and DNAMAN.

The primer sequences for the forward primers were as follows, written 5' to 3':
AMEL-fwd GTCTCTYYTAATGTKAACAATTGCAT
AMELse TGTKAACAATTGCATATTGACTTAATC.
FI GGCACCCTGGTTATATCAACTTCA.

The primer sequences for the reverse primers were as follows, written 5' to 3':
AMEL-rev CCAACCATCAGAGCTTAAACTG
AMELas CACTATTCTTTACAGAGCCCAGG.
RI CCATCAGAGCTTAAACTGGGAAGC.

On the AMELX gene, the forward primer begins at the 3855 bp and the reverse primer ends on the 4187 bp of the gene. On the AMELY gene, the forward primer begins at 4463 bp and the reverse primer ends on the 4800 bp of the gene.

Table 7.1: Highly conserved sex specific polymorphic sites in the isolated section of the amelogenin gene. Compiled using information gathered from Ensembl Genome Browser.

Polymorphism	**Base Change**	**AMELX bp position**	**AMELY bp position**
1	T/C	3906	4514
2	T/A	3917	4525
3	A/C	3940	4548
4	G/A	3960	4568
5	C/T	4041	4649
6	T/C	4044	4652
7	G/A	4052	4660
8	A/G	4056	4664
9	A/G	4059	4667
10	G/C	4061	4669
11	T/G	4086	4694
12	T/G	4089	4697
13	T/C	4097	4705
14	G/A	4104	4712

Table 7.2: Indel on the amelogenin gene. Compiled using information gathered from Ensembl Genome Browser.

	AMELX bp position	**AMELY bp position**
Begins	4107	4715
Ends	4107	4720

In a preliminary study, a number of sex determination methods were attempted on modern human blood and bone controls from both sexes. However, the two systems elected here gave the most reliable results, while the other systems tested failed during optimisation.

A method tested early on in this study was a hybridisation probe designed on the focus area of the gene, at the polymorphic site 4 according to table 7.1. This method while extensively tested on human blood controls, failed to discriminate the X and Y chromosomes using melting curve analysis. Therefore, sex could not be determined using this method. Sequencing of these PCR products, displayed that sequencing was a optimal way to distinguish sex.

Another method attempted was a (Geneprint STR Promega) sex identification kit based on gel electrophoresis band separation. Band separation was assessed on various percentages of agarose gels (1-4%). However, due to lack of band separation on any of the agarose gels at different percentages, it was proposed that products might be resolved using the more sensitive 8% polyacrylamide silver stained gel. After extensive testing this system was not considered reliable as it failed to distinguish sex.

During the evolution of the novel molecular sex determination methods for the present study these two systems of sex identification were designed using both modern blood and bone controls. The rationale here was that one method isolates an indel that can be distinguished with the Experion system, while the other system gives very consistent and reliable results through the sequencing of 10 polymorphic regions.

Conventional PCR was used to conduct a nested PCR reaction. This technique uses two sets of primers, in two successive PCR reactions. The second set of primers are designed to amplify a secondary area within the first round products. This additional round of PCR is used to generate smaller strands of the DNA before the final run and is used when a PCR product generates low yields of DNA. As the specimens in the present study were sourced from archaeological bone, there is a good possibility that the DNA is degraded. The chances of isolating an amplicon are improved by performing a nested PCR.

As previously mentioned contamination is a serious concern during PCR amplification as contaminant DNA can infiltrate the PCR product by various means, through the air, reagents, labware, or the sample itself (Schmidt *et al.* 1995).

In order to reduce the risk of sample contamination in this study, the laboratories were physically separated. There were four different laboratory rooms: preparation (pre PCR), PCR, gel electrophoresis area and sequencing areas (post PCR). These were completely equipped including: disposables, chemicals, pipettes, reagents and labware (Schmidt *et al.* 1995). In each room there were separate working benches dedicated to these experiments only. Gloves were changed when switching rooms, and the pipettes were sterilised with hypochlorite and ethanol between reactions. PCR blanks and positive controls were always included with each PCR amplification to ensure that no contamination infiltrated the sample, while setting up the PCR reaction (Machugh *et al.* 2000). A blank and a positive control were carried out in parallel with both of the molecular systems to monitor possible contamination.

7.4.1 PCR Optimisation

Due to the poor quality of the starting material of archDNA samples, PCR optimisation is a crucial component of this research to maximise DNA amplification. These two systems of sex identification were first optimised extensively using modern blood and bone controls representing both sexes. Only once these methods were consistently working with 100% efficiency they were optimised for arch/aDNA samples using pooled DNA from the *ex-situ* collection. When these procedures worked effectively on the pooled DNA, they were applied to the *ex-situ* collection.

7.4.1.1 Primer Compatibility

As mentioned previously, a nested PCR reaction was used. The primers used in the second round isolate a fragment within the first PCR product. Four sets of primers were designed on this area of the gene. To test the different primer combinations and their compatibility, one round of conventional PCR was run on the modern blood and bone controls, to determine which primer combinations (both outer and inner designed primers) gave the best result (table 7.3

PCR cycling conditions). The PCR product was run on a 1.5% agarose gel with ethidium staining (appendix D) and visualised using transilluminated UV light (Biorad gel/chemi doc). The best result was represented by the brightest band with the correct molecular weight dependent on the primers used, without non-specific bands and an absence of bands in the negative control. At this stage, one can also detect if there were any primer dimers. To assess the compatibility of the best first PCR primer combination in a nested PCR reaction, the PCR product with best first round (outer) primer combination were run using the best PCR product for the second round (inner) set of primers in a conventional PCR. The most compatible primer combinations were the first round primers 'amel fwd' and 'amel rvs' with the second round primers being 'se' and 'as'; and the first round primers 'amel fwd' and 'amel rvs' with the second round primers being 'fi' and 'ri' (listed above).

7.4.1.2 Cycling Conditions

The PCR reagents and cycling conditions were optimised for each method. The annealing temperatures for each set of primers were analysed at the designed optimal melting temperature, in addition to 5 °C intervals above and below it. For primers 'amel fwd', 'amel rvs', 'as' and 'se' the melting temperature was 55 °C, and for primers 'fi' and 'ri' the melting temperature was 65 °C.

7.4.1.3 Real-Time PCR

Both sex determination systems were tested using SYBR green (table E.1), a nucleic acid stain that specifically binds to double stranded DNA and during amplification the fluorescence emission is detected by the real-time software. In this study the ABI 7500 real-time PCR machine was used with melting curve analysis to assess their applicability for the present study.

The use of melting curve analysis on the system spanning the indel proved unsuccessful, as the 6 bp shift between the X and Y amplicons could not be differentiated.

Real-time technology is theoretically more sensitive and accurate compared to conventional PCR. After a nested PCR amplification in system 1, real-time PCR was evaluated to see if more archaeological samples could be sexed. Due to the low level of DNA in the starting material a third round of PCR amplification on the real-time instrument failed to render more results, therefore it was not used here.

7.4.1.4 Taq Polymerase

Three different types of Taq polymerase were evaluated: Ampli-Taq Gold (Applied BioSystems), Sahara Taq (Bio-Line) and Roche Expand High Fidelity PCR System. Sahara and Expand DNA Taq polymerases are robust enzymes specifically designed for low copy number and very high PCR sensitivity. Each of these enzymes were supplied with their own optimised system and reagents that included magnesium and PCR buffer. A dNTP mix (Gene Amp 10 mM dNTP mix from Applied Biosystems) was added to the PCR reaction mix. All three Taq polymerases were tested running the same samples (a mix of modern bone, archbone and blood samples). The products obtained were assessed against one another, based on band visibility on a 1.5% agrose gel and visualised under UV light. The PCR set-up and cycling conditions for Bio-line Sahara Taq and Roche Expand High Fidelity PCR System examined here were included in appendix E. All three enzymes gave equally good results. Due to availability, the Ampli-Taq Gold Core Reagent Kit from Applied Biosystems was used. This is a hot start enzyme which assists in avoiding primer dimers and non-specific binding of the primers, as it is only activated at 94 °C.

7.4.1.5 Magnesium Titration

As magnesium ions are essential for the PCR reaction, a magnesium titration designed for a nested PCR reaction was performed with both systems, to determine the optimal amount of $MgCl_2$ (appendix E).

7.4.1.6 Gel Electrophoresis

The primers designed here spanning the indel did not yield band separation using either an agarose gel or a low melting temperature agarose gel. In addition, a sensitive staining process was attempted using SYBR gold nucleic stain. An experiment was set-up to compare SYBR Gold versus ethidium staining. This was not found to be superior and ethidium staining was used. However, the newly marketed Bio-rad Experion automated gel electrophoresis microfluidic system, did separate the two PCR fragments and consequently this was elected as the best technique to use.

7.4.1.7 DNA Precipitation

To increase the DNA yield (concentrate) and the chances of amplifying DNA from the archaeological specimens, a DNA precipitation test was carried out. DNA from 3 random archaeological samples were amplified using nested PCR and visualised with UV light on an agarose gel. Following this, the remaining extracted DNA was precipitated (appendix E), for these same three specimens and amplified using a nested conventional PCR. The results of both PCR products were compared. DNA precipitation did not change the outcome and was not used in the present study.

7.4.1.8 Bovine Serum Albumen

Bovine Serum Albumen (BSA) (Promega) was added to the PCR reaction to assess if it could improve on the PCR amplification result, as many studies (Hagelberg *et al.* 1991; Höss & Pääbo 1993; Schmidt *et al.* 1995; Faerman *et al.* 2000; Kolman & Tuross 2000; Fletcher *et al.* 2003; Papagrigorakis *et al.* 2006) have shown its use to increase DNA yields. BSA stabilises the PCR reaction by binding to the PCR inhibitors haeme and gelatin (Richards *et al.* 1993).

Two studies were set up in parallel using the same 6 blood controls and 3 random archaeological bone extracts (cycling conditions and reaction mix are

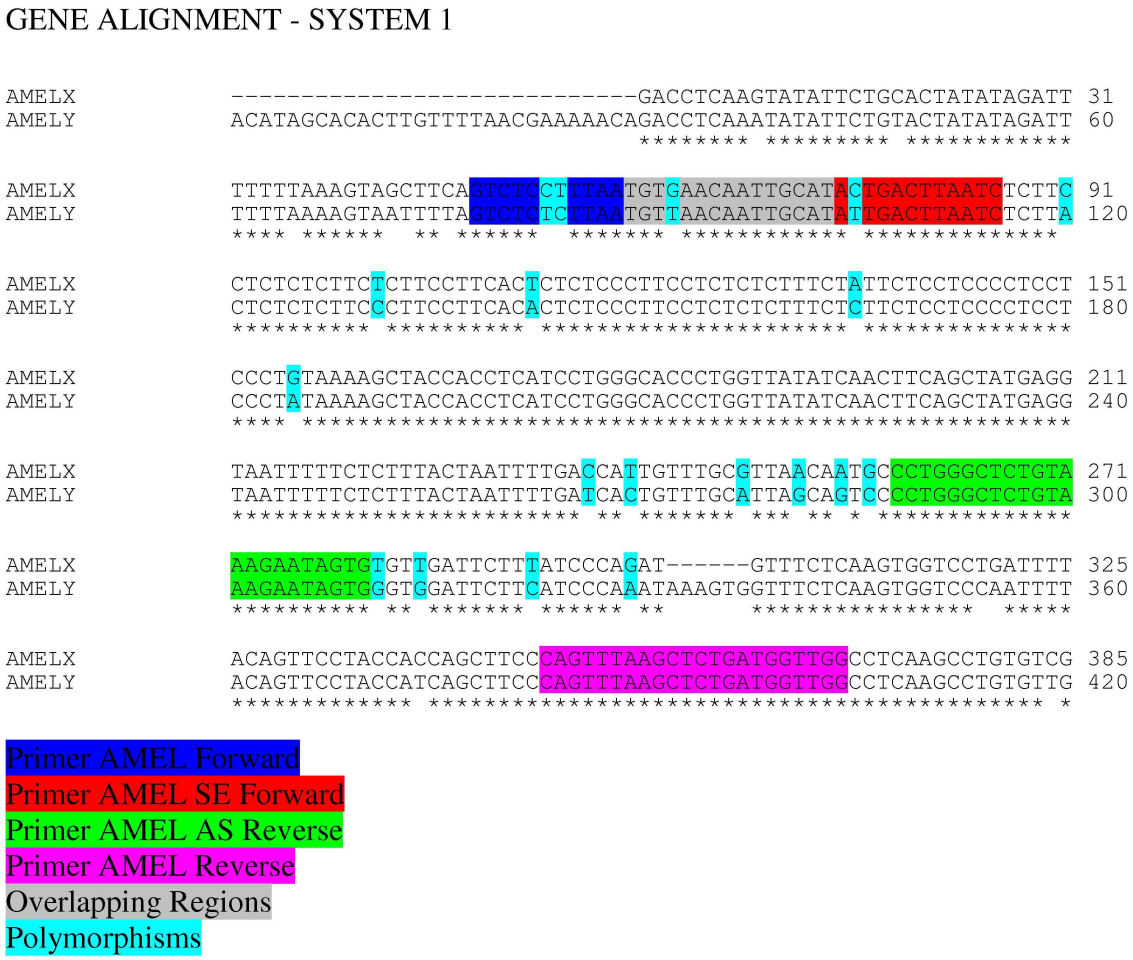

Figure 7.2: System 1: Amelogenin X and Y gene alignment mapped with primers, polymorphisms and the homologous regions.

in table E.8). Study one included 30 mg of BSA and study two was without BSA. The PCR products from both studies were compared using an agarose gel. As the addition of BSA did not make any difference in the results of the PCR amplification, it was not included in this study.

The final PCR reaction mix and cycling conditions for these two systems were optimised on good controls using the above experiments. Only once these methods were working with 100% effeciency on the modern controls (male and female blood and bone samples) were they applied to the archaeological sample.

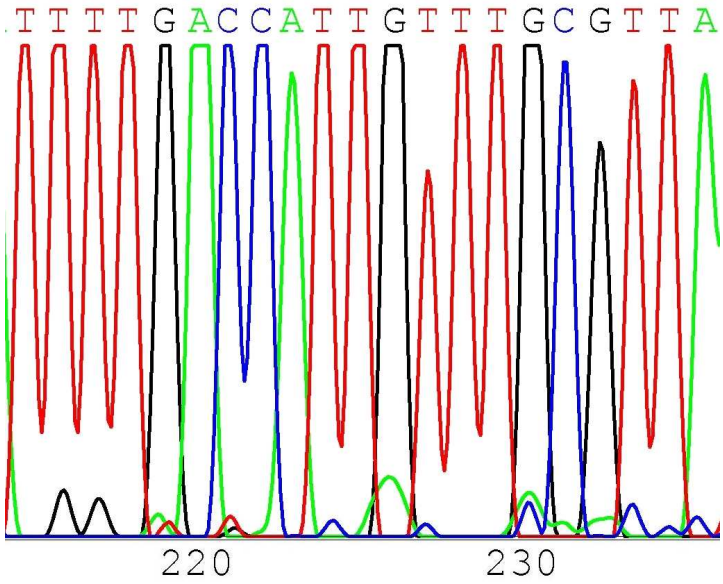

Figure 7.3: An example of the female chromatogram spanning polymorphisms 5, 6 and 7 according to table 7.1, with only the X chromosome represented.

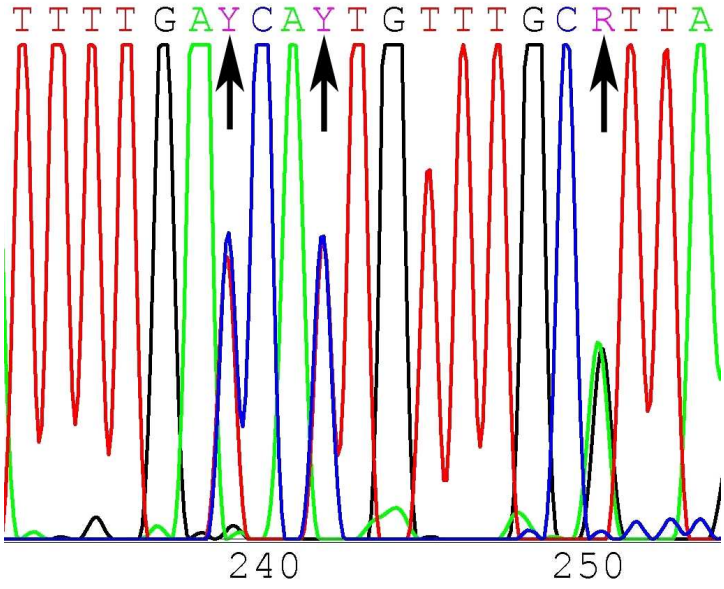

Figure 7.4: An example of the male chromatogram spanning polymorphisms 5, 6 and 7 according to table 7.1, notice three polymorphic sites demonstrating the co-dominance of the X and Y alleles, indicated by the shaded nucleotides (arrows).

7.5 System 1 - Sequencing

In the first system a nested conventional PCR was used with sequencing in order to determine sex (figure 7.2). It consists of two forward primers (AMEL-fw and AMEL-se) and two reverse primers (AMEL-rev and AMEL-as), the

final PCR amplicon being 199 bp long.

The classification of sex using this system was determined by analysing the sequence for 10 sex specific polymorphic regions (1-10 table 7.1) to determine whether both the X and Y (male) was represented or only the X (female) was represented. The results for each sample were displayed in a chromatogram file. An example of a full unedited (raw) chromatogram file for both a male and female sample are represented in figures H.1 and H.2. Edited examples of chromatograms exported from a 'Chromas Light file' clearly show how sex was diagnosed; a female is represented in figure 7.3 and a male in figure 7.4.

7.6 System 2 - Indel Analysis

In system two, a nested conventional PCR product that spans an indel was analysed with the Experion automated gel electrophoresis system and verified with sequencing. The primer design on the amelogenin gene for system two (figure 7.5) consists of two forward primers (AMEL-fwd and AMEL-fi) and two reverse primers (AMEL-rev and AMEL-ri). The molecular weight for the X is 188 bp and for the Y is 194 bp.

The Experion electrophoresis station (Bio-Rad) uses microfluidic separation technology to transport very small volumes of liquid that allow rapid sample separation, the results are displayed on a electropherogram and a simulated gel. To determine sex, the samples were examined for band separation; wherein the male should have two bands 6 bp apart and the female should only be represented by one band (figure 7.6). In addition to a simulated gel, each sample was represented by an electropherogram (for examples of these refer to figures H.4, H.5 and H.6). The results were confirmed using sequencing (Inqaba Biotechnical Industries). The sequence was analysed for 10 sex specific polymorphic regions (polymorphisms 5-14 according to table 7.1), in addition to the indel (table 7.2) to determine whether both the X and Y chromosomes were represented, or if only the X (female) was represented. An example of a full unedited (raw) chromatogram file for a male is displayed in figure H.3.

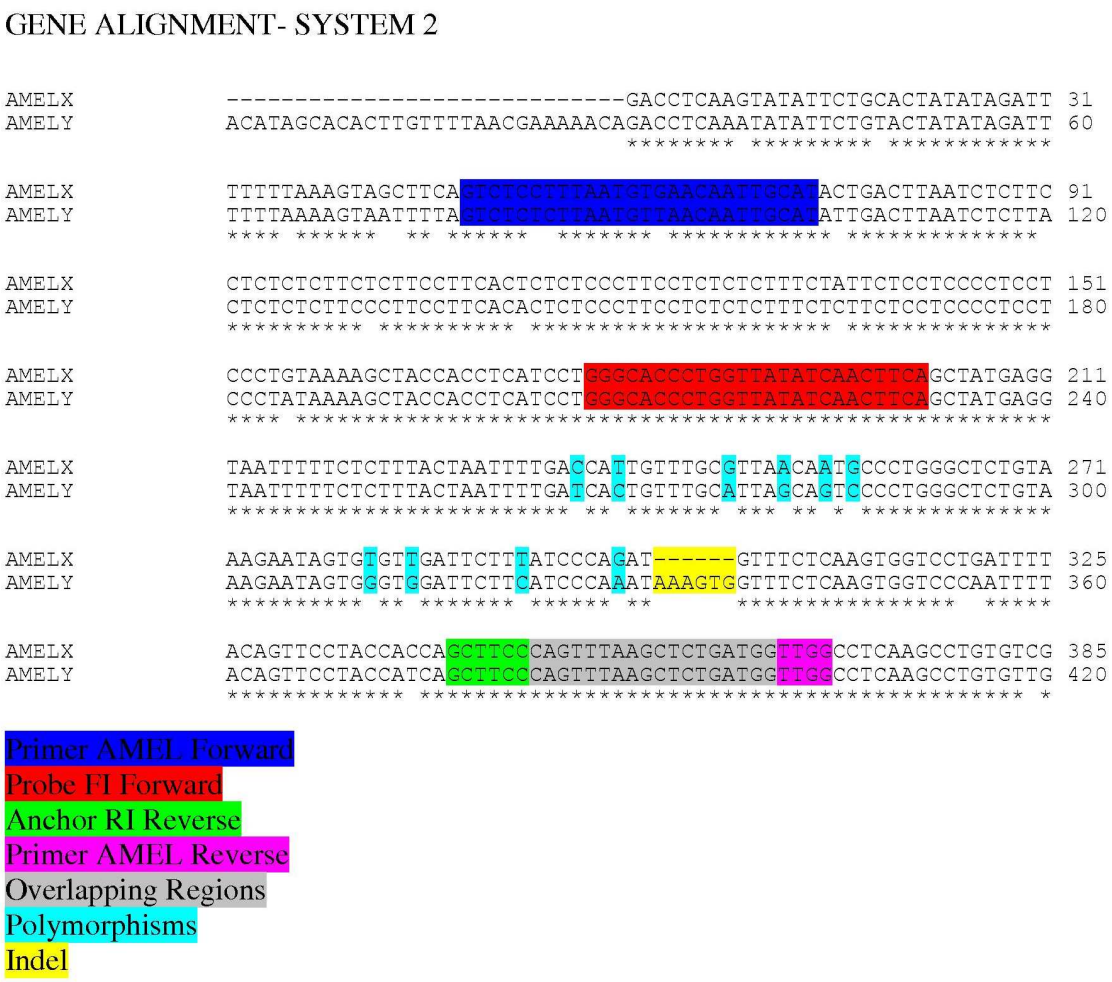

Figure 7.5: System 2 Amelogenin X and Y gene alignment mapped with primers, polymorphisms and the indel.

7.7 Methods

7.7.1 PCR Amplification

In order to reduce the risk of contamination in the sample, the PCR master mix was prepared in a separate designated area. After this, the DNA was added in a separate room. Each of these areas were equipped with dedicated equipment. Pipettes were sterilised with 0.6% hypochlorite and 70% ethanol between reactions. PCR blanks and positive controls were included with each PCR amplification in order to monitor the possibility of contamination while preparing the PCR reaction.

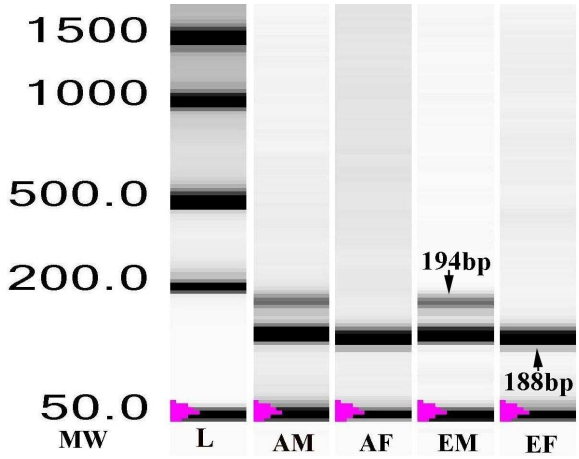

Figure 7.6: Example of sex determination system on the Bio-rad automated gel electrophoresis station. The 194 bp product is the X amplicon, and the 188 bp product is the Y amplicon. The PCR product obtained for the males were represented by 2 bands 6 bp apart while the females only have one band. Key: MW- Molecular Weight Marker, L-Ladder, AM- Asian Male, AF-Asian Female, EM-European Male and EF-European Female.

The nested PCR reaction requires outer primers for the first round of PCR and the second round requires primers that bind within the first PCR fragment. For the second round of PCR, the volume of the PCR reaction mix was almost double the first, this was to dilute any primers remaining from the initial reaction.

The samples were placed in the Hybaid Thermal Cycler 9600 PCR Sprint (PCR reaction mix and cycling conditions for each set of primers were shown in table 7.3).

7.7.2 System 1-Sequencing

The PCR product was run on a 1.5% agarose gel stained with ethidium bromide and visualised using UV light. The PCR product was purified using a Qiagen purification kit (QIAquick PCR purification Kit (50)).

PCR Purification Protocol From QIAquick Spin Hand book 2003 (pg 19)

Table 7.3: PCR Master Mix Applied Bio-Systems Core Reagent Kit

	1st Rd	**2nd Rd**
	1x	1x
25 mM Magnesium	5 μl	5 μl
10X Buffer	3 μl	5 μl
10 mM dNTPs	0.6 μl	1 μl
Fwd Primer 2 μM	3 μl	5 μl
Rvs primer 2 μM	3 μl	5 μl
Taq Polymerase 5 u/μl	0.2 μl	0.35 μl
H_2O	5.2 μl	28.65 μl
DNA	10 μl	0 μl
Total volume	30 μl	50 μl

Initial incubation of 94 °C for 10 minutes. Cycling conditions: 94 °C for 1 minute, outer primers 55 °C for 45 seconds and inner primers 65 °C for 45 seconds, 72 °C 1 minute for 35 cycles.

1. Add 200 μl of Buffer PBI to 40 μl of the PCR sample and mix.
2. Check that the colour of the mixture is yellow.
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge at 13 000 rpm for 30-60 seconds.
5. Discard flow-through. Place the QIAquick column back into the same tube.
6. To wash, add 750 μl buffer PE to the QIAquick column and centrifuge at 13 000 rpm for 30-60 seconds.
7. Discard flow-through and place the QIAquick column back in the same tube.

8. Centrifuge at 13 000 rpm the column for 1 minute.

9. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

10. To elute DNA: Add 50 μl H_2O to the centre of the QIAquick membrane and centrifuge at 13 000 rpm the column for 5 minutes.

11. Check purification on a agarose gel (appendix D) and Biorad gel/chemi doc.

Each sample was run on the sequencer twice, once with the forward and once with the reverse primer. The samples were sequenced with a Big Dye v3.1 Terminator Sequencing Kit (Applied Biosystems) in a GeneAmp PCR System 9700 Perkin and Elmer/Applied Biosystems PCR machine. The sequencing reactions were subsequently cleaned up using an ethanol precipitation.

Table 7.4: Reaction Mix: Big Dye v3.1 Terminator Sequencing Kit (Applied Biosystems)

	x1	**x50**
Reaction Mix	4 μl	200 μl
Buffer	2 μl	100 μl
Primer (3.2 pmol)	1 μl	50 μl
Template and H_2O	5 μl	400 μl

Cycling conditions: 96 °C for 1 minute x1 cycle, 96 °C for 10 seconds, 52 °C for 10 seconds, 60 °C for 4 minutes for 25 cycles, finally a hold step of 4 °C.

96 Well Ethanol Precipitation Protocol

1. Mix: 2.5 ml of absolute ethanol (EtOH) with 100 μl of 3 M sodium acetate (NaOAc pH 4.6).

2. Add 25 μl of the EtOH/NaOAc mix to each reaction.

3. Replace strip caps or full plate cover and mix on vortex.

4. Centrifuge for 30 minutes at 1500-3000 g.

5. Immediately invert tray on paper towels, place inverted tray and paper towel in centrifuge for 1 minute at 150 g to remove the supernatant.

6. Add 50 μl of cold 70% ethanol to wells, centrifuge for 5 minutes at 1500-3000 g.

7. Immediately invert tray on paper towels, place inverted tray and paper towel in centrifuge for 1 minute at 150 g to remove the supernatant.

8. Dry the pellet: by placing 96 well plate without a cover on PCR machine at 63 °C for 3 minutes.

9. Resuspend pellet in 10 μl of Hi-Di Formamide (Applied Biosystem).

10. Heat sample with cover in PCR machine at 96 °C for 2 minutes.

The sample was placed in the 3100 Genetic Analyser (Applied Biosystem) machine for electrophoresis of the sequenced products. The 10 sex specific polymorphic regions on the X and Y amelogenin gene were determined, and the classification was analysed by viewing the sequence of each sample in a chromatogram file in the Chromas Lite program and comparing it to the known sequence of the gene.

7.7.3 System 2- Indel analysis

The PCR product for each sample was analysed using the Bio-Rad Experion DNA 1K system for indel analysis. This system runs 11 samples at a time, one of which was always a negative (H_2O) and positive (human blood) control.

Experion DNA 1K Analysis Protocol

1. 30 minutes before use, remove DNA gel, DNA stain and loading buffer from cold storage and equilibrate to room temperature. Vortex and briefly centrifuge. Protect stain, and gel stain solutions from light. Using aluminium foil cover the ladder and keep on ice while working.

2. Cleaning the electrodes before analysis:

   800 μl Electrode cleaner in cleaning chip for 2 minutes.

   850 μl DEPC H_2O (appendix E) in cleaning chip for 5 minutes.

   850 μl DEPC H_2O in cleaning chip for 5 minutes.

   Leave lid open for 1 minute, remove chip then close it.

3. Preparation of Gel Stain (GS):

   Add 12.5 μl Stain in a full tube of G (250 μl).

   Transfer to a spin filter tube.

   Centrifuge for 15 minutes at 2400 g, or until all the gel has passed through the filter.

   Filtered gel stain can be stored for up to one month at 4 °C.

4. Priming the Chip:

   Pipette 9 μl of GS in the priming GS well, using the priming code C3.

5. Loading:

   9 μl of GS in the priming station.

   Prepare 65 μl of loading buffer in a tube.

   Pipette in order:

   5 μl of loading buffer in well 1 to 11 and L.

   1 μl of sample in well 1 to 11.

   1 μl of ladder in L.

   Pipette 1 μl of DNase free H_2O in non-used sample wells.

6. Vortex:

   Place chip in Experion vortex station, turn on vortex, which will automatically stop.

7. Electrophoresis:

   Put chip on the chip platform and start the run by initialising the software.

8. Cleaning electrodes between two runs or at the end:

   800 μl of DNase free H_2O in the cleaning chip 1 minute.

Leave lid open for 1 minute, remove chip then close it.

To confirm that the correct gene has been amplified the PCR product was purified using a Qiagen purification kit and sequenced by Inqaba Biotechnical Industries Pty. Ltd., South Africa.

7.8 Results

Due to the way in which the results were displayed through large graphic files (such as; chromatograms, gels, simulated gels and electropherograms) for simplicity they were all analysed and compiled into tables and summaries. There are examples of how these results are originally created and displayed in figures H.1, H.2, H.3, H.4, H.5 and H.6, and specific edited examples are presented within the text below.

7.8.1 Controls

Both of these methods were first extensively optimised on 6 male and female human blood and then on male and female modern bone controls. Only once these methods worked consistently with 100% efficiency were they applied to archDNA sourced from the *ex-situ* collection. Prior to their application to the *ex-situ* sample, DNA was first pooled from this sample. The purpose of this is for further optimisation as pooled DNA was representative of the entire collection.

7.8.2 System 1 - Sequencing

Post amplification, each sample was analysed on an agarose gel (table H.2). The sequence for each sample was analysed, and for the samples with readable sequence, the 10 polymorphic regions were examined and recorded and compiled in table H.1. Only 6 (20%) of the specimens possess a result, 4 (66.66%)

of these were male and 2 (33.33%) were female. The results of the sequencing versus those that have post PCR bands are shown in table 7.5. Using system 1 figures 7.7 and 7.8 show edited examples of chromatograms for two *ex-situ* specimens, and figure 7.9 is an example of an agarose gel with *ex-situ* samples.

The method of sequencing used here was very effective, as with some samples it gave a sequence even when no band was detected. Obtaining a sequence result without a band is unexpected, since a band is seen as a predictor of the sequence. Due to the low level of DNA in archDNA it is not surprising that no band was visualised post amplification, even though a small amount of sequence was present (the minimal amount of DNA for sequencing is 50 ng/μl). A successive round of PCR amplification was assessed during the optimisation period and it did not increase the DNA yield, it only resulted in primer dimers. As discussed previously, agarose gel electrophoresis is not the most sensitive way to visualise DNA, although none of the more sensitive methods gave better results, except for the Experion system used in system 2.

Table 7.5: System 1: Summary of results for the *Ex-situ* specimens

Sample ID	**Seq-sex**	**Band**	**Sex**
A75	*		F
A240		*	
A293	*		M
A302	*	*	M
A312	*		F
A379	*		M
A427		*	
A908			M
A1096		*	

Seq- sequence result; Band- electrophoresis result; Blank- no result. M = male and F = female.

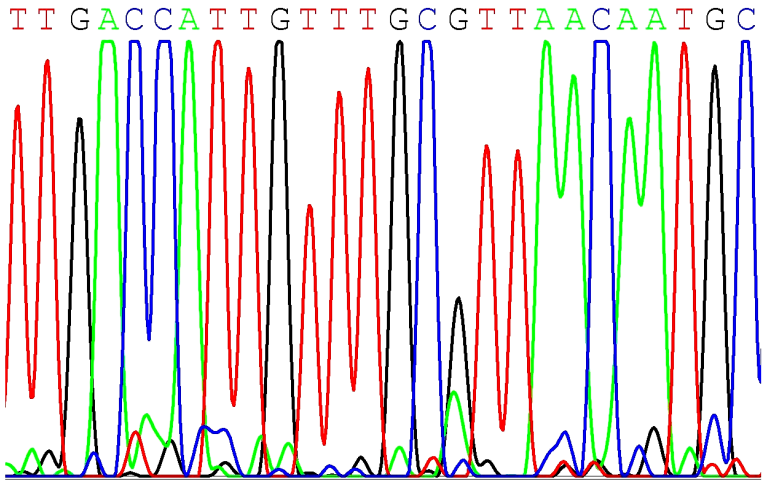

Figure 7.7: Specimen A75, a female chromatogram spanning polymorphisms 5-10 according to table 7.1, with only the X chromosome represented in system 1. The nomenclature for the base pairs is available in appendix F.

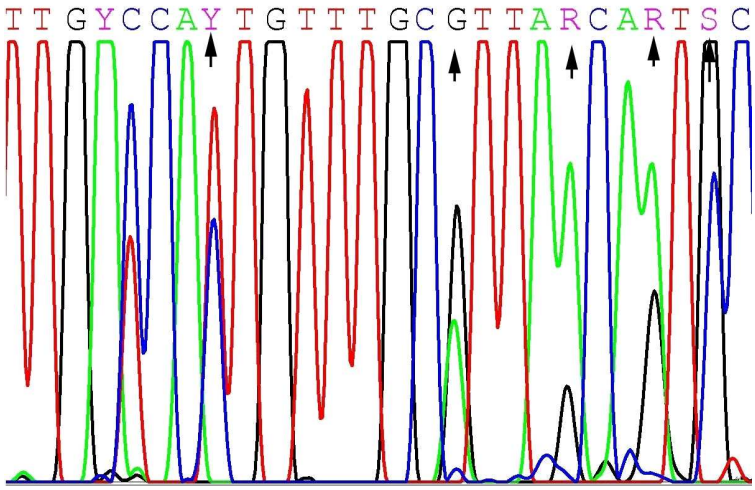

Figure 7.8: Specimen A908, a male chromatogram spanning polymorphisms 5-10 according to table 7.1, with the X and Y chromosomes represented in system 1 (arrows). The nomenclature for the base pairs is available in appendix F.

7.8.3 System 2 - Indel Analysis

Each sample was analysed on the DNA 1K Experion electrophoresis system and on each gel both a negative and positive control were included. The results of the gels were analysed for no bands, one band or two bands (compiled in table H.3). The samples were prepared for sequencing, the results of which are recorded in table H.4.

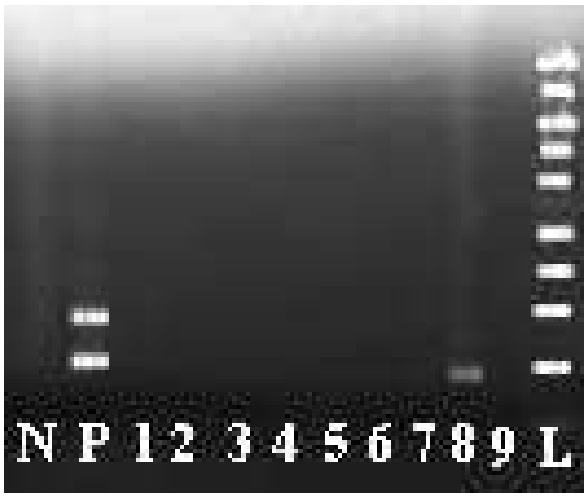

Figure 7.9: Representative agarose gel electrophoresis for 9 *ex-situ* specimens using system 1. Key: N-Negative control, P-Positive control, 1- A909, 2- A123, 3- A293, 4- A78, 5- A330, 6- A326, 7- A2227, 8- A75, 9- A312 and L-100bp Ladder.

Using system 2, 16 (53%) specimens gave a result that were compared in table 7.6. In analysing these results it was notable that four of the specimens (A302, A379, A658 and A908) with determined sex for the gel and sequence methods do not match (table 7.6). This can be explained in the following manner: specimen A658 was only represented by one band on the gel system and when it was sequenced only the Y chromosome amplified. The existence of the Y is indicative of a male and as only the Y amplified this justifies the presence of only one band on the gel. This shows how only using a gel based system sex can be incorrectly interpreted. In some samples it was noted from the sequence analyses that the Y chromosome was more prominent than the X, which can also explain such results. In these samples the sex determination via sequencing was more reliable and consequently only these were used in the final results, which decreased the results from 16 (53%) to 12 (40%) specimens with determined sex, all of which were male. Using system 2, figures 7.10 and 7.11 show chromatograms for two *ex-situ* specimens, and figure 7.12 is an example of a microfludic chip gel with *ex-situ* samples.

It can also be noted that four (A268, A427, A675 and A907) specimens gave a result on the gel system and did not provide readable sequence, preventing

confirmation by this method. In each of these samples sequence was produced, however due to excessive background in the sequence, it was not possible to decipher an accurate diagnosis of sex (see figure 7.13 and compare to figures 7.11 and 7.7). These samples were re-extracted and run through the amplification process again, to ensure there was not a problem with the original extraction, however similar sequence results were found. In view of the consistency for the negative and positive controls, this background sequence is not an artefact and it is unlikely to be non-specific amplification. The most probable explanation for this is DNA breakage, due to the original state of the archaeological specimens.

In addition, two samples (A76 and A293) gave a result using sequencing that did not render a band on the gel, as previously discussed this could be a result of a low level of DNA in the sample, being undetectable on the gel even though it was adequate for sequencing.

It must be noted that from a practical perspective with regards to laboratory procedures and expense, the Experion system is costly and has a short shelf life of six months. In addition to this, once the gel and staining solution are mixed together they must be used within a month; this is enough gel and stain for five chips. Each chip can hold 11 samples and if a well is not used it cannot be used again. Therefore, in order to use the 1K DNA Experion kit, efficiently it is important to have a strict plan to maximise the use of the entire kit within a two month period. For these reasons the Experion system is only viable when large sample sizes are being run within a short period of time.

7.8.4 System 1 and System 2

Using both systems of sex determination, 18 (60%) specimens in the sample produced a result and of these, 15 (83.33%) were male and 3 (16.66%) were female (table 7.7). These raw data include the specimens that had sex determined in system two based on the Experion station and not confirmed with sequencing. For accuracy these have been omitted from the analyses of the two

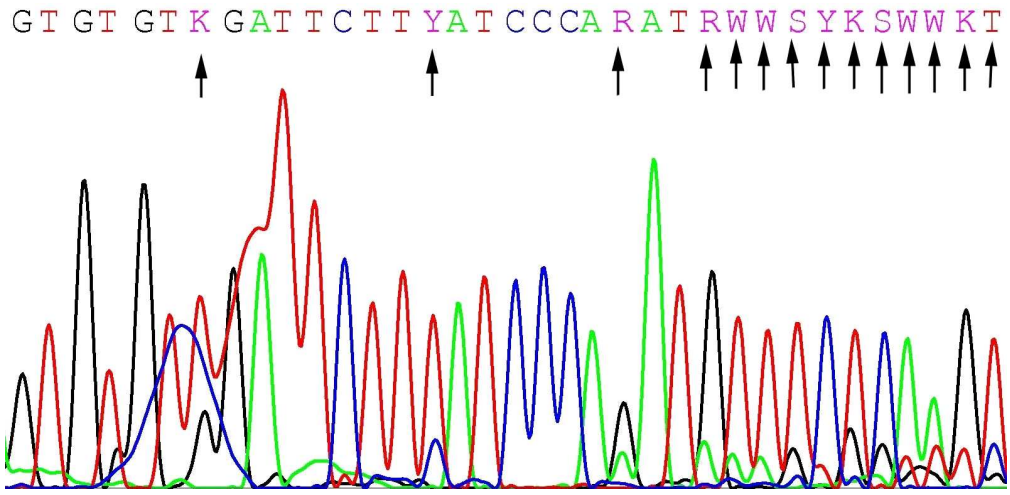

Figure 7.10: Specimen A26, a male chromatogram spanning polymorphisms 11-14 and the indel according to tables 7.1 and 7.2, with the X and Y chromosomes represented in system 2 (arrows). The nomenclature for the base pairs is available in appendix F.

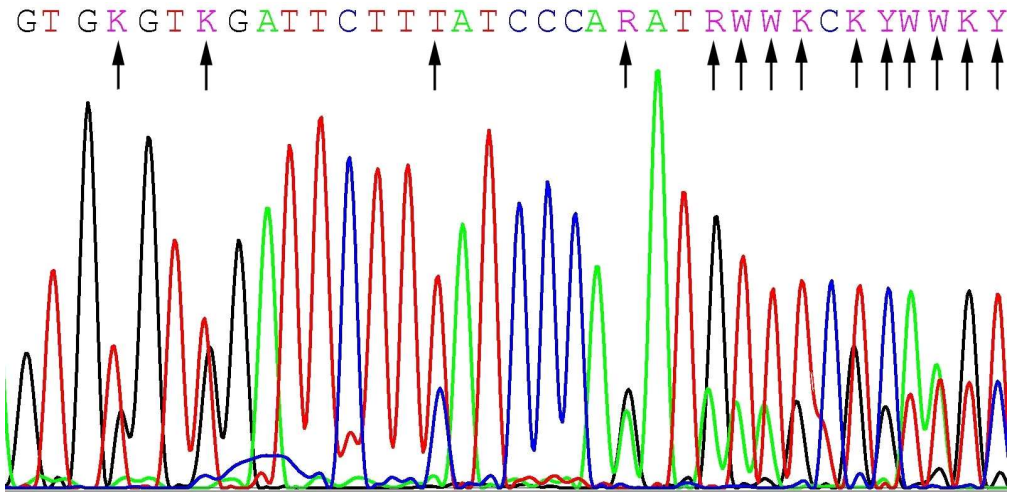

Figure 7.11: Specimen A908, a male chromatogram spanning polymorphisms 11-14 and the indel according to tables 7.1 and 7.2, with the X and Y chromosomes represented in system 2 (arrows). The nomenclature for the base pairs is available in appendix F.

systems. This takes the total sample with determined sex results using both systems to 14 (46.66%), and of these 12 (85.71%) were male and 2 (14.28%) were female. Based on this data, 4 (28.57%) of the specimens in the sample possess the same result in both methods. Method one produced 2 (14.28%)

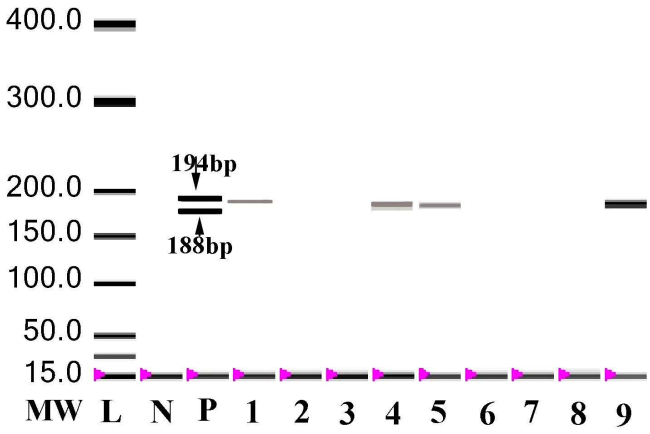

Figure 7.12: Representative microfluidic electrophoresis gel for 9 *ex-situ* specimens using system 2. Key: MW-Molecular Weight Marker, L-Ladder, N-Negative control, P-Positive control, 1- A908, 2- A2227, 3- A909, 4- A77, 5- A26, 6- A326, 7- A74, 8- A79 and 9- A294.

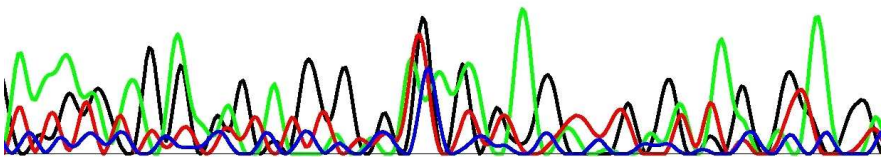

Figure 7.13: Specimen A675, an example of a sample with a low level of sequence and high background sequence (compare to figures 7.11 and 7.7). Specimens such as this produced a band on the gel and undecipherable sequence preventing an accurate diagnosis of sex.

specimens and 8 (57.14%) specimens were achieved using system two. In comparing the results of the two methods, it would appear that system two was more effective, as it produced a higher precentage of sex determination results.

7.9 Conclusion

Both of these novel methods of molecular sex determination developed here using the amelogenin gene were optimal for sex determination as they yielded results for nearly half the analysed skeletal sample, an outcome that was within the norm for studies using arch/aDNA. The second system doubled the results of the first method and it was clearly the better method for sex determination from skeletal tissue. The positive outcome of a sample is ultimately reliant on the quality of the DNA in the *ex-situ* collection.

Table 7.6: System 2: Summary of results for *Ex-situ* specimens

Sample ID	**Gel**	**Seq**
A26	M	M
A76		M
A77	M	M
A268	M	
A293		M
A294	M	M
A302	F	M
A319	M	M
A379	F	M
A427	M	
A453		M
A658	F	M
A675	M	
A907	F	
A908	F	M
A1096	M	M

M = male and F = female, blank indicates sex was not determined.

Seq- sequence result; Gel- Experion result.

Table 7.7: Summary of molecular sex determination results for the *Ex-situ* specimens using both systems 1 and 2

Sample ID	**System 1**	**System 2**
A26		M
A75	F	
A76		M
A77		M
A268		M*
A293	M	M
A294		M
A302	M	M
A312	F	
A319		M
A379	M	M
A427		M*
A453		M
A658		M
A675		M*
A907		F*
A908	M	M
A1096		M

* Represent specimens in system 2, where sex was determined by the Experion gel only and not confirmed with sequencing. M = male and F = female, blank indicates sex was not determined.

Chapter 8

Discussion

8.1 Bone extraction

The bone extraction methods used for molecular studies from skeletal tissue are not always adequately described in the literature. However, there are other minimally invasive bone extraction methods available, some using drill-like instruments (Faerman & Bar-Gal 1998; Greenwood *et al.* 1999; Adcock *et al.* 2001). Those that use drilling do so inconsistently and on different aspects of the skeleton. There is also a minimally invasive method that uses a buffered solution to dissolve the bone (Asher & Hofreiter 2006). The majority of these methods are not used on human remains, and as of yet there is no method that takes into consideration damage to morphological and metrical areas on the skeleton. As developed here, it is important to have a published method that can be replicated in any laboratory.

The novel bone extraction method devised here is particularly important as it is minimally invasive to the specimen and does not interfere with any morphometric points of identification. For these reasons, the curators of the Dart Collection granted permission to conduct molecular work on the *ex-situ* collection. This method uses a drill and creates a small hole in the intercondylar fossa, retrieving enough bone powder from the internal surface of the bone for molecular studies. This method of bone extraction is applicable for molecular studies on both human and animal skeletal material. Its use is not limited

to sex determination, it can be used for extraction of mtDNA or any gene of interest in the genome.

8.2 DNA Extraction

The DNA extraction method using the silica column system is not novel, as it has been applied in other studies, as has the addition of proteinase K and EDTA to the lysis buffer to maximise the DNA yield. The samples with a pure ratio (of 1.8 to 1.99) all yielded results. However, there was no observed pattern according to the samples with levels of purity both above or below this standard ratio of DNA purity. As some samples with RNA contamination produced results, while others did not. This observation was similar for contamination of DNA samples by aromatic substances and proteins.

8.3 Molecular Sex Determination

As demonstrated in this study the use of a nested conventional PCR with the amelogenin gene is a useful technique for sex determination. The two systems of molecular sex determination designed here proved successful and yielded consistently reliable results with 100% efficiency on both the modern blood and bone controls, demonstrating that this area on the amelogenin gene is optimal for sex determination. Further these newly developed procedures for diagnosing sex developed here are unique compared to the available methods of sex identification.

Previously it has been shown that polymorphic sites could be used for sexing, although, this has only been conducted to confirm the sex results from a method of band separation (Zoledziewska *et al.* 2004). System one is unique in that it uses sequencing to determine sex by isolating a specific area on the amelogenin gene, over 10 conserved sex specific polymorphic regions; this system more importantly isolates an area not previously used for sex determination.

The isolation of an indel, as in system two, is the more commonly used method for sex determination and has been used in other studies. Moreover, the specific indel isolated here has been used previously both for MGB probes and for primers spanning the indel, with an observed shift of 6 bp resolved on a polyacrylamide gel system (Mannucci *et al.* 1994; Cattaneo *et al.* 1997; Meyer *et al.* 2000*a*; Zoledziewska *et al.* 2004; Alonso *et al.* 2004; Arnay-de-le-Rosa *et al.* 2007). Although the indel focused on in the present study has been previously isolated, the second system of sex identification designed here is unique. It uses the Experion station, which relies on microfluidic chip technology. This electrophoresis based-chip gel is used in conjunction with sequencing to verify the presence of the indel. Therefore this system, through sequencing has an inherent control besides the indel, as it additionally isolates 10 sex specific polymorphic regions. Further, all the primers designed and used here were unique, except for AMEL 'as'.

The success of determining sex by examining many polymorphic regions is higher, compared to methods that only localise one or two polymorphic sites or only an indel (Mannucci *et al.* 1994; Cattaneo *et al.* 1997; Meyer *et al.* 2000*a*; Zoledziewska *et al.* 2004; Alonso *et al.* 2004; Arnay-de-le-Rosa *et al.* 2007). The great advantage of these methods of sex determination designed here, should one polymorphic region or the indel not amplify, then there are other amplified areas on the gene distinctive for sex determination.

A study recently released by Fontanesi *et al.* (2008) uses a similar method, but on the porcine amelogenin gene. An indel was isolated in addition to polymorphic sites to diagnose sex. However, the indel in this study is 9-10 bp in length and relies on the amplification of a 400 bp fragment. This system is limited as it is designed for fresh tissues, also a 400 bp fragment would not be suitable for arch/aDNA samples, as amplicons over 200 bp rarely amplify. However, this system of sex diagnosis also has the advantage of those methods developed here, with the inherent control of isolating both an indel and sex specific polymorphic sites.

Of the 30 specimens sourced from the *ex-situ* sample, the first method (system

1) produced satisfactory results, 6 (20%) specimens with sex determination. The second method (system 2) was significantly superior, yielding 12 (40%) specimens in the sample with determined sex. This is interesting as the only difference in the two systems were the primers used in the second round of the PCR amplification, which shifts the amplified area slightly. The most logical explanation for the difference in these results would be that they were caused by chemical and physical degradation of the DNA molecule in these archDNA samples. This implies that the desired binding site of the primers was affected by chemical degradation. The primers were therefore, either not binding or not binding effectively to the archDNA, resulting in minimal or no amplification. The scope of this specific study was not to examine DNA degradation and the techniques to repair these defects, however this is a valid area of research for the future. Additionally, an advantage of the second system of sex determination is that it amplifies a smaller fragment size, which preferentially amplify in arch/aDNA samples (Pääbo *et al.* 2004).

Combining both methods of sex determination 14 (46.66%) specimens from the *ex-situ* collection yielded results, which corresponds well with other studies that attained a range of 30-80% of sex determination from their analysed skeletal samples (Cattaneo *et al.* 1997; Faerman & Bar-Gal 1998; Meyer *et al.* 2000*a*; Alonso *et al.* 2004; Arnay-de-le-Rosa *et al.* 2007). An advantage with the methods developed here was the use of both systems together, which served to increase the number of samples with determined sex. Where results of the two methods of sex determination overlapped for sex diagnosis, they correlated positively with one another.

While the majority (12 specimens or 85.71%) of the *ex-situ* specimens were male, it should be noted. In a collection sourced from a specific archaeological location or context, a 1:1 ratio of males and females is expected. However, the sex ratio of this collection is not relevant and there were no predictable expectations for the sex outcome. This is due to the randomly selected nature of this *ex-situ* collection, these specimens are sourced from various places, differing time periods and conditions. The only factor these specimens have in common

with one another is that they decomposed naturally in South Africa. As previously stated after optimisation of these methods on modern controls, they were optimised for archaeologically derived specimens using this collection.

8.4 Assessment of Bone and Molecular Sex Determination

Prior to and during the bone extraction process the specimens were analysed for condition of bone, colour of femur, colour of the produced bone powder and for weight of the yielded bone powder. When these data were correlated with the samples that yielded positive results (tables B.1 and 7.7), no pattern was found with these variables. There were, however, a few notable correlations to recorded conditions of the specimens. Sample A293 even though it has thin and fragile compact bone, nevertheless produced a result, suggesting that the physical nature of the bone did not influence the end product using molecular sex determination methods. Sample A312 (figure 4.2), the only specimen not fully fused in the sample, was successfully sexed. Lack of fusion was obviously not a limiting factor for DNA retrieval using these methods. In the case of specimens A173 and A240, where both were missing the proximal end of the femur, in addition to a crack in the mid-shaft, neither of these specimens produced a result. Specimens A294 and A302 with evidence of natural mummification (figure 4.3) had sex successfully determined. This was not unexpected as natural mummification as opposed to artificial (chemical), is often associated with molecular preservation. This is obviously dependent on how long the specimen has been exposed to the environmental conditions responsible for the mummification.

Clearly, longer exposure to the elements results in reduced chances of a positive PCR amplification and, with regards to the *ex-situ* collection, the post mortem interval is unknown. From the above correlations in the data, it is clear that condition of the bone based on cracking, fragmentation and mummification

were important predictors of either a positive or negative PCR amplification. However, while 26 (86%) specimens in this collection were considered whole and well preserved, only 18 (69%) of these produced a positive result. Despite good physical preservation of the specimen an intact specimen is not necessarily indicative of molecular preservation. In the absence of a post mortem interval and many unknown variables for these specimens it is difficult to interpret these data.

While the methods developed here are suitable for molecular sex determination, it is important to understand the limitation of these methods. They are completely dependent on the quality and integrity of the DNA molecule preserved in the skeletal remains, which in turn relies on factors associated with the post mortem interval and the taphonomic conditions surrounding the burial. With regards to the *ex-situ* collection used here, neither of these sources of information were known. It is important to apply the novel methods developed here to a collection of archaeological value to emphasise their capabilities in archaeology.

Chapter 9

Conclusion

The novel methods developed from the bone extraction through to the diagnosis of sex, were thoroughly designed and optimised on appropriate controls prior to being implemented on the *ex-situ* collection. In addition, decontamination measures for aDNA research were followed throughout the development of these procedures.

The novel bone extraction method designed here is optimal for molecular studies and due to its minimally invasive nature, it permits permission to be obtained for molecular studies on skeletal tissue. This method provides the opportunity for future molecular studies as the collection is not destroyed and can still be used in other studies.

In using the silica column based DNA extraction method with the addition of EDTA to the lysis buffer, this produced the maximum yield of DNA from the bone samples. The recorded DNA purity readings for each sample were not reflective of a positive or a negative sexing outcome.

The amelogenin gene is the best gene to use for sex determination based on its characteristics on the X and Y chromosome, which allow sex identification through PCR amplification in one reaction. The novel methods of molecular sex determination developed in this study determined sex for nearly half the *ex-situ* sample. The second method using the Experion system is clearly a better method of sex determination, as it produced double the results of the

first method. However, while the Experion system produced reliable and consistent results using the blood controls, in archDNA samples it was difficult to separate the two bands. Due to its sensitivity the Experion system resolved bands that were not visualised with conventional agarose gel electropohoresis. It is shown here that the Experion system should be used to confirm the presence or absence of archDNA. It is therefore, optimal to use sequencing for sex determination and the Experion system as a predictor of the PCR amplicon in arch/aDNA samples. These novel methods have led the way for numerous applications in molecular research using skeletal material.

Although, the condition of the bone was found to be an indicator of preservation there was no guarantee of molecular preservation. It was evident from the molecular analysis on this collection that the quality and the integrity of the starting material are the most important factors influencing a positive or negative molecular sexing outcome. The burial conditions and the post mortem interval are further indicators of molecular preservation. While, the collection used here was suitable for the optimisation of the above methods on skeletal tissue, due to the lack of information associated with these skeletal remains it was not a good collection for compiling data on past populations. Therefore, it is important to apply these methods on a skeletal collection of archaeological value.

Part II

Chinese Indentured Miners

Chapter 10

Introduction

The second aim of the present study was to apply the novel methods of molecular sex determination from skeletal tissue designed in part one, to an archaeological sample with good provenance: the Chinese indentured miners sourced from the Dart Collection. These techniques were used here to address questions regarding the sex profile of this sample.

In 1951, while the Boksburg municipality in the greater Johannesburg area were laying a main water line, they discovered a Chinese cemetery on the site of the old Witwatersrand Deep Mine (Dart 1952). It contained remains of 36 individuals who worked for the gold mines on the Witwatersrand at the beginning of the twentieth century. The former Transvaal Provincial Administration allowed the graves to be exhumed and the skeletons were transferred to the University of the Witwatersrand for permanent custody, and are currently stored in the Dart Collection (Dart 1952). When Dart (1952) originally analysed this sample, he noted that each of the skeletons were buried with mining apparel. The rule on the mines was to bury the Chinese miners in separate cemeteries. As Dart knew at this time period, only African and Chinese labourers were employed on the gold mines, he deciphered that the skeletal features of the specimens were not African. Through the process of elimination he concluded that they must all be Asiatic in origin.

There is a great amount of detail in the historical records relating to the employment of Chinese labour by the gold mines however, there is virtually no

information on this specific collection of 36 indentured labourers. This collection has no associated records containing the demographic variables of the individuals represented, nor information regarding the excavation. During a period of labour shortage in the South African gold mines, men were brought from Northern China to work on the mines. This collection is the skeletal remains from 36 of those indentured miners that died while working for the Witwatersrand Deep Mine. This mine was owned by S. Neuman and Company and in October 1906, the mine employed 861 African miners and 2942 Chinese miners (Richardson 1982).

This specific collection has been previously studied at the Univeristy of the Witwatersrand. In 1952 Dart carried out morphological analyses on this collection in order to conduct a comparison between Asiatic and African skeletal features. More recently in 2004, a BSc Honours student, School of Anatomical Sciences carried out a study on these 36 Chinese skeletons focusing on morphometric analysis (Edwards 2004). The following biological variables were examined; ageing, sexing, population affinity and stature estimation. The results for sexing through morphological assessment were that 57.14% were male and 42.86% are female. However, metrical analysis suggested that 78.50% of the sample were male and 21.50% were female (Edwards 2004). The results for age estimation based on closure of the cranial sutures was 28.6-60 years of age (Edwards 2004). The mean stature of this collection was 169.21 cm and the population affinity was found to be mongoloid, based on a high number of mongoloid features (Edwards 2004).

It is surprising that Edwards (2004) morphometric study shows such a high level of female characteristics, because between 1904 and 1907, 63 935 Chinese miners were imported to work on the South Africa gold mines. Of these, only 14 women and children are recorded to have accompanied miners to the Witwatersrand (Reeves 1954; Sacks 1967). Therefore it seems improbable to have a female in this sample, and this raises questions regarding the sex profile of this collection. Could more females have been brought to the Witwatersrand than are documented in the historical record or perhaps Edwards (2004) study

incorrectly diagnosed sex for some individuals?

Chapter 11

History of Chinese Indentured Labour in South Africa

In 1898 the city of Johannesburg was the largest single source of gold in the world and the South African economy relied on the gold mining industry as gold was the basis of international trade (Richardson 1982; Richardson & Van-Helten 1984; Saunders 1994; Ndlovu *et al.* 2006). During this gold mining period the unskilled labour force was African men from countries in the region of Southern Africa, working short contracts before returning home (Ndlovu *et al.* 2006).

After the Anglo-Boer War (1899-1902) there was a major shortage of unskilled labour for the Johannesburg gold mines because the supply of African labourers that had gathered on the Rand prior to the war had returned home (Markham 1904; Reeves 1954; Sacks 1967; Richardson & Van-Helten 1984; Silver 2003; Ndlovu *et al.* 2006). During the war, mining activities were forced to stop, which led to debt accumulation by the mines (Van-Helten 1982; Reeves 1954; Ndlovu *et al.* 2006). A labour commission was appointed to investigate the labour shortage and options for unskilled labour globally (Jeeves 1983).

The labour commission looked to China for cheap labour to solve their labour shortage, especially as Chinese labour had been successfully employed in other countries such as Canada, Singapore, Australia, New Zealand and the United States of America (Sacks 1967; Richardson 1982; Jeeves 1983; Richardson &

Van-Helten 1984).

On May 13 1904, the Anglo-Chinese Labour Convention was signed in London. (Sacks 1967; Jeeves 1983; Richardson & Van-Helten 1984; Yap & Man 1996). The British government had imposed the labour importation ordinance of 1904, which regulated recruiting, shipping and the conditions of the service contract under the Anglo-Chinese convention (Richardson & Van-Helten 1984).

The Chinese labourers were employed to do hard manual labour as hand drillers, tramming and shovelling underground, while unskilled African labour were used on the surface (Reeves 1954; Richardson 1982; Yap & Man 1996; Ndlovu *et al.* 2006).

As very little information is known about the 36 individuals being studied here other than they are representative of Chinese indentured labourers from the gold mining era in the early 1900's, it is important to understand the conditions of their labour, which may have contributed to their fatality.

11.1 Chinese Labour: Conditions of Contract

Chinese labour was employed by 35 mines in the Witwatersrand district and regulated by conditions laid out by the mining industry (Richardson 1982; Kynoch 2003). The 3 year contracts were subject to a renewal for a further 3 years (Campbell 1923; Reeves 1954; Sacks 1967; Richardson & Van-Helten 1984; Ndlovu *et al.* 2006). The Chinese had strict conditions of labour; they could only work on the gold mines in the Witwatersrand district, could not become citizens, and could not purchase property (Campbell 1923; Sacks 1967; Richardson & Van-Helten 1984; Yap & Man 1996). They were allowed to bring their families, were provided with cash wages, food, lodging and clothing (Reeves 1954; Sacks 1967). The mines had no right to administer corporal punishment and were responsible for the cost of repatriation at the termination of the labour contract (Campbell 1923; Sacks 1967).

Medical examinations were necessary throughout the recruiting process to ensure that the labourers would be able to stay on the mines for the duration of their three year contracts. Preliminary medical examinations were done at the ports in China to rule out unsuitable workers (Richardson 1982; Richardson & Van-Helten 1984). After this they were brought on board the ships and inspected daily by the on-ship medical inspector (Richardson 1982). Upon arrival at the port in Durban, the living conditions on the ship were assessed by a Chinese inspector to ensure that the workers were treated well (Richardson 1982). There was also a Transvaal emigrant agent, along with a doctor to explain the conditions of labour, and to complete another medical examination prior to reaching the mines (Sacks 1967; Ndlovu *et al.* 2006).

Between May 1904-1907, 34 shiploads brought 63 695 Chinese indentured miners to the Witwatersrand gold mines; of these 62 006 were recruited from North China and the remainder from South China (Reeves 1954; Richardson 1982; Saunders 1994; Yap & Man 1996; Ndlovu *et al.* 2006).

Under the contracts the indentured workers could bring their families. In total only 2 wives and 12 children came to the Witwatersrand area. By 1907 only 4000 out of 43 000 Chinese labourers had registered their families for later passage (Reeves 1954; Sacks 1967). Although the government would pay for transport for the family members, the labourers were responsible for the subsistence of their family while in South Africa and this was relatively expensive (Yap & Man 1996).

11.1.1 Living Conditions

The living conditions for labourers were extremely restrictive as they were confined to the mine premises (Kynoch 2003; Ndlovu *et al.* 2006). They could only leave the compound with a permit; with a red permit they could leave until sundown, with a white permit they were allowed to exit overnight, not exceeding 48 hours. By law they were forbidden to leave the Witwatersrand magisterial district (Yap & Man 1996; Kynoch 2003). Accommodation within

Figure 11.1: A photograph taken at the Simmer & Jack Gold Mining Company showing the compounds on South African gold mines in the 1900's for Chinese indentured miners (Copyright MS 429/15: The Brenthurst Library, Johannesburg).

the compounds were wire mesh bunks divided by curtains, which were well lit, ventilated and sanitary (Reeves 1954).

11.1.2 Communication

There were major communication difficulties on the mines as the mine management and supervisors did not speak Chinese (Yap & Man 1996; Kynoch 2003). The head policeman was a Chinese speaking person in charge of the men on

Figure 11.2: A photograph taken at the Simmer & Jack Gold Mining Company showing Chinese indentured miners bunk rooms situated inside the compounds in the 1900's (Copyright MS 429/13: The Brenthurst Library, Johannesburg).

the mines and interpretation, but they were out numbered by miners (Yap & Man 1996).

11.1.3 Food Allowance

The Chinese miners were provided with a daily food ration consisting of 0.22 kg rice, 0.22 kg meat, 0.22 kg vegetables, 15 g of tea, half an ounce of nut oil and salt (Reeves 1954; Yap & Man 1996).

11.1.4 Recreation

For recreation they were reported to gamble, have drilling competitions, consort with prostitutes and on special occasions have Chinese theatre (Reeves 1954; Yap & Man 1996). Also, opium was reportedly used in large quantities (Sacks 1967).

11.1.5 Health Status

It was in the interest of the mines to keep the Chinese miners in good health in order to renew their contracts, therefore, the mines provided free Chinese and Western medical treatment (Reeves 1954; Yap & Man 1996; Ndlovu *et al.* 2006). Despite access to medical facilities there was a high rate of diseases such as scurvy, silicosis, venereal syphilis and beri beri. In addition opium-poisoning, suicide and murder were common (Reeves 1954; Yap & Man 1996; Ndlovu *et al.* 2006). Mining is hazardous and miners were exposed to dust, poor ventilation, high temperatures, lack of latrine facilities, polluted water and overcrowding, making the mines a breeding ground for disease (Richardson 1982; Ndlovu *et al.* 2006). Injuries and fatalities were frequent amongst the Chinese miners, often caused by mining accidents such as digging and drilling in unexploded holes, snapping of cage rope and inexperience with explosives (Reeves 1954; Richardson 1982). In 1904 to 1905, 1167 labourers were repatriated to China due to incapacity for work by physical infirmity or disease (Campbell 1923). It is estimated that between the years 1904 to 1910, 3192 Chinese people died on the Witwatersrand (Reeves 1954; Richardson 1982; Yap & Man 1996).

11.1.6 Restrictions of Labour

Due to the oppressive labour restrictions and practices of the gold mines many criminal charges were laid against the Chinese miners. Flogging was a regular punishment for offences against the ordinance, until it received public attention causing outrage (Reeves 1954; Sacks 1967; Richardson 1982; Yap & Man 1996;

Kynoch 2003).

Reports of torture and abuse were published in various South African and British newspapers forcing the mines to deal with the problem of assault by supervisors (Kynoch 2003). This led to the decision to stop recruitment. The indentured labour laws Act of 1907 provided time for the completion of current contracts and to phase out the labour over a period of 2.5 years (Campbell 1923; Sacks 1967; Richardson & Van-Helten 1984; Yap & Man 1996; Ndlovu *et al.* 2006). Prior to repatriation, many of the labourers submitted urgent appeals for the remains of those who died on the mine to be exhumed and cremated for their return to China, which was granted, provided the graves were older than twelve months (Yap & Man 1996). The last shipment of repatriated miners was returned to China at the beginning of 1910 (Richardson 1982; Ndlovu *et al.* 2006).

11.2 After Repatriation

After repatriation, African labour returned to the Rand and maintained the mine production levels reached by the Chinese. By 1910, only 2000 Chinese miners remained in South Africa and never returned home.

Chapter 12

Materials and Methods

The methods developed and optimised in the initial stages of this study were applied to a historical archaeological skeletal collection of 36 Chinese indentured miners sourced from the Dart Collection in the School of Anatomical Sciences at the University of the Witwatersrand. Ethical clearance for this collection falls under the Human Tissues Act of 1983. Permission was granted from the Collections Committee, School of Anatomical Sciences, University of the Witwatersrand.

The methods detailed in part one of this thesis were followed: bone powder was extracted by drilling into the distal end of the left femur; DNA was purified and sex was determined by analysing data gained through sequencing analyses and the Experion electrophoresis system. All of the recommended precautions and procedures for working with arch/aDNA were followed.

12.1 Assessment of Collection

During the initial assessment of the Chinese indentured sample, it was noted that the specimens have varying degrees of preservation, ranging from extremely well-preserved to a high level of fragmentation. Due to fragmentation (A1021, A1025 and A1029), or missing both femora (A1024 and A1026) 5 specimens were not suitable for use in the present study (figure 12.1). Further, 5

of the specimens had an unusable left femur and for these specimens, the right femur was used instead (table I.1). Therefore, in total only 31 specimens from the Chinese collection were analysed for molecular sex determination. In table I.1, the condition and colour of bone was noted based on this investigator's criteria of observation found in tables A.1 & A.2 . One notable specimen (A1020) used in this study was unfused on the distal end of the left femur (figure 12.2).

Figure 12.1: Chinese specimen A1025, demonstrating fragmentation on the distal end of both femora (arrows). Scale=1 cm.

Figure 12.2: Chinese specimen A1020, demonstrating an unfused metaphysis (arrow) on the distal end of the left femur. This is indicative of the individual still growing. Scale=1 cm.

Chapter 13

Results

Due to the way in which the results are created through large graphic files (chromatograms, gels, simulated gels and electropherograms) for simplicity they were analysed and compiled into tables and summaries. There are examples of how these results are displayed figures H.1, H.2, H.3, H.4, H.5 and H.6, and specific edited examples are displayed within the text below.

13.1 Bone Extraction and DNA Extraction

The bone extraction result from the 31 specimens sourced from the Chinese sample produced a fine to medium bone powder with an average yield of 0.96 g (table I.1). As with the *ex-situ* specimens, the Chinese sample shows varying DNA concentrations (table I.2).

13.2 Molecular sex determination

13.2.1 System 1

In the first system, after amplification the PCR product was resolved on an agarose gel and visualised (table I.4). Sex classification was determined by analysing each sequence for the 10 polymorphic regions to determine whether

the sequence corresponds to the X and Y (male), or if only the X (female) was represented (1-10 table 7.1). The sequence for each sample was examined for those with readable sequence the 10 polymorphic regions are compiled and recorded in table I.3.

Only 10 (32.25%) of the specimens produced a result 9 (90%) of these were male and 1 (10%) was female. A comparison of both the results from the agarose gel and the sequencing for system one are recorded in table 13.1. In analysing the polymorphic sites one notable specimen, A1028 has co-dominance of the X and Y chromosome in some sites and in other sites only the X was visible. As this particular sample has both the X and Y chromosome, it was classified as a male (table I.3). Using system 1, figures 13.3 and 13.2 show edited chromatograms for two Chinese specimens, and figure 13.1 is an example of an agarose gel with Chinese samples.

Figure 13.1: Representative agarose gel electrophoresis for 9 Chinese specimens using system 1. Key: L-100 bp Ladder, P-Positive control, N-Negative control, 1- A997, 2- A908, 3- A999, 4- A1000, 5- A1001, 6-A1002, 7- A1003, 8- A1004, 9- A1005, 10- A1006 and 11- A1008.

13.2.2 System 2

In the second system, the results from the Experion gel were analysed for either no bands, or one band or two bands (table I.5). The corresponding samples

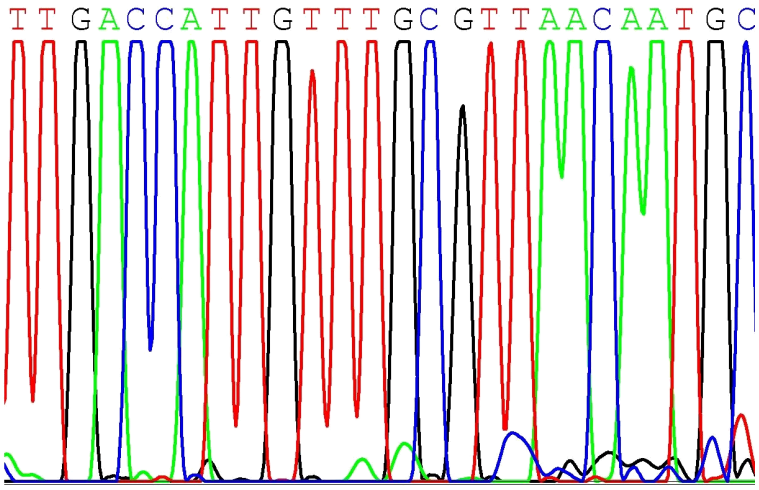

Figure 13.2: Specimen A1008, a female chromatogram spanning polymorphisms 5-10 according to table 7.1, with only the X chromosome represented in system 1. The nomenclature for the base pairs is available in appendix F.

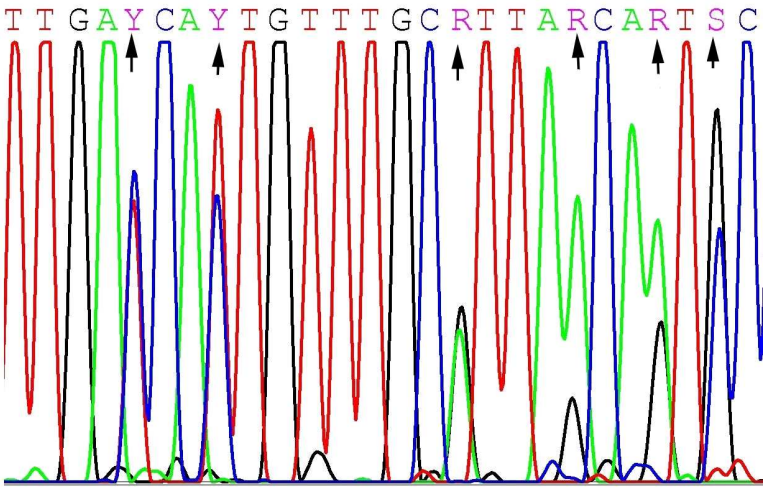

Figure 13.3: Specimen A997, a male chromatogram spanning polymorphisms 5-10 according to table 7.1, with the X and Y chromosomes represented in system 1 (arrows). The nomenclature for the base pairs is available in appendix F.

were subsequently prepared for sequencing (I.6). With both sequencing and the Experion gel methods 18 (58%) specimens in the sample produced a positive result. The combined results for both the sequencing and the Experion station are compared in table 13.2.

In analysing these results, 6 of the specimens (A996, A998, A1003, A1004,

Table 13.1: Summary of results for Chinese specimens for system 1

Sample ID	**Seq-sex**	**Band**	**Sex**
A996		*	
A997	*	*	M
A1000	*		M
A1001	*	*	M
A1002		*	
A1003	*	*	M
A1004		*	
A1007	*	*	M
A1008	*	*	F
A1009		*	
A1015	*		M
A1016	*		M
A1028	*	*	M
A1030	*	*	M

Seq- sequence result; Band- electrophoresis result; Blank- no result. M = male and F = female.

A1016 and A1028) with determined sex using both the Experion and Sequencing methods do not correspond. Upon further analysis, specimen A1008, for example, was only represented by one band on the Experion gel station and with sequencing, only the Y chromosome was represented. The existence of the Y chromosome explains the single band on the gel; a Y chromosome was indicative of a male. This shows how only using a gel based system, sex can be incorrectly diagnosed. Similarly, when analysing the sequence in some samples the Y was more prominent than the X. For all of these samples, the sex determined through sequencing was more reliable.

While a total of 8 (44.44%) of the specimens produced a positive result using the Experion system, not all of these produced readable sequence. In each of

Table 13.2: Summary of results for Chinese specimens for System 2

Sample ID	**Gel**	**Seq**
A996	F	M
A997	M	
A998	F	M
A1000		M
A1003	F	M
A1004	F	M
A1007	M	M
A1008	M	M
A1013	F	
A1014	F	
A1015	F	
A1016	F	M
A1022	F	
A1023	F	
A1027	F	
A1028	F	M
A1030	M	M
A1031	M	

M = male and F = female, blank indicates that sex was not determined. Seq- sequence result; Gel- electrophoresis result.

these samples sequence was produced, however due to excessive background, it was too difficult to decipher an accurate diagnosis of sex (figure 13.7), as discussed previously on the ex-situ collection. Consquently, sex could not be confirmed using sequencing. Only one sample gave a result using sequencing that did not render a band on the gel. This demonstrates that there was an insuffcient level of DNA in this sample to be detected electrophoretically, even though it was adequate for sequencing.

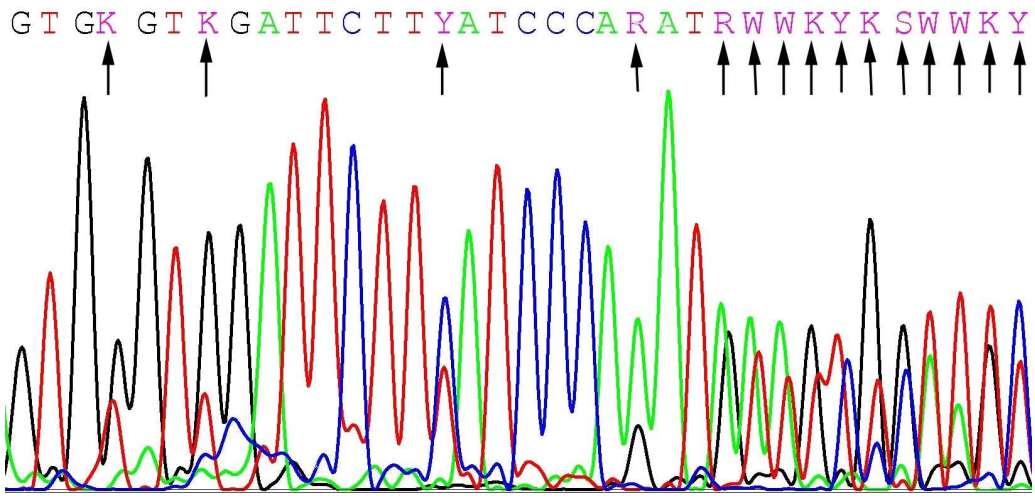

Figure 13.4: Specimen A1003, a male chromatogram spanning polymorphisms 11-14 and the indel according to tables 7.1 and 7.2, with the X and Y chromosomes represented in system 2 (arrows). The nomenclature for the base pairs is available in appendix F.

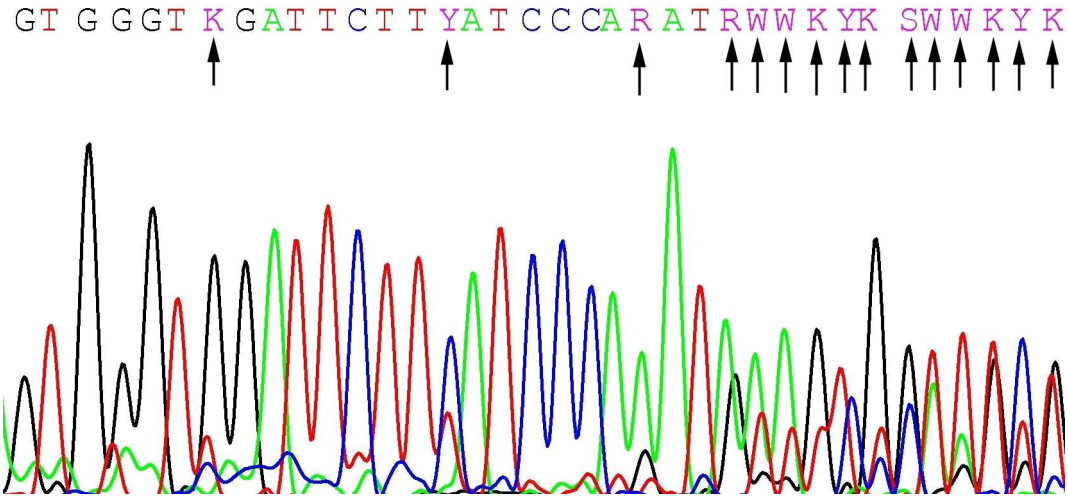

Figure 13.5: Specimen A1016, a male chromatogram spanning polymorphisms 11-14 and the indel according to tables 7.1 and 7.2, with the X and Y chromosomes represented in system 2 (arrows). The nomenclature for the base pairs is available in appendix F.

In the final analysis of system 2, the samples that gave a positive result on the Experion station and not confirmed with sequencing were omitted. Consequently, only 10 (32%) specimens in the sample gave reliable results through sequencing and they were classified as male. Using system 2 figures 13.4 and 13.5 show edited chromatograms for two *ex-situ* specimens, and figure 13.6 is

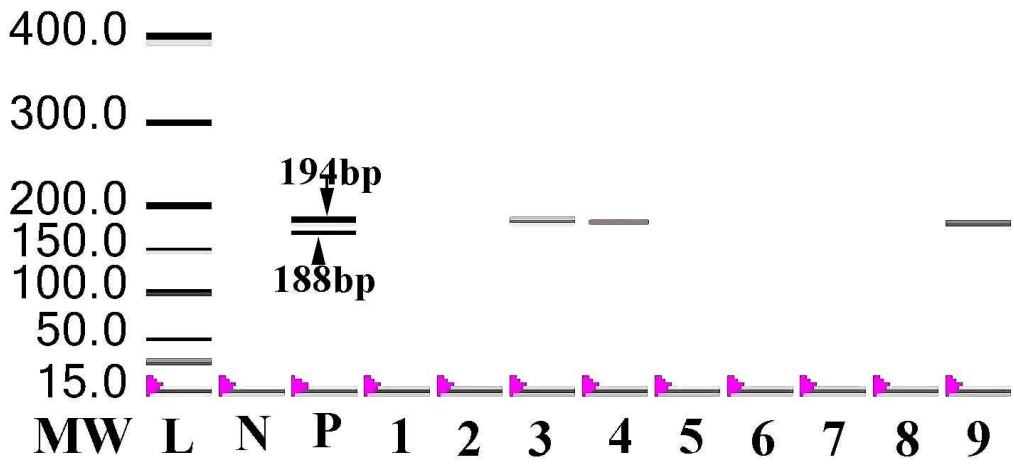

Figure 13.6: Representative microfluidic electrophoresis for 9 Chinese specimens using system 2. Key: MW-Molecular Weight Marker, L-Ladder, N-Negative control, P-Positive control, 1- A1021, 2-A996, 3-A997, 4- A998, 5-A999, 6- A1000, 7- A1001, 8- A1002 and 9- A1003.

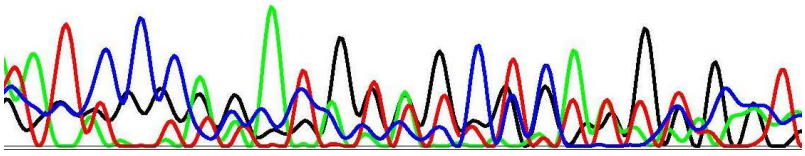

Figure 13.7: Specimen A76, an example of a sample with a low level of sequence and high background (compare to figures 13.3 and 13.4). Specimens such as this produced a band on the gel and undecipherable sequence preventing an accurate diagnosis of sex.

an example of a microfludic chip gel with Chinese samples.

13.2.3 System 1 and System 2

Specimen A1008 yielded different results in the two systems of sex determination. In system one, the sequence only produced the X chromosome, and similarly using the Experion gel in system two, an X was also visualised. However, when the sample was sequenced in system two it produced a mix of polymorphic regions corresponding to both the X and Y chromosomes. Even though the amplification of the Y chromosome was low, this sample was clearly a male.

Using both systems of sex determination, 19 (61.29%) specimens in the sample produced a result, wherein 14 (73.68%) were male and 5 (26.31%) were female (table 13.3). This raw data includes the specimens that had sex determined in system two based only on the Experion gel and not confirmed with sequencing. For accuracy these were left out of the analysis of the two systems, which takes the total sample with determined sex results using both systems to 13 (41.93%), all of which were male.

The reason no females are represented in the compiled results is due to the low level detection of the Y chromosome. In system 1, A1008 was the only female result and was found to actually be a male in system 2; as discussed above the Y chromosome was amplified in very low-copy number. Similarly in system 2, the specimens that were diagnosed as a female on the Experion gel system when sequenced, a low-level Y chromosome was represented. The females diagnosed solely through the Experion gel system were considered to be untrustworthy results on their own, therefore, all of the results obtained from only the Experion system were omitted. Only the samples verified with sequencing were used. However, the results from sequencing in cases where the Experion gel diagnosed them as female were all diagnosed as male, due to the detection of a low level Y chromosome.

Based on this data, 7 (53.84%) specimens in the sample produced the same result using both methods and 2 (15.38%) specimens produced different results for the same specimens. Using system one, 1 (7.69%) specimen generated a

result and 3 (23.07%) specimens produced results using system two. Based on the results of the two methods, it appears that system two was more sensitive as it diagnosed sex for a higher percentage of samples.

Table 13.3: Summary of molecular sex determination results for Chinese specimens using both systems 1 and 2

Sample ID	**System 1**	**System 2**
A996		M
A997	M	M*
A998		M
A1000	M	M
A1001	M	
A1003	M	M
A1004		M
A1007	M	M
A1008	F	M
A1013		F*
A1014		F*
A1015	M	F*
A1016	M	M
A1022		F*
A1023		F*
A1027		F*
A1028	M	M
A1030	M	M
A1031		M*

* Represent specimens in system 2 where sex was determined by the Experion gel only and not confirmed with sequencing. M = male and F = female, blank = sex was not determined.

Chapter 14

Discussion

The employment of Chinese indentured labour in South Africa was filled with controversy, these individuals died working for the gold mining industry on the Witwatersrand. As previously mentioned prior to their arrival on the mines, the miners health was examined by two medical assessments. It is therefore, acceptable to conclude that upon arrival in South Africa, they were of relatively good health (Ndlovu *et al.* 2006). The cause of death for these 36 individuals is unknown. Mining is hazardous work, in addition, unhealthy living and working conditions may account for these fatalities (Reeves 1954; Richardson 1982; Yap & Man 1996; Ndlovu *et al.* 2006).

This collection of Chinese indentured miners were all buried at the same location on the site of the Witwatersrand Deep Mine, within the six year period that Chinese labour was employed on the gold mines. These individuals were all exposed to the same taphonomic conditions and have approximately the same post mortem interval (PMI). Since the Chinese indentured labour was employed from 1904 and the final shipment of repatriated miners was in 1910, the PMI for these individuals is 98 to 104 years old. The remains of Chinese miners were exhumed and cremated provided the graves were older than a year (Yap & Man 1996). Based on this the PMI is closer to 98 years old. Either way this is not an old collection. In comparison the recent publications of the Neandertal where researchers extracted small fragments of genomic DNA from an individual with a PMI of 30 000 years old (Green *et al.* 2006; Noonan

et al. 2006). In the ground, DNA degradation is characteristically slower due to the cooler conditions and decreased exposure to oxidative damage (Mitchell *et al.* 2005). To preserve and maintain remaining DNA in the Neandertal specimen, it was carefully excavated in sterile conditions and placed directly into controlled cooler conditions (Green *et al.* 2006; Noonan *et al.* 2006). The Chinese samples were accessioned into the Dart Collection in 1951 and consequently for 57 years they have been kept at warmer conditions that are conducive for molecular degradation via oxidation and microbial activity. This could attribute for the reasons certain samples did not yield a result.

These data gathered on the Chinese specimens throughout the method, ranging from the conditions and colour of the bone to the DNA concentrations obtained for each specimen, were compiled and compared with the determined sex results. As with the *ex-situ* collection, none of these factors except for the condition of bone were reflective of molecular preservation. Two specimens were recorded as being in poor condition and with neither of these was sex determined. In this collection fragmented specimens were not included in the study. While all of the specimens were intact not all of them produced a result. This was similar to the results obtained in the *ex-situ* collection, showing that intactness of the specimen was not necessarily indicative of molecular preservation.

Due to fragmentation of the femora, 5 specimens were omitted from the molecular study conducted on the Chinese sample. However, if the investigator was not replicating the designed methods based on the *ex-situ* samples, other complete skeletal elements using the criteria described in the bone extraction method could have been used, which may have produced more results.

For the Chinese sample both systems produced results for the same number of specimens, this being 10 (32.25%). However, there were some differences between these results. This finding was similarly reflected in the results of the developed methodologies on the *ex-situ* specimens, and was accounted for through a variety of reasons. These were most likely due to physical and chemical degradation of the DNA molecule in these archDNA samples, that

disable the desired binding site of the primers. The primers were either not binding or not binding effectively to the archDNA, resulting in low or no amplification. In addition, the second system has the advanatge of amplifying a smaller DNA fragment and these preferentially amplify in arch/aDNA samples (Pääbo *et al.* 2004).

Using both systems, 13 (41.93%) specimens in the sample produced results, all of which were male. This reflects the level of molecular preservation of the DNA molecule in the area of interest on the amelogenin gene. The only concern when comparing the results obtained from both systems on this collection, was that 2 (15.38%) specimens in the sample gave conflicting results. One of these specimens A1015, could not be confirmed by sequencing. The second sample A1008, was problematic as in each system it produced a different sex result; in system 1 only the X chromosome was seen and in system 2 only the Y chromosome was detected. This could be due to primer mis-binding as a result of DNA degradation.

Based on the historical records only 2 women out of 63 935 individuals were brought to the Rand (Reeves 1954; Richardson 1982; Saunders 1994; Yap & Man 1996; Ndlovu *et al.* 2006). It seems improbable that one of these 36 specimens is female. In addition, 12 children came to the Rand. Specimen A1020 does show evidence of non-fusion of the distal end of the femur and tibia, as well as to the distal and proximal ends of the humerus (figure 12.2). Based on the use of standards of epiphyseal fusion of the primary ossification centres in relationship to chronological age (Buikstra and Ubelaker 1994), the age of the specimen is between 17 and 21 years. One specimen from the sample could be the child of a miner and therefore, could also be a female. This would account for 3 women out of 63 935 miners.

As expected the data gathered here using molecular sex determination on the Chinese sample yielded only male results, and this correlates with what is documented in the historical record. However, the morphometric study conducted by Edwards (2004) has a high level of female characteristics. The molecular

methods yielded more accurate results which were in accordance with the historical record.

The morphometric study conducted by Edwards (2004) had certain limitations. The population standard used was based on a Japanese population and this may have influenced the results. Moreover, as many demographic variables besides sex were being assessed and due to time constraints only the crania were used, and not the pelvis from which sex is most accurately diagnosed. Due to fragmentation on some of the specimens, only 28 skulls were suitable for this study and of these only 9 were well-preserved, while the others had differing states of fragmentation.

It would be of scientific interest to apply ageing and stature estimates based on the diagnoses of sex gained through the molecular methods developed here, and compare this to the data gathered by Edwards (2004). This would demonstrate how molecular methods could be used in conjunction with the traditional methods to add information to this archaeological collection. Moreover, molecular methods are not reliant on population specific standards, nor are they restricted in the same way by fragmentation. Therefore, the application of more methods to this collection would result in a more complete set of data.

Edwards did not reveal which specimens were used for the study by the Dart Collection number, nor which samples were excluded or included and which of these were identified as male and female. It was due to these limitations in the data gathered by Edwards that it was impossible to conduct comparisons with the data collected in the present study. In addition, statistical comparisons of the data gathered between both the traditional and the molecular methods of sex determination could not be conducted. The study conducted by Edwards (2004) on the Chinese sample should be repeated using the entire skeleton, as this would more accurately determine sex through morphometric methods.

Chapter 15

Conclusion

The methods developed in part one were used to examine the sex profile of the Chinese indentured miner collection dating back to the gold mining era in the early 1900's. These 36 Chinese indentured miners were brought to South Africa during a labour shortage after the Anglo-Boer war, and they died while working for the Witwatersrand Gold Mine.

Using both systems of molecular sex determination, 41.93% of the Chinese sample produced a result and the sex profile was all male individuals, which correlates well with what is documented in the historical records. This however conflicts with the study by Edwards' (2004) that showed a high level of female characteristics for this sample. Edwards (2004) study has limitations, it is suggested that morphometrics should be redone on this collection, to better understand the population and to add information to the results of the molecular analysis obtained here.

Both the traditional morphometric and molecular methods of sex identification have their advantages and disadvantages. Biological anthropologists should apply all the available approaches to understand a given individual or a population represented, as this results in the most complete and accurate set of demographic variables.

Chapter 16

Final Conclusions

The purpose of this study was to develop novel methods of molecular sex determination from skeletal tissue and to apply these techniques to examine the sex profile of the Chinese indentured miners sourced from the Dart Collection.

The novel bone extraction method designed here uses the area between the medial and lateral condyles- in the intercondylar fossa of the left femur. This procedure only forms a small hole on the surface and does not interfere with any known anthropometric landmarks and provides enough bony tissue for DNA analysis. In addition, due to the marginally invasive nature of this method, it does not physically compromise the femur's use in future studies. Permission is easily obtained for molecular studies on skeletal tissue due to these characteristics and the efficacy of this novel method, which provides the opportunity for future molecular studies.

The maximum yield of DNA from bone samples is produced using the silica column based DNA extraction method with the addition of EDTA to the lysis buffer. No distinct pattern was found from the A260/A280 ratios of the DNA, other than for the samples with a purity ranging between 1.8 and 1.99, all of which produced sex determination results.

The methods of bone extraction and DNA extraction designed here can be used to isolate any desired area on the genome. In addition, these methods can be used on skeletal material derived from any animal or human.

Two novel molecular sex determination methods were developed using an amplicon on the amelogenin gene that isolates 14 sex-specific polymorphic regions and an indel. The first system uses sequencing of 10 sex-specific polymorphic regions, and the second system in addition to using sequencing uses microfluidic technology with automated gel electrophoresis.

These methods performed with 100% success on the modern bone and blood controls. It is unexpected for the application of these methods to archaeologically derived samples on also yield results for 100% of the sample. If this did occur it would not only be incredibly rare, but also a good reason to question the authenticity of such results.

These methods were then optimised on pooled DNA from the *ex-situ* collection and only after they produced optimal results, were they applied to the *ex-situ* collection. Together these methods diagnosed sex for 46.66% of the *ex-situ* sample. The second method with microfluidic technology was a superior method as it doubled the results of the first method. Due to its sensitivity it is proposed that the Experion system should be used as a predictor of the PCR amplicon in arch/aDNA samples and sequencing for sex determination. In those instances where a result was not attained, the most logical reason was due to chemical and physical degradation of the target gene.

These methods were subsequently applied to the collection of Chinese indentured miners to address questions regarding the sex profile of this sample. Sex was successfully determined from 41.93% of this sample and the sex profile revealed that all of these individuals were male. Unlike the previous morphometric study conducted on this collection that produced results with a high number of female characteristics, the results gained in this research substantiate the information in the historical records. However, one should bear in mind that the study conducted by Edwards (2004) had a number of limitations and should be redone.

In both of the collections studied here the molecular methods did not render results for every specimen in the sample. Therefore, it is important to also

use traditional morphometric methods. With the use of technology, biological anthropology is constantly evolving in methodology and their applications to answer questions previously thought to be impossible. Despite these new advances within the field, the traditional methods of morphometrics must be used with the technology as they complement one another, where one fails the other succeeds. The sex determination results gained in the present study provide a platform for further research in morphometric studies, specifically for ageing and stature estimations.

16.1 Future Research

The method of bone extraction developed in this study coupled with the silica-based DNA extraction method have numerous applications for the field of anthropological genetics. These methods were adapted for skeletal tissue, which can now be applied to the abundant collections of skeletal remains in museum and university collections to examine anthropological questions.

The novel methods of molecular sex determination designed in this study can be applied to other archaeological samples to examine sex related questions. It must be noted that these methods are not limited to use with only the femur for bone extraction. They may for example, be applied to DNA extracted from teeth (Grimoud *et al.* 2002) or any bony element that yields DNA. In addition, these methods are useful for materials derived from both forensic and archaeological contexts.

An excellent South African example where the application of these methods of sex identification would be of great importance is to the world heritage site of Mapungubwe, the first stratified state society in the region of Southern Africa. The knowledge of sex for the 12 royal graves is of great importance to understand this state society. However due to fragmentation, an accurate sex profile for this collection based on morphometrics is virtually impossible. The sex of the royals has been hypothesised based on the archaeological pattern derived through the examination of taphonomy and artefacts that were distinct for

males and females. Due to the high level of fragmentation in this collection the bone extraction method is not applicable. However, in this instance the teeth are well preserved and there is a minimally invasive method of dental enamel extraction developed by Grimoud *et al.* (2002) that could be used instead. The molecular methods developed here can certainly be used to confirm the sex profile of the royals.

With regards to the Chinese collection it would be of scientific interest to conduct further investigation into the cause of death for these individuals. This could be done through a pathological assessment of each skeleton in relationship to information gained from the historical records. In addition, as previously stated the morphometrics on this collection was limited and this should be repeated using a Chinese standard on the entire skeleton. Further, mitochondrial DNA could be extracted from this Chinese sample, in order to confirm population affinity through population specific markers.

As in this study, DNA degradation greatly affected the number of positive results. It would be valuable to further investigate the degradative factors impinging on DNA integrity and also to develop means of repairing these defects. Such techniques would certainly improve on the attainment of molecular results.

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Appendix A

Bone Assessment

Table A.1: Condition of bone

Good	**Fair**
No Broken Ends	Slightly Damaged Distal end

Table A.2: Colour table for bone

Light	**Light with dark**	**Light/Dark**	**Dark with light**	**Dark**
1	2	3	4	5

Appendix B

Bone Extraction

Table B.1: Bone extraction results from the *ex-situ* specimens

Specimen number	**Condition of bone**	**Weight of powder sample**	**Colour of femur**	**Colour of bone powder**
A26	Good	0.655 g	5	4
A74	Good	0.59 g	5	4
A75	Fair	1.04 g	3	4
A76	Good	0.58 g	3	2
A77	Good	0.527 g	2	1
A78	Fair	0.45 g	2	5
A79	Good	0.587 g	4	5
A123	Fair	0.84 g	2	3
***A173	Fair	2.1 g	1	3
***A240	Fair	1.14 g	1	1
A268	Fair	0.96 g	2	1
**A293	Fair	0.52 g	3	2
*A294	Good	0.95 g	3	4
A299	Good	0.97 g	3	1
*A302	Good	0.85 g	4	2
++A312	Fair	0.39 g	4	2

Table B.1: Bone extraction results from the *ex-situ* specimens (continued)

Specimen number	**Condition of bone**	**Weight of powder sample**	**Colour of femur**	**Colour of bone powder**
A319	Good	0.76 g	4	2
A320	Fair	0.81 g	4	4
A326	Fair	0.814 g	2	2
A330	Fair	1.31 g	2	3
A379	Fair	0.86 g	2	3
A427	Good	0.67 g	1	2
A453	Fair	0.96 g	2	5
A658	Good	1.39 g	1	1
A675	Good	0.48 g	1	1
A907	Fair	1.02 g	1	1
A908	Good	0.62 g	1	2
A909	Good	0.3 g	3	1
+A1096	Good	0.65 g	3	1
A2227	Fair	1.82 g	2	3

* Mummified soft tissue present on distal ends. ** Thin fragile layer of compact bone. *** Proximal end missing, distal end not fully fused, crack in midshaft. + Proximal end missing, most likely due to carnivore activity. ++ Proximal end missing and unfused, distal end not fully fused.

B.1 Materials and Methods for Temperature Experiment

The temperature of the bone extraction procedure was recorded through two experiments. The temperature in both experiments was measured using two copper-constantan thermocouple probes connected to an laboratory digital thermometer (Physitemp, Model BAT-12; Sensortek Inc., Clifton, NJ, USA). The wires were held in plastic forceps, as not to transfer any heat from the researcher to the probe. A conventional hand held power drill (Bosch PSB 650RE) with a 4.5 mm titanium masonry drill bit was used. The temperature changes were assessed using 5 femora from the X-collection.

In experiment one, prior to drilling the ambient temperature of both the drill bit and bone were recorded. The temperature was then recorded on both the drill bit and hole, upon initial breakthrough of the cortical bone and also during pulverisation.

In experiment two, a temperature probe was placed inside a pre-drilled hole in the femur and the ambient temperature was recorded. Then while drilling another adjacent hole, the temperature was recorded from inside the distal end of the femur.

Table B.2: Experiment 1 recording temperature in °C for the bone extraction method on the drill bit

	1	**2**	**3**	**4**	**5**
Before Drilling	18.7	18.7	18.8	18.8	18.9
Initial breakthrough	20	19.2	19	19.4	19.4
Pulverise	19.4	19.3	19	19.3	19.8

Table B.3: Experiment 1 recording temperature in °C for the bone extraction method in the bone hole

	1	**2**	**3**	**4**	**5**
Before Drilling	18.4	18.4	18.6	18.5	18.4
Initial breakthrough	18.8	18.4	18.8	18.8	18.9
Pulverise	19.1	18.8	18.8	18.9	19.2

Table B.4: Experiment 2 recording temperature in °C for the drilling procedure with a temperature probe within a previously drilled hole in the distal end of the femur

Probe temp.	**1**	**2**	**3**	**4**	**5**
Before Drilling	19	18.9	18.9	18.7	18.6
While Drilling	19.8	19.5	19	19.3	20.7
After Drilling	19.7	19.5	19.1	19.3	20.7

Appendix C

DNA Extraction

All the chemicals used in this section of this study were sourced from Saarchem unless otherwise stated.

C.1 Recipe for Sucrose-Triton X Lysing Buffer (Miller *et al.* 1988)

For 1 L:
10 ml 1 M Tris-HCL (pH 8.0)
5 ml 1 M $MgCl_2$
10 ml Triton X-100
Make up to one litre. Autoclave. Store at 4 °C
Add 109.5 g/L sucrose (Merck) just before use and dissolve.
*Only add sucrose when ready to use as once added, the solution does not keep (need 70 ml per sample).

C.2 Recipe for T20E5 Buffer (Miller *et al.* 1988)

For 500 ml:

20 mM Tris-HCL (10 ml 1 M)

5 mM EDTA (5 ml 0.5 EDTA)

Make up to 500 ml autoclave store at room temperature.

C.3 Recipe for Saturated NaCl (Miller *et al.* 1988)

For 100 ml:

Slowly add 40 g NaCl to 100 ml sterile water

When completely saturated there should be some precipitate in the solution.

Store at room temperature.

C.4 Recipe for 10% SDS (Merck) (Miller *et al.* 1988)

For 10 ml:

1 g in 10 ml sterile water.

C.5 Recipe for 10 mg/ml Proteinase K (Roche) (Miller *et al.* 1988)

For 50 ml:

Add 50 ml sterile water to bottle continue 500 mg

Aliquot into 1 ml tubes.

Table C.1: Proteinase K Mix (Miller *et al.* 1988)

	Stock Solution	**2 ml (4/sam)**	**4 ml (8/sam)**	**8 ml (16/sam)**
1% SDS	10% SDS	200 μl	400 μl	800 μl
2 mM EDTA	0.5 M EDTA	8 μl	16 μl	32 μl
2 mg/ml P.K	10 mg/ml	400 μl	800 μl	1600 μl
Water		200 μl	1400 μl	2800 μl

Make fresh each time, need 500 μl per sample.

P.K is proteinase K

Table C.2: Purity and concentration of DNA extracted from the *ex-situ* specimens

Specimen Number	**260**	**280**	**Ratio**	**Concentration ng/μl**
A26	0.560	0.277	2.02	28.00
A74	0.900	0.511	1.76	45.00
A75	1.624	0.891	1.82	81.20
A76	0.515	0.268	1.92	25.70
A77	0.715	0.441	1.62	35.70
A78	1.694	1.124	1.51	84.70
A79	1.246	0.698	1.78	62.30
A123	0.843	0.516	1.63	42.10
A173	0.590	1.570	1.53	119.71
A240	1.584	0.972	1.63	79.20
A268	1.637	0.944	1.73	81.90
A293	0.871	0.470	1.85	43.60
A294	0.587	0.326	1.80	29.40
A299	0.641	0.281	2.28	32.00

Table C.2: Purity and concentration of DNA extracted from the *ex-situ* specimens (continued)

Specimen Number	**260**	**280**	**Ratio**	**Concentration ng/μl**
A302	1.746	1.022	1.71	87.30
A312	0.520	0.286	1.82	26.00
A319	0.451	0.267	1.69	22.50
A320	0.973	0.562	1.73	48.70
A326	1.984	1.120	1.77	99.20
A330	2.140	1.202	1.79	107.40
A379	0.306	0.190	1.61	15.30
A427	0.496	0.261	1.90	24.80
A453	1.757	1.183	1.49	87.80
A658	0.290	2.091	1.42	179.74
A675	0.605	0.268	2.26	30.30
A907	3.514	1.768	1.99	175.70
A908	3.356	2.126	1.58	167.80
A909	1.533	1.091	1.41	76.70
A1096	0.672	0.328	2.05	33.60
A2227	1.233	0.785	1.57	61.70

Appendix D

Agarose Gel

All the chemicals used in this part of the research were sourced from Saarchem unless otherwise stated.

D.1 Recipe for Tris-borate (TBE) (Perbal 1988)

10X stock solution 1 L. Mix for 30 minutes, then pH to 8.3.

108 g Tris

9.3 g EDTA

55 g Boric acid

1X working solution 1 L

100 ml 10X stock

900 ml dH_2O

D.2 Recipe for Loading Buffer (Perbal 1988)

For 250 ml: Mix in 500 ml bottle

0.63 g Bromophenol Blue (Merck)

150 ml H_2O

Stir for 60 minutes

Add 25 ml of 100% anhydrous glycerol using a syringe

Stir for 30 minutes until mixed

Add dH_2O up to 250 ml

Store in 50 ml aliquots at -20 °C.

D.3 Ethidium Bromide (Perbal 1988)

10 mg in 1 ml dH_2O or

1 g in 100 ml dH_2O

Table D.1: Agarose Gel (Perbal 1988)

% Gel	**1%**	**1.5%**	**2%**	**3%**
Agarose Powder (Bio-Rad)	1 g	1.5 g	2 g	3 g
1X TBE	100 ml	100 ml	100 ml	100 ml
Ethidium Bromide	3 μl	3 μl	3 μl	3 μl
Normal Gel				Separation

Microwave for 1 minute. Mix. Microwave for another minute. Mix. Add ethidium bromide. Pour into casting tray.

D.4 Loading Gel (Perbal 1988)

Tank buffer 1X TBE

5 μl of 100 bp ladder (Promega) including dye

2.5 μl of loading dye (Promega) per sample

10 μl of sample

Run at 80 volts for 1.5 hours.

View with Bio-Rad Gel Docking System under UV light.

Running conditions:

Electrophoresis was carried out at a constant voltage of 80 volts for 1.5 hours.

Appendix E

PCR Optimisation

E.1 SYBR Green with Real-time PCR

Table E.1: SYBR Green PCR Master Mix

	1x
SYBR green Master mix	5 μl
Fwd Primer 2 μM	1 μl
Rvs primer 2 μM	1 μl
DNA	3 μl
Total volume	10 μl

Initial incubation of 94 °C for 10 minutes. Cycling conditions: 94 °C for 15 seconds, 55 °C for 15 seconds, 72 °C for 45 seconds for 45 cycles.

E.2 Taq Polymerase

E.2.1 Sahara DNA polymerase (Bioline)

This was tested with 'Amel fwd' and 'Amel Rvs', with outer primers of 'As' and 'SE', on blood controls and six archaeological samples that produced varying results with Amplitaq Gold (Applied Biosystems).

E.2.2 Roche Expand High Fidelity PCR System

Roche Expand High Fidelity PCR System: three mixes per round of PCR

1. First Round of PCR: primers, amel fwd and amel rvs
2. Work on ice
3. Make mix one and two according to table E.3 in separate tubes.
4. For the final mix, add both mixes and aliquot 15 μl per PCR tube and then add 10 μl of DNA.
5. Set up second round mixes according to table E.4 in separate tubes.
6. For the final mix, add both mixes 25 μl from each then add 50 μl per tube.

Table E.2: PCR Master Mix for Bioline Sahara DNA Polymerase

	1st Rd	**2nd Rd**
50 mM Magnesium	2.4 μl	4 μl
10X Buffer	3 μl	5 μl
10mM dNTPs	0.6 μl	1 μl
Fwd Primer 2 μM	3 μl	5 μl
Rvs primer 2 μM	3 μl	5 μl
Taq Polymerase 4 u/μl	0.3 μl	0.5 μl
H_2O	7.7 μl	29.5 μl
DNA	10 μl	0 μl
Total volume	30 μl	50 μl

Initial incubation of 95 °C for 8 minutes. Cycling conditions: 94 °C for 1 minute, outer primers 55 °C for 45 seconds and inner primers 65 °C for 45 seconds, 72 °C for 1 minute for 35 cycles.

Table E.3: Roche Expand High Fidelity PCR System: First Round PCR

	Mix 1	**Mix 2**
10 mM dNTPs	0.5 μl	
Fwd Primer 2 μM	5 μl	
Rvs primer 2 μM	5 μl	
H_2O	0 μl	9.625 μl
Buffer and Magnesium		2.5 μl
Taq Polymerase 5 u/μl		.375 μl
Total volume	12.5 μl	12.5 μl

Initial incubation of 94 °C for 2 minutes. Cycling conditions: 94 °C for 30 seconds, outer primers 55 °C for 45 seconds and inner primers 65 °C for 45 seconds, 72 °C for 1 minute for 35 cycles. Final elongation 72 °C for 7 minutes and a 4 °C hold.

Table E.4: Roche Expand High Fidelity PCR System: Second Round PCR

	Mix 1	**Mix 2**
10 mM dNTPs	1 μl	
Fwd Primer 2 μM	2 μl	
Rvs primer 2 μM	2 μl	
H_2O	20 μl	19.25 μl
Buffer and Magnesium		5 μl
Taq Polymerase 5 u/μl		0.75 μl
Total volume	25 μl	25 μl

Initial incubation of 94 °C for 2 minutes. Cycling conditions: 94 °C for 30 seconds, outer primers 55 °C for 45 seconds and inner primers 65 °C for 45 seconds, 72 °C for 1 minute for 35 cycles. Final elongation 72 °C for 7 minutes and a 4 °C hold.

E.3 Magnesium Titration

For the magnesium titration the AmpliTaq Gold Core reagent kit was used (Applied Biosystems).

Optimisation of $MgCl_2$ for nested PCR (4 steps)

1. **Step 1:**
2. Set up 10 first round 20 μl tubes with a dilution series of $MgCl_2$.
3. Make master mix (table E.5) add 7 μl of pooled DNA for each tube. Add 70 μl of pooled DNA to master mix then add 10 μl to each tube.
4. Aliquots of $MgCl_2$ and H_20 according to table E.6.
5. **Step 2:**
6. Make master mix table E.5.
7. When first round of PCR is complete, add 80 μl of second round master mix, then cycle again.
8. Run 10 μl of each second round PCR product on gel (appendix C).
9. The tube with the brightest band (Biorad gel/chemi doc) and no non-specific bands has the optimum $MgCl_2$ for the first round.
10. **Step 3:**
11. Set up 10 first round 20 μl tubes with the optimum $MgCl_2$ from step 2.
12. Make master mix (table E.5), add 7 μl of pooled DNA for each tube. Add 70 μl of pooled DNA to master mix then add 20 μl to each tube.
13. Spin down and cycle.
14. **Step 4:**
15. Set up 10 second round 100 μl reactions with a dilution series.

16. Make master mix according to table E.5.

17. When first round is complete, add all 10 second round tubes to the 660 μl master mix, mix well, spin down and use 86 μl for the second round.

18. Aliquot of $MgCl_2$ and H_2O according to table E.7.

19. Cycle. Run 10 μl of the PCR product on a gel (appendix C).

20. The brightest band (Biorad gel/chemi doc) lacking non-specific bands is the optimum $MgCl_2$ for the second round of PCR.

Table E.5: Master Mix's for $MgCl_2$ Titration

	Step 1 x1	**Step 2 x1**	**Step 3 x1**	**Step 4 x1**
25 mM Magnesium	0	6 μl	X μl	0 μl
10X Buffer	2 μl	10 μl	2 μl	10 μl
10 mM dNTPs	0.4 μl	2 μl	0.4 μl	2 μl
Fwd Primer 10 pmol/μl	0.2 μl	1 μl	0.2 μl	1 μl
Rvs primer 10 pmol/μl	0.2 μl	1 μl	0.2 μl	1 μl
Taq Polymerase 5 u/μl	0.2 μl	0.8 μl	0.2 μl	0.8 μl
H_2O	0 μl	59.2 μl	X μl	52 μl
Total volume	3 μl	80 μl	13 μl	66 μl

Initial incubation of 94 °C for 10 minutes. Cycling conditions: 94 °C 1 minute, 55 °C 45 seconds, 72 °C 1 minute for 35 cycles. Final extension of 72 °C for 10 minutes.

E.4 DNA Precipitation Test

DNA Precipitation

2x volume 100% cold ethanol.

Table E.6: $MgCl_2$ Step 1 Dilution Series

Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mixture	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl	10 μl
$MgCl_2$ (25 mM)	1 μl	2 μl	3 μl	4 μl	5 μl	6 μl	7 μl	8 μl	9 μl	10 μl
H_2O	9 μl	8 μl	7 μl	6 μl	5 μl	4 μl	3 μl	2 μl	1 μl	0 μl

Table E.7: $MgCl_2$ Step 4 Dilution Series

Tube	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Mixture	86 μl	86 μl	86 μl	86 μl	86 μl	86 μl	86 μl	86 μl	86 μl	86 μl
$MgCl_2$ (25 mM)	5 μl	6 μl	7 μl	8 μl	9 μl	10 μl	11 μl	12 μl	13 μl	14 μl
H_2O	9 μl	8 μl	7 μl	6 μl	5 μl	4 μl	3 μl	2 μl	1 μl	0 μl

2 hours precipitate at -20 °C.

Spin at 12 000 rpm for 15 minutes at 4 °C.

Wash pellet with 70% ethanol.

Air dry pellet.

Resuspend in 10 μl H_2O.

E.5 Bovine Serum Albumen

Table E.8: BSA PCR Reaction Mix

	1x
25 mM Magnesium	5 μl
10X Buffer	3 μl
10 mM DNTPs	0.6 μl
Fwd Primer 2 μM	3 μl
Rvs primer 2 μM	3 μl
Taq Polymerase 5 u/μl	0.2 μl
H_2O	4.2 μl
BSA	1 μl
DNA	10 μl
Total volume	30 μl

Initial incubation of 94 °C for 10 minutes. Cycling conditions: 94 °C for 15 seconds, 55 °C for 15 seconds, 72 °C for 45 seconds for 45 cycles.

Appendix F

Nomenclature for Nucleotide Bases

G- Guanine

A- Adenine

T- Thymine

C- Cytosine

R- Adenine and guanine

Y- Thymine and cytosine

W- Adenine and thymine

S- Guanine and cytosine

M- Adenine and cytosine

K- Guanine and thymine

H- Adenine, thymine and cytosine

B- Guanine, cytosine and thymine

V- Guanine, adenine and cytosine

D- Guanine, adenine and thymine

N- Guanine, adenine, thymine and cytosine

(Moss 1984)

Appendix G

Experion Electrophoresis

G.1 Diethylpypocarbonate (DEPC) Treated Water

Autoclave 2 L flask and a 1 L bottle.

Add 1 ml DEPC (Sigma Aldrich) to 1 L distilled water.

Shake overnight at 37 °C in flask.

Pour into 1 L bottle and autoclave.

Appendix H

Molecular Sex Determination

H.1 System 1

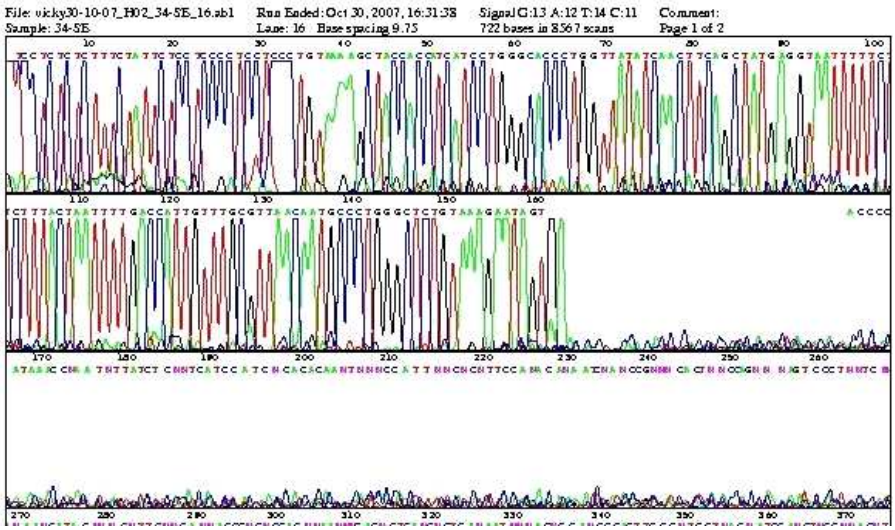

Figure H.1: An example of a full unedited chromatogram sequence, for a female specimen A75, using primers from system 1. The data recorded in the tables below was gathered from chromatograms such as this.

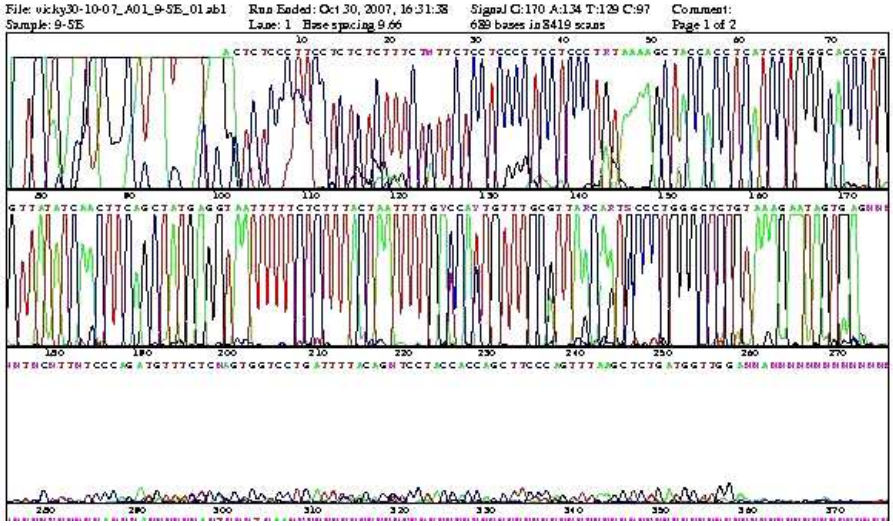

Figure H.2: An example of a full unedited chromatogram sequence, for a male specimen A908, using primers from system 1. The data recorded in the tables below was collected from chromatograms such as this.

Table H.1: System 1: Record of nucleotides at the polymorphic sites and the indel, correlating to tables 7.1 and 7.2 for the *ex-situ* specimens

Sample ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A26										
A74										
A75	T	T	A	G	C	T	G	A	A	G
A76										
A77										
A78										
A79										
A123										
A173										
A240										
A268										
A293			A/C	A/G	C/T	T/C	A/G	A/G	A/G	G/C
A294										

Table H.1: System 1: Record of nucleotides at the polymorphic sites and the indel, correlating to tables 7.1 and 7.2 for the *ex-situ* specimens (continued)

Sample ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A299										
A302				A/G	C/T	C/T	C/A	A/G	A/G	G/C
A312			A	G	C	T	G			
A319										
A320										
A326										
A330										
A379		T/A	C/A	G	C/T	T/C	A/G	A/G	A/G	G/C
A427										
A453										
A658										
A675										
A907										
A908	T/C	T/A	A/C	G/A	C/T	T/C	G/A	A/G	A/G	G/C
A909										
A1096										
A2227										

Table H.2: System 1: Using the presence or absence of bands on agarose gel electrophoresis for the *Ex-situ* specimens as an indicator of PCR amplification

Sample ID	**No Bands**	**Post PCR Band**	**Post Clean-Up Band**
A26	*		
A74	*		
A75	*		
A76	*		
A77	*		
A78	*		
A79	*		
A123	*		
A173	*		
A240		*	
A268	*		
A293	*		
A294	*		
A299	*		
A302		*	
A312	*		
A319	*		
A320	*		
A326	*		
A330	*		
A379	*		
A427		*	
A453	*		

Table H.2: System 1: Using the presence or absence of bands on agarose gel electrophoresis for the *Ex-situ* specimens as an indicator of PCR amplification (continued)

Sample ID	**No Bands**	**Post PCR Band**	**Post Clean-Up Band**
A658	*		
A675	*		
A907	*		
A908	*		
A909	*		
A1096		*	
A2227	*		

H.2 System 2

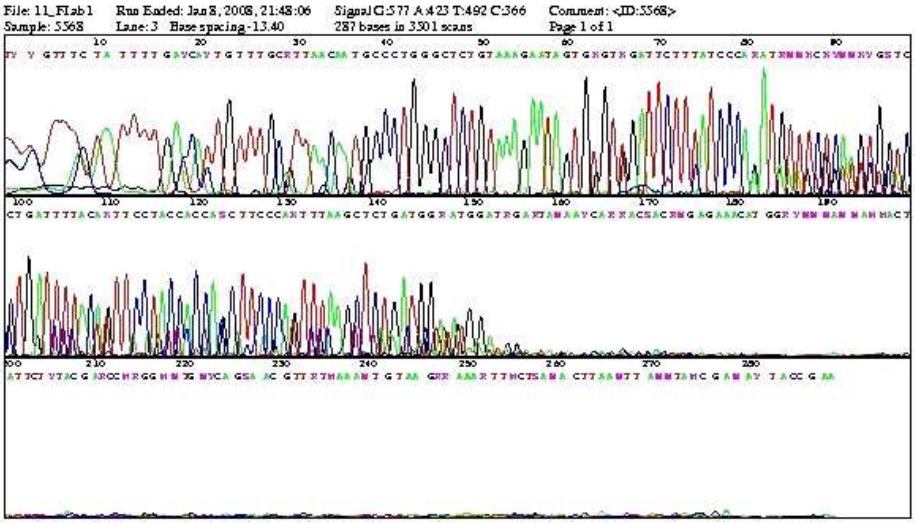

Figure H.3: An example of an unedited chromatogram sequence, for a male specimen A908, using primers from system 2. The data recorded in the tables below was gathered from chromatograms such as this.

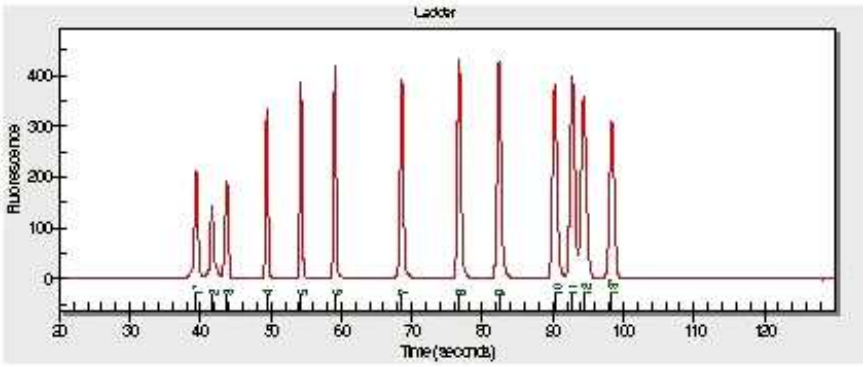

Figure H.4: An example of a electropherogram generated from the Experion system for the 100 bp ladder.

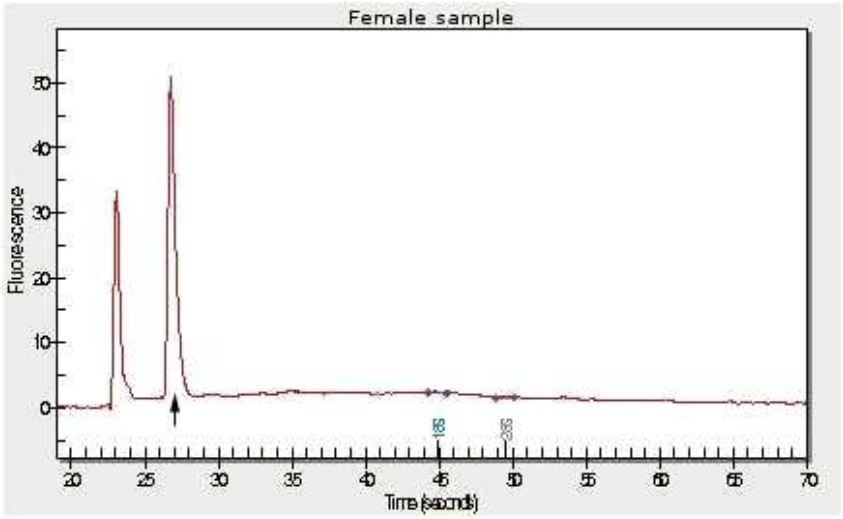

Figure H.5: An example of a electropherogram generated from the Experion system for a female sample, with one peak (arrow) representing the amplification of one band on the simulated gel. The first peak is a control peak generated by the system to show that the well did not fail.

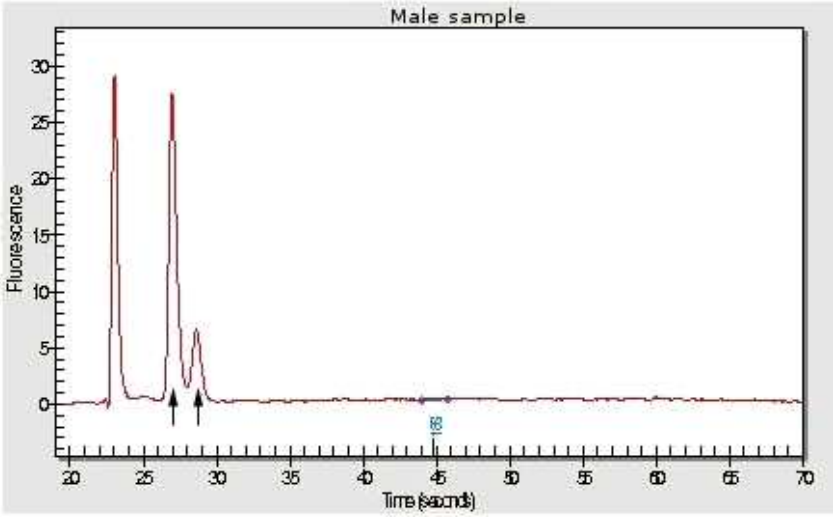

Figure H.6: An example of a electropherogram generated from the Experion system for a male sample with two peaks (arrows), representing the amplification of one band on the simulated gel. The first peak is a control peak generated by the system to show that the well did not fail.

Table H.3: System 2: Using the presence or absence of bands on the Experion automated gel electrophoresis for the *Ex-situ* specimens as an indicator of PCR amplification

Sample ID	**No Bands**	**1 band**	**2 band**
A26			*
A74	*		
A75	*		
A76	*		
A77			*
A78	*		
A79	*		
A123	*		
A173	*		
A240	*		
A268			*
A293	*		
A294			*
A299	*		
A302		*	
A312	*		
A319			*
A320	*		
A326	*		
A330	*		
A379		*	
A427			*
A453	*		
A658		*	
A675			*

Table H.3: System 2: Using the presence or absence of bands on the Experion automated gel electrophoresis for the *Ex-situ* specimens as an indicator of PCR amplification (continued)

Sample ID	**No Bands**	**1 band**	**2 band**
A907		*	
A908		*	
A909	*		
A1096			*
A2227	*		

Table H.4: System 2: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the *Ex-situ* specimens

Sample ID	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Indel**
A26			G/A	A/G	A/G	G/C	T/G	T/G	T/C	G/A	XY
A74											
A75											
A76	C/T	T/C	G/A	A/G	A	G	G				
A77	C/T	T/C	G/A	A/G	A/G	G/C	T/G	T/G	T/C	G/A	XY
A78											
A79											
A123											
A173											
A240											
A268											
A293	T	C	A	G/A	G	G/C	T/G	T/G	T/C	G/A	XY
A294	C/T	C/T	G/A	A/G	G	G/C	T/G	T/G	T/C	G/A	XY

Table H.4: System 1: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the *Ex-situ* specimens (continued)

Sample ID	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Indel**
A299											
A302						G/C	T/G	T/G	T/G	G/A	XY
A312											
A319	T	C	A	G	G	C	G	G	C	A	Y
A320											
A326											
A330											
A379		C/T	G/A	A/G	A/G	G	T/G	T/G	T/C	G/A	XY
A427											
A453					G	C	G	G	C	A	Y
A658	T	C	A	A/G	G	C	G	G	C	A	Y
A675											
A907											
A908	C/T	T/C	G/A	A/G	A/G	G/C	T/G	T/G	T/C	G/A	XY
A909											
A1096		T	G	A	A/G	G/C	T	T	T	G/A	
A2227											

Appendix I

Chinese Indentured Miners

I.1 Bone Extraction

Table I.1: Bone extraction results for the Chinese indentured miners

Specimen Number	**Condition of Bone**	**Weight of Powder Sample**	**Colour of Femur**	**Colour of Powder Bone**	**Right or Left Femur**
A996	Good	0.81 g	2	3	L
A997	Good	0.67 g	2	3	L
A998	Fair	0.74 g	2	4	L
A999	Good	2.50 g	2	4 S	L
A1000	Good	0.87 g	1	1	L
A1001	Good	0.81 g	1	3	L
A1002	Fair	0.33 g	2	2	L
A1003	Good	0.98 g	1	3	L
A1004	Fair	0.68 g	1	2	L
A1005	Fair	0.49 g	1	3	L
A1006	Fair	3.00 g	2	3 S	L
A1007	Fair	0.62 g	2	2	L
A1008	Good	0.82 g	1	1	L

Table I.1: Bone extraction results for the Chinese indentured miners (continued)

Specimen Number	**Condition of Bone**	**Weight of Powder Sample**	**Colour of Femur**	**Colour of Powder Bone**	**Right or Left Femur**
A1009	Fair	0.78 g	2	3	R
A1010	Fair	0.82 g	2	3	L
A1011	Fair	0.62 g	2	3	R
A1012	Bad	0.59 g	2	5	L
A1013	Good	2.30 g	1	3 S	L
A1014	Good	0.77 g	2	3	L
A1015	Fair	0.91 g	1	1	R
A1016	Fair	0.73 g	2	5	L
A1017	Bad	0.65 g	4	3	L
A1018	Fair	0.59 g	5	4	L
A1019	Fair	0.78 g	1	1	L
***A1020	Fair	1.20 g	1	2 S	L
**A1021	Bad		3		
A1022	Fair	0.63 g	2	1	L
A1023	Fair	1.30 g	1	1	R
*A1024	Good		4		
**A1025	Bad		3		
*A1026	Fair		2	1	
A1027	Fair	0.53 g	1	2 S	L
A1028	Fair	0.55 g	3	5	R
**A1029	Fair		3		
A1030	Fair	0.84 g	2	2	L
A1031	Fair	1.80 g	2	1	L

Table I.1: Bone extraction results for the Chinese indentured miners (continued)

Specimen Number	**Condition of Bone**	**Weight of Powder Sample**	**Colour of Femur**	**Colour of Powder Bone**	**Right or Left Femur**

* Missing both femora, **Both femora Highly fragmented,*** Unfused distal epiphyses of the femur, S sample appeared to have some red soil

I.2 DNA Extraction

Table I.2: Purity and concentration of DNA extracted from the Chinese indentured miners

Specimen Number	**260**	**280**	**Ratio**	**Concentration ng/μl**
A996	0.896	0.513	1.75	44.8
A997	1.056	0.625	1.69	52.8
A998	0.894	0.649	1.38	44.7
A999	2.315	1.540	1.5	115.7
A1000	0.447	0.230	1.94	22.3
A1001	0.656	0.403	1.63	32.8
A1002	0.569	0.354	1.61	28.5
A1003	0.495	0.289	1.71	24.8
A1004	0.632	0.426	1.49	31.6
A1005	1.00	0.556	1.8	50
A1006	4.445	3.010	1.48	222.3
A1007	0.609	0.359	1.70	30.5
A1008	0.616	0.206	2.99	30.8
A1009	0.473	0.205	2.31	23.6
A1010	0.875	0.491	1.78	43.7
A1011	0.626	0.301	2.08	31.3
A1012	0.717	0.304	2.11	35.8
A1013	1.208	0.786	1.54	60.4
A1014	1.022	0.569	1.80	51.1
A1015	0.409	0.208	1.96	20.5
A1016	0.886	0.495	1.79	44.3
A1017	0.390	0.163	2.39	19.5
A1018	1.115	0.636	1.75	55.8
A1019	0.343	0.193	1.76	17.1
A1020	2.571	1.789	1.44	128.6

Table I.2: Purity and concentration of DNA extracted from the Chinese indentured miners (continued)

Specimen Number	**260**	**280**	**Ratio**	**Concentration ng/μl**
A1022	0.285	0.135	2.11	14.2
A1023	1.380	0.897	1.54	69
A1027	0.758	0.377	2.01	37.9
A1028	6.136	3.974	1.54	306.8
A1030	0.489	0.324	1.51	24.4
A1031	0.575	0.290	1.99	28.8

I.3 Molecular Sex Determination

Table I.3: System 1: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the Chinese specimens

Sample ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A996										
A997			A/C	G/A	C/T	T/C	G/A	G/A	A/G	G/C
A998										
A999										
A1000				G/A	C/T	C/T	G/A			
A1001			A/C	G/A	C/T	C/T	G/A	A/G	A/G	G/C
A1002										
A1003			A/C	G/A	C/T	C/T	G/A	A/G	A/G	G/C
A1004										
A1005										
A1006										
A1007			A/C	G/A	C/T	C/T	G/A	A/G	A/G	G/C
A1008			A	G	C	T	G	A	A	G
A1009										
A1010										
A1011										
A1012										
A1013										
A1014										
A1015				G	C/T	C/T	G/A	A/G	A/G	G/C
A1016			A/C	G/A	C/T	C/T	A	A/G	A/G	G/C
A1017										
A1018										
A1019										

Table I.3: System 1: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the Chinese specimens (continued)

Sample ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A1020										
A1022										
A1023										
A1027										
A1028			A	G	C/T	C/T	A/G	A		
A1030			A/C	G/A	C/T	C/T	G/A	A/G	A/G	G/C
A1031										

Table I.4: System 1: Using the presence or absence of bands on agarose gel electrophoresis for the Chinese specimens as an indicator of PCR amplification

Sample ID	**No Bands**	**Post PCR Band**	**Post Clean-up Band**
A996		*	
A997		*	
A998	*		
A999	*		
A1000	*		
A1001			*
A1002		*	
A1003			*
A1004		*	
A1005	*		
A1006	*		
A1007			*
A1008		*	*
A1009		*	
A1010	*		
A1011	*		
A1012	*		
A1013	*		
A1014	*		
A1015	*		
A1016	*		
A1017	*		
A1018	*		

Table I.4: System 1: Using the presence or absence of bands on agarose gel electrophoresis for the Chinese specimens as an indicator of PCR amplification (continued)

Sample ID	**No Bands**	**Post PCR Band**	**Post Clean-up Band**
A1019	*		
A1020	*		
A1022	*		
A1023	*		
A1027	*		
A1028		*	*
A1030		*	*
A1031	*		

Table I.5: System 2: Using the presence or absence of bands the Experion automated gel electrophoresis for the Chinese specimens as an indicator of PCR amplification

Sample ID	**No Bands**	**1 band**	**2 band**
A996		*	
A997			*
A998	*		
A999	*		
A1000	*		
A1001	*		
A1002	*		
A1003		*	
A1004		*	
A1005	*		
A1006	*		
A1007			*
A1008			*
A1009	*		
A1010	*		
A1011	*		
A1012	*		
A1013		*	
A1014		*	
A1015		*	
A1016		*	
A1017	*		
A1018	*		
A1019	*		
A1020	*		

Table I.5: System 2: Using the presence or absence of bands the Experion automated gel electrophoresis for the Chinese specimens as an indicator of PCR amplification (continued)

Sample ID	**No Bands**	**1 band**	**2 band**
A1022		*	
A1023		*	
A1027		*	
A1028		*	
A1030			*
A1031			*

Table I.6: System 2: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the Chinese specimens

Sample ID	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Indel**
A996	C/T	T/C	A/G	A/G	G	G/C	T/G	T/G	T/C	G/A	XY
A997											
A998	C/T	T/C	A	A/G	G	G/C	T/G	T/G	T/C	G/A	XY
A999											
A1000				A/G	G	C	T/G	G	G	A/G	XY
A1001											
A1002											
A1003	C/T	T/C	G/A	A/G	G	G/C	T/G	T/G	T/C	G/A	XY
A1004	T	T/C	A	A/G	G	C	T/G	G	T/C	A	XY
A1005											
A1006											
A1007	T	C	A	A/G	G	C	G	G	T/C	A	Y
A1008	C	C	A	A/G	G	C	G	G	C	A	Y
A1009											
A1010											
A1011											
A1012											
A1013											
A1014											
A1015											
A1016	C/T	T/C	A/G	A/G	G	G/C	T/G	T/G	T/C	A/G	XY
A1017											
A1018											
A1019											
A1020											

Table I.6: System 2: Record of nucleotides at the polymorphic sites and the indel correlating to tables 7.1 and 7.2 for the Chinese specimens (continued)

Sample ID	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Indel**
A1022											
A1023											
A1027											
A1028	C/T	T/C	A	A/G	G	G/C	T/G	T/G	T/C	A/G	XY
A1030	C/T	C/T	A	A/G	G	G/C	T/G	T/G	T/C	A/G	XY
A1031											

Appendix J

Published Work

The abstract below has been published in the American Journal of Physical Anthropology. 2008. 135 (S46). pg.103.

Novel methods of molecular sex determination utilising the amelogenin gene.

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[1]*School of Anatomical Sciences,* [2]*Department of Medicine, University of the Witwatersrand, Johannesburg,* [3]*Department of Health and Chiropractic, Macquarie University.*

This study presents novel methods of molecular sex determination using PCR amplification of the sex chromosome linked amelogenin gene. These methods were first optimised on blood and bone controls and then applied to skeletons sourced from the Raymond Dart Collection of Human Skeletons (University of the Witwatersrand, Johannesburg). The DNA was extracted using a silica based spin column (Qiagen Micro kit 50) that binds the DNA to the silica membrane. For the purposes of extracting DNA from bone obtained from the Dart collection, an additional step of 0.5 M EDTA (pH 8.0) and proteinase K was added to the lysis step in the protocol; together, these two chemicals assist in the breakdown of the mineral aspect of bone, which then increases the yield of DNA. The classification of sex was specifically determined using a nested conventional PCR reaction through the amplification of the amelogenin gene.

The amplification isolates 16 highly conserved sex specific variations, in addition to a 6 bp deletion on the X chromosome. The amelX amplicon is 188 bp and the amelY is 194 bp in length. The deletion was visualised using the highly sensitive Bio-Rad Experion DNA 1K analysis kit. The classification of sex was subsequently confirmed through sequencing the PCR product by examining both the sex specific variations and the deletion. Each of these methods using the new area on the amelogenin gene proved to be reliable, easily replicable and successful in sex determination.

The bone extraction method developed here has been published under my maiden name, Woodward. South African Archaeological Bulletin in 2006: 61 (183), 96-97. The paper was converted from PDF and is copied below.

Field and Technical Report

INTERCONDYLAR FOSSA OF THE FEMUR: A NOVEL REGION FOR DNA EXTRACTION

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Human skeletal material found in archaeological localities provides a wealth of bio-cultural data on the individuals and populations represented. These data were until recently collected exclusively through the traditional methods of physical anthropology. In the 1980s, the amount and variety of information dramatically increased as new techniques in molecular biology allowed the analysis of genetic material from deceased organisms (Kaestle & Horsburgh 2002).

Although DNA has been successfully extracted from bones of humans and other animal species of various ages (including fossil material) and differing states of preservation, several challenges still face molecular anthropologists. Three main concerns face the field of DNA extraction: contamination from modern DNA, degraded molecular mass, and the destructive nature of the procedure to the skeleton (O'Rourke *et al.* 2000). While the first problem might be successfully avoided through the implementation of strict laboratory measures, the other two (being related to the preservation of the material) represent a constant impediment within this field of research. Obtaining DNA, therefore, often represents a compromise between the level of destruction and the amount of bone needed for successful extraction. This paper proposes a previously non-utilized region on the femur for DNA extraction. The use of this region limits destruction of the bone and at the same time provides enough bony tissue for DNA analysis.

Bone is often considered one of the best DNA sources due to the binding of DNA to hydroxyapatite (a calcium phosphate), which slows DNA degradation over time (O'Rouke *et al.* 2000). Historically, DNA extraction from skeletal remains has been detrimental to the holistic value of the specimen, because large sections of bone were destroyed, often leaving a mutilated skeleton with missing elements or sections. This clearly limited the use of the specimen in anthropological analysis, which requires the examination of the skeleton to reconstruct the life history of an individual.

The skeletal elements that have commonly been used for DNA extraction are the femur (Hagelberg *et al.* 1991; Cattaneo *et al.* 1995; Kolman & Tuross 2000; Wurmb-Schwark *et al.* 2003; Gilbert *et al.* 2005), ribs (Kemp & Smith 2005), vertebrae (Cattaneo *et al.* 1995) and teeth (Meyer *et al.* 2000; Cobb 2002; Gilbert *et al.* 2005). Vertebrae, ribs, and teeth are utilized because of their numbers, the rationale being that should one be damaged, more are available for data collection. In addition, teeth are thought to be extremely valuable as they are more robust than bones, thus making them a good element for preserving DNA. The most commonly used bone, however, is the femur. Usually, a femoral head or a section of the mid-shaft is used. However, the destructive nature of the processes typically involved in DNA extraction may preclude subsequent detailed metrical and morphological analysis of the femur, resulting in a reluctance of curators of skeletal collections to allow for such studies.

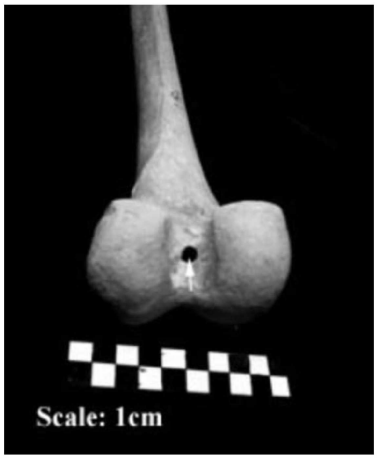

FIG. 1. *Femur specimen showing point of extraction (see arrow) in the intercondylar fossa .*

There are specific criteria to determine the ideal place on the skeleton to retrieve a bone sample suitable for DNA analysis. First, the bone extraction method should not interfere with any known metrical or morphological sites used for individual reconstruction. The second criterion concerns choosing a bone with an ideal amount of cancellous bone. The third criterion is that the skeletal element must not be destroyed. The fourth consideration is bone preservation, as it is associated with DNA survival (Haynes & Searle 2002). Preservation is defined as the outward appearance of the bone based on softness, cracking and flaking. Damaged surfaces and lesions provide avenues for contamination and consequently no bone with any damage or lesions on the external surface, distal or proximal end should be utilized. Therefore, prior to the extraction the appropriate skeletal element should be chosen. Lee *et al.* (1991) stated that spongy bone can yield 10- to 20-fold more DNA than compact bone. The compact outer layer of bones protects DNA and therefore is usually less degraded after a certain period (Machugh *et al.* 2000; Wurmb-Schwark *et al.* 2003). The chosen element must have a good yield of cancellous bone as it is much easier to pulverize and is generally protected by robust, compact bone. This rules out the ribs and smaller bones that do not contain enough cancellous bone, resulting in the final options being the distal and proximal ends of the long bones.

Bearing this in mind, it appears that the best area of the skeleton for bone extraction is the distal end of the femur in the intercondylar fossa (Fig. 1). This region of the femur contains a good store of cancellous bone protected from environmental contamination by a hard layer of compact bone. This technique

is marginally invasive and does not interfere with morphological or metrical data collection.

This new approach was tested using 30 archaeological specimens from the Raymond Dart Collection of Human Skeletons (University of the Witwatersrand). During sample preparation, recommended procedures were followed to prevent DNA contamination. A contaminant-free power drill (Bosch PSB 650RE) with a 4.5 mm sterilized titanium drill bit was used; once through the cortical surface a rotating action was employed to break up the cancellous bone on the inner table. A fine- to medium-grained bone powder with an average yield of 0.77 g (ample for DNA extraction) was obtained from the samples. While there was individual variation in bone yields, this probably related to a decrease in bone mass as a result of the ageing process.

The technique of bone removal for the purpose of DNA extraction from the distal end of the femur in the intercondylar fossa leaves only a small hole on the surface and does not interfere with known metrical or morphological points. Therefore, this ensures that the future use of the specimen is not negatively affected. This approach is potentially useful for studying the genetic history of archaeological specimens.

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