THE EFFICACY AND OPTIMIZATION OF MOLECULAR TECHNIQUES FOR SEXING SKELETONIZED HUMAN REMAINS

BY

CURTIS HILDEBRANDT ©

A THESIS

PRESENTED IN PARTIAL FULLFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY,

LAKEHEAD UNIVERSITY,

THUNDER BAY, ONTARIO

SEPTEMBER 2003

Your file Votre référence
ISBN: 0-612-92244-8
Our file Notre référence
ISBN: 0-612-92244-8

Abstract

Forensic anthropologists and bioarchaeologists are often confronted with the problem of sex determination in poorly preserved, skeletonized, adult, human remains. This thesis tests the ability of newly developed DNA methods to address this problem. Using a blind research design, four molecular DNA techniques - amelogenin, alphoid repeats, SRY (sex determining region of the Y-chromosome) and Y-STRs (short tandem repeats) - are assessed for their ability to determine the sex of 19 skeletons with known morphological and/or documented sex. The skeletons represent both historic and ancient human remains. The latter are from a Roman cemetery in the Dakhleh Oasis, Egypt (n=13), while the former are from historic 19th century cemeteries in London, Ontario (n=4) and Thunder Bay, Ontario (n=1); as well as, an unidentified skeleton from a forensic cold case (CFS file # A-475-95).

It was hypothesized that the alphoid repeat technique should yield the best results due to their position near the chromosomal ends and the nature of DNA degradation. The results show that although the alphoid repeats typed for all but one sample, the amelogenin had a superior performance, especially for the historic samples. The SRY method was very poor, appearing for only two samples, and the Y-STR method did not yield amplification at all. The latter was unexpected and disappointing, because it Y-STRs have the potential to aid in male individuation in addition to its inherent ability to identify sex. The implications for both forensic and bioarchaeological research are far reaching.

Based on the data from this thesis, two important conclusions emerge. The first is that the results of the amelogenin locus on 100-year-old samples are encouraging. This

shows that even in samples with relatively high degradation, the sex of an individual is still ascertainable and this is important for forensics, especially where cold cases are concerned. The second conclusion is that more research is definitely required given the poor performance of the alphoid repeat and Y-STR methods. These include studies to determine more stringent contamination checks, such as mtDNA sequencing of all samples yielding results for ancient DNA (aDNA).

Acknowledgements

A special thanks to my advisor Dr. El Molto for the time and effort he put forward to bring this project to fruition. To my fellow student colleagues Arlene Lahti, Jenny Brown, Carolyn Murray, Laura Smith, Amy Junila, and Christine Beaulne, and the staff of the Paleo-DNA Laboratory for their advice and support. The samples for the project were generously provided by: Dr. Molto of the Anthropology Department at Lakehead University (Egyptian samples and River Street Burial), Dr. Spence of the Anthropology Department at the University of Western Ontario (London cemetery samples) and Dr. Cathy Gruspier of the Center of Forensic Science, Toronto (Danforth Doe sample). Also, the time and advice offered to me by my thesis committee, which includes my external examiner, Dr. Jack Ballantyne and the core committee of Dr. Kam Leung, and Dr. Janice Hughes, was very helpful and appreciated. Finally, I would like to thank Lynn, my parents, and my sister for the encouragement and moral support they provided through this endeavour. This work was made possible by a grant from SSHRC (Social Sciences and Humanities Research Council #410-2002-1337) which was awarded to my mentor Dr. El Molto.

Table of Contents

List of Figures

List of Tables

Chapter1: Introduction

Statement of Problem

A long standing research problem in the bioarchaeological and forensic sciences is the sex determination of pre-pubertal and poorly preserved adult remains (Dorion 1973). The lack of sexual dimorphism in the skeletal morphology of pre-adults and the destructive environmental exigencies of select regions of the skeleton of adults (i.e. Os pubis) are the key respective reasons for these problems. The fact that in well represented 'normal' ancient population samples, approximately half the population are subadults (Molto 2000), and children are often victims of homicides (Faerman and Bar-gal 1998), underscores the importance of improving our ability to determine sex in skeletonized, unidentified human remains. In recent years a number of molecular techniques utilizing specific DNA loci have shown great potential to address this issue (Witt and Erickson 1989; Sullivan *et al.* 1993; Santos *et al.* 1998). These techniques are possible because of a number of breakthrough molecular technologies, particularly the polymerase chain reaction (PCR) (Mullis K. *et al.* 1986). This technique makes possible the amplification of highly degraded samples, and has been successfully applied to tissues several thousand years old (Higuchi R.G *et al.* 1987; Paabo S *et al.* 1988; Woodward *et al.* 1994). This amplification rests on the stability of nucleic acids, particularly in calcified tissue (i.e. bone and dentin) from diverse taphonomic circumstances. The specificity of DNA when properly extracted and amplified makes it an invaluable tool in gender individuation for all age groups. This thesis tests the efficacy of several recently developed DNA based methods using a blind research design on excavated skeletal remains of which sex is known from morphological and/or

documentary evidence. The importance of the blind study design in this area of DNA research cannot be overstated. If the sex of a skeleton is known prior to attempting the molecular analysis, inherent biases can enter unintentionally into the research. The temptation for the researcher to re-analyze a sample that is mistyped is great and if this were to occur, the results and ultimately the methodology will be influenced. This is especially true for forensic sample because they are not retested until you get the result you want. The samples are analyzed and the results are reported.

This thesis incorporates four systems for identifying the genetic sex of an individual. They are the sex determing region of the Y-chromosome (SRY), the amelogenin loci, the alphoid repeats, and Y-chromosome short tandem repeats (Y-STR). The SRY and Y-STR systems both identify male specific DNA (i.e. found only on the Y-chromosome) and thus will identify a male sample in the event that these regions of DNA are present. The other two loci (amelogenin and alphoid repeats) are found in both male and female DNA. The reason these genetic regions are used is because it has been demonstrated the length of DNA in these regions differs between males and females. With this in mind, the working hypothesis for this thesis suggests that the alphoid repeats should yield the best results when analyzing ancient or degraded tissue (Matheson and Loy 2001). This hypothesis is based on the knowledge that due to chromosomal location, the alphoid repeats should have greater protection from degradation. Replication and by inference, degradation usually starts at the telomeres (ends of the chromosomes) and works its way in (Medina 2003), thereby degrading the most peripheral loci first. The samples used herein span a considerable time frame, from ancient historic to modern historic. The ancient historic samples are from the Dakhleh Oasis, Egypt, while the

historic samples are from several cemeteries; one from London, Ontario and one from Thunder Bay, Ontario. The final sample is also modern historic and is from a forensic cold case (CFS file # A-475-95). The information derived from this research is used to assess the feasibility of sex determination in subadult skeletons from an ancient Egyptian population. The implications of this research for the fields of forensics and ancient DNA are profound.

The thesis is organized in the following chapters. Chapter 2 provides a historical overview of the theoretical foundation of sex determination in mammalian cells including the recent advancements involving DNA research. Chapter 3 overviews the field of ancient DNA (aDNA) research as it pertains to early attempts at sex determination and the use of Y-chromosome STRs in ancient studies. Chapter 4 describes the skeletal samples and molecular techniques used in the analysis, while Chapter 5 covers the results and Chapter 6 the discussion and conclusions respectively.

Chapter 2: History of Cytological/Molecular Sex Determination

Theory and Methods in Cellular Biology

As this thesis is concerned with testing and optimizing new molecular methods in sex determination, a review of sex determination methodology is required. This research is rooted in traditional cellular and reproductive biology. Sexual reproduction began when organisms that reproduced asexually via fission (mitotic divisions) started to mix genetic information from different individuals. The origin(s) of this is still debated, but what is certain is that this new form of reproduction had advantages over asexual reproduction despite some costs (Daly and Wilson 1983). One of the costs, in fact, was the evolution of sex with sexes. An enormous amount of energy is utilized to differentiate males and females developmentally, but the widespread success of this across the animal kingdom is testimony to the selective advantages that it must have provided, in addition to the advantages of sexual reproduction.

In theory, the evolution of sexes is thought to arise from gamete size competition; with the female producing larger gametes being the one constant that separates males from females in all species (Daly and Wilson 1983). It is argued that sexual reproduction evolved as a competitive advantage during times of environmental stress so that the organisms with favourable genes would survive to pass them on to their offspring. Mixing "parents" with certain favourable genes could potentially pass on combinations of these "good" genes, or pool these advantageous genes in a single genome, thereby increasing the fitness of their progeny in uncertain and stressful environments (Becker 1986; Anderson 1992). Organisms that evolved this strategy thus developed a competitive reproductive advantage, especially in times of duress.

The benefits of sexual reproduction have been selected over many millennia and have produced numerous independently designed and interesting variants of sex determination. This includes the temperature regulation systems found in some reptiles, the ZW system found in birds, some fishes and moths and the XY system found in most mammals. The latter, of course, is most appropriate to this thesis although the evolution of the XY system is lost in antiquity; one hypothesis however, states that the X and Y chromosomes arose independently from different autosomal pairs in a common ancestor (Roldan and Gomendio 1999).

This DNA based research is therefore predicated on the fact that the human genome, like that of most mammalian genomes, utilizes the XY system of sex determination. The XY male and XX female genotypes have long been differentiated at the cellular level and many techniques have utilized the inherent properties of the human karyotype to determine sex (e.g., Barr body technique based on the Lyon Hypothesis and H-Y antigen levels). These systems will be briefly overviewed as they are pertinent to understanding the theoretical basis and specificity of the DNA methodologies.

Barr Bodies and the Lyon Hypothesis

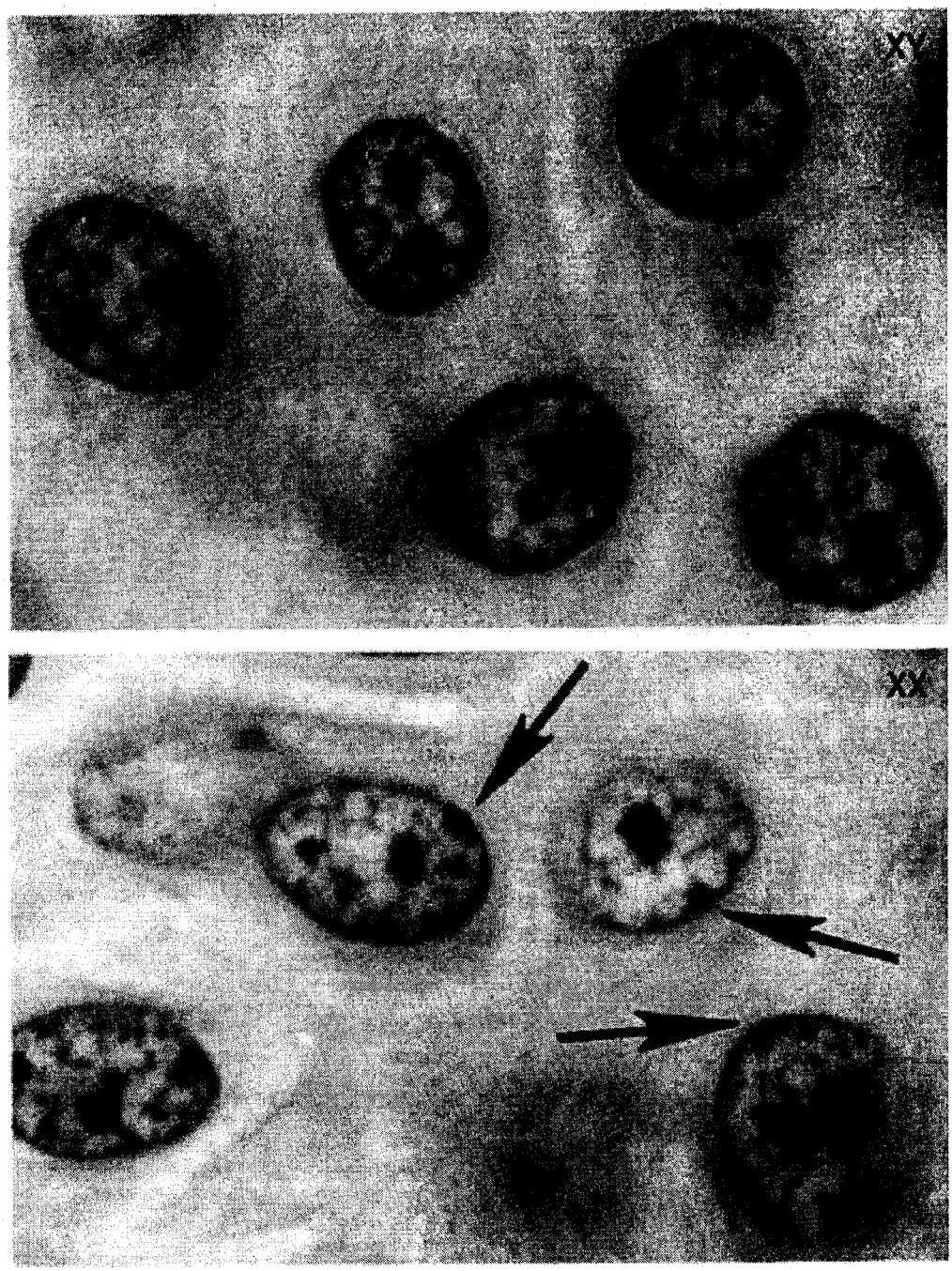
The sex chromatin body (better known today as the "Barr body") was initially discovered in 1909 by Ramon Y Cajal, a Spanish neuroanatomist, who researched the cells of cats, dogs, and humans. However, he was unaware of how these bodies related to sex determination. Significant progress with respect to the sex chromatin was not made until 1949. In that year, the Canadian researchers Murray Barr and Mike Bertram at the University of Western Ontario in London, reported that the nuclei in nerve cells of cats in the two sexes were characteristically different (in so doing they provided meaningful

insight into Cajal's work). They noted that often in normal female cats a darkened area occurs adjacent to the nucleus during interphase, whereas they are absent in males (Figure 1). This area became known as a Barr body, named after lead researcher. Continued research demonstrated that Barr bodies form in all placental mammals including humans. In fact, it was discovered that in buccal epithelial cells of human females, the same darkly staining bodies occur in 30% to 40% of the cells but were absent in males. The number of cells positive for the Barr bodies in female samples corresponded to the number of cells in the interphase state. The other 60% to 70% of the cells were actively undergoing mitosis, and therefore, all the chromosomes were in a condensed state thereby obscuring the detection of the Barr body. It took another 10 years before both Russel and Ohno independently postulated that the Barr body was in fact an inactivated X-chromosome. Later, Mary Lyon working at the University of Western Ontario showed that a Barr body is an inactive X chromosome that could originate from either parent. This became known as the Lyon hypothesis, which has since been confirmed. The inactivation of the X-chromosome occurs relatively early in embryogenesis and results in groups of cells with the same X remaining active. The X-chromosomes are visible as Barr bodies because they are in a highly condensed state and thus are usually the last to replicate (acting as dosage compensation). The Lyon Theory explains the absence of sex chromatin in males and its incomplete penetrance in females (Lyon 1961, 1974).

In females, it was subsequently found that near the 100 cell stage of human development, the X chromosomes that are to be reduced to a Barr body are nearly completely inactivated and all their descendants share this condition.

Figure 1: This figure shows the presence of Barr bodies in female cells and the absence in male cells. The Barr bodies are located adjacent to the nuclear membrane and are only found in cells during interphase. Therefore, at any given moment in female cells, there are approximately 30-40% of all cells in the interphase state that will exhibit a Barr Body.

(http://www.mun.ca/biology/scarr/Barr_Bodies.jpg)



This "shut down" of the X chromosome is hypothesized to be a purely random event (Mermoud *et al.* 2002) except in the case of an abnormal X (via deletion, insertion, inversion, etc.) The inactivation actually requires a gene found on the inactivated X called XIST (X-inactivation specific transcript-2). The XIST gene codes for a large molecule of RNA, which when produced, accumulates on the X chromosome housing the XIST gene. Therefore, the production of the RNA inactivates all or nearly all of the hundreds of genes on that X chromosome by binding and inhibiting transcription factors. However, this process does not affect the sister X chromosome. The X chromosome that remains active does so via the methylation of the XIST gene, thus repressing gene transcription (Brown *et al.* 1991; Penny *et al.* 1996; Willard 1996; Hendrich *et al.* 1997; Lyon 1999).

The resulting inactivated X chromosome or Barr body with its conglomeration of RNA is thus visible under the microscope using common staining methods. This provides us with a method capable of sexing an individual via cellular microscopy (Valenti *et al.* 1972; Bakharev 1976; Peter *et al.* 1977; Slozina *et al.* 1977). Its usefulness in detection of chromosomal variants had been a cornerstone of medical genetics for many years (Mange 1980).

H-Y Antigen and TDF

A breakthrough in our understanding of sexual determination in cellular biology was posited by Fisher in his seminal (don't mind the pun) paper entitled "The Genetical Theory of Natural Selection" (Fisher 1930). Fisher hypothesized that male characteristics develop in one of two ways; the first is strictly under the control of sex-linked genes on the Y-chromosome, and the second is through sex limited autosomal genes. The second

hypothesis was favoured by Fisher, but was not proven until Eichwald and Silmser provided the supporting experiment in 1955. They used skin grafts to show that a testicular hormone that is regulated by a single Y-linked gene in fact activates autosomal genes (Eichwald and Silmser 1955). Research continued and, in 1959, Liane Russell and colleagues showed that the mechanism for determining maleness was located somewhere on the Y-chromosome (Welshons and Brauch 1959). This research predicted that the testis-determining factor (TDF) was a new protein (H-Y antigen) encoded by a gene on the Y-chromosome. The H-Y antigen, was discovered by conducting cross-sex skin grafts between highly related mice resulting in graft rejection due to the lack of the antibodies in female cells.

This led to the hypothesis that during fetal maturation the presence of the H-Y antigen and its receptor initiates the development of the testis and subsequent male differentiation. This hypothesis enjoyed continued support in the mid-1960's to the late 1970's from experiments attempting to isolate the H-Y antigen in humans. These showed that the levels of the antigen differed among the sexes and in various transsexuals (Eicher *et al.* 1979). For example, differences in H-Y antigen levels were found between two forms of transsexuals and normal males. Male-to-female transsexuals exhibited lower levels of the antigen than those found in both normal males and female-to-male transsexuals. The latter two individuals would have very similar HY-antigen levels. Moreover, Simpson et al. (1986) also showed that in experiments with mice lacking the H-Y antigen during development, no testes are formed.

The intellectual environment during this phase of our knowledge of sex determination in mammals knew that regardless of the genotype all individuals start out

with identical primordial gonads capable of becoming ovaries or testis. As previously mentioned, it was believed that only in the presence of the H-Y antigen do testis develop and almost exclusively the H-Y antigen is only present when a Y-chromosome is part of the genetic complement. If the H-Y antigen is not secreted then the fetus will ultimately become female.

With the knowledge that the H-Y antigen is a product of a gene found on the Y-chromosome it was obvious that if the protein can be detected in a cell culture, the donor was a male. In fact, once the H-Y antigen is produced, it can be detected in all males. Thus, testing the levels of the antigen in various human karyotypes showed that the protein level mirrored the number of Y-chromosomes present. Therefore, in XYY males the protein level was double that of XY males whereas no protein product was found in normal XX females (Wachtel *et al.* 1975). Another use for the detection of this protein includes testing females for the antigen who exhibit masculinization and may have had a translocation of the specific gene from the Y to the X-chromosome. Thus the discovery of the H-Y antigen led to further investigation of the Y-chromosome and its genetic content. To re-iterate, it was still believed that the H-Y antigen was in fact the testis determining factor so this is where most studies were focused. However, it would soon become apparent that the TDF was in fact situated on a different region of the Y-chromosome (Sinclair *et al.* 1990).

It was ultimately demonstrated that a portion of the Y genetic material could in fact be translocated to the end of the short arm of the X-chromosome (Xp), resulting in XX males (Anderson 1986). One year later investigations following this line of inquiry demonstrated that the genetic loci for the H-Y antigen and the testis determining factor

were in fact different and separated on the Y-chromosome (on the long arm (Yq) and the short arm (Yp) respectively) (Simpson *et al.* 1987).

Subsequently, the H-Y antigen has been implicated as a possible factor in spermatogenesis. Because the TDF and H-Y antigen were located on different arms of the Y-chromosome, a paper describing six men suffering from azoospermia was revisited. This study (Tiepolo and Zuffardi 1976) demonstrated that the six individuals were indeed lacking the region mapped to Yq11 (distal part of the Y chromosome long arm), providing proof that the accepted location of the sex determining region was incorrect. Based on their information as to where the gene could not be, scientists went on a search for the true location of the TDF, leading them to first investigate the zinc finger gene (ZFY) then the sex determining region of the Y-chromosome (SRY).

DNA and the SRY gene

Future investigations into the formation of testes in male individuals produced some contradictory results pertaining to sex determination. The problem occurred when XX males tested negative for the H-Y antigen.

By this time, so many individuals were discovered with the sex-reversal genotypes that geneticists were able to study the phenomenon (Seton 1990). In fact, they were able to detect that the translocation took place between the short arms of the X and Y-chromosomes without gross deletions or rearrangements. This meant that the genetic content remained the same except for those genes crucial for testis determination. In 1986, deletion analysis helped narrow the search to interval 1 of Yp near the pseudo-autosomal X-Y pairing and exchange region. The following year both a male and a female patient were found with the same region translocated and thus it was believed that

region 1A2 of the Y-chromosome was the TDF (Figure 2). The region contained a gene coding for a zinc finger protein on the Y-chromosome (ZFY) that was thought to be the TDF. However, in 1989 there were three XX males discovered who lacked the ZFY gene but did have the 1A1 region (Figure 2; Seton 1990). Therefore the exact location of the TDF had eluded scientists yet again.

Ultimately, this initiated a search using oligonucleotide DNA probes specific for the Y-chromosome to detect pieces of the chromosome that may be found on one of the two X-chromosomes identified in the male individual (Sinclair *et al.* 1990). These DNA probes eventually identified the presence of small homologous regions on the Y-chromosome and X-chromosomes of the male. Ironically, the same region could not be found on a Y-chromosome of an XY female. In fact, nearly all male mammals tested have the Y-chromosomal loci found in XX males.

The region identified in these translocations was discovered in 1990 by Sinclair and his associates, which they named the sex-determining region Y (SRY) and found it to be located on the short (p) arm near the pseudoautosomal region (Figure 3; Sinclair *et al.* 1990). Since then it has been shown that the SRY gene and not the H-Y antigen acts as a switch in embryological development, leading to a male embryo. Some of the evidence in support of the SRY acting as the male "trigger" has come from studies on rare occasions of aneuploidy in humans. Even genetic mosaics with karyotypes such as XXY, XXXY, and XXXXY are all male despite their abundance of X-chromosomes

Figure 2: This figure depicts the timeline for the search for the testis-determining factor (TDF) on the Y chromosome. As time and research progressed, the resolution of the Y chromosome increased dramatically. It was ultimately discovered that the TDF was linked to the SRY gene after it was proposed to have been part of the Zinc Finger genes.

(Sinclair *et al.* 1990)

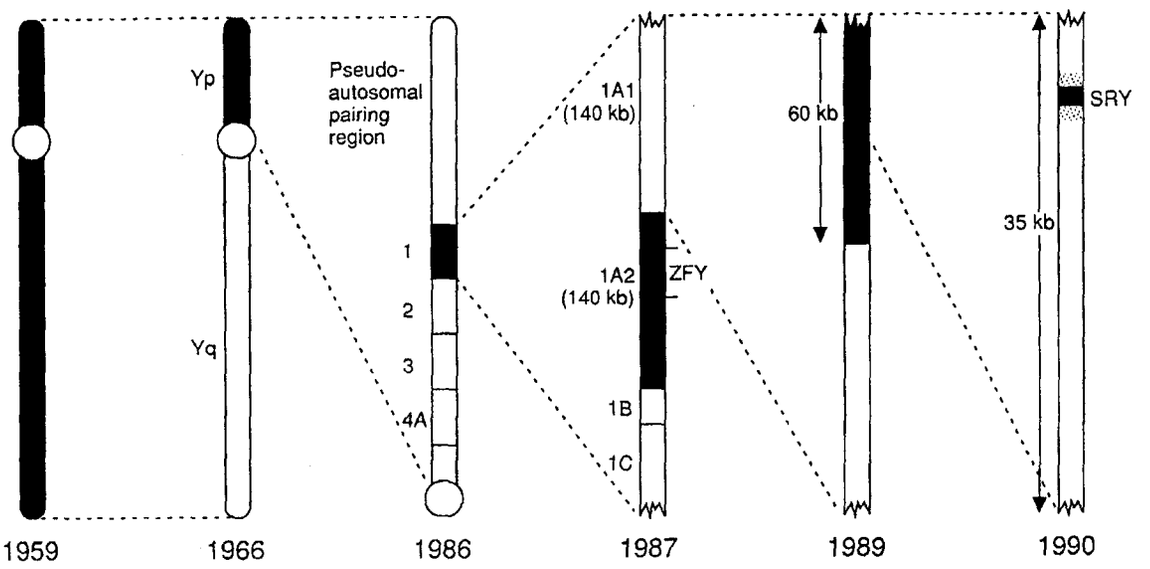
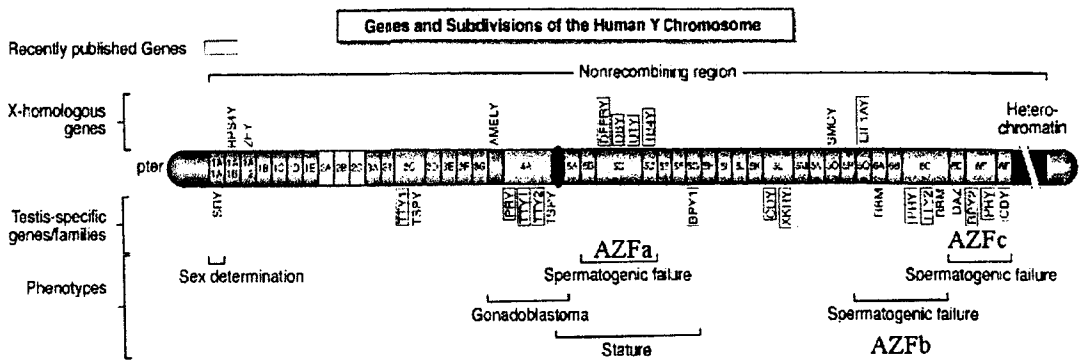


Figure 3: This figure represents a detailed map of the Y chromosome. It identifies the major gene region involved in sex determination (i.e. SRY gene) as well as genes involved in other various aspects of testis development.

(Lahn and Page 1997)



(Lovell-Badge 1992). The premise or function of the SRY gene is to reconfigure the DNA structure in order to change or alter the expression of a number of genes, leading to testis formation. The SRY gene product is a protein that acts like a transcription factor binding to a specific DNA sequence and inserting itself into the minor groove of the double helix and contorting the helix by approximately 80 degrees. The reconfiguration brings the regions flanking either end of the SRY gene into close proximity, allowing other transcription factors to bind and express other downstream genes (i.e. Sox9 and Dax1–nuclear hormone-receptor superfamily member).

Knowledge of the sequence of the SRY gene has led to many different practical applications of SRY. For example, it is now possible to use the SRY gene along with PCR techniques to provide an effective method of solving sexual crimes. This is the case even in the absence of ejaculation, and works by retrieving Y-chromosome DNA from the diploid epithelial cells that were shed during intercourse (Dziegelewski *et al.* 2002; Sibille *et al.* 2002). The SRY gene has also been used to test potential competitors in women's events during the 1996 Atlanta Olympics (Serrat and Garcia de Herreros 1996).

Summary

The cytological and molecular history of sex determination in mammals provides a detailed story underpinning the difficulties that had to be overcome to find accurate sexing methodologies for unknown individuals. Based on the research laid forth in the past century, we can now use more sophisticated (capillary electrophoresis) and more accurate methods (molecular testing of SRY, Amelogenin, Alphoid Repeats and Y-STRs) to identify the sex of an individual rather quickly and easily. We hope this will also be true of ancient or problematic samples for the fields of aDNA and forensics respectively.

This work however, would not be possible without the foundation laid by Mary Lyon and others who first discovered the presence and purpose of sex chromatin. The Barr body technique was instrumental in the advancement of cytology in relation to sex determination. It paved the way for research into the Y-chromosome and its genetic content, without which, none of this work would be possible.

Chapter 3: Ancient DNA

Background in Ancient DNA (aDNA)

In recent years the authenticity of DNA extractions from numerous ancient samples including plants, feces, hair, feathers, mummified tissue, blood, bloodstain residues and blood smears and bone has been established (Heller *et al.* 1992; Leeton *et al.* 1992; Hoss and Paabo 1993; Fridez and Coquoz 1996; Cattaneo *et al.* 1997; Kalmar *et al.* 2000). It is also known that some of these samples (specifically bone) will survive internment conditions and remain relatively intact for prolonged periods of time (Gurfinkel and Franklin 1988; Higuchi *et al.* 1988; Golenberg *et al.* 1990; Hagelberg *et al.* 1991a; Hoss *et al.* 1992; Loy and Hardy 1992; Cano and Poinar 1993; Hagelberg 1993; Hoss and Paabo 1993; Brown T.A and Brown K.A 1994). With the advent of the polymerase chain reaction (PCR), it is now possible to increase minimal DNA copy numbers to a sufficient quantity where reliable analysis can be performed. In fact, it is theoretically possible to amplify as little as a single molecule of target DNA using the PCR technique to levels suitable for analysis (Saiki *et al.* 1988; Paabo *et al.* 1989; Chou *et al.* 1992). This proves very useful due to the fact that upon the death of an organism, chemical and autolytic processes begin to act on and change the DNA (Perry *et al.* 1988). These alterations commonly fragment the double stranded helix and can alter the bonds between the strands or the bonds within the sugar/phosphate backbone of the DNA (Hofreiter *et al.* 2001). These degradation processes are the limiting factors in the field of aDNA and act by reducing the amount of amplifiable DNA from a given sample. The problem lies in the fact that most ancient samples after fragmentation will not yield products greater than 450 base pairs (bp); in fact, some researchers believe that 200bp may be the limit (Jehaes

et al. 2001) to amplify aDNA (Hagelberg and Clegg 1991; Cooper *et al.* 1992). Upon the successful retrieval or extraction of DNA, information such as; sex identification, species identification, genetic disease or defect identification and population or sub-population studies (mitochondrial, and Y-chromosome) can be obtained (Loy and Wood 1989; Rogan and Salvo 1990; Hagelberg *et al.* 1991b; Vigilant *et al.* 1991; Faerman *et al.* 1995; Stone *et al.* 1996).

Background to Sex Identification

Sex identification has routinely been performed on skeletal material via osteometric and osteoscopic analyses of the pelvic girdle and skull (Phenice 1969; Bass 1971) as well as discriminant-function analysis based on bone and tooth measurements (Ditch 1972). However, these methods are limited particularly since they require fairly complete mature skeletons (Ubelaker 1978; Meindl 1985; Krogman 1986). Problems with adult skeletons arise when they are incomplete. Many burial practices and post-depositional conditions may alter the state of the bone sample. For example, low pH may result in very little and/or poorly preserved bone which can greatly impede the identification of sex (Matheson 2001). However, the accuracy of skeletal sexing that can be as high as 98% on a complete adult skeleton is greatly reduced when sub-adults are considered. Although sexual dimorphic traits may be visible prior to birth (Ubelaker 1978) - males tend to be longer and heavier than females - these differences depend on exact chronological age (Weaver 1998), a factor not ascertainable from the archeological record. Furthermore, sex designation by size alone is troublesome due to the fact that sexual dimorphism differs between and within populations depending on environmental stresses (Hall 1978). The problem is compounded when fetal remains are to be analyzed.

According to Warren (1997), there are no significant differences in the measurements of long bones in fetal remains. There have been a hand-full of papers published stating that sub-adult sexing is possible from a combination of morphometric measurements (i.e. width of the sciatic notch and sub-pubic angle of the *os coxae* and length of the pubic bone) (Reynolds 1945; Boucher 1957; Fazekas and Kosa 1978); however, newer studies have produced contrasting results. Therefore, current thinking in human skeletal research views the determination of sex on sub-adults as unreliable at best (Krogman 1973; Ubelaker 1978; France 1998).

The field of molecular genetics has been evolving quickly in recent years and can now provide viable alternatives to the morphometric and chemical sexing of adults and, more importantly, sub-adults. These alternatives utilize the surviving DNA found within the bone and bone fragments commonly discovered at archeological sites (Faerman *et al.* 1995; Stone and Stoneking 1996). To sex an individual via genetic means requires the ability to precisely measure differences between males and females at the molecular level. As described in chapter two, there is a great difference between males and females at the genetic level because of the XY sex determination system. Past studies limited themselves to utilizing one or two different genetic sex tests (Colson *et al.* 1997b; Cunha *et al.* 2000; Faerman and Bar-gal 1998; Mays 2001; Sullivan *et al.* 1993a) which may, depending on the target being sought, have inherent problems prior to beginning the studies. One such problem involves a rare mutation in the amelogenin gene that essentially eliminates the presence of Y-chromosome "alleles" from genetically male individuals (Roffey *et al.* 2000). Recent studies provide differing results as to how common this mutation is. Steinlechner *et al.* (2002) showed a prevalence of 0.018% in

the Austrian National Database; however, Thangaraj *et al.* (2002) provided evidence that the mutation may be more common, at 1.85% in Indian males. Interestingly, in identifying a genetic mutation in human populations, even these low rates of mutation can produce significant errors in results. For this reason, this study has incorporated two additional genetic sex tests. This is in addition to the amelogenin loci that uses specific primers to amplify a portion of the amelogenin gene (involved in enamel formation) resulting in X and Y-chromosome specific fragments of 106bp and 112bp respectively (Sullivan *et al.* 1993). The alphoid repeats are found on the X and Y chromosomes in different copy numbers while the SRY locus is found solely on the Y chromosome. Although the alphoid repeats are spacer regions found on all human chromosomes near the pericentromeric region, the primers used for sex determination are specific to the X and Y chromosomes and, in this case, produce fragments of 130bp and 170bp respectively (Sutton 1996). The SRY gene is found only on the Y chromosome (involved in determining maleness) and therefore any amplicon that corresponds to the 93bp specific primers (Santos *et al.* 1998) results in a sample being identified as genetically male.

Background to Y Chromosome STRs

Y chromosome genetics is still a relatively young discipline and its importance has only recently been recognized. Until the multicenter study evaluating the use of Y chromosomal STRs was published (de Knijff *et al.* 1997), the analysis of male-specific DNA was limited to sex identification. Only towards the end of the 1990's did the use of short tandem repeats (STR) on the Y chromosome find a niche in the forensic arena (Gill 2001) for the analysis of paternity (in deficiency cases - no father available) and mixed-

stains (male/female DNA combinations). Recently however, there have been a multitude of papers published on Y STR haplotypes of various populations (Anslinger *et al.* 2000; Brinkmann *et al.* 1999; Das *et al.* 2002; Fernandes *et al.* 2001; Gavrilidis and Luisa Ashdown 2001; Gusmao *et al.* 2002; Hou *et al.* 2001; Kayser *et al.* 2002). These studies now make it possible to analyze the STRs found on the Y chromosome and possibly assign an individual to one of the major ethnic groups (i.e. Amerindians, Asians, Europeans, and Africans).

This type of information was once sought solely by means of the maternally inherited mtDNA (Easton R.D *et al.* 1996; Merriwether A.D *et al.* 1996; Mesa *et al.* 2000; Naumova O.Y and Rychkov S.Y 1998; Torroni *et al.* 1993; Torroni *et al.* 2000). However, the maternally contributed genome only focuses on one aspect of ancestry and thus a complete picture of population genetics and ancestry cannot be obtained.

By incorporating Y chromosome STRs in this study, the goal is twofold. One is to assess if the Y-STRs are sensitive enough to use as a sexing test to discern the male fraction of a population. The second aspect is more ambitious and follows on the success of the first objective. With the Y chromosome we would like, in the future, to be able to complete an ancestral picture complementing mtDNA data. This may be possible because the Y-STRs are a uniquely inherited portion of the Y chromosome which does not recombine with any other chromosome during meiosis and thus remains virtually identical from generation to generation (Naito *et al.* 2001). Furthermore, this unique non-recombining portion of the Y chromosome means that any variation among Y chromosomes is due to the accumulation of new mutations (Scozzari 2001). This allows

us to study aspects of paternal lineages such as founder populations and their migrations (Lell and Wallace 2000).

Summary

In this chapter a framework for DNA studies, within which this thesis can rest, has been established. The realities of doing ancient DNA work were identified. This included the tissues from which DNA has been extracted (mummies, bones, teeth, etc.), the minute quantities required (as little as a single molecule), and the limitations of amplifying this DNA (up to 200 base pairs).

Although sex determination has been done on ancient samples before, rarely have they been done in blind and furthermore, previous studies limited themselves to one or two molecular sex determining assays. This study extends previous research by using the blind design on a range of "old samples" with multiple genetic sexing loci being key. A new approach to ancient DNA sex determination was also introduced. This involved Y-STRs and their ability to identify the male fraction of DNA. In the event that any of the samples should amplify at the Y-STR loci, the resulting information could provide insight into paternal relationships and the social implications surrounding them.

Chapter 4: Samples and Methods

Origin of Samples

The samples tested in this thesis are listed in Table 1 (for in depth description of the samples, refer to appendix A). As noted, they all derive from archaeological (excavation) contexts, although they represent disjunct temporal and spatial circumstances. All burials are well-preserved adult remains. In each case skeletal sex was determined using hip criteria according to Phenice (1969). This method utilizes three osteoscopic characteristics of the *Os pubis*; namely, the ventral arc (broader in females; Figure 4), the lateral recurve (shallower in females; Figure 5), and the thickness of the sub-symphyseal border (sharper and narrower in females; Figure 6). The former two characteristics are limited to female hips, which also have thin sub-symphyseal faces as opposed to the broader and thicker borders of males. The Phenice method has proven successful in sexing well preserved skeletons, since the differences in the *Os pubis* directly relate to the adaptation of the female hip bone to parturition. In addition to skeletal sex, four burials have known personal identification.

Thirteen burials (A-M) are from Kellis 2 (K2), a large Roman period cemetery located in the central part of the Dakhleh Oasis, Egypt (Molto 2001). These are all primary extended burials placed in mud-brick crypts interred in the classic 'Christian' position with bodies oriented east-west and the head in the latter direction. Archaeological evidence suggests that K2 was likely in use from 280 AD to circa 390 AD although radiocarbon dating suggests a range of 100-460 AD (Stewart et al. in press).

Table 1: Brief description of samples (A-S) analyzed in this study

Label	No.	Type of Sample	Age	Comments	Skeletal Sex	Legend
A	D189	rib	50	slight osteoporosis, no pathology	F	D = Dakhleh Sample
B	D377	humerus	55	spinal tuberculosis, bones slightly osteoportoic	F	L= London cemetery sample
C	D392	rib	40	Lepromatous leprosy, bone condition excellent	M	RSB = River St. Burial
D	D261	rib	61	very osteoporotic, healed femoral neck fracture,	F	DD = Danforth Doe
E	D265	rib	55	spinal tuberculosis, bone condition excellent	M	MC = metacarpal
F	D213	rib	54	slight perisoteal newbone, psortici arthritis, bone condition excellent	M	MT = metatarsal
G	D116	rib	23	Lepromatous leprosy, bone condition excellent	M	
H	D322	rib	60	Widespread osteoporosis, slight infection (periosteal newbone)	F	
I	D6	rib	29	Lepromatous leprosy, bone condition excellent	M	
J	D280	humerus	65	spinal tuberculosis, slight to moderate osteoporosis	F	
K	D410	MT	27	bone condition excellent, evidence of infection (periosteal newbone)	F	
L	D269	rib	55	very osteoporotic,unhealed femoral neck fracture, no infection	F	
M	D437	rib	40	Lepromatous leprosy, bone condition excellent	M	
N(11)	L6	MC5	61	Sam Green, died Oct. 12, 1855;	M	
O(7)	L7	MT3	81	Sarah Lawrence, died Sept , 1878	F	
P(18)	L10	MT3	45	William Hussey, died Aug. 13, 1883	M	
Q(10)	L18	MT3	84	Helen Robotham, died Dec. 19, 1881	F	
R(6)	RSB	rib	40	This person died between 1870-1884, bone condition excellent	M	
S(77)	DD	tibia	35	This person died likely died in the 1940s (dental appliance), bone excellent	F	

Figure 4: This figure depicts the first criteria used in the Phenice method to sex an individual based on hip morphology (i.e. the ventral arc). As is clearly visible, the angle between the white line and the sample (ventral arc) is substantially less on the male than it is on the female.

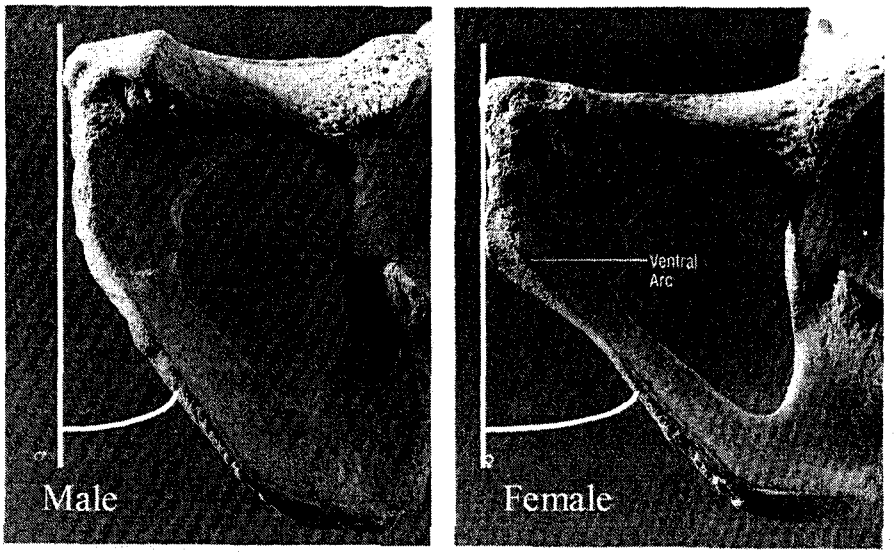


Figure 5: This figure depicts the second criteria used in the Phenice method to sex an individual based on hip morphology (i.e. lateral recurve). The figure illustrates that on the male sample, the subpubic concavity is much deeper than it is on the male. The white arrows indicate the area of interest.

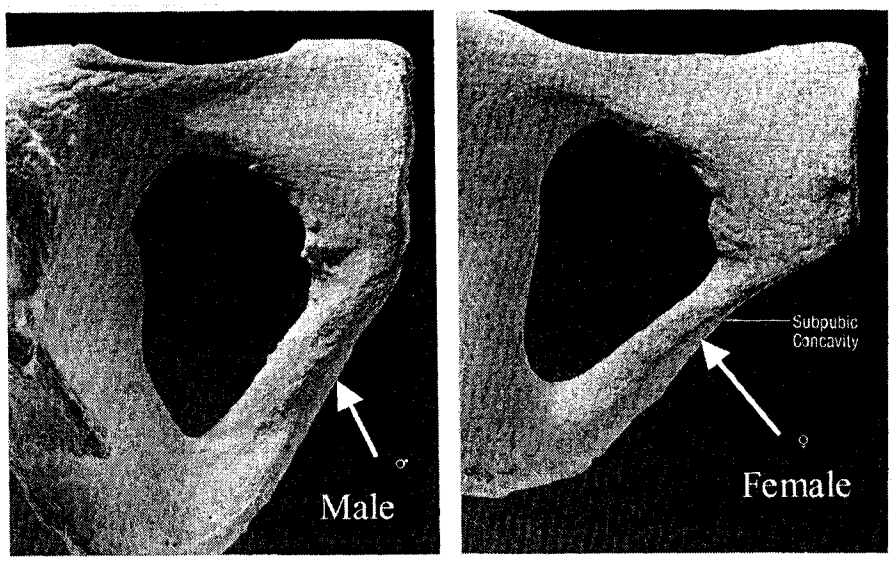
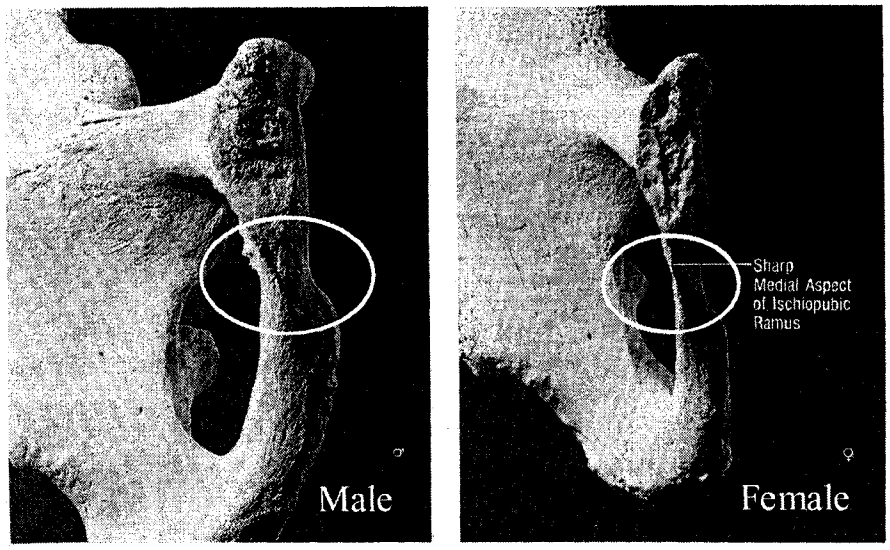


Figure 6: This figure demonstrates the third criteria used by the Phenice method for sexing an individual based on hip morphology (i.e. sub-symphyseal margin). The difference is found between the thickness of the male and female sub-symphyseal margin, and is visible in the white outlines encircling the medial aspect of the ischiopubic ramus. As can be seen, the sub-symphyseal margin of the male is much broader than that of the female.



The hyperarid conditions now and in the past have resulted in exceptional skeletal and soft tissue preservation (Fairgrieve and Molto 2000; Molto 2002). Previous studies have authenticated the preservation and extraction of mtDNA in the Kellis 2 cemetery (Graver *et al.* 2001; Parr 2002) and one unpublished report successfully sexed a Kellis 2 burial using the amelogenin technique (Schmerer 2001). To date no autosomal STRs have been attempted on K2 remains.

Four historical burials (samples N-Q) are from the late 19th century Stirrup Court (SC) cemetery in London, Ontario. The samples from the SC cemetery were provided by Dr. Michael Spence (Department of Anthropology, University of Western Ontario). Stirrup court was a large family based cemetery that was in use from 1859-1890 (Cook 1986). The cemetery, which is located on the campus of the University of Western Ontario, was found and excavated in 1982 (Cook 1986). As shown in Table 1 most of the SC burials used in this thesis represent older individuals with known identities. The sex of the individuals, inferred from their names, has also been confirmed by the Phenice method (Personal Communication, Spence, 2003). The fact that they were interred in coffins may have provided some protection from the taphonomic conditions known to degrade DNA, particularly soil pH and hydrolysis. In fact, of the 20 graves excavated at this site, 13 of the adult skeletons were over 90% complete and were found intact in their respective coffins (Parish 2000). The four samples used in this study were from the 13 excellently preserved skeletons. Some of the individuals were so well preserved that hair, brain, and skin are still available for several individuals (Parish 2000). Although the samples are very well preserved, this is the first DNA analysis of any type to be attempted on these remains.

Burial R is from the former St. Andrews cemetery, which was in use from 1870 to 1884 in Thunder Bay, Ontario, Canada (Brown 2003). In 1883 relatives were notified of the cemetery's relocation to Oliver Road and had the opportunity to exhume and re-inter the remains at the new location. This burial, herein called the River Street Burial (RSB) after the 'original' street location, was discovered during a housing development in 1979. How this rather statused individual (housed in an ornate coffin) was missed during the relocation of the cemetery remains a mystery, although it may be because he died of a highly infectious disease (the coffin had a full-length plate-glass covering which facilitated viewing without risk of disease transmission). The coffin was very fragmented and the burial had been exposed to the highly acidic and damp soil. However, mtDNA has been successfully recovered from the RSB for the continuing research on individuation (Brown 2003). The individuation process first involves identifying possible fits for this 35-45 year old man with the cemetery records and then finding potential relatives to match with the DNA.

Burial S represents an individual called Danforth Doe (DD) whose remains were discovered during a construction project on Danforth Avenue, Toronto, Ontario, Canada in 1995 (Melbye *et al.* 1999). Her violent death led to an intensive investigation that produced the presumptive ID of Doddy Cox, a 40-year-old women who apparently left her family in disgrace in the 1940s. Ultimately the presumptive identification was made from a craniofacial reconstruction witnessed by her son. The time period of her disappearance was backed by a dental appliance and thus linked the burial to the alleged son. Dr. Cathy Gruspier of the Centre of Forensic Sciences, Toronto, sent remains of the burial and comparative samples from two sons to Lakehead University in January of

2003. MtDNA analysis confirmed a match between the sons but not with the skeleton (Murray 2003). Moreover, previous analysis of 4 STR loci of the bone and one of the sons by Dr. Sweet, Department of Dentistry, University of British Columbia, were also an exclusion (not one matching allele). Amelogenin analysis done at Dr. Sweet's laboratory did, however, support the skeletal sex as female.

It should be restated that all information about these burials was unknown to the writer during the research. This blind design ensured objectivity during the treatment of each sample.

Extraction

Several extant extraction protocols have now proven successful for yielding aDNA from bone (Matheson 2001). This study uses a modified GuSCN (guanidinium thiocyante) method optimized for the retrieval of DNA from less than 100 mg of bone powder. To remove and protect the ancient material from contamination, many precautions delineated in the literature were followed (Ou *et al.* 1991; Cooper 1992; Van Helden *et al.*;1992 Sarkar and Sommer 1993). All extraction procedures were conducted in a dedicated "clean room". At each stage of preparation and extraction the samples were separated both physically and temporally as well as using dedicated hoods for each step in the extraction process. Moreover all reagents were prepared with ultra-pure, diamond distilled and UV irradiated water.

Contamination has been the key issue where aDNA research is concerned; therefore, even at the excavation stage an attempt to control this problem was initiated. All samples were carefully collected with DNA analysis in mind, thus, great care was

taken to avoid extraneous DNA contamination by over handling in the field. For example, the collectors wore disposable, sterilized latex gloves, and masks.

Once the samples were entered into the lab, further precautions were taken for the removal of surface contamination from depositional and/or post-depositional handling. This was done via UV irradiation of bone surfaces for a minimum of 24 hours as UV has been proven to help reduce contamination and false positive results from polymerase chain reaction (PCR) procedures (Ou *et al.* 1991). This cross-links any surface DNA, creating thymine dimmers, which render the DNA unamplifiable. Subsequently the samples were reduced to a fine powder of 35 microns in an oscillating speed mixer mill (Retsch/Brinkman). Separate ball and mortars were used for each sample and all were initially sterilized by washing with a tergazyme solution, ultra-sonication, UV irradiation and autoclaving. All other equipment followed the same precautions when possible and, at minimum, received a washing with a 10% hypochlorite bleach solution and UV irradiation (as in the case of the extraction hoods). The bone powder was then transferred to UV-sterilized and autoclaved 1.5mL or 2.0mL tubes for use or storage at -20°C. Any extra bone was repacked into separate sterilized plastic bags, which in turn were each placed into another sterilized plastic bag.

For extraction and purification, a minimum of 50 mg powdered bone was first incubated in 500uL of GuSCN (4M Guanidinium Thiocyanate: 0.1M Tris-HCl pH 6.4, 0.02M EDTA pH 8.0, 1.3% Triton X-100, sterile H_20) for a minimum of eight hours at 56°C at 900 rpm on an Eppendorf thermomixer. After the samples incubated overnight, a 10-minute boil at 94°C under gentle agitation (400 rpm) was performed prior to a one-minute centrifugation at 12000 rpm. The supernatant was then drawn off and transferred

to a new sterile 1.5mL tube. An organic solution (OS) separation was then performed according to the standard protocols.

An attempt to extract DNA was also made using modifications to an enzymatic extraction method (Yang *et al.* 1998) using 50μL of Proteinase K (Qiagen) with 1000μL of the Lysis buffer (EDTA 0.5Mm, Tris 1M, Tween). The samples were thoroughly mixed, then placed on an Eppendorf Thermomixer to incubate overnight at 56° C and 750 rpm. After the overnight incubation the samples were centrifuged for two minutes at 12000 rpm to separate the supernatent from bone powder. Upon completion of the extractions, all samples were treated to the same purification protocols regardless of extraction method.

Purification

Following the extraction process a silica bead purification was conducted on each sample by adding 900uL of GuSCN and 10uL of silica bead solution. The samples were then mixed and placed on ice for one hour in order to bind the DNA to the silica. A zip spin was then performed and the supernatant drawn off in order to add 500uL of wash buffer (0.2M Tris-HCl (pH 7.5), 1.0M NaCl, 20mM EDTA, Sterile H_20 and EtOH) to wash the silica. Upon mixing, centrifuging and drawing off the supernatant, 200uL of 99% EtOH was added in order to further wash the silica and allow for easier desiccation of the samples. A 100uL volume of sterile ddH_20 was then added to elute the DNA off the silica during a one-hour incubation at 56°C and 700 rpm agitation. Prior to using the solution for PCR (polymerase chain reaction) the samples were subjected to one more purification system (BIO RAD, P-30 Tris Chromatography) in order to reduce to the possibility of PCR inhibition.

Primer Design

Sexing Primers:

Four previously established primer sets (Table 2) were used to sex the bone samples via PCR amplification. The alphoid repeat primers (two separate sets) were designed to amplify a 130bp specific fragment from the X chromosome and a 170bp specific fragment from the Y chromosome (Witt and Erickson 1989). The third set of primers amplified a region of the amelogenin gene located on both the X and Y chromosomes (Sullivan *et al.* 1993). The amelogenin regions of the sex chromosomes are distinguishable from one another due to a six base pair difference in amplicon size (X=106bp and Y=112bp). The SRY gene is the target of the fourth set of primers strictly for sexing purposes. This previously established primer set specifically targets the Y-chromosome and produces an amplicon of 93bp (Santos *et al.* 1998).

The suitability of these primers dictated they be used in a multiplex system. The Amplify computer software program for the Macintosh Computer systems was used to assess virtual product size and the presence of both non-specific amplicons and primer dimerization. This check was performed because the non-specific products and/or primer dimmers can singly or tandemly lower the yield of PCR reactions. The primers were then subjected to the Oligo Toolkit software provided by Operon (www.operon.com) to verify their corresponding annealing temperatures (approximately 60°C). As a result of the significant size difference in amplicons, only two fluorescent dyes were required. For both the alphoid repeat and amelogenin primers 6-FAM was incorporated, while for the SRY primers a TAMRA label was added. The primers were subjected to a set pattern of

Table 2: List of sexing primers used and their respective amplicon sizes for both male and female loci

Primer Set	Amplicon Size	Reference
Amelogenin	Male = 112 Female = 106	(Sullivan *et al.* 1993)
Alaphoid Repeat Male	170	(Witt and Erickson 1989)
Alphoid Repeat Female	130	(Witt and Erickson 1989)
SRY	93	(Santos *et al.* 1998)

changes in order to optimize them to the capacity where they produced concise and consistent results on modern DNA samples. The first step was to use the primers in equimolar concentrations and then to vary them accordingly (primer concentrations of weaker amplicons were increased while stronger amplicons remained the same or were decreased) (Henegariu *et al.* 1997). The next major step was to remove non-specific products, which according to Henegariu et al. (1997) is accomplished by lowering the annealing temperature by 4-6°C. This technique may lead to an increase in non-specific primer annealing in single loci amplifications; however, in multiplex PCR it seems that the increased number of loci will bind more easily to the template and thus non-specific product remains invisible (Henegariu *et al.* 1997). Therefore, the primers with single-plex annealing temperatures of 60°C were set to anneal at 56°C under multiplex conditions.

Y-STR Primers:

Upon the completion of sex typing, Y-STR analysis was conducted using various primer sets developed for use by the Y-forensic user group (Table 3). The primers used to analyze these regions vary throughout the literature. It was imperative to try and find primers that amplify regions of DNA conducive to ancient studies because over time the degradation of DNA via autolytic processes tends to be detrimental to DNA amplicon size. For this reason, the largest amplicon is found at the DYS 389 loci and has a maximum size of 387bp. It may prove that this amplicon is in fact too large a fragment to be amplified in ancient tissues. It has been shown that authentic ancient DNA is usually no larger than about 300-350bp (Hagelberg and Clegg 1991). However, this

Table 3: List of Y-STR Primer Sets and Their Respective Amplicon Sizes

Primer Set	Amplicon Size	Reference
DYS 19	174-210	(de Knijff *et al.* 1997; Schneider *et al.* 1998)
DYS 385	352-416	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 389I DYS 389II	I. 235-267 II. 355-387	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 390	187-231	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 391	271-299	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 392	95-122	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 393	108-136	(Kayser *et al.* 1997; Schneider *et al.* 1998)
DYS 434	110-122	(Ayub 2000)
DYS 437	184-196	(Ayub 2000)
DYS 439	116-136	(Ayub 2000)

locus is incorporated because it amplifies a different region of the Y-chromosome with an allele size of 267bp.

Once the primers were chosen, the annealing temperatures were tested and it was determined that only four of the 11 primer sets were designed with markedly lower temperatures in respect to the others. Thus, the Y-STR primers were divided into two groups in an attempt to create two multiplexes: one consisting of DYS 390, DYS 391, DYS 393, DYS 434, and DYS 437 and one consisting of DYS 19, DYS 385, DYS 389-I/ II, DYS 392 and DYS 439. The Y-STR system was setup in this manner in order to facilitate future work. To incorporate all the primer sets into a single multiplex would require that certain primers from the second multiplex be redesigned in order to raise their annealing temperatures to correspond to those of the first multiplex.

All primers were fluorescently labeled to avoid any problems with overlapping of amplicon sizes. This entails that no two primer sets will produce amplicons that could potentially be ambiguous during the analysis of the Y-STRs. The fluorescent labels were chosen according to several criteria. One was to use currently established fluorescent technology (i.e. five-dye system from Applied Biosystems). The second was to consider that this system might be run in conjunction with autosomal kits currently labeled with the same fluorescent dyes. This allows two separate tests to be run together and time to be saved. The third criteria was to have a dye set flexible enough to increase the number of primer sets for possible future expansion of the Y-STR system.

PCR Amplification

Amplifications of 2-7.5uL purified sample were conducted using an Eppendorf Mastercycler or Mastercycler Gradient in 10uL reaction volumes for sex identification.

For haplotype analysis of the Y chromosome, 5-20uL of purified sample was added in 25uL reaction volumes for amplification in the thermalcyclers noted above. All amplifications used the Qiagen Platinum Taq kit and were used according to the manufacturers protocols (10X PCR buffer, 10mM dNTP, 50mM $MgCl_2$, 10uM primers and 5 units of Qiagen Platinum Taq polymerase). An initial denaturation at 94°C for 2 minutes is required to activate the thermostable Taq polymerase. The PCR is then run for 50 cycles under the following conditions. For the sexing multiplex and Y-STR set 1 a 94°C denaturation of 30sec to 1 min was followd by a 56°C annealing step for 30sec to 1min, and 72°C elongation for 1 to 2 min. For Y-STR set 2, only the annealing temperature was changed from 56°C to 50°C.

The fluorescently labeled primers of the various multiplex systems allowed for the omission of PCR product detection via polyacrylamide gel systems, and direct analysis through the ABI 310 CE (capillary electrophoresis) system due to its increased sensitivity. The labeled primers also provide greater feasibility for the reliable separation of amplicons of similar sizes (Ziegle *et al.* 1992), such as with the amelogenin primers. Therefore, upon amplification all PCR products (including both positive and negative controls) were prepared for loading on the ABI 310 (Applied Biosystems).

Genetic Analysis

The analysis was performed by capillary electrophoresis (CE) on the ABI 310 System (Applied Biosystems). CE detection was conducted by adding 0.5uL of GeneScan size standard (Rox 350 or Tamra 500 for sexing PCR and Liz 500 for Y-STR), 2uL of PCR product and 12uL of formamide. The reagents were then treated to a five minute denaturation at 95°C followed by a five minute cold shock on ice (in order to keep the

DNA single stranded). The samples were then loaded into the ABI 310 and run for 24 minutes under the following conditions: 5 sec injection, 15.0 Kv injection, 15.0 Kv run, 60°C run on module GS STR POP4 (1mL) D for sexing. For STR analysis the ABI 310 parameters were as follows: 5 sec injection time, 15.0 KV injection, 15.0 KV run, 60°C run form 28 minutes on module GS STR POP4 (1mL) G5.

The use of differing fluorescent tags and amplicon size in the sexing multiplex system should provide an internal control for the discrepancy of not producing Y-chromosome products when the individual is in fact male. The triplex system allows for three tests to be performed simultaneously in order to determine maleness while two tests are performed to provide a female determination. This methodology should help overcome the possibility of Y allelic drop out. Nevertheless, any amplification of the Y chromosome is sufficient enough to type the sample as male (Sutton 1996). In conjunction all experiments included both positive (modern DNA) and negative (no DNA) controls in order to confirm the validity of the results and ensure that typing was not the result of contamination.

The Y-STR multiplex systems also provide a means of identifying individual samples as male. Due to the Y-chromosome specificity of the primers used in these systems only Y-DNA is amplified and thus any result will indicate a male individual. Furthermore, even if only one of the 11 markers is successfully amplified, this suffices to determine the individual as male. However, a problem arises when no DNA is amplified. We can not say with 100% certainty that the result is female because, perhaps the DNA is absent or fragmented to a point where amplification is no longer possible. In addition to the sexing ability of the Y-STR multiplexes, there is also the potential of identifying

paternal lineages and population ancestries based on the Y-chromosome haplotypes identified.

Summary

The samples used in this project provide a broad spectrum of factors against which the sexing methods can be tested. Not only do the samples offer varying time and environmental conditions, but they also exhibit varying degrees of preservation. This allows for a more complete assessment of the efficacy of the varying sexing loci.

However prior to analysis, the samples had to be extracted and purified. This was accomplished using a guanidinium based method for extraction and a silica bead purification. The resulting "DNA-solution" was further purified (P-30 chromatography) to try and eliminate possible inhibitors, which adversely affect the polymerase chain reaction.

Once the DNA is extracted, it must be amplified to a level suitable for analysis. To accomplish this, primers were chosen (specific for sex determination and/or the Y-chromosome) and fluorescently labeled. The primers were then optimized in order to function as multiplexes. Amplification of DNA using the primers was accomplished via PCR in Eppendorf Mastercyclers. The resulting, amplified DNA was then subjected to analysis using the ABI 310 capillary electrophoresis system.

Chapter 5: Results

As noted in chapter 4, the initial first step in this analysis was the testing and validation of the sexing protocols on modern samples prior to their application to archaeological remains. The results proved to be variably successful. The sexing primers were fully optimized and worked very well when typing modern males and females. For example, all loci (SRY, amelogenin, and the alphoid repeats) show clear and very similar levels of amplification (relative peak heights) when typing a modern male (Figure 7). Furthermore, the SRY loci (93bp) did not amplify in the modern female sample and neither did the 112bp or 170bp loci (Figure 8), which correspond to the male fraction (Y-chromosome) of a sample. Therefore it is clear that this sample is that of a female.

The Y-STR multiplexes did not optimize to the same degree. The Y-STR multiplex containing DYS 390, DYS 391, DYS 393, DYS 434, and DYS 437 amplified most loci (Figure 9) with some non-specific products, but was reproducible on modern samples. The non-specific products most likely relate to a couple of factors. First, the theoretical optimal annealing temperatures for the primers selected were not identical. This means that at PCR operating temperatures (annealing temperature specifically), some loci will be preferentially amplified. Secondly, some of the primers may form secondary amplicons by interacting during the PCR process. Although screened for using various computer software programs (i.e. Oligotoolkit by Operon and the Amplify program for the Mac OS), this may have occurred because of polymerase buffer levels and/or $MgCl_2$ levels which can alter primer binding and amplification depending on the size (bp) of the amplicon.

Figure 7: This electropherogram depicts the efficacy of the sexing multiplex on a modern male sample. Amplification at all three loci is well demonstrated with the 93bp peak of the SRY loci, the 106/112bp peaks of the amelogenin loci, and the 130/170bp peaks of the alphoid repeat loci. The red peaks represent one of the size standards that must be incorporated into every run as a sizing reference.

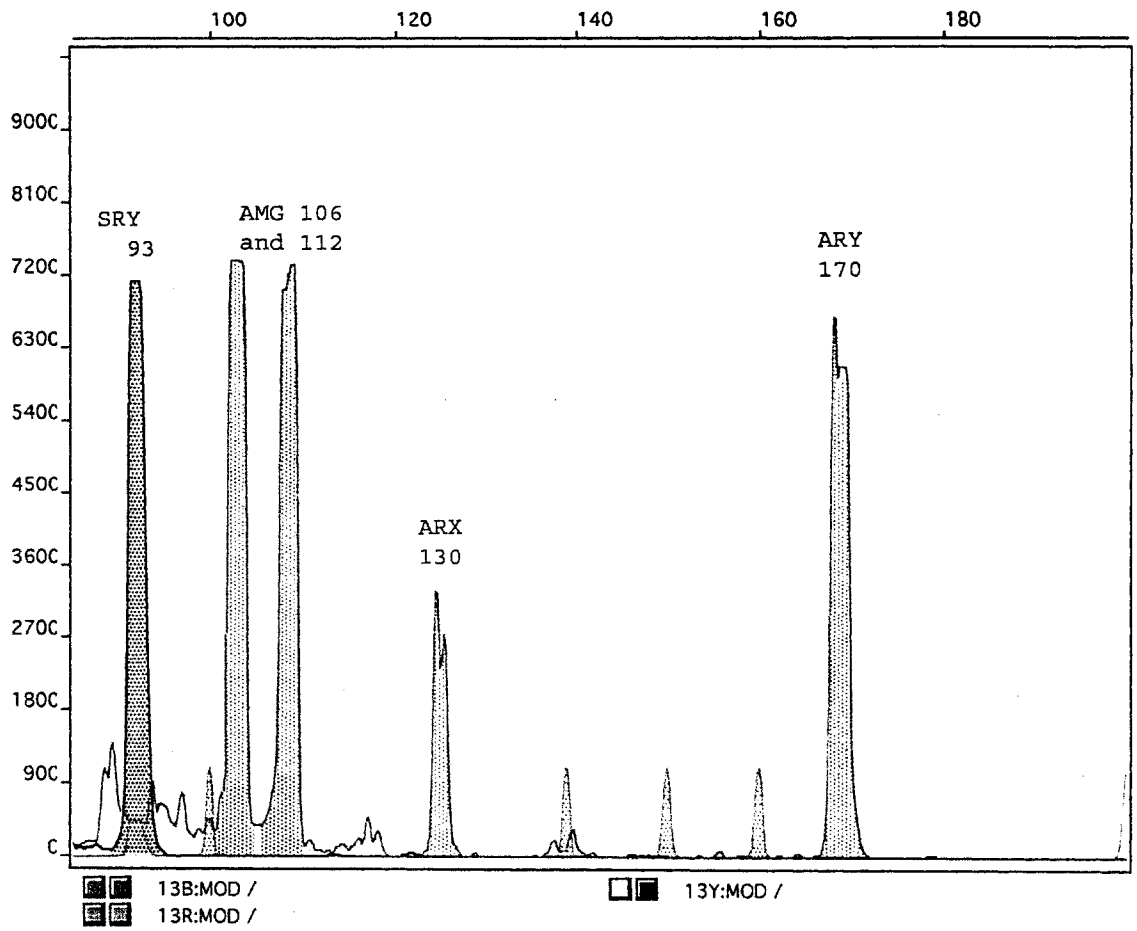


Figure 8: This electropherogram illustrates the efficacy of the sexing multiplex on a modern female sample. Amplification at two of the three loci is expected (due to the male specificity of the SRY loci) and is well demonstrated with the 106bp peak of the amelogenin loci, and the 130bp peak of the alphoid repeat loci. The black peaks represent one of the size standards that must be incorporated into every run as a sizing reference.

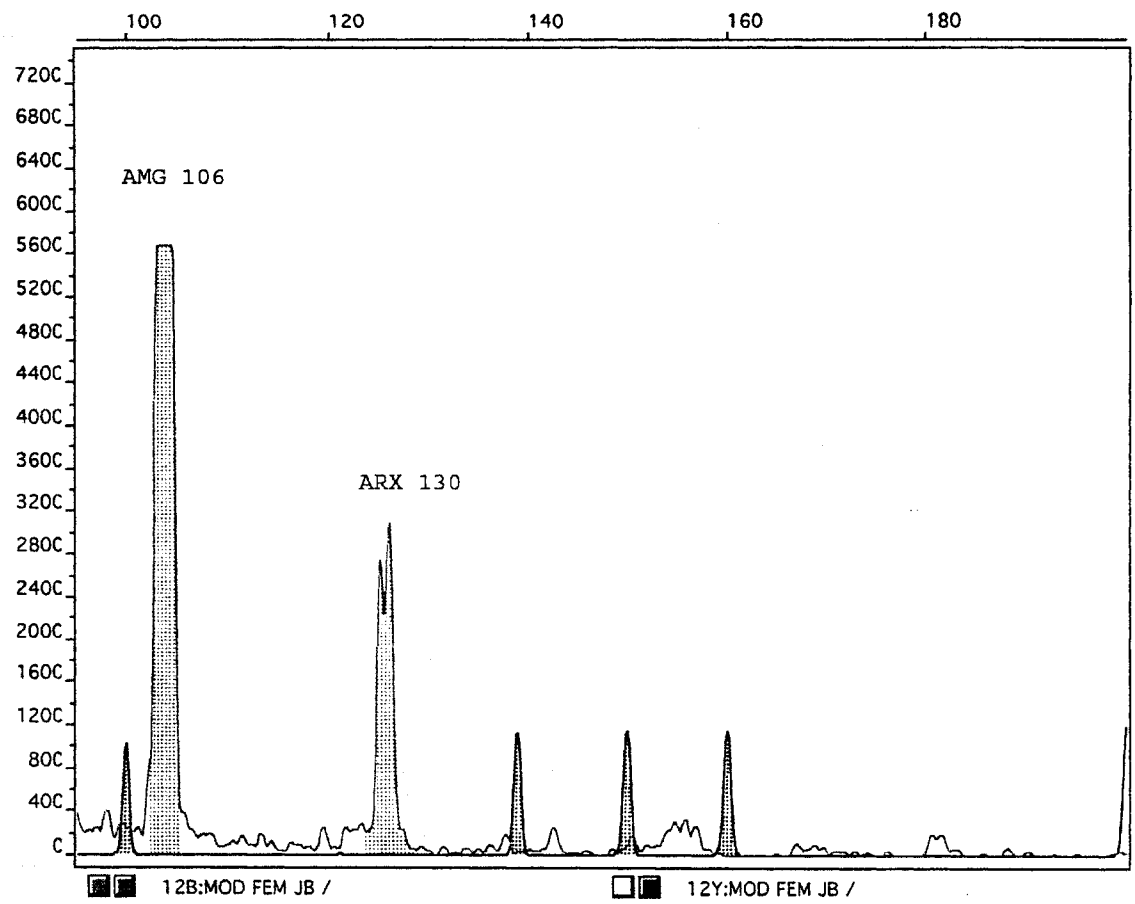
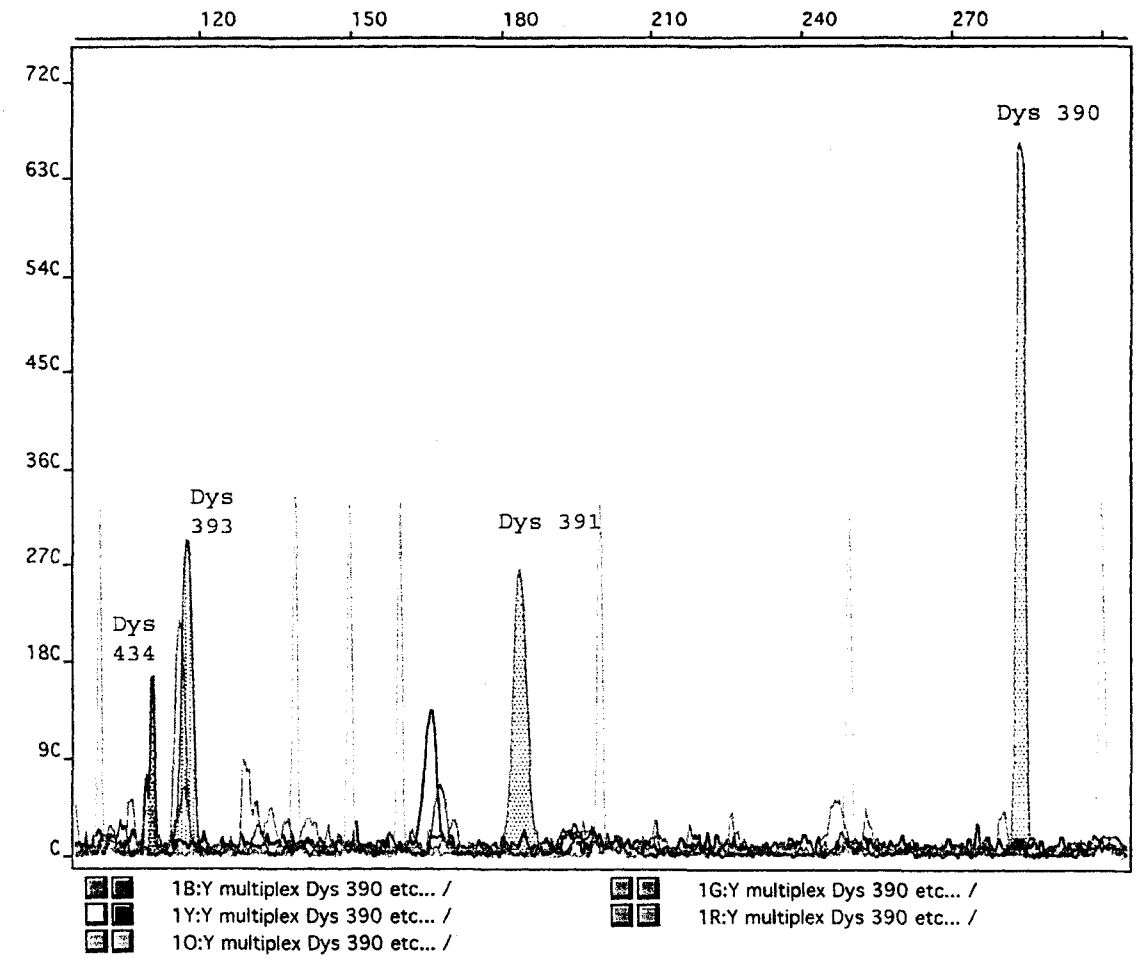


Figure 9: This is the electropherogram of a modern sample that has been amplified with Y-STR multiplex 1 (DYS 390, DYS 391, DYS 393, DYS 434, and DYS 437). As can be seen in the figure, all but one of the loci are amplified (DYS 437 not present). The peak intensity varies with DYS 390 working the best; moreover, there are also several smaller peaks of unspecific product. The non-specific product however, does not interfere with the calling of the Y-STR amplicons.



The second Y-STR multiplex (containing DYS 19, DYS 385, DYS 389, DYS 392, and DYS 439) did not optimize to a satisfactory degree and, as a result, did not perform consistently well, producing only sporadic results on modern samples. This is most likely attributable to the same factors mentioned above, as well as to an even greater variance in primer annealing temperatures. Nevertheless, amplification of the ancient samples was attempted using the Y-STR multiplexes because it was theorized that a single amplicon would be sufficient to identify a sample as male.

Amplification of the historical samples yielded DNA for all individuals. The molecular sexing of the historical samples was for the most part in agreement with the osteoscopic analysis. Of the four London samples (N, O, P, and Q; Table 4), two (N and P) agreed completely at all amplified loci. Of the other two London samples, O agreed at the amelogenin locus but not at the alphoid repeat loci; whereas, Q matched at the amelogenin locus but not at the other two loci (alphoid repeat and SRY). The final two historic samples (R and S), the River Street burial and Danforth Doe, were correctly identified. The River Street burial typed conclusively as male for the amelogenin, alphoid repeat and SRY loci. For the Danforth Doe, results at the amelogenin and alphoid repeat loci typed the individual as female. The Y-STRs did not amplify for any of the historic samples.

The ancient samples (A to M; Table 4) from the Roman period cemetery in Dakhleh, Egypt yielded inconsistent results. Of the 13 ancient samples, 12 amplified at the Alphoid Repeat loci; however, five were correctly sexed (B,F,H,J and M) and seven were incorrectly sexed (A,C,D,E,G,I and K). Only one electropherogram was obtained for sample L, it could not be re-amplified and because confirmation was not obtained the

Table 4: Comparison of the results obtained from the individual loci used in the molecular analysis of the ancient samples

Label		Skeletal Sex	Amelogenin	SRY	Alphoid Repeat	Y-STR
A	*	F	M	---	M	---
B	*	F	F	---	F	---
C	*	M	F	---	F	---
D	*	F	---	---	M	---
E	*	M	---	---	F	---
F	*	M	F	---	M	---
G	*	M	---	---	F	---
H	*	F	---	---	F	---
I	*	M	---	---	F	---
J	*	F	F	---	F	---
K	*	F	F	---	M	---
L	*	F	---	---	---	---
M	*	M	---	---	M	---
N	+	M	M	---	M	---
O	+	F	F	---	M	---
P	+	M	M	---	M	---
Q	+	F	F	M	M	---
R	+	M	M	M	M	---
S	+	F	F	---	F	---

Legend

M= male

F = female

--- = no amplification

* = ancient samples

+ = historic samples

sample was entered as 'no result'. The remaining loci were equally poor in determining the sex of the samples. Amelogenin amplified in only six of the thirteen individuals and typed correctly three times. Neither the SRY nor the Y-STRs amplified for any of the ancient Egyptian samples.

Overall, the fact that aDNA was recovered for all but one sample is encouraging given the temporal span covered. However, not all samples yielded correct results gauged against 'known' sex using reliable morphological and documentary evidence. The rate of error was particularly evident in the older samples from Egypt. The poor and/or disparate performance of these molecular techniques may be attributable to a number of factors that will be discussed in the following chapter. The recommendations arising from these results are invariably tied to the 'blind research design', a design that is 'method' not 'result' oriented.

Chapter 6: Discussion and Conclusion

Discussion

In 1976 Dennison introduced a new biochemical sexing technique which received considerable interest among bioarchaeologists. The method was based on the differential accumulation of citrate in male and female skeletons, with it being considerably more concentrated in the females. Unfortunately his study, which involved archaeological samples, was not done in blind and future researchers were unable to validate his results. The study however was important in that it demonstrated the interest among bioarchaeologists for new and more sensitive inter-population techniques for sexing human remains. Also in postscript it exposed the weakness of studies that do not utilize blind research designs. The lack of validation of Dennison's method resulted in considerable skepticism among researchers about biochemical methods in bioarchaoelogical science (Gibbs 1987).

The new molecular techniques investigated in this thesis have considerable potential for the bioarchaeological and forensic sciences in that they can address sex determination, not only in poorly preserved adult remains, but also in subadult remains. Moreover, these data are specific and not prone to the types of errors that can be introduced by nonspecific techniques, such as the fluorescent methods used in Dennison's research. The use of a blind design ensures that the methodology is being examined critically and prospectively. This allows future research to address the inevitable problems outlined.

The results obtained in this thesis show that it is in fact possible to obtain amplifiable DNA for sex determination loci from bone samples that range in age from

approximately 100 years old to just under 2000 years old. Moreover, this was achieved using very little bone (< 100mg of bone powder) which, is an advancement over previous research protocols that used more osseous tissue. This improvement can be seen when the extraction protocols used by Matheson and Loy (2001) or Mays and Faerman (2001) are compared to those used by Hummel and Herman (1996). The former studies were able to obtain DNA from as little as 5-25mg of bone powder (Matheson and Loy 2001; Mays and Faerman 2001) whereas the latter study (incorporating an automated system) used 0.3g (Hummel and Herrmann 1996). Advances in extraction technique allow for advancements in sampling, such as, the possibility of attempting to extract DNA from more prestigious and or sacred individuals or from trace evidence remaining at crime scenes. Moreover, in an archeological context, a sample less than 100mg could go unnoticed upon removal, while in the forensic arena this means that other portions of a sample could be sent off for other types of investigation. These two points will undoubtedly facilitate the process of obtaining permission to conduct DNA research on small fragments of important samples.

Clearly the amount of bone required is of critical importance as is the issue of performance. The latter has proven to be a major problem with the available techniques. I found that error rate was inversely correlated with the age of the sample, and the most ancient samples from Egypt essentially matched probability estimates (50/50) and the hypothesized 'best' method (Alphoid repeat) actually was out performed by the amelogenin technique.

It is imperative that a number of precautionary measures be taken when conducting ancient DNA research. One, of course, involves measures to reduce and/or

eliminate extraneous (modern) DNA that may contaminate the samples. Although many precautions were taken to try and avoid this issue (i.e. UV irradiation of bone fragments and washing with bleach and ethanol, full Tyvek suits, double gloves, hair nets, face masks, and washing all equipment and work stations prior to use) it seems that this is the most plausible explanation for some of the samples that were incorrectly typed (A, D, K, O, and Q). All of the aforementioned samples were identified as male for at least one of the loci when the skeletal sex designation was female. This will occur if the samples are contaminated by exogenous male DNA because not all archeological remains will contain endogenous DNA (Poinar and Stankiewicz 1999). It must be noted that without amplification of the Y-STRs for the ancient samples, a comparison could not be made between my DNA (male) and that of the male results obtained. This only validates the importance of obtaining a method to verify the Y chromosome DNA as not being the result of contamination by handlers or analysts (especially males).

It is difficult to determine exactly at which stage the contamination was introduced. However, it can be hypothesized that because the negative controls were clean, the contamination was either introduced directly to sample prior to extraction or to the sample tubes prior to PCR. The latter is a more plausible explanation because with some samples the correct result was obtained with the amelogenin but not with the alphoid repeats (samples O and Q).

Due to the inherent difficulties in resolving this issue, a suggestion to control for this problem would be to do mtDNA sequencing on PCR products that have yielded sex results. The sequence obtained could then be compared to those of all personnel involved with the laboratory and/or samples. If the sequences do not match any of the personnel

then the results can be substantiated. This could also have been accomplished for all male results had the Y-STRs yielded results. The Y-STR profile of the sample could have been compared to the profiles of all male individuals who worked with the samples to see if they matched. If the samples matched, then statistical analysis could be conducted to show the likelihood that the result was from contamination. Due to the lack of efficient Y-STR methodologies currently available for ancient DNA, mtDNA sequencing would be a much better alternative.

Another reason why some of the samples (e.g. samples F, K, and Q) typed incorrectly and certain loci (e.g. SRY) did not type at all is also problematic. A possible explanation includes differential degradation and low copy number. This encompasses the processes involved in the destruction of DNA immediately post-mortem by endogenous nucleases and by slower processes afterwards (oxidation, background radiation, deamination, depurination and hydrolysis) (Hofreiter *et al.* 2001). This includes degradation that starts at the telomeres working its way inwards (Medina 2003). This may be able to explain the differential amplification of the various loci because as the DNA is degraded, the initial concentration or copy number of DNA is decreased. This may ultimately lead to an insufficient level of DNA for PCR amplification. For example, the SRY primer set binds just inside the telomere region and thus would be the first to suffer degradation. This would be followed by the amelogenin loci and lastly the alphoid repeat loci. This probably explains why better results were obtained for the historic samples than for the ancient Dakhleh samples. The older samples have had more time for the degradation process to make its way further into the chromosome rendering it more difficult to obtain the remaining fragmentary DNA.

One unexpected result found in this research was that the amelogenin method typed correctly for historic samples when the alphoid repeats should have been the least affected by the degradation process. A possible explanation is that if the DNA survives the immediate autolytic actions postmortem, it is then subjected to the most common form of degradation via hydrolysis and oxidation (Richards and Sykes 1995). Therefore, a factor that should be investigated involves the soil types in which the samples were found. The samples from Egypt are from a desert environment and will dessicate relatively quickly, reducing the amount of hydrolytic and, in turn, oxidative damage to the DNA (Matsuo *et al.* 1995). In contrast, the soils of Ontario, Canada are more water-saturated. This increases hydrolytic degradation resulting in more sporadic or random degradation in the DNA structure (Perry *et al.* 1988). Due to the age of the samples, DNA is still present in abundant amounts to allow for the amplification of nearly all the loci in the sexing multiplex. However, depending on the hydrolytic damage that has occurred, the damaged areas may be sporadic and thus may not follow the telomere pattern of degradation. This could explain why the alphoid repeats typed incorrectly for some of the historic samples while the amelogenin was correct for all.

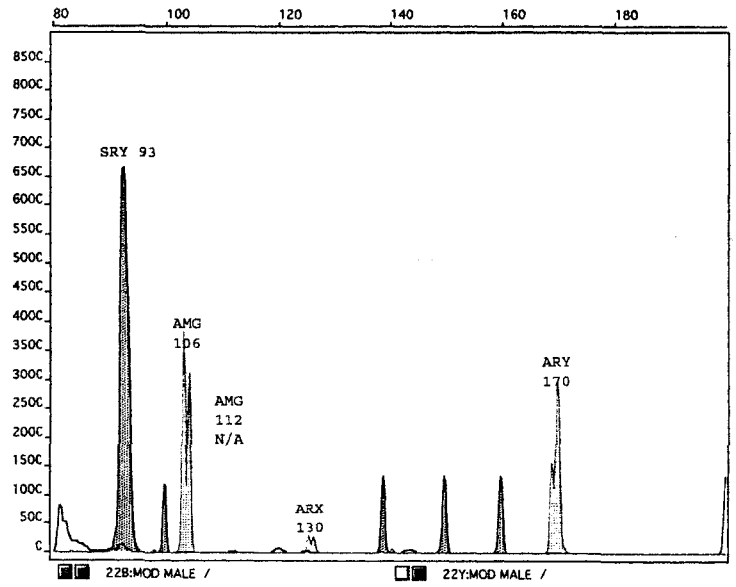
An explanation for the incorrect typing of samples C, E, G, and I involves allelic drop-out (Boutrand *et al.* 2001), which occurs when the primer binding sites are damaged or when there is a mutation in that area. If the primer binding sites are damaged sufficiently then the primer that is supposed to bind to that region can not. This results in only one of the two primers binding and, therefore, amplification will not ensue. The allelic drop out phenomena presented itself in two of the modern control samples (Figure 10). Therefore, it can be predicted that in DNA that is much older and more degraded,

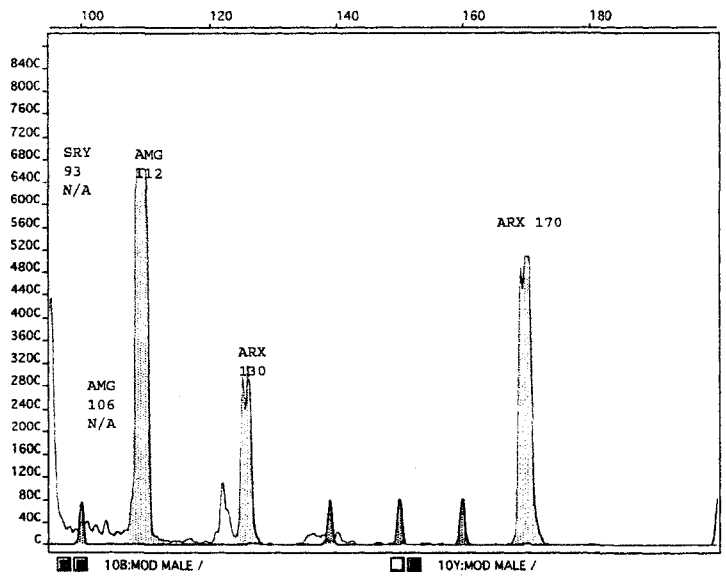
allele drop-out may occur more frequently and will yield female results when in fact the sample is actually male. Allelic drop-out is not male to female specific; it can occur from female to male readings and thus may have affected some of the sample discrepancies discussed in relation to contamination issues. Therefore, if it is to be used as an explanation more research is required to substantiate it as a legitimate cause of failure from expected results because it has always been portrayed as a rare phenomena. An additional important point to consider herein is that samples that type as male via molecular methods, definitely contain Y-chromosome DNA (be it endogenous or exogenous). However, if a sample types as female there is always a possibility that the sample may be male, and compromised by allele drop-out or some other form of Y-chromosome inhibition not yet described. Schmidt et al (2003) have attempted to address this problem by implementing a X-chromosome STR system that will identify females accurately but only if they are heterozygous for the loci in question. However, the only way to completely resolve this problem will be to find a gene or loci that is unique to the female genome (Matheson and Loy 2001).

The Y-STRs were hypothesized to be an important component to this study. They unfortunately did not amplify any of the samples and this could be due to a number of factors. First, only Y-STR set 1 was sufficiently optimized during the time of this study.

Although it produced results for modern samples even at relatively high dilutions (1:1000 of modern extract), this quantity of DNA may still be too high for it to be

Figure 10: Two electropherograms from modern male samples depicting the phenomenon known as allelic drop-out. The first electropherogram (top) illustrates the allelic drop-out of the 112bp amelogenin peak and, if it were to have been run alone, this individual may have been identified as female. However, due to the concurrent amplification of the SRY loci (93bp peak) and the alphoid repeat loci (130/170bp peaks) the individual can be correctly identified as male. The second electropherogram (bottom) is another example of allelic drop-out. This time the SRY loci has not been amplified; however, we know the individual is male based on the results from both the amelogenin 106/112bp peaks and the alphoid repeat 130/170bp peaks.





compared to an ancient sample. Furthermore, exact quantification of aDNA has to date proved impossible due to the minute quantities of endogenous DNA and the insensitivity of the current quantification methods. Only in special instances has quantification using spectrophotometers and/or fluorometers identified DNA in ancient samples in this lab. The second Y-STR set was used in the hope that it may amplify some DNA from the samples; although, given its inconsistency with the modern samples the alternative hypothesis was more likely.

Finally, Y-chromosome work and its relation to STRs is still a relatively new field. Although the Y-STR analysis did not function properly, there are new systems currently being developed (Butler 2002) and, for future success, a pre-optimized system may be in order. By no means should further research be discouraged. At this stage however, I suggest that Y-STRs may have to be used as individual primer sets to yield aDNA, although continued research on developing more robust multiplex systems should be attempted.

In this regard the recent work on single nucleotide polymorphisms (SNP) is encouraging for both Y-chromosome and mitochondrial lineage work. As noted the molecular sexing techniques using Y-chromosomes have the additional potential for lineage delineation. Y-chromosome SNPs can provide the same information (Jobling 2001) but have smaller oligonucleotides and, identifying any SNP that is not homologous to the X chromosome would yield an assay that is male specific. This type of test may eventually lead to the replacement of autosomal STRs (Stoneking 2001) let alone Y-chromosme STRs; however, a lot of work is needed before this becomes reality.

In summary, the results obtained show that it is possible to obtain DNA from relatively old to ancient samples. Although it has been argued that survival of DNA is not correlated to the age of the sample (Colson *et al.* 1997a), the results from this thesis provide evidence that there may be a declining gradient for the robusticity of DNA as sample age increases. Better results were obtained for the historic samples with 100% typing at the amelogenin locus with approximately 67% being correctly identified at the alphoid repeat loci. This was not the case for the ancient Egyptian samples. The success rate for these samples drops dramatically to approximately 40% at the alphoid repeat loci. Relative to the amelogenin locus, only six results were obtained with a success rate of 50% which is still only what probability alone would dictate. This information may substantiate claims that as samples get older, the DNA within becomes more degraded, harder to extract, and more problematic to amplify.

The samples represent two distinctly separate periods, one approximately 100 years ago and one approximately 2000 years ago. Moreover, the samples represent two drastically different environmental regions. Further analysis of samples that bridge the gap between the two temporally isolated sample groups should be conducted. This type of research could potentially yield information as to when certain markers will no longer amplify. Another avenue for future work involves studying populations from similar environments and from the same time periods represented here. The analysis of this work could help determine the extent to which time and/or environment plays a role in the survival of DNA.

A corollary to this work is then to test the relationship between these studies (nuclear DNA) and those from mtDNA studies. If work is done on the same populations,

where there is the potential to find a correlation between the two types of DNA and when they start to lose their capacities to be amplified. It is worth restating that this is a significant problem because it may be that although we can amplify mtDNA, nuclear DNA from the same sample may no longer be present because of the lower copy number per cell when compared to mtDNA.

The excellent performance of the molecular sexing techniques on the more modern samples is encouraging from the standpoint of forensic DNA applications. Perpetrators of crimes often attempt to destroy skeletal evidence, which, often only results in fragmentary material (e.g. the attempts to destroy a skeleton by cremation). That such samples can yield verifiable sexing results and potentially individuation using y-chromosome markers is encouraging. Although this thesis did not involve subadult bone samples, future research using skeletal remains of recent victims of crimes in a blind design would definitely be a plausible research venue.

Finally, it is worth reemphasizing that this research was conducted using a blind design. The problems outlined for these molecular sexing techniques can be investigated thoroughly using a similar approach but with more specific hypotheses, such as, comparing the process of degradation and the relative performance of the alphoid repeat loci to the amelogenin loci. This would enable us to see how well each loci functions on degraded samples resulting in the application of the proper technique. Furthermore, we would be able elucidate the drawbacks to using one loci over the other (e.g. amelogenin mutations and mis-typing) and determine if the drawbacks out-way the benefits (e.g. amelogenin types correctly more often on relatively old samples). Hypotheses such as

these need to be tested in order for the best "tool" to be used under varying circumstances.

Conclusion

The fact that DNA can be amplified from archaeological bone has been demonstrated yet again in this thesis. Concerning the specific problem herein, namely the use of molecular techniques for sex assignment, the results are mixed but promising. I conclude that with some refinement, both historic-anthropological and forensic investigations will have a reliable 'stand alone' method for sexing fragmentary and/or subadult remains. However, any refinements will necessarily require testing against known data using a blind research design. I recommend, therefore, that the molecular methods be used in conjunction with other traditional methods until such time as the protocols have a level of precision and accuracy that will instill confidence among the academic and forensic users. This recommendation follows because of the inevitable fragmentation that happens to all DNA in post-depositional environments. That the very well-preserved, oldest samples in this study (Roman Period Egypt) were inconsistently sexed, according with the individuals 'known morphological sex' suggests, that the reliability of these methods is dependent on the archaeological (depositional) age. Future studies should continue to work with these older materials in a blind design as they will provide the ultimate testing ground of the method because the DNA in such samples is invariably less well preserved than in more recent samples. It follows that if refined, existing (e.g. Y-STR multiplexes) and new (e.g. SNPs on Y-chromosomes) protocols can be successful on very old bone then they will have increased precision on more modern materials. While this conclusion may contrast the existing literature in terms of

preservation, it is important to note that none of the current research has focused specifically on the sex determination problem using a blind research design. Without the latter, we do not know for certain the number of tests performed and the validation criteria will remain unconvincing. Therefore, a concluding opinion on the value of 'blind designs' in aDNA research is warranted. I should note that it was a humbling experience for me to reveal the results (Table 4) of this thesis to my core committee and fellow graduate students when I was the only one not knowing the actual sex of the subject burials. As the results were unfolded in sequence, anxiety was paramount. Burial 1 - molecular consensus male ...skeletal sex female, Burial 2 - molecular consensus female ...skeletal sex female, Burial 3 - molecular consensus female ....skeletal sex male. My initial reaction was disbelief; however, as the unfolding of the results continued and the success to failure rate increased, I realized that it was the methods on trial not me. The experience clearly demonstrated that the problems of interperting archaeological DNA samples are unique and a blind design is the only way to examine this issue.

Moreover, to find that my core hypothesis stating that the alphoid repeat method should outperform the others was rejected was again unexpected. Had a blind design not been invoked, the test differences found might have lead unknowingly to preferential treatment (e.g. extra extractions etc.) for individual samples. In many respects, the non-corresponding results of this thesis are in fact more beneficial to the science of sex determination because it will lead to improved protocol development. We know the aDNA is in fact present, we just need to improve on the methods of pulling specific and intact DNA out of its matrix. It is our future goal to improve the quality and product of

the amplified DNA through continued testing of hypotheses that are generated from good observations.

As noted, key problems include research on contamination checks, temporal studies, environmental factors, and DNA type comparisons. Although aDNA researchers pride themselves on their research record in these areas relative to other types of DNA testing facilities, it is clear that aDNA laboratories have to improve their intra- and inter-laboratory validation standards (Richards and Sykes 1995) that, which at present, should be the mainstay of forensic DNA laboratories. Also, since aDNA research is such a young science, publications and research reports should avoid broad categorical conclusions and assumptions, which could impede the science's progress. Two examples directly relating to this thesis are noteworthy; (1) that post-depositional age is not a determining factor in aDNA preservation and (2) that aDNA is not present in Egyptian bones older than 800 years B.P. The latter stems for a research article recently published in the prestigious American Journal of Physical Anthropology (Marota *et al.* 2002). Rather than making such a broad sweeping conclusion that contradict a recently published article on samples from pre-Roman (circa 800 B.C.) and Roman period Dakhleh remains (Graver *et al.* 2001), they should have concluded that "...with the protocols used in our research we were unable to amplify aDNA from Egyptian bones greater than 800 years B.P. Our work contrasts that reported by Graver et al (2001) for the recovery of aDNA from Roman and Pre-Roman Period Bones from the Dakhleh Oasis, Egypt. Future collaborative research between our laboratories, designed to test and refine aDNA protocols would seem to be in order." From the perspective of this thesis we know that it is possible to recover aDNA from old and new archaeological bone

for 'sexing' purposes. Moreover, I envision a time when it will be possible to study large populations from remote time periods and reliably sex both subadult and fragmentary remains using molecular techniques. As noted these advancements will also aid in validation for forensic cases.

Appendix A

This appendix provides detailed descriptions of the burials used in this thesis. As noted in the text, burials A to M are from the Roman Period Cemetery at Kellis 2; Dakhleh, Egypt; burials N-Q are historic period (1860-1890) remains from the Stirrup Court Cemetery in London, Ontario, Canada; Burial R is from Thunder Bay, Ontario Canada and dates to 1870-1884; and burial S is from Toronto Ontario and likely dates to the 1940s.

Sample A:

Burial 6 is a male in his late 20's (symphysis pubis score of 28.7± 6.5 years). The dental pathology agrees with this mean age estimate. Bone condition is excellent. His anterior nasal spine, and palate show signs of erosion with loss of the alveolar bone, the incisors, left canine and premolar. The palatal erosion primarily involves the maxillae and in the dried bone has produced a small lesion on the left side that is patent with the nasal septum. Considerable porous hypervascular reactive periostitis is present on the metacarpals and metatarsals. The distal surfaces of the proximal phalanges show distinct transverse grooves indicating the claw hand deformity. The tibia also show hypervascular periosteal reactions. These skeletal changes suggest this individual suffered from lepromatous leprosy. On the lingual surface of the anterior mandibular teeth there is a band of thick tartar that likely developed due to a chronic lack of abrasion from the absence of an anterior maxillary teeth. Additional osteopathology is represented by slight erosive pitting of the articular facets of T6, T7 and T9. A sternal foramen, an

unfused acromial epiphysis, and a large bregmatic bone are key nonmetric traits present. He is the only person with the latter trait in this population sample.

Sample B:

Burial 116 is the nearly complete skeleton of a male with a symphysis pubis age estimate of 23.4 $\pm$ 3.6 years. The pattern of dental pathology agrees with the mean age estimate. This individual has osteological changes that are consistent with a diagnosis of leprosy. The nasal spine is atrophied and while the palate does not show extensive erosion, the adjacent area to the incisive foramen is hypervascular and pitted which is generally considered as an early manifestation of infection. In addition, the right inferior nasal concha bone has reactive changes with sequel atrophication. In the long bones there is slight 'active' periosteal new bone on the distal right ulna and radius, and the left distal tibia and fibula. The latter bone is particularly involved as diffuse and striated periostitis occurs throughout the diaphysis. There is also a faint trace of periostitis on the right distal anterior tibia. By far the most dramatic changes occur on the feet with the left calcaneus virtually unrecognizable do to the infection, as are several of the phalanges which show the classic needle point configuration. While there is no other evidence of osteopathology, the femora show an unusual torsion that likely reflects difficulties in gait as a sequel to the infection in the feet. Several important nonmetric traits are present including bilateral pterygo-alar bridges, agenesis of the right anterior calcaneal facet, a bipartite left superior facet of the atlas and a right lateral foramen of the pterygoid plate.

Sample C:

Burial 189 is the complete skeleton of a female with a symphysis pubis age estimate of 38.2 ± 10.9 years. The advanced dental pathology (6 teeth lost antemortem), the presence of degenerative change in many joints particularly the patellae, left hand bones and vertebral column (particularly the C1-C2 articulation) , plus widespread but slight osteoporosis (no vertebral collapse) suggest an age estimate at least in the upper limit of the symphysis pubis range (i.e. approximately 50 years old). The only other macroscopic pathology present is several small button osteomas on the left frontal bone. Nonmetric variants present include, a left open foramen spinosum, a right infraorbital suture, a small left dehiscence of the tympanic plate. A key anomaly present is the frontal temporal articulation on the right side.

Sample D:

Burial 213 is the incomplete skeleton (skull and atlas missing) of a male with a symphysis pubis age estimate of 45.6 ± 10.4 and 61.2 ± 12.2 years. Without dental observations it is difficult to estimate his age, although the presence of moderate degenerative rib and spinal changes (osteoarthritis in lower cervical and thoracic), advanced bilateral chondromalacia patella, and the presence of ossified costal cartilages of the first and seventh ribs suggest that the mean of the two symphyses estimate is a reasonable estimate (i.e. an age of 54 years). Of note is the fact that both first metatarsal-phalangeal joints have exuberant new bone formation that is not fully ossified to the adjoining cortical and synovial bone areas. The bony buildup may represent gout or possibly psoriatic arthritis. The fact that it is limited to the first metatarsals is more indicative of the former, although SEM analysis did not confirm that the bony products

contained sodium urate crystals, which is pathognomonic of gout. Also both hands are very arthritic, with an erosive lesion occurring on the right MC IV. Plaque-like periostitis occurs on the anterior diaphyses of the femora while huge exostoses (spurs) occur on the popliteal lines. This individual is the tallest burial yet found in the Oasis population attaining a stature of 6'2" $\pm$ 1.4 inches. Future testing of his DNA will be used to test if this individual was in fact an import to the region. The only nonmetric trait of note was the bilateral presence of septal apertures.

Sample E:

Burial 261 is the complete skeleton of an osteoporotic female with a symphysis pubis age estimate of 60.0 $\pm$ 12.4 years. The extensive toothloss (22 teeth lost antemortem), the osteoporotic related fractures of the right hip (long healed femoral neck) and T12-L1, and the advanced degenerative changes in the synovial joints and spine, support at least the mean value, and likely suggest an age in the upper limits of the symphysis pubis range (70 $\pm$ 5 years). Nonmetric traits present include: L. Os Japonicum, a left divided hypoglossal canal, bilateral fronto-temporal articulations, a left 3rd trochanter and a right incomplete foramen transversarium of the axis.

Sample F:

Burial 265 is the complete skeleton of a robust adult male with symphysis pubis age estimate of 45.6 $\pm$ 10.4 years. The dental pathology agrees with this age estimate, although the presence of extensive osteophytosis with ankylosis (without compression fractures) occurring in two regions (T8-T9 and L2-L5) suggest this individual is in the upper range of the symphysis pubis estimate. The osteophytosis is likely a concomitant of longstanding spondylitis deformans, although DISH is a good differential diagnosis

candidate. The best estimate of age is 55 $\pm$ 5 years. Apart from some very slight AO in his left MC I, the only evidence of osteopathology is an infectious process in the ankylosed vertebrae and the anterior surface of S1 and S2. There is macroscopic and or radiologic evidence of lytic destruction of all the centra (T8,T9, L2-L5 and S1 and S2) with and limited or no evidence of host response. This is most clearly seen in L2 in which a large cloaca drains from the body. Several infectious diseases could be involved with TB being the most likely candidate. Noteworthy is that the ribs in this person are normal. This individual also has a long healed fracture of the left nasal bone, likely a result of fisticuffs.

Sample G:

Burial 269 is the complete very osteoporotic skeleton of a female with a symphysis pubis age estimate of 48.1 $\pm$ 14.6 years. All skeletal and dental indicators of age support this mean estimate, although the severity of the osteoporosis with concomitant fractures of the femoral neck (unhealed) suggest a person at least in her mid-fifties. There is soft tissue imbedded in between the femoral head and neck, which indicates that this fracture was sustained shortly before the person's death. In addition there is a long healed fracture of the right hip ischium and sacrum and the left ulna. The latter fracture is located between the midshaft and distal end, which presents a problem in diagnosis (malintent versus accidental). It is possible that the healed fracture of the sacrum/hip and ulna represent an earlier fall in this person's life. The only other evidence of osteopathology is chondromalacia in both patellae. Key nonmetric metric traits present include: a left fronto-temporal articulation, a left intermediate condylar canal, a notochord remnant (medium development) and bilateral carotico-clinoid bridges.

Sample H:

Burial 280 is the complete skeleton of a female estimated to be 65 ± 5 years of age. This estimate is based on the Loth Iscan rib age (7 score or 59-71 years) and dental pathology, which resulted in at least 22 teeth being lost antemortem. The bones are also moderately osteoporotic which is consistent with an older individual. Well healed Colles fractures occur bilaterally. Whether they are a concomitant of the osteoporosis is impossible to determine. The symphysis pubis score (V or 48 ± 14.6 years) underestimates the age. Osteolytic lesions occur on the cranial vault (singular lesion on mid-frontal), C1 (r. interarticular region) and T1-T2. There is very limited osteoblastic response. Tuberculosis is most likely disease involved. Nonmetric variants present present include: a right trochlear spur, an incomplete bipartition of the left hypoglossal canal, a right divided jugular canal, a large pharyngeal fossa and a trace of the notochord remnant.

Sample I:

Burial 322 is the complete skeleton of a female with respective symphysis pubis and rib age estimates of 48.1 ± 14.6 and 43-58 years. The bones are very demineralized and several of the vertebrae and key joints have slight degenerative changes. The extensive antemortem toothloss (28 teeth including an edentulous maxilla) suggests an advanced age which is estimated at 60 ± 5 years. There is considerable trauma present including healed left clavicle, a right femoral neck fracture, a healed fracture of right ischium, a healed fracture of the right MT II, healed fractures of the 5^{th} to 8^{th} ribs and a compression fracture of the L2. Slight periosteal newbone is present on the medial surfaces of the tibia. Key nonmetric traits present include; a metopic suture, bilateral slight (+) tympanic dehiscences, a right divided hypoglossal canal, absent posterior condylar canals, and a

right fronto-temporal articulation, a moderately developed pharyngeal fossa, and bilateral accessory ossicles in the lambdoidal suture, at asterion and in the parietal notch.

Sample J:

Burial 377 is the complete skeleton of a female with respective symphysis pubis and rib age estimates of 48.1 $\pm$ 14.6 and 59-71 years. There is considerable antemortem tooth loss and advanced attrition and the bones are only slightly osteoporotic. Age is estimate at 55 $\pm$ 5 years. Osteolytic lesions occur in the centra of C5 and C6, and L5 and S1-S2. In the cervical vertebrae, osseous destruction of the centra has led to the collapse of both vertebrae anteriorly with early evidence of ankylosing. The left articular facets have ankylosed possibly due to a previous trauma. Osteoblastic response is limited to the anterior surface of the atlas suoeruior to the osteolytic focus. In the lumbar sacral region the anterior centrum of L5 shows numerous focal points of infection with several draining cloaca but the vertebrae has not collapsed. In S1, there are three major osteolytic foci of infection; the external surface of anterior centrum immediately inferior to the sacral promontory and large circular osteolytic defects on the ala immediately adjacent to the lateral margins of the centrum. The left anterior surface of S2 presents of large cloaca. The only evidence of osteoblastic reactive bone formation is in the perifocal area surrounding the lesion on the anterior S1 body. Osteophytosis occurs between the L5-S1 bodies particularly on the left side where it has ankylosed. Given the age of the skeleton this process is likely separate from the infection. Key nonmetric traits present include; bilateral open foramen spinosum, a right infraorbital suture, a left divided hypoglossal canal, bilateral absence of the posterior condylar canal, a left intermediate condylar canal and an incipiently developed pharyngeal fossa.

Sample K:

Burial 392 is partially disturbed (head, neck and thorax to T10) remains of a male estimated to be 40 ± 5 years of age on the basis dental evidence (dental attrition 35-40; 7 teeth lost antemortem) symphysis pubis (45.6 ± 10.4) and rib (43-55). Evidence of infection is present in rhinomaxillary region, the hands, and the tibia and fibulae. In the skull, alveolar resorption and pitting is present at prosthion, which extends to the incisive foramen. The anterior nasal spine has undergone significant resorption as has the margins piriform aperature, particularly on the right side. In the nasal septum, the concha bones have evidence of osteolysis and osteoblastic response, while nasal floor and the right frontal process of maxilla also show evidence of reactive changes. The palate is normal. In both hands extensive destruction of the distal (achroosteolsysis) and middle phalanges has occurred. Unfortunately, only two distal phalanges (one showing a beret-shaped appearance) were present; they may have been missed during excavation or possibly were completely destroyed from achroosteolysis. The latter is a likely scenario given that all the middle phalanges were lysed and/resorbed virtually to their bases. Even the distal three quarters of the proximal phalanx of the right thumb is destroyed. The left shows some reactive perisoteal changes particularly on its dorsal surface. The proximal phalanges showing varying degrees of periosteal reactive changes which are most pronounced on the margins of the volar surfaces of both 4th digits and the second digit on the right hand. The latter proximal phalanx has considerable bone thickening and periosteal newbone on the volar surface margins and the whole dorsal surface. Of note is the presence of extensive pitting with groove formation on the distal volar surface of the right 3rd digit. The metacarpophalangeal joints and the carpal bones appear normal.

Overall a 'mitten hand appearance' would have characterized this individual accompanied by slight claw-hand deformity on the right hand. With the amount of destruction to the hand it is unexpected to limited changes (sight periostitis) to the distal ulnae and radii. However, the opposite occurs in the lower limb where the feet appear normal except for some osteopenia, but both the tibia and fibulae have extensive and irregular perisoteal changes with bone thickening. Unfortunately, radiographs were not possible so it is unknown if these changes also involved the medullary cavity.

Sample L:

Burial 410 is the partially disturbed but almost complete and well preserved skeleton of a female with estimated skeletal-dental age of 27± 3 years. Attrition is slight, one tooth has been lost antemortem, all the epiphyses are fused, S1-S2 is open, and the rib (24-32) and symphysis pubis (30.7 ± 8) age estimates overlap. The only evidence of osteopathology is active and diffuse periosteal new bone on the diaphyses of the tibia and fibulae, and the mid to superior anterior diaphysis of the left femur. The bones are well mineralized. Several nonmetric traits are present including; bilateral fronto-temporal articulations, a right paracondylar process, a left partially divided (+ spur) of the hypoglossal canal, a left intermediate condylar canal, and a moderately developed pharyngeal fossa.

Sample M:

Burial 437 is the complete remains (undisturbed burial) of a male with respective symphysis pubis and rib age estimates of 35.2 ± 9.4 and 37 ± 5 years. The pattern of dental pathology and attrition and antemortem toothloss suggest an individual at least 40 years of age or within the upper range of the symphysis pubis estimate. The presence of chondromalacia patella, calcification of the thyroid cartilage and lack of osteoporosis also

agree with dental age estimate. Collectively, an age of 45 ± 5 years is suggested. There is slight degenerative joint disease in both acetabuli and the lumbar vertebrae (osteophytosis L2-L5) and long healed fractures occur on the right clavicle and right 3rd rib which could have been from the same event. This individual has pathognomonic evidence of lepromatous leprosy. In the skull the palate is extremely thinned and has several perforations patent with the floor of the nasal cavity. In the nasal cavity inflammatory changes involve all the concha bones and the vomer though the floor of the nasal cavity is not involved. Resorptive changes are present in the borders of the piriform aperture though the anterior nasal spine is intact. The anterior alveolus has resorptive changes and some antemortem toothloss (all incisors) that could be concomitants of the infective process.

Samples N, O, P, and Q:

Burials 6, 7, 10, and 18 are the nearly complete skeletons of a male, female, male, and female respectively. Their age estimations were bases on four skeletal age methodologies where possible. These included the pubis symphysis method, the sternal rib method, the auricular surface method, and the cranial suture closure method. The best age estimates for burials 6, 7, 10, and 18 are: 61 years, 81 years, 45 years, and 84 years respectively. The skeletons were nearly complete with over 90% of the skeletal elements being collected and accounted for. All individuals were found intact in their respective coffins with relatively little damage to the remains. Cortical bone was found in varying states of preservation (from no damage to none present) with root damage being the major cause of destruction, leaving marks of vermification on the surface. All individuals were also identified with names. Burial 6 was identified as Sampson Green,

while burial 7 was identified as Sarah Lawrence. The other two burials, 10 and 18, were identified as William Hussey and Helen Robotham, respectively (Parish 2000).

Sample R:

In 1884, River street cemetery was closed and many coffins and caskets were moved to St. Andrew's R.C. Cemetery on Oliver Road. In 1979 a backhoe operator was excavating where the cemetery once was for a new building when a casket was unearthed. The ornate casket was found on the edge of the cemetery, which is said to be the infectious disease section.

The police and coroner were then notified. Police were instructed to recover every bit of evidence at the site. Only a quarter of the casket was found along with an incomplete skeleton. Skeletal analysis of River Street skeleton concluded that the individual was of medium build and was between 5 feet 5 inches and 5 feet 10 inches. Some textiles were recovered such as, frilly shirt with intricate needlework and padded shoulders. A heavy, black, felt material was also found over the shirt with only the collar area intact. The casket was constructed from rolled zinc with cast iron top-frame and plate glass atop. The handles were nickel plated and covered with a unique design. The casket itself must then have been inserted into a pine box with charcoal placed around it (Brown 2003).

Sample S:

In mid May 1995, human remains were found during the demolition of an auto dealership on Danforth Avenue in Toronto, Ontario (Melbye *et al.* 1999). The remains were found approximately 1 metre beneath the cement showroom floor. A human skull and torso remained in situ and infracranial bones were scattered throughout the immediate area.

No soft tissue was present. Artifacts associated with the body, included fragments of clothing, a pair of women's shoes, and a vulcanite dental plate.

Forensic analysis included; odontological and skeletal analyses. The former was conducted by Dr. R. Woods, revealing that the vulcanite denture present in the maxilla was popular in the Toronto area during the 1940's and 1950's and thus the burial must have occurred within that time period. The latter was performed by Dr. Jerry Melbye (consultant to the Office of the Chief Coroner) and included determination of sex, age and race respectively. Morphological traits of the hip bones (e.g. ventral arc, scars of parturition) and cranium (e.g. brow ridge), indicated that the remains were that of a female individual. Age at death was determined using several methods including pubic symphysis analysis (Brooks and Suchey 1990), resulting in an estimate of 28-40 years of age at the time of death. Race, although difficult to assess in skeletal remains, was suggested to be Caucasian. This was based on various traits; such as, dentition (carabelli's cusp), hair colour (red) and the FORDISC analysis of (Ousley and Jantz 1996). Moreover, FORDISC 2.0 also provided an estimation of stature between 5'2.5''-5'8''. Forensic analysis also indicated the presence of seven perimortem traumas in the cranium. The injuries consisted of a series of wounds which all shared similar characteristics (radiating out; beveling in), consistent with blunt force (Murray 2003).

Appendix B

This appendix provides graphical representations of the amplified DNA for the samples that yielded results. The data herein is presented in electropherogram format or raw data for the respective samples and their amplified loci.

Electropherograms for each sample identified by the SRY marker:

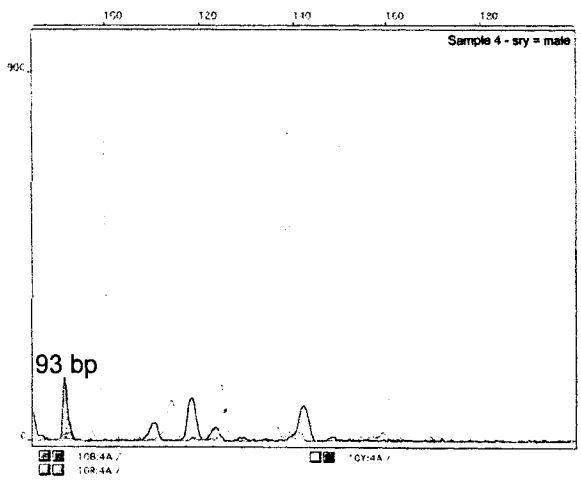


Figure B1: Sample Q – SRY = male

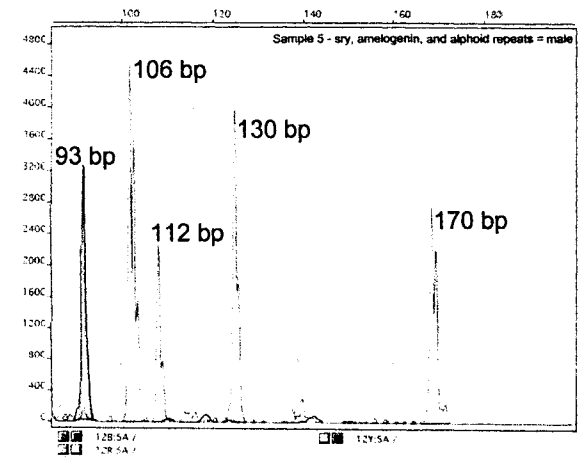


Figure B2: Sample R – SRY = male

Electrophergrams for each sample identified by the amelogenin markers:

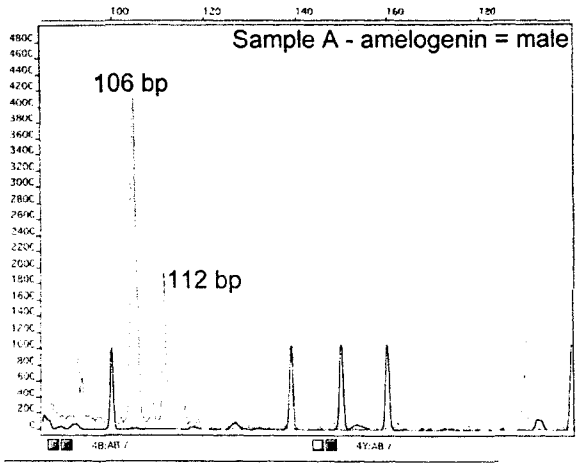


Figure B3: Sample A - amelogenin = male

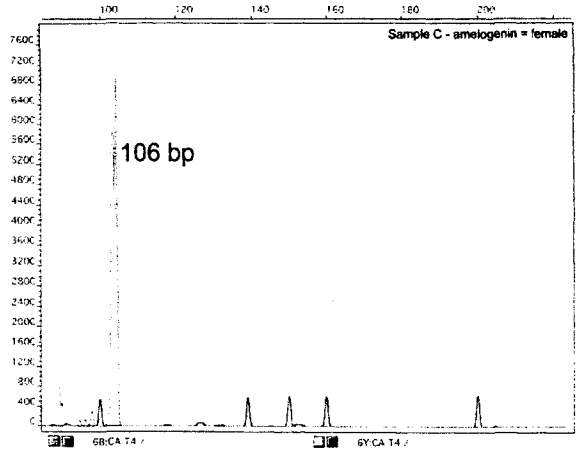


Figure B4: Sample C - amelogenin = female

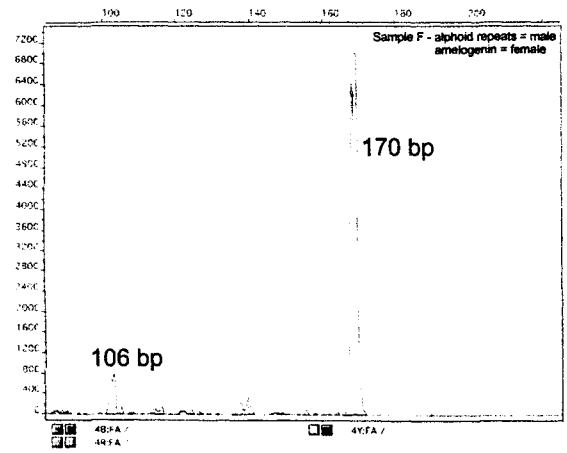


Figure B5: Sample F – amelogenin = female

Electrophergrams for each sample identified by the amelogenin markers: (continued)

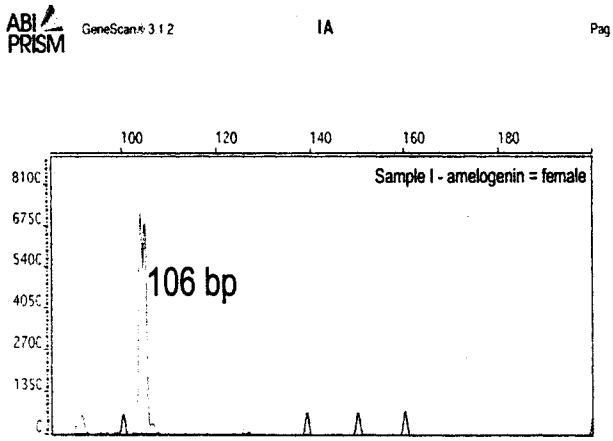


Figure B6: Sample I – amelogenin = female

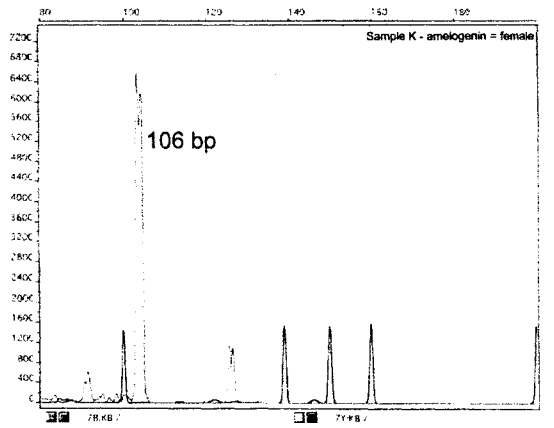


Figure B7: Sample K – amelogenin = female

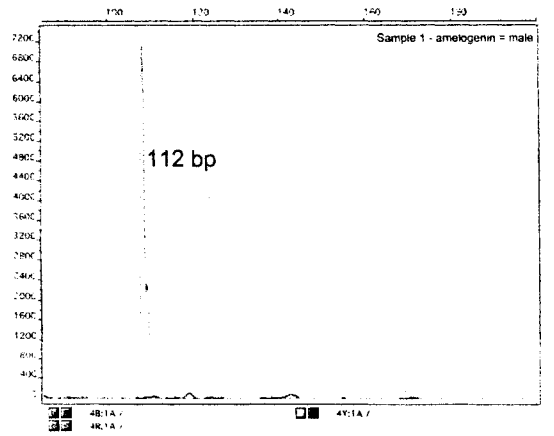


Figure B8: Sample N – amelogenin = male

Electrophergrams for each sample identified by the amelogenin markers: (continued)

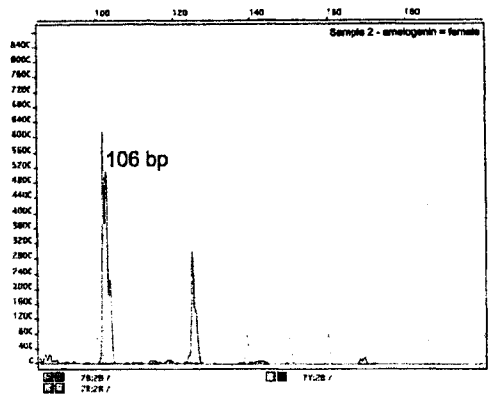


Figure B9: Sample O – amelogenin = female

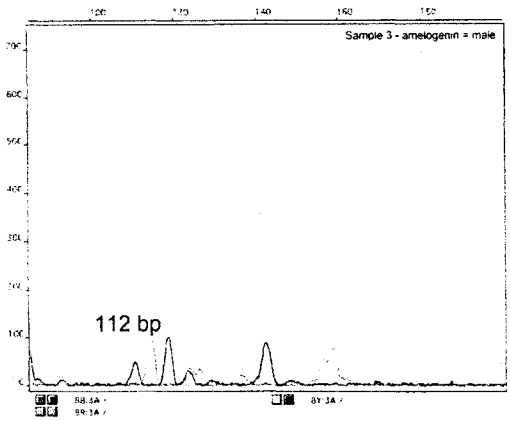


Figure B10: Sample P – amelogenin = male

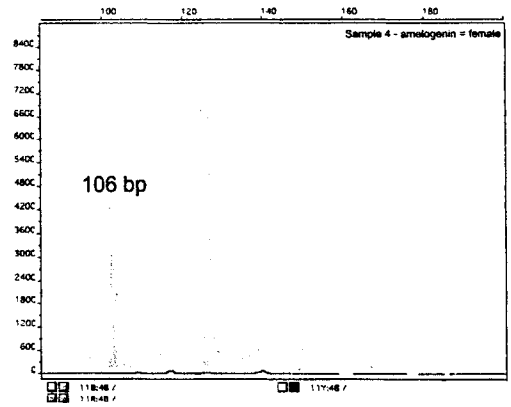


Figure B11: Sample Q – amelogenin = female

Electrophergrams for each sample identified by the amelogenin markers: (continued)

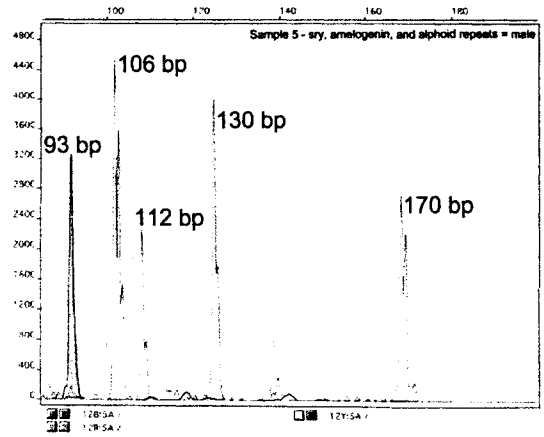


Figure B12: Sample R – amelogenin = male

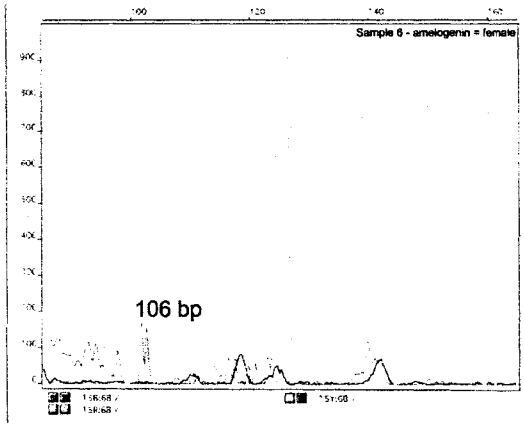


Figure B13: Sample S – amelogenin = female

Electrophergrams for each sample identified by the alphoid repeat markers:

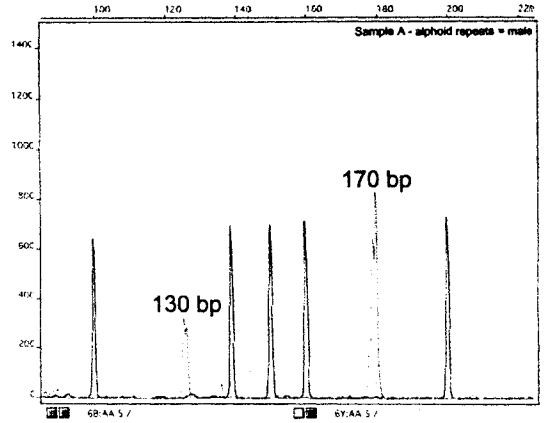


Figure B14: Sampel A – alphoid repeats = male

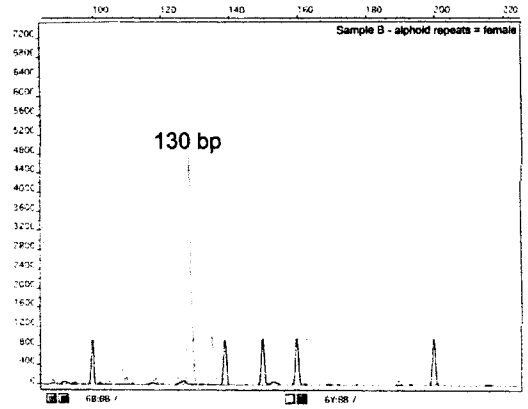


Figure B15: Sample B – alphoid repeats = female

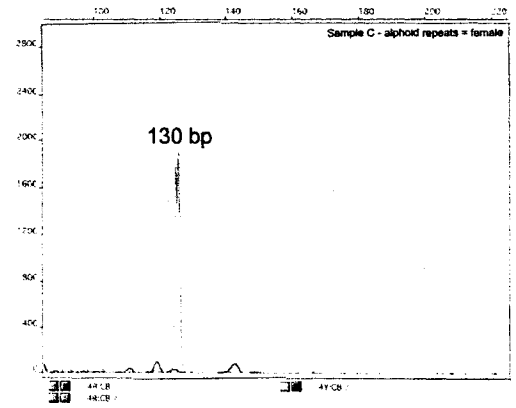


Figure B16: Sample C – alphoid repeats = female

Electrophergrams for each sample identified by the alphoid repeat markers: (continued)

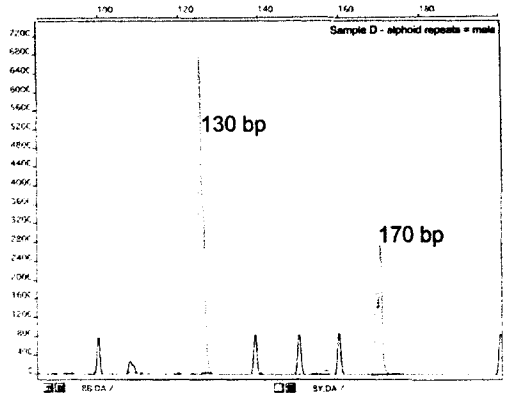


Figure B17: Sample D – alphoid repeats = male

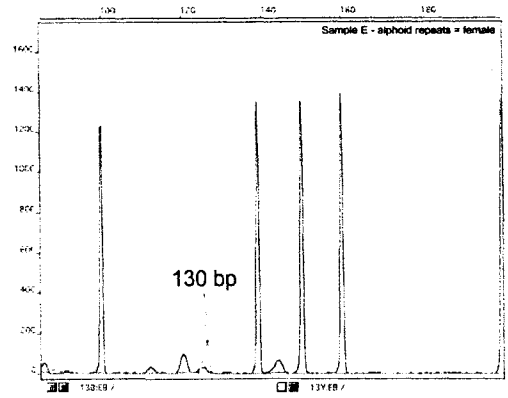


Figure B18: Sample E – alphoid repeats = female

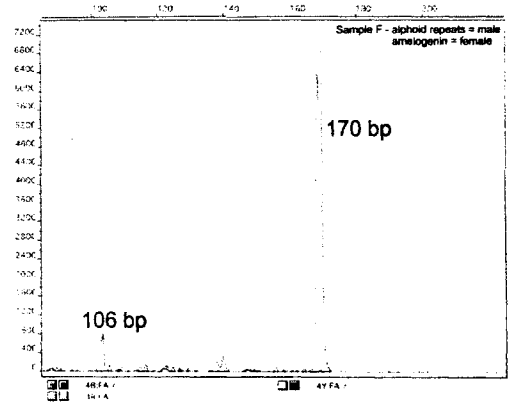


Figure B19: Sample F – alphoid repeats = male

Electrophergrams for each sample identified by the alphoid repeat markers: (continued)

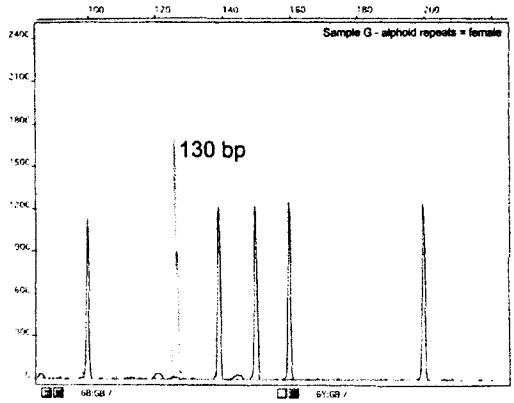


Figure B20: Sample G – alphoid repeats = female

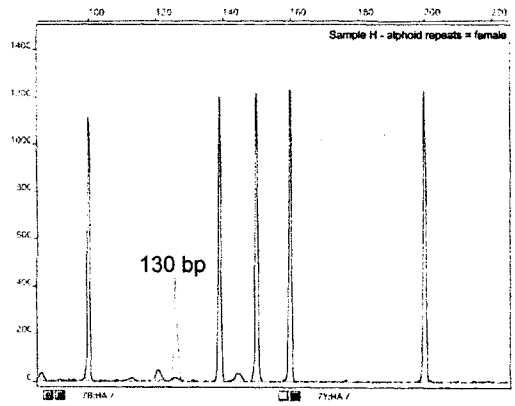


Figure B21: Sample H – alphoid repeats = female

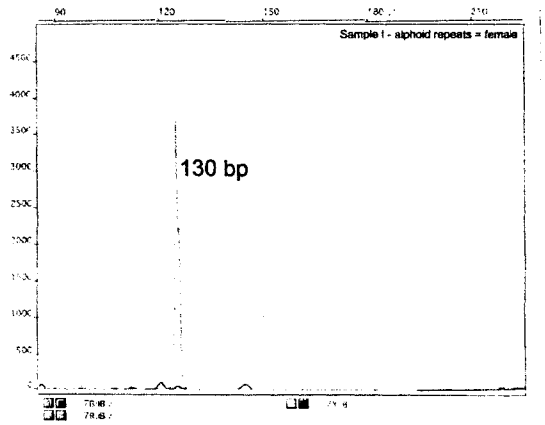


Figure B22: Sample I – alphoid repeats = female

Electrophergrams for each sample identified by the alphoid repeat markers: (continued)

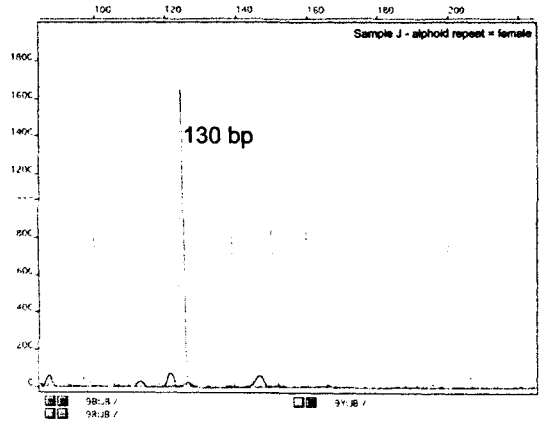


Figure B23: Sample J – alphoid repeats = female

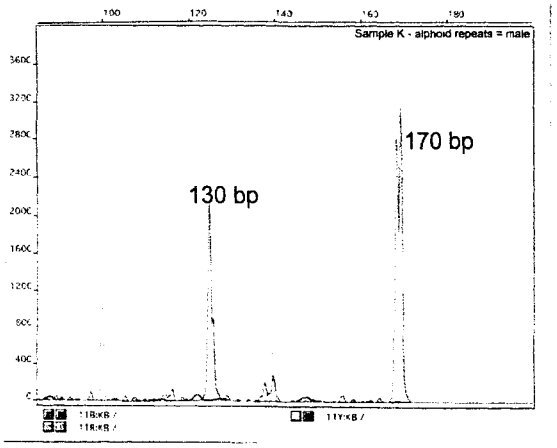


Figure B24: Sample K – alphoid repeats = male

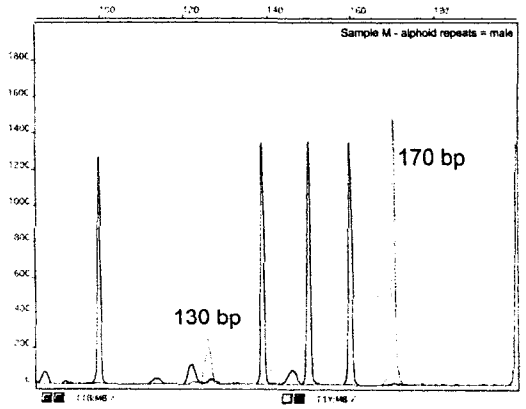


Figure B25: Sample M – alphoid repeats = male

Electropherograms for each sample identified by the alphoid repeat markers: (continued)

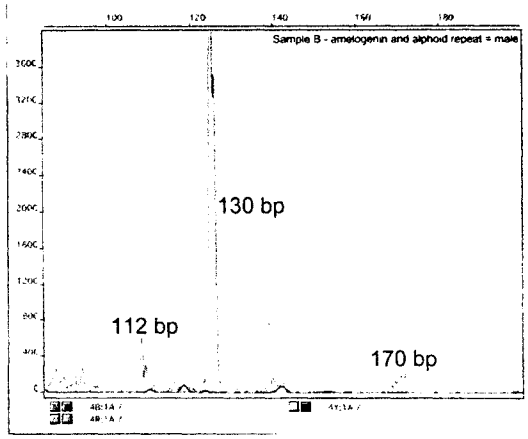


Figure B26: Sample N – alphoid repeats = male

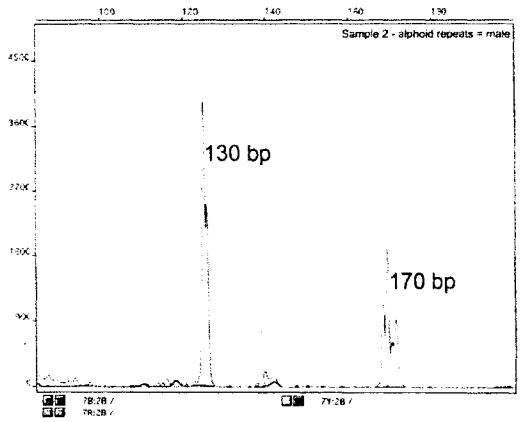


Figure B27: Sample O – alphoid repeats = male

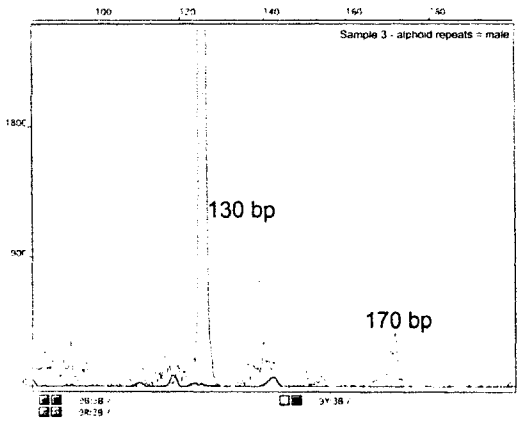


Figure B28: Sample P – alphoid repeats = male

Electrophergrams for each sample identified by the alphoid repeat markers: (continued)

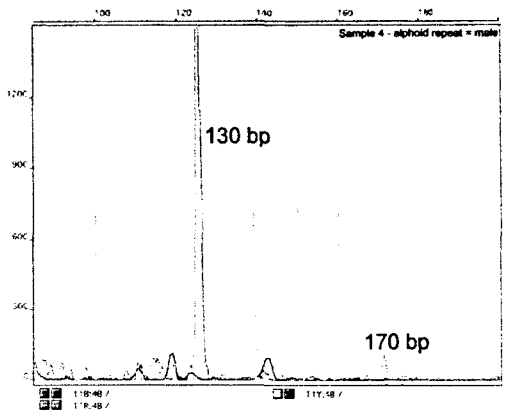


Figure B29: Sample Q – alphoid repeats = male

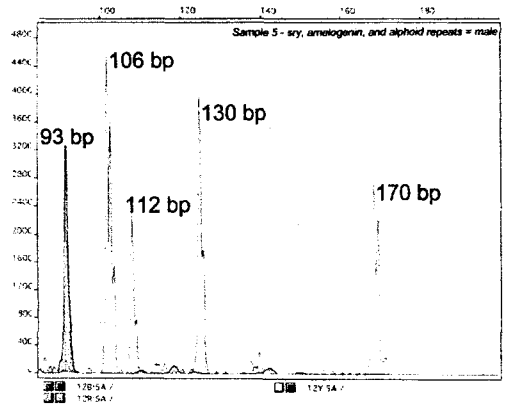


Figure B30: Sample R – alphoid repeats = male

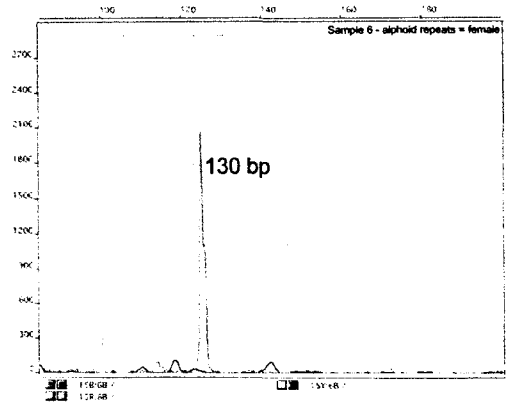


Figure B31: Sample S – alphoid repeats = female

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