SEX ESTIMATION OF UNIDENTIFIED HUMAN REMAINS: CONCORDANCE BETWEEN MORPHOLOGICAL ANTHROPOLOGICAL ASSESSMENT AND DNA ANALYSIS

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Declaration

I, **Céleste Esther Leggett**, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the university to reproduce for the purpose of research either the whole or any portion of the content.

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Abstract

Identifying skeletonised human remains is a challenge worldwide, and sex determination is an important part of the process. Recently in Cape Town, there have been two medico-legal death investigations involving unidentified skeletonised remains who were estimated to be female anthropologically but were biologically sexed male based on DNA analyses. This study aimed to assess the agreement of sexing methods in a Western Cape South African forensic sample of skeletonised individuals (n=126), who were morphologically estimated to have biological sex as female (n=41). Of the 41 anthropologically estimated to be female cases, 19 were excluded for being probable or likely 'archaeological'. DNA was extracted from hard tissue samples from the remaining 22 individuals and biological sex was assessed by quantitative real-time PCR. DNA from thirteen cases (13/22; 59%) were amplified, with six showing evidence of Y-chromosomal DNA and inferred male sex. However, since the DNA concentrations were below the validated dynamic range, these results were suggestive only. DNA profiling confirmed that one case was male but did not provide further clarity on the biological sex of the remainder of cases due to low copy number (LCN) DNA. This study presents another confirmed case report of sex discordance between anthropological and DNA analysis for a sample of decedents from the Western Cape, South Africa. While qPCR suggested 54% (7/13) agreement and 46% (6/13) disagreement, confirmation in most cases was hindered by LCN DNA. The Western Cape population, influenced by San and Khoe ancestry, tends to have smaller body size and gracility, which may impact the accuracy of morphology-based assessments of male robusticity as they may appear more female or ambiguous. These results highlight the need to update anthropological data for the diverse South African population and implement improved molecular techniques for reliable DNA profiling. This study underscores the limitations of both anthropological and molecular sex methods and stresses the importance of interdisciplinary collaboration for accurate forensic identification.

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I am dedicating this thesis to my beloved departed father, Frank Leggett. I hope I have made you proud.

"The bones of the fallen should be treated with respect and accorded the dignity that was their proper due. If only the living were also granted such grace, what a wonder our world could become." ― Daniel Thorman, Mayhem at the Mill

Table of Contents

List of Figures

List of Tables

Abbreviations

Abbreviation	Meaning
CODIS	Combined DNA Index System
DHA	Department of Home Affairs
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACT	Forensic Anthropology Cape Town
FBI	Federal Bureau of Investigation
FPS	Forensic Pathology Service
FSL	Forensic Science Laboratory
g	Gram
Hz	Hertz
LDF	Linear Discriminant Functions
LCN	Low copy number
LOD	Limit of detection
MBG	Molecular biology grade water
mg	milligram
ml	Millilitre
mm	Millimetre
NGS	Next-Generation Sequencing (NGS)
PMI	Post-mortem interval

PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
QS	Quality sensor
rpm	Revolutions per minute
SAPS	South African Police Service
SDS	Sodium dodecyl sulphate
STR	Short tandem repeats
SWGANTH	Scientific Working Group for Forensic Anthropology
SWGDAM	Scientific Working Group on DNA Analysis Methods
USA	United States of America
VIC	Victim Identification Centre
ViSUN	Victim Identification @ Stellenbosch University

Chapter 1: Introduction and literature review

1.1 Introduction

The identification of human remains is crucial and has wide-ranging implications, involving social, ethical, legal, and financial factors. (Naidoo, 2007; Krysztofiak, 2017; Mazzarelli *et al.*, 2021; Reid *et al.*, 2023). Determining the biological sex of the individual is a vital step in forensic identification and can assist in reducing potential matches in missing-person searches and differentiating commingled skeletal remains (Saukko & Knight, 2015a; Rowbotham, 2016; Mokoena *et al.*, 2019; Tawha *et al.*, 2020). However, identifying the biological sex of skeletonized remains presents challenges for forensic scientists (Bruzek & Murail, 2006; Bidmos *et al.*, 2010). Anthropological and genetics-based methods are commonly used to assess the sex of such medico-legal death investigations, with anthropological methods often preferred for their non-invasiveness, cost-effectiveness, and time efficiency in local context (Gibbon *et al.*, 2009; Bidmos *et al.*, 2010; Krishan *et al.*, 2016; Gibbon, 2020).

Recent literature has presented contradictory findings regarding the results obtained from anthropological and molecular analyses of skeletal individuals, with the latter considered the most reliable method of sex identification (Gibbon *et al.*, 2009; Ubelaker, 2018; Adel *et al.*, 2019). Case reports of contradictory sex results in two medicolegal death investigations in Cape Town, South Africa, will be discussed later in this chapter. However, a study investigating the discordance on a larger sample size has never been done for a South African sample. Such a study is important as it would provide insight regarding the reliability, standardisation and challenges of current sex estimation methods, which may need to be tailored according to the population or medico-legal context (DeSilva *et al.*, 2014; Peckmann, Logar & Meek, 2016; Colman *et al.*, 2018; Manthey *et al.*, 2018).

The literature review that follows describes the global and local burden of unidentified bodies, an overview of the identification processes followed in South Africa, its role players, and the legislation governing them. Sex estimation of skeletonised remains is then discussed, particularly biological anthropological sex estimation methods and the impact of sex misclassification and possible factors mitigating these discrepancies in medicolegal death investigations in the Western Cape. This will be followed by molecular sex determination using short tandem repeat (STR) typing, the use of the Amelogenin sex marker and other secondary sex-determining markers, and limitations of molecular testing. Finally, this study's rationale,

research aims, and objectives will be provided. In the subsequent sections of this thesis, the term "sex" pertains to an individual's biologically assigned sex, ascertained through morphometric or molecular techniques.

1.2 The burden of unidentified bodies

Unidentified bodies are a global issue, but rates differ between countries (Mazzarelli *et al.*, 2021). In South Africa, there have been three reports regarding the numbers and proportions of unidentified bodies. Approximately 9% of bodies were unidentified each year at Salt River Mortuary in Cape Town from 2010-2017 (Reid *et al.*, 2020; Reid *et al.*, 2023). Similarly, in Gauteng, the Pretoria Medico-Legal Laboratory reported that approximately 10% remain unidentified, with 4000 bodies remained unidentified in Gauteng from 2012-2015 (Evert, 2012; Mabotja, 2015). At the Salt River Mortuary, 14.1% of unidentified bodies had signs of decomposition, skeletonisation or other physical restrictions inhibiting visual sex determination. These rates are comparable to other developing countries such as India, which reported 16% of their caseload being unidentified and about 1.7% of skeletonised bodies yearly from 2010-2014 (Yadav *et al.*, 2017). On the other hand, developed countries report much lower rates of unidentified human remains, as reviewed by Mazzarelli *et al.*, (2021). For example, in Fulton County, USA, 0.2% of cases remained unidentified after a one-year investigation (Hanzlick & Smith, 2006). A study by Reid *et al.*, (2023) found that developing countries, on average, had more than twice the proportion (9.56%) of unidentified bodies compared to developed nations (4.40%). The disproportion of unidentified human bodies between developed and developing countries could be attributed to population densities, socioeconomic conditions and mortality rates (Yadav *et al.*, 2017; Reid *et al.*, 2020).

1.3 Human identification procedure in South Africa

In South Africa, it is required by law that all deaths, whether natural or unnatural, must be reported to the Department of Home Affairs (DHA) (*Births and Deaths Registration Act 51 of 1992*, n.d.). The process of determining the cause, time, place, and manner of death, as well as identifying the deceased, is mandated by the *The Inquests Act 58 of 1959*. This act requires a comprehensive investigation to establish whether any individual bears criminal responsibility for the death.

When it comes to identifying the deceased, the South African Police Service (SAPS) and the Forensic Pathology Service (FPS) are responsible (*National Health Act 61 of 2003. Regulations: Rendering of Forensic Pathology Service. Government Gazette No. 30075*, n.d.). If a preliminary identity is available, the next-of-kin must confirm the identity of the deceased through visual identification. If no identity can be confirmed, the Victim Identification Centre (VIC), a specialized unit within SAPS, is responsible for assisting with identification. The VIC collects fingerprints from the deceased and searches various local and national fingerprint databases. When a family reports a missing person, authorities gather comprehensive information about the individual, including personal details and any relevant ante-mortem data. They also seek biological samples from the missing person's belongings, if available. specifically for DNA profiling. Additionally, samples are collected from living family members or biological relatives to aid in the DNA comparison process, helping to establish identity and facilitate investigations into the disappearance. The samples collected by the mortuary are sent to the SAPS's Forensic Science Laboratory (FSL) for analysis. However, due to recent and current backlogs in DNA analyses, some mortuaries also submit samples to universities or private laboratories for expedited DNA analyses.

In cases of skeletonised remains, the primary method of visual identification is not feasible. Anthropological analyses of skeletal remains may be required to narrow down the search from a group to an individual, to ultimately aid with identification (Bidmos *et al.* 2010; Saukko & Knight, 2015b; Tawha *et al.,* 2020). In South Africa, there are four anthropology service providers utilized by FPS, most of which are associated with universities. There are two anthropology laboratories in the Western Cape. These include Forensic Anthropology Cape Town (FACT) at the University of Cape Town (UCT), which receives the majority of cases from Salt River Mortuary in Cape Town (Baliso *et al.,* 2019) and the other being 'Victim Identification @ Stellenbosch University' (ViSUN) (Baliso *et al.,* 2023).

1.4 Anthropological sex estimation

Forensic anthropology utilises principles of biological anthropology to construct an individual biological profile to help identify individual tissue skeletal elements for legal purposes (Ubelaker, 1989; Rowbotham, 2016; Rissech, 2021). This includes estimating age, sex, ancestry and stature. Sex estimation is crucial in facilitating identification by enabling the comparison of estimates with ante-mortem records. These techniques are based on "sex

differences", which refer to the biological variations between males and females, primarily observed in skeletal structure, reproductive systems, and secondary sexual characteristics. However, contemporary research highlights that sex is not strictly binary but exists along a spectrum, shaped by the interaction of genetic, hormonal, and environmental factors (Flaherty *et al.*, 2023). Typically, males are larger in stature and muscularity and exhibit more robust cranial and facial features than females, who are more gracile (Frayer & Wolpoff, 1985). These differences are influenced by genetics, sexual selection, and sex-specific differences in energy intake, nutrition, body composition and environment (Loth & Henneberg, 1996; Rogers, 2005; Kimmerle, Ross & Slice, 2008; Sierp & Henneberg, 2015; Nikitovic, 2018; Del Bove *et al.*, 2023), which can make sex estimations challenging.

Sex estimation relies on two principle factors: features of the pelvis due to female child-bearing functions and those based on the overall body size and robusticity (Ubelaker & DeGaglia, 2017). Where cases include a pelvis, accurate sex estimation is integral in estimating age, ancestry, and stature, as there are distinguishable differences in growth patterns and aging between sexes, as well as variations in morphological traits linked to ancestry (White, 1979; White & Folkens, 2005; (Scientific Working Group for Forensic Anthropology (SWGANTH), 2010). Hence, the accuracy of sex estimates is influenced by several factors, including inter- and intra-population differences, age, and pathological and taphonomic changes (Scientific Working Group for Forensic Anthropology (SWGANTH), 2010). Thus, sexual dimorphism differs between groups and is demonstrated in the literature where certain methods or sexually dimorphic traits may prove accurate in one population but not in others (MacLaughlin & Bruce, 1990; Maat *et al*., 1997; Bidmos & Dayal, 2004; Walker, 2005; Spradley & Jantz, 2011; Franklin *et al.*, 2013; Kotěrová *et al.*, 2017; Swift *et al.*, 2024).

1.4.1 Overall Performance of Sex Estimation Methods

Sex estimation method accuracy depends on the anatomical region of the skeleton and the bones' preservation state. The pelvis is the most accurate part of the skeleton (90-95%) for sex estimation (80-90%) (Krogman & İşcan, 1986; Klales *et al.*, 2012). Studies have shown that after the pelvis, measurements of long bones are most reliable for sex estimation. These findings challenge earlier perspectives that prioritized cranial traits, emphasizing the superior accuracy of long bone metrics (Spradley & Jantz, 2011; Patterson & Tallman, 2019). Other regions of the skeleton have also been studied, including long bones (Steyn & İşcan, 1997; İşcan *et al.*, 1998), ribs (Íşcan, 1985; Wiredu *et al.*, 1999) and vertebrae (Rohmani, *et al*., 2021)

amongst others. Due to population differences, population-specific reference data are required for reliable interpretation of demographic variables (Byers, 2002; Krishan *et al.*, 2016). While forensic anthropology has traditionally emphasized population-specific methods, there is increasing discussion around population-inclusive approaches (Tallman & Bird, 2022). These methods are particularly relevant in demographically diverse regions or when forensic anthropologists opt not to conduct ancestry, race, or population affinity assessments. The shift aims to improve the applicability and accuracy of sex estimation across broader population groups, minimizing biases inherent in population-specific frameworks. Sexual differences have enabled the development of various approaches to sex estimation methodology. Metrical and morphological (non-metrical) approaches are the two main conventional sex estimation techniques, each with strengths and limitations that depend on the analysis context. Anthropologists must carefully consider these various factors before selecting the most appropriate sex estimation method.

1.4.2 Methods of sex estimation

1.4.2.1 Morphological (non-metrical) sex estimation

Morphological sex estimation relies on visual observation of sexually dimorphic traits, making it ideal for preliminary assessments. Researchers commonly use standardised ordinal scoring methods to classify morphological sex traits popularized by Buikstra & Ubelaker (1994). These traits are scored on a five-grade scale which ranges from a lower score indicating a female's gracility to a higher score indicating a male's robusticity (Buikstra & Ubelaker, 1994). An intermediate score of three means the outcome is inconclusive. However, this scoring system is subjective and dependant on the observer's interpretation and experience (Sierp & Henneberg, 2015; Klales, 2020). Measuring features may be challenging due to inter and intra-observer errors, classification difficulties, and analysis problems (Byrnes *et al*., 2017; Daniels, 2018; Slone, 2023). Contemporary morphological sex estimation methods increasingly incorporate statistical models to improve accuracy. Walker (2008) employs population-specific logistic regression equations tailored to U.S. Black, White, and Native American groups. These equations use the combined scores of five cranial traits—nuchal crest, mastoid process, supraorbital margin, glabella, and mental eminence. For this study, Logistic regression correctly classified 88% of contemporary crania with minimal sex bias, while accuracy dropped to 78% for ancient Native Americans, highlighting the need for population-specific models due to differences in sex differences. Similarly, Klales *et al.*, (2012) apply a logistic regression

model to pelvic morphology, focusing on three sexually dimorphic traits: the ventral arc, subpubic concavity, and the medial aspect of the ischiopubic ramus. The study achieved a mean classification accuracy of 94.5%, with low intra- and interobserver error, and 86.2% accuracy in an independent validation sample. In both methods, each trait is scored on a five-point scale, with 1 indicating a strongly female expression, 2 to 3 representing intermediate forms, and 4 to 5 indicating a strongly male expression. These scores are then used to calculate the probability of the individual being male or female, providing a systematic and statistically robust framework for sex estimation. Morphological assessment provides more accurate results with intact bones, while its accuracy tends to decrease with fragmented or incomplete skeletons or skeletal elements. Morphological sex estimation is preferred because it is easy to use, inexpensive, and does not require specialised equipment. As previously mentioned, the pelvis and cranium are amongst the most reliable skeletal elements for sex estimation. Therefore, forensic sex estimation is generally done morphologically, using these elements (Spradley & Jantz, 2011).

1.4.2.2 Metrical sex estimation

Metrical sex estimation is based on quantitative and measurable differences between male and female anatomical dimensions of skeletal elements. This method involves statistical approaches that use derived models or equations, which may consist of discriminant function analysis, logistic regression, indices and proportions (Krishan *et al.*, 2016; Klales *et al.* 2020). These methods can provide objective and reproducible results that are suitable for statistical analyses (Bidmos *et al.*, 2010). However, these methods require calibrated specialised instruments (*e.g.*, a pair of measuring callipers), are time-consuming, and can be prone to observer error if unclear landmarks are measured *(Krishan et al., 2016; Oikonomopoulou et al.*, 2017). They are also limited when applied to fragmentary remains, as they often require measurements of several intact bones or dimensions spanning several bones. (Rogers, 2005). Additionally, similar to non-metric sex differences, some metrical sex estimation methods are linked to known ancestry. The most reliable method, discriminant function analysis, is highly population-specific and requires periodic revision (Rogers, 2005; Sierp & Henneberg, 2015). Developing such population-specific equations for a particular population group under investigation requires large-scale datasets of individuals of known sex, which are often unavailable (Rogers, 2005; Oikonomopoulou *et al.*, 2017). For these reasons, morphological sex estimation methods are more commonly relied upon in South Africa, however in the United States of America (USA), both metric and nonmetric approaches are commonly employed.

1.4.3 Erroneous sex estimation

Anthropological sex estimation faces complexities when dealing with contemporary populations that deviate from typical sexual dimorphism generalizations (Gibbon, 2008). This variability is influenced by temporal, geographical, and genetic factors, with genetic admixture playing a significant role (Inskip *et al.*, 2019). A stark example of how historical and social factors shape biological traits can be seen in South Africa's Apartheid era, which severely restricted gene flow, resulting in significant inter-population differences (McDowell, *et al.*, &2012; Stull *et al.*, 2014; McDowell *et al.*, 2015; Maass & Friedling, 2019; Tawha *et al.*, 2020). This illustrates how intrinsic and extrinsic factors, intertwined with social history, complicate the analysis of ancestry beyond conventional typological method (Serre & Pääbo, 2004; McDowell *et al*., 2012).

An illustrative case is the 'Mixed Ancestry (MA) population' of South Africa, bureaucratically categorized as 'coloured'. Described as "biologically heterogeneous" (Tawha *et al.*, 2020), this group traces its origins to various ancestries including indigenous African San and Khoekhoe, European, Bantu-speaking African, Asian, and Madagascan Cape slaves/migrants (Kittles & Keita, 1999; de Wit *et al.*, 2010; Patterson *et al.*, 2010; Montinaro *et al.*, 2017). The Western Cape Province, where the Mixed Ancestry population is largest, has the country's second highest murder rate, influencing their disproportionate representation in forensic investigations (Mokoena *et al.*, 2019). A study by de Wit *et al.*, (2010) demonstrated that the primary ancestral elements of the Mixed Ancestry population in the Western Cape population consist predominantly of San and Khoekhoe (32–43%), Bantu-speaking Africans (20–36%), Europeans (21–28%), and a smaller Asian contribution (9–11%). Despite many individuals being genetically homogeneous to a single origin, the term 'mixed' acknowledges that they were historically grouped under a single racial label during apartheid, which still persists today (Tawha *et al.*, 2020). Previous studies estimating the biological profile of the South African population have primarily focused on the Bantu-speaking and European ancestries, with less emphasis on the Mixed Ancestry population (Mokoena *et al.*, 2019).

Sex estimation in San and Khoekhoe populations is challenging due to their small stature and gracile skeletal traits, which exhibit less sexual dimorphism than other populations (Pfeiffer, 2012; Morris, 2014; Malek *et al.*, 2023). Moreover, both males and females in these groups led physically active lives, resulting in robust muscle attachment sites on bones despite their small

frames (Malek *et al.*, 2023). Consequently, male skeletal characteristics may appear more female, complicating the accurate assessment of sex based solely on morphological criteria. Malek *et al.*, (2023) conducted a study on sex estimation methods in southern African Holocene San and Khoekhoe populations, found that cranial and mandibular traits like the mastoid process and mandibular shape had the highest agreement with pelvic-based sex estimates (73% and 72%), while traits like mental eminence and nuchal crest showed lower agreement (53%). Discriminant function equations provided the highest overall agreement, ranging from 73% to 77%. Interestingly, a study by Liebenberg *et al.* (2019) measured postcranial features and found that Mixed Ancestry males had the lowest classification accuracy (47%) amongst their South African counterparts.

Several studies and case reports have reported erroneous anthropological sex estimations using molecular analyses, emphasizing the need for population-specific anthropological methods that are reliable for use in forensic contexts. For example, Stone *et al.*, 1996 reported that the sex of one of 20 contemporary Indonesian individuals was incorrectly sexed as male using morphological methods, but was confirmed as female using DNA analysis. In another study, eight archaeological English male individuals were incorrectly sexed as females, with the combined estimate accuracy (crania and pelvis) of males being 100% and females 93.7% (Inskip *et al.*, 2019), illustrating the limitations of traditional anthropological approaches. Similarly, Thomas *et al*., (2016) analysed 360 contemporary Federal Bureau of Investigation (FBI) forensic cases in the United States of America (USA) and found a 94.7% overall accuracy in sex estimation, with errors more likely when only one skeletal element was available. This disparity emphasises the need for population-specific anthropological methods.

In Cape Town, two medico-legal death investigations revealed errors in morphometric sex estimation, where individuals were initially identified as female using South African population data but were later confirmed as male through DNA analysis (Finaughty *et al.*, 2020; Bennett *et al.*, 2021). In the first case, remains labelled as "Case A," including a pelvic bone, were thought to be female due to a shoe strap and initial forensic assessments. Nearby, a hand, "Case B," was discovered. However, DNA profiling and Next-Generation Sequencing (NGS) later identified both remains as belonging to the same male, highlighting potential cognitive bias in the initial assessments. In the second case, skeletal remains found in a field were estimated as female using the Phenice method, shape of the greater sciatic notch and gracility of the cranium, despite male-associated clothing being present. DNA analysis

ultimately confirmed the remains were male. Initial attempts by state and private labs failed, but DNA extracted from a molar tooth at the UCT revealed Y-chromosome amplification, confirming male sex. Both these abovementioned studies underscore the need for updated anthropological data relevant to South Africa's diverse population.

The examples provided above demonstrate that molecular analysis of skeletal evidence may be a more reliable method of determining sex (Bruzek & Murail, 2006; Ubelaker, 2018). Molecular analyses are particularly invaluable for investigations involving juveniles, fragmented remains, and adult skeletons that exhibit both male and female morphometric variation, leading to inconclusive sex results (Stone *et al.*, 1996; Bidmos *et al.*, 2010). Therefore, it is essential that the accuracy of anthropological sex estimation is assessed against molecular methods in various population groups. However, molecular methods of sex determination are also not without limitations, necessitating a balanced approach in forensic practice, which will be discussed below.

1.5 Molecular-based sex determination

1.5.1 Forensic DNA profiling using STR typing

Forensic DNA testing is used for human identification by analysing genetic variation to differentiate between individuals. This process generates a 'forensic DNA profile' for an unknown individual or sample, which is then compared to a DNA profile from a known individual. The process of DNA profiling targets specific regions within the human genome that vary between individuals, known as short tandem repeats (STRs) (Butler, 2011). These regions contain core repeat motifs of two to seven nucleotides in length that are repeated in tandem, ranging from six to several dozen times. Alleles are named according to the number of repeat motifs within an STR. For example, if a motif of DNA repeats itself seven times, the allele is referred to as seven (Goodwin *et al*., 2011).

To establish if an unknown DNA sample matches a reference sample, the alleles at all STR loci must match. This provides strong evidence that the sample has a common source. The number of repeat units is highly variable among individuals, providing a high power of discrimination when analysed for identification purposes. However, before a DNA profile can be presented as evidence in a court of law, statistical analyses must be conducted to determine the probability of this match occurring by chance. This is known as the 'random match

probability' and it is calculated based on expected allele frequencies from the relevant background population (Scientific Working Group on DNA Analysis Methods (SWGDAM), 2017).

Although the human genome contains countless STR markers, less than 30 STR loci are typically used in forensic DNA testing with commercial STR kits (Butler, 2006; Dashnow *et al.*, 2018). These kits are widely accepted and validated for use in forensic casework to compare DNA profiles between different laboratories. STR multiplex kits can analyse several loci simultaneously and require less than one nanogram of input DNA (Butler, 2011; Goodwin *et al.*, 2011). Polymerase chain reaction (PCR) is used to amplify specific STR loci, producing PCR products of varying sizes.

Most of the STRs analysed in DNA profiles do not impart any phenotypic information related to the individual's physical appearance, behaviour, or medical information, except for the biological sex. The biological sex can be determined by analysing the sex chromosomes, X and Y. Females have two copies of the X chromosome, while males have an X and a Y chromosome. The amelogenin marker, present on both sex chromosomes, is commonly used as a sex-determining marker in forensic DNA profiling kits (Chang *et al.*, 2007; Westen *et al.*, 2014). It is also the most frequently used sex-typing marker in forensic casework and is included in all DNA profiles added to the US National DNA Index System (NDIS) and Combined DNA Index System (CODIS) (Hares, 2015).

1.5.2 Amelogenin sex marker

The amelogenin (AMEL) locus is responsible for producing a matrix protein that plays a vital role in the development of tooth enamel (Butler & Li, 2014). The AMEL locus has two homologous genes: AMELX on the X-chromosome and AMELY on the Y-chromosome (Salido *et al.*, 1992). The use of amelogenin as a sex-determining marker is based on the length variation between AMELX and AMELY (Nakahori *et al.*, 1991). AMELX contains a 6 base pair deletion in intron three, which contributes to the variation in size between genes. If only the smaller AMELX amplicon is present, it is interpreted as a female individual containing only X chromosomes. On the other hand, if the longer AMELY amplicon and the AMELX fragment are present, it is interpreted as a male individual containing both X and Y chromosomes (Butler & Li, 2014).

There have been inconsistencies reported in forensic casework regarding the amelogenin marker. One of these discrepancies is due to variants in the primer binding region for the

AMELY locus, which prevents the primer from binding and thus there is no amplification of the Y-target (Shadrach *et al.*, 2004). The failure of AMELY to amplify (referred to as a null allele) may be perceived as there being no Y-DNA present when, in fact, a Y chromosome is present but could not be amplified. Hence, males who have deletions and variants in the AMELY primer-binding region would be incorrectly identified as female. The null allele is most often neutral but may indicate infertility in the male, depending how big the deletion is and if surrounding genes have also been deleted (Chang *et al.*, 2007). Although rare, biological males who genuinely possess the XX karyotype but with a translocated sex-determining region Y (SRY) would also be interpreted as female (Butler & Li, 2014; Nicolae *et al.*, 2019).

Published data on the presence of AMELY null alleles varies by population, ranging from as low as 0.008% to as high as 8%, with the highest frequencies occurring in populations originating from the Indian subcontinent (Butler & Li, 2014; Dash *et al.*, 2020). The null alleles of amelogenin are also most common when using the primer sets manufactured by Thermo Fisher Scientific (Cadenas *et al.*, 2007).

1.5.3 Other molecular methods of determining sex

To improve the accuracy of sex determination in forensic cases, alternative markers and techniques have been developed beyond the amelogenin marker. Y-chromosome STRs (Y-STRs) like DXYS156 and DYS391 are often used alongside amelogenin to enhance reliability (Butler & Li, 2014). The DYS391 marker, for example, is included in newer forensic DNA profiling kits such as the GlobalFiler™ PCR Amplification Kit by Thermo Fisher Scientific. X-STR markers on the X chromosome offer an additional tool for forensic genetic analysis alongside Y-STRs. Identifying skeletal remains can be challenging due to prolonged exposure to the environment and extensive degradation of DNA. Short amplicons representing X-STR markers are well-suited for the fragmented DNA typically found in skeletal remains. Several X-STR kits are validated for forensic casework, with the Investigator Argus X-12 QS from Qiagen Corporation being the most common, amplifying 12 X-STR loci and amelogenin (Sahajpal & Ambers, 2023).

Another technique used to determine sex is quantitative PCR (qPCR), used to amplify and quantify specific segments of DNA. It detects and measures the presence of targeted DNA sequences, such as those found on the Y chromosome. By identifying and quantifying unique Y-chromosome markers absent on the X chromosome, qPCR can effectively confirm the presence of male DNA in a sample. If these Y-chromosome markers are detected, it indicates

that male DNA is present in the sample. Commercially available kits, such as the Quantifiler® Trio DNA Quantification Kit by Applied Biosystems, are commonly used for this purpose. For the presence of Y-chromosomal DNA to be confirmed, the concentration of the Y-chromosome target must fall within the validated range (dynamic range) of the assay.

While perhaps not routine in forensic DNA analyses, other molecular methods can also be used for sex determination such as analysis of the *sex-determining region Y* (*SRY*) gene, the *Y-encoded testis-specific protein* (*TSPY*) and *STS* gene (Butler & Li, 2014). Additionally, chromosome karyotype analysis, the use of single nucleotide polymorphisms (SNPs) in the Y-chromosome, next generation sequencing (NGS) and pyrosequencing can also be used for this purpose, as reviewed by Dash *et al.* (2020).

1.5.4 Limitations of molecular testing

Molecular testing is considered the gold standard and the most accurate method available for sex determination (Bruzek & Murail, 2006; Bašić *et al.*, 2017; Adel *et al.*, 2019). Nonetheless, they also present several practical limitations, such as the chromosomal and genetic anomalies described above. Additionally, extracting usable DNA from hard tissue from diverse taphonomic circumstances can be challenging (Latham *et al.*, 2021). Similar to STR markers, the quality of DNA can also be limiting to obtaining results, with PCR inhibitors potentially impeding DNA amplification (including sex markers), leading to allele dropouts (Dash *et al.*, 2020). Analyzing DNA samples with low quantities, known as "low copy number" (LCN) or low template DNA, introduces further complexities. Stochastic effects during PCR can cause imbalances in peak heights, allele dropouts, drop-ins, and increased stutter peaks (Marshall *et al*., 2015; Meintjes-Van der Walt, 2021). Other challenges with LCN DNA are alleles or amplification being below the threshold or the dynamic range of the assay used, thus being insufficient to reliably confirm sex. These challenges make interpreting LCN profiles difficult, compounded by degraded samples that can result in ambiguous amelogenin sex results and heterozygous imbalance in DNA profiles (Hansson *et al*., 2017).

Moreover, sampling of bone material for molecular analysis is destructive to human bodies and poses several health and safety risks through the grinding, drilling and sanding of bone tissue (Pluim *et al*., 2019). Moreover, DNA testing is time-consuming and costly, and is often only performed when other methods of identification are not useful (Pierce, 2017). Additionally, obtaining ante-mortem comparative DNA samples can be challenging as the family may be untraceable, unknown or hesitant to come forward to authorities (Baliso *et al*., 2019; Reid *et*

al., 2019). In a local context, there were considerable backlogs at the SAPS forensic science laboratories that hindered the effective utilisation of DNA profiling in South Africa (Zuzile, 2020; "SA'S DNA backlog crisis", 2024). Hence, locally, anthropological methods may be relied upon for identification. Improved sex estimation could aid in identifying the many unclaimed individuals in mortuaries annually, emphasizing the necessity of interdisciplinary collaboration in forensic investigations.

1.6 Rationale

The identification of deceased individuals is becoming increasingly problematic in South Africa, affecting forensic practitioners, the legal system, and the families of the deceased (Mazzarelli *et al.,* 2021). This is particularly challenging when dealing with skeletonised, decomposed or burnt remains. Anthropological sex estimation plays an important role in forensic human identification, however this is only useful if results are accurate (Klales *et al.* 2020). Forensic anthropology methodologies have evolved significantly, but there are still several challenges that forensic anthropologists face, such as analysing fragmented or poorly preserved skeletal remains, sub-adult skeletal remains, and population specificity (Saukko & Knight, 2015a; Krishan *et al.,* 2016).

There are individuals in contemporary populations who do not conform to the same degrees of sexual dimorphism. These include males exhibiting more gracile features and females being more robust, which result in erroneous conclusions (Gibbon, 2008). An example is the Mixed Ancestry population of South Africa. The Mixed Ancestry population is a biologically heterogeneous group with variable and complex admixture and includes origins from indigenous African Khoesan, Bantu-speaking African, European, Asian (Malaysian, Indonesian and Indian descent) and Madagascan Cape slaves/migrants (Adhikari, 2005; de Wit *et al.*, 2010; Tawha *et al.*, 2020), although many individuals belonging to this group are genetically homogeneous to a single origin (Tawha *et al.*, 2020). Thus, forensic identification of human skeletonised remains is particularly challenging in the Western Cape Province of South Africa due to the heterogeneous genetic and ancestral make-up of the population (Tawha *et al.,* 2020).

Although DNA testing may not be the most cost-effective identification method, it accommodates anthropological methods' shortcomings. This includes being independent of

ancestry and eliminating issues of population variation, which is particularly advantageous for the heterogeneous genetic and ancestral makeup of the Western Cape population. Also, unlike anthropological methods of sex estimation, molecular methods are not affected by individual variation in size and structure of bones, and can therefore, be used to determine the sex of neonate and sub-adult skeletal remains. Additionally, molecular sex testing is also not hindered by bone fragmentation, as with anthropological analyses (Bidmos *et al*., 2010).While there have been several case reports of discordance, it is not known how common this is in a South African population. Reports of discordance in other countries have been reported but it is known that this varies between population groups. In Cape Town, specifically, two males have been anthropologically estimated to be female in recent forensic casework. This suggests that the anthropological population data for the South African population may be under-represented. Therefore, it is important to investigate how many other skeletons estimated to be female using anthropological methods may be male. A study of this nature that involves comparisons of both molecular-based sex determination and morphological data in forensic cases has not been done in South Africa before but has important consequences for forensic casework and may identify the extent of the problem, making more accurate identifications possible.

1.7 Research aims and objectives

The aim of this dissertation was to assess the concordance between morphological and DNA-based sex results in a forensic case series of morphologically female estimated individuals in the Western Cape province of South Africa.

The key research question was how many skeletons which have been estimated to be female using anthropological methods are actually biologically male?

The objectives included:

- Estimate sex using morphological assessments of the pelvis, cranium and mandible.
- Determine the inter- and intra-rater reliability of morphological sex estimations.
- Perform DNA-based sex analysis on individuals which have been estimated to be female using anthropological methods.
- Determine the accuracies of the morphological methods used compared to molecular sex analyses.

1.8 Study overview and structure of minor dissertation

The study includes human remains of a forensic context from the University of Cape Town's Human Skeletal Repository. The repository is housed and curated under the Division of Clinical Anatomy and Biological Anthropology, Faculty of Health Sciences. It comprises 1059 skeletons of which 160 (14%) are of forensic context (Gibbon & Morris, 2021). Skeletal remains that fall within the forensic category are cases of unidentified human skeletal remains recovered by the South African Police Service (SAPS) and the Forensic Pathology Services (FPS) and are submitted for biological profiling. These skeletons are stored at UCT until identification can be ascertained or the inspector of Anatomy authorises the use of the remains for teaching and research purposes. The majority of the skeletal remains stem from South Africans of Mixed Ancestry, usually unclaimed individuals from a forensic mortuary or Groote Schuur Hospital. Ethical clearance for this study was approved by the Human Research Ethics Committee of the Faculty of Health Science within the University of Cape Town (HREC: 269/2021) (Appendix A) and is linked to the umbrella study under Prof Victoria Gibbon (HREC: 035/2021), which aims to conduct osteobiographic analyses for the purpose of providing a biological profile of unknown individuals.

The overall structure of the study takes the form of four chapters, including this introductory chapter. Chapter Two covers biological anthropological sex estimation, the methodology used for this study, and the anthropological results followed by a brief discussion. Similarly, the third chapter deals with molecular sex determination, the methodology used, the results obtained, and a brief discussion. The final chapter draws upon the entire dissertation, including the critique of the findings and its implications for further research into this area. Finally, the conclusion summarises the findings.

2 Chapter 2: Morphological sex estimation

2.1 Introduction

Morphological sex estimation involves assessing sex-related differences of traits in skeletal remains to estimate biological sex. It is a critical tool in forensic anthropology and bioarchaeology, providing valuable information for identifying individuals. Due to pronounced sex-related differences, sex estimation was conducted using the crania, mandibles, and pelves. The accuracy of sex estimation methods is influenced not only by the anatomical region of the skeleton, but also by the preservation state and condition of the bones, and any changes induced by taphonomy (Klales, 2021). In addition, skeletal inventories were created for the crania, mandibles and pelves evaluated in this study.

2.2 Materials and methods

2.2.1 Study sample

This study examined the skeletal remains of 145 individuals from UCT's Human Skeletal Repository (Appendix B: Table B1). Sexual dimorphism only becomes significant on the skeleton after adolescence. The inclusion criteria, therefore, were adult individuals, as evidenced by the complete epiphyseal fusion of all long bones, excluding the clavicle.

2.2.2 Observer error assessment

To ensure consistent and reliable data collection, tests were conducted for intra-observer and inter-observer errors prior to data collection. Intra-observer error tests were performed on 43 individuals as per the study's methodology, and the process was repeated after approximately 24 hours to obtain a second set of recordings. On the same 43 individuals, inter-observer error tests were performed by a doctoral student. The participating researcher received a description of the data collection procedure and then gathered data independently using the same methods as the study.

To assess the level of agreement, we conducted statistical analyses on the morphological and categorical data to evaluate both intra- and inter-rater reliability. This analysis was carried out using SPSS Statistics 27 (IBM© Corp, 2021). Cohen's kappa (κ) was used to assess observer agreement, which considers the possibility of chance agreement and is interpreted as follows:

Table 2.1: Interpretation of Cohen's kappa value level of agreement and percentage of data reliability as interpreted by McHugh (2012).

Kappa (κ) value range	Level of agreement	Percentage of data reliability (%)
0 – 0.20	None	0 – 4
0.21 – 0.39	Minimal	4 – 15
0.40 – 0.59	Weak	15 – 35
0.60 – 0.79	Moderate	35 – 63
0.80 – 0.90	Strong	64 – 81
Above 0.9	Almost perfect	82 – 100

2.2.3 Morphological sex estimation methods

Morphological methods were selected due to ease of use, cost-effectiveness, and rapid results. The selection of skeletal elements for sex estimation was determined by the degree of sexual dimorphism present in the bones and the frequency at which these elements were recovered. Moreover, the methods selected are established practices in the field, and included, but not limited to methods routinely used by FACT for sex estimation (Baliso *et al.*, 2019). As such, morphological analysis of the pelvis, cranium and mandible were selected for this study. These methods were used on both complete and fragmented skeletal elements where possible.

2.2.3.1 Pelvic sex estimation

Phenice's (1969) study reported a 95% classification accuracy for sex estimation using three morphological traits of the anterior pelvis: the ventral arc, sub-pubic concavity, and ischio-pubic ramus. This method, commonly referred to as the "Phenice method," has become a standard approach for sex estimation due to its ease of use and reported high accuracy, even though subsequent studies have demonstrated lower accuracies than the original study (Johnstone-Belford *et al*., 2018; Jager & Eliopoulos, 2023; Rojas González *et al*., 2024) (Figure 2.1A). According to Phenice (1969), only females possess a ventral arc. Females also have a more pronounced sub-pubic concavity than their male counterparts. The ridge on the face of the medial aspect of the ischio-pubic ramus is more pronounced in female, while males have a broad flat appearance. The Phenice method, later revised by Klales *et al.* (2012), expands the scoring of the three traits into five ordered character states and has demonstrated to have high

classification accuracies in South African populations (Kenyhercz, 2012; Stull, Kenyhercz & L'Abbe, 2013; Kenyhercz *et al.*, 2017).

Other popular morphological pelvic sex traits are the greater sciatic notch (Figure 2.1B) and the pre-auricular sulcus (Figure 2.1C) (Ubelaker & DeGaglia, 2017; Alizadeh *et al.*, 2013; Selliah *et al.*, 2020). The greater sciatic notch is particularly useful in cases where the fragile anterior portion of the pubic region is damaged or destroyed, and the Phenice method of sexing cannot be used (Walker, 2005). The greater sciatic notch exhibits high levels of sexual dimorphism and is more resistant to damage (Walker, 2005). The greater sciatic notch of females is broader than those of males. The female greater sciatic notch is also more open, with a lower width to depth ratio, when compared to males, the greater sciatic notch is more narrow and U-shaped (Walker, 2005). Likewise, several studies on the pre-auricular sulcus show that it could be used as a reliable indicator for sex estimation (Novak *et al*., 2012; Karsten, 2018; Selliah *et al.*, 2020). The pre-auricular sulcus of males is typically more narrow and short, whereas females are more likely to have a broad long sulcus (Karsten, 2018).

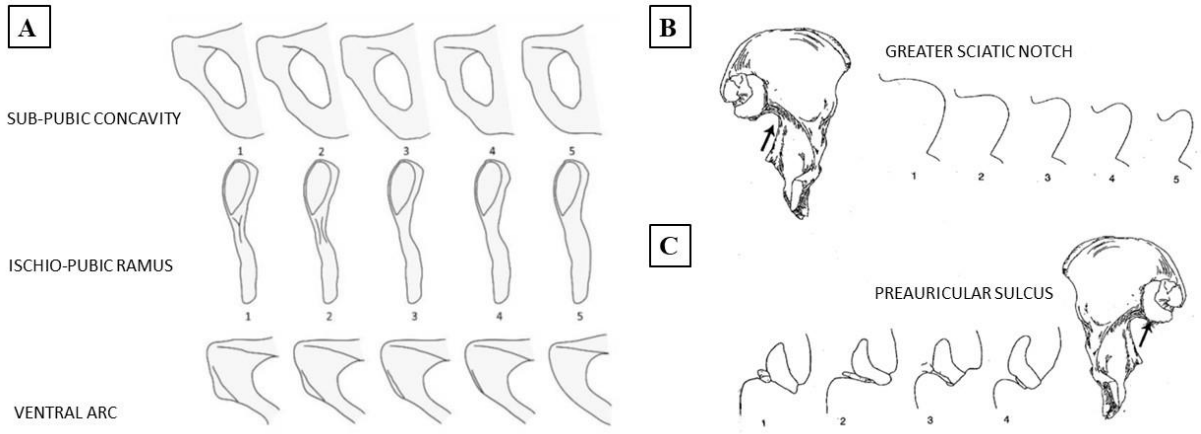

Figure 2.1: Pelvis morphological sex traits assessed: **A)** Diagrams of Phenice (1969) method traits described by Klales *et al.* (2012) using the 5-grade scoring system. Phenice traits from top to bottom: sub-pubic concavity, medial aspect of the ischio-pubic ramus, and the ventral arc. **B)** Diagrams of the 5-grade scoring system of the greater sciatic notch of the pelvis, indicated by arrow on the left (Buikstra & Ubelaker, 1994) described by Walker (2005). Trait scoring key for A and B: 1 – 2: female, 3: indeterminate, 4-5: male. **C)** Diagrams of 4-grade scoring system of the pre-auricular sulcus described by Walker (2005) after Milner (1992) obtained from Buikstra and Ubelaker (1994). Trait scoring key: (1 – 2: female, 3-4: male)

2.2.3.2 Cranial and mandibular sex estimation

In forensic cases in South Africa, the cranium is the skeletal element most frequently recovered (*2012 Annual Report, Forensic Anthropology Research Centre, University of Pretoria, South*

Africa, 2012). One of the most common morphological methods of sexing crania is using the five traits included in the "Standards for Data Collection from Human Skeletal Remains" by Buikstra & Ubelaker (1994). These five traits include the glabella-supra-orbital ridge, mastoid process, nuchal crest, super orbital margin, and mental eminence (Figure 2.2A). The illustrations by Walker (2008) were used to score these five traits. The mandible is the largest, strongest, durable, and most sexually dimorphic facial bone, shown to estimate sex with high accuracy. (Loth & Henneberg, 1996; Franklin *et al.*, 2006; Hu *et al.*, 2006). Mandibular traits visually assessed include mandibular shape and gonial flaring (Loth & Henneberg, 1996; Franklin *et al.*, 2006; Hu *et al.*, 2006).

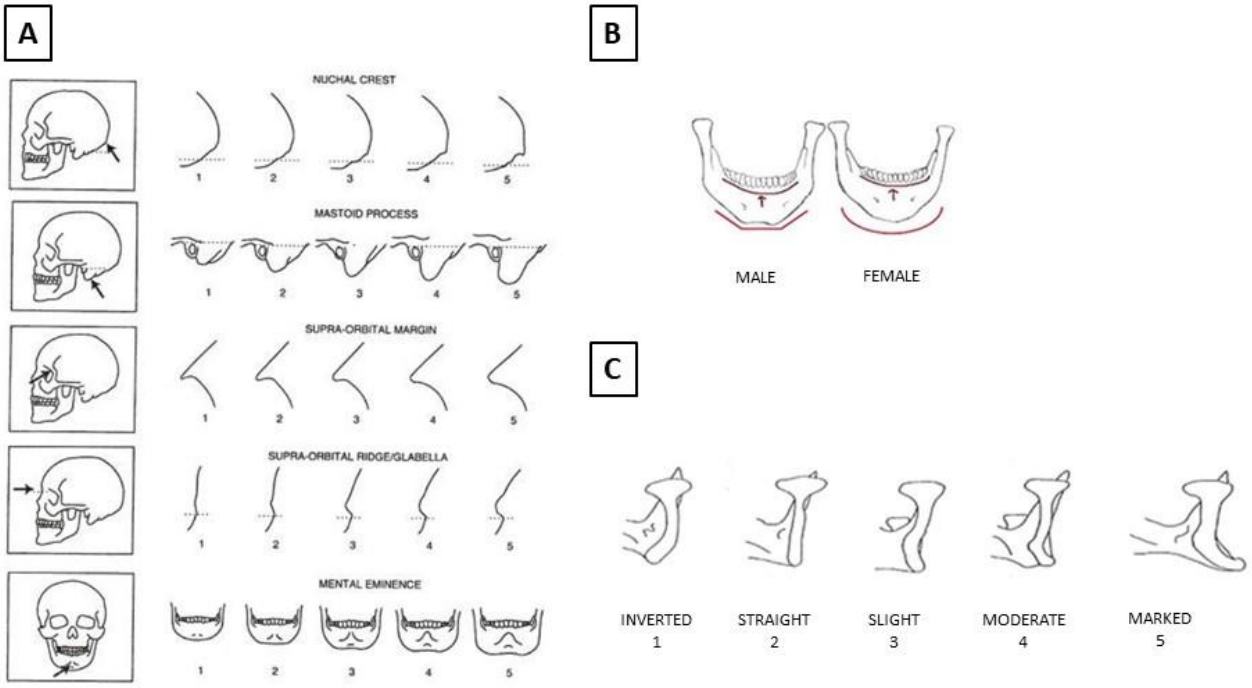

Figure 2.2: Cranial and mandibular morphological sex traits assessed: **A)** Diagrams of ordinal 5-grade scoring system illustrated by Walker (2008) for the five morphological cranial traits described by Buikstra and Ubelaker (1994), *i.e.* nuchal crest, mastoid process, supra-orbital margin, glabellar and mental eminence. The numbers beneath the diagrams represent the scores assigned to individuals that exhibit morphological features most closely aligned with the illustrated examples; **B)** Diagrams illustrating the contour of the body of the mandible from the front, squared mandibular body shape (male), rounded mandibular body shape (female) adapted by Hu *et al.* (2006). **C)** Diagrams of the 5-grade scoring system of the mandibular ramus flexure, located on the posterior border of the ramus at the occlusal plane, after Loth & Henneberg (1996).

These traits were evaluated based on a 5-grade ordinal scoring system (refer to Table 2.2), except for the pre-auricular sulcus, which was rated on a 4-grade scoring system (1 – 2: female, 3 –4: male). The traits were scored as follows:

Table 2.2: Five-grade scoring system for observed sex traits.

Score	Description
1	Female. There is little doubt that the structures represent a female.
2	Probable female. The structures are more likely female than male.
3	Ambiguous. Sexually diagnostic traits are ambiguous.
4	Probable male. The structures are more likely male than female.
5	Male. There is little doubt that the structures represent a male.

All recorded morphological data were entered into a spreadsheet for subsequent statistical analysis.

2.2.4 Conditions of interment

In forensic anthropology, the initial analysis focuses on determining whether a case holds medico-legal significance or is of archaeological or historical interest. This assessment considers several factors, including whether the individual died within the last 50 to 100 years. Key indicators such as the state of preservation, taphonomic conditions (e.g., burial environment), and skeletal element coloration or staining are analysed; for example, red ochre or green copper staining often points to archaeological remains. The presence of soft tissue typically suggests more recent, contemporary remains. Additionally, personal belongings, such as clothing and artifacts, can provide valuable clues about the time period.

2.2.5 Data capture and analyses

Sub-adults and individuals who did not have any of the skeletal elements necessary for morphological assessment, *i.e.*, crania, mandible and pelvis were excluded from analyses. Sex estimates were finalised by analysing the scores on cranial, mandibular and pelvic traits collectively. Hence, a higher frequency of traits on either side of the spectrum would indicate the applicable sex. If the scores were not weighted towards either male or female, the individual was assigned as having an ambiguous sex. The percentage of male, female and ambiguous observations were calculated. Skeletal inventories were created for the crania and pelves of each individual to determine the preservation/recovery rates of skeletal elements. The recorded data were tabulated into Microsoft® Excel® for subsequent statistical analyses and creation of tables and graphs.

2.3 Results

Out of the 145 forensic cases analyed, eight individuals could not be sexed because of fragmentation or the absence of skeletal elements necessary for sex estimation, *i.e.*, lacked assessable cranium, mandible, and pelvis. A further 11 cases were sub-adult and could not be sexed using traditional methods. Therefore, these cases were collectively excluded from subsequent analyses (Appendix B: Table B1). Of the 126 individuals included for sexing, the majority were morphologically estimated as male (56%; 71/126), while 33% were female. Sex was ambiguous for 11% of individuals (Figure 2.3). The morphological scores for intra-observer error analysis demonstrated strong agreement with Cohen's kappa values exceeding 0.8. In contrast, inter-observer error analysis revealed moderate agreement with Cohen's kappa values above 0.6 (Appendix C). These high levels of agreement in both types of assessments indicate the reliability and consistency of the methods used.

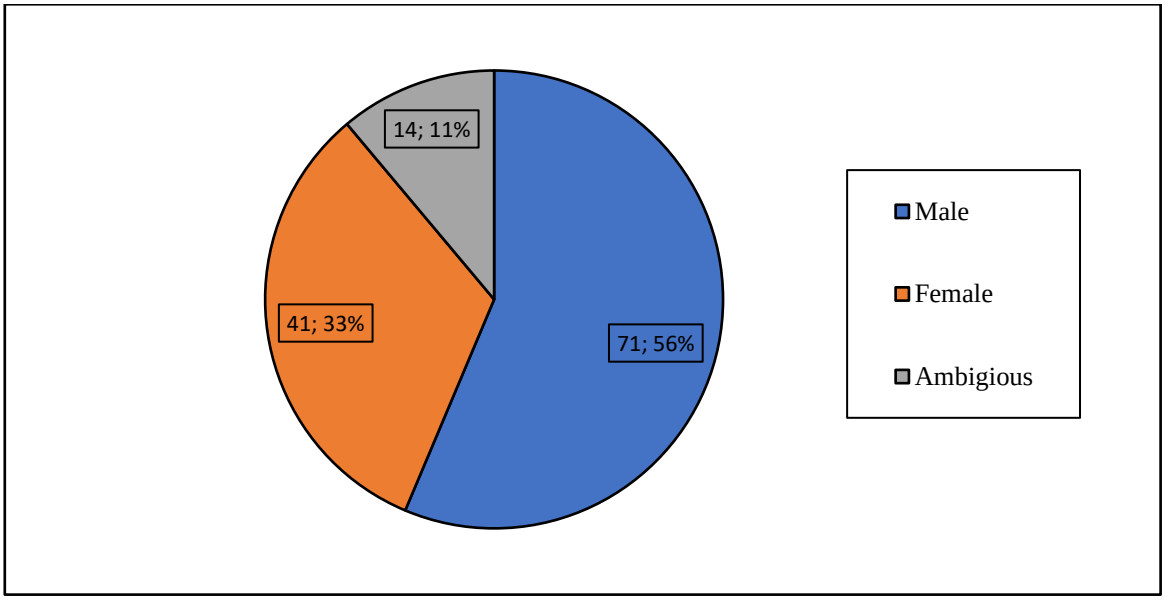

Figure 2.3: Sex distribution of adult forensic cases in the UCT Skeletal Repository.

Skeletal inventories were created for the crania, mandibles and pelves of each morphologically sexed individual (n = 126) (Appendix B: Table B3). Forty-six (36.5%) individuals were sexed based on the cranium, mandible, and pelvis and 30 (24%) individuals were assessed based on the cranium and mandible. Twenty-eight individuals (22%) were assessed on the cranium alone, twelve individuals (10%) were assessed on the pelvis, and one (0.8%) individual on the mandible. Nine (7%) individuals were assessed using the cranium and pelvis only. Among the three skeletal elements, the cranium was the most frequently recovered, appearing in 89.7% of

the individuals. This is followed by the mandible, present for 77 (61.1%) cases and the pelvis for 67 (53.2%) (Figure 2.4).

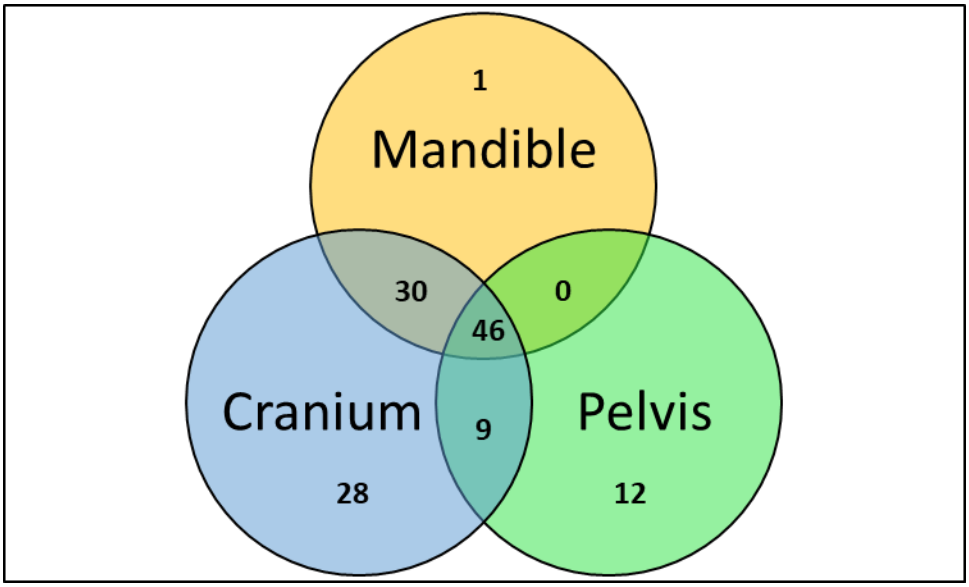

Figure 2.4: Skeletal elements present for estimated male, female and ambiguous individuals.

2.3.1 Considerations for molecular analyses

Due to the requirements of the MPhil minor dissertation, the sample size of 126 individuals for genetic analyses was too large. Therefore, the subset of anthropologically female cases was chosen for molecular analyses. Only anthropologically assessed female cases were included, there were 41 in total. These 41 cases were assessed for conditions of interment according to the criteria mentioned above to determine whether they were of forensic significance. This included duplicate graves (*i.e.*, cases pertaining to more than one individual, sharing a repository accession number), which were excluded from any subsequent molecular analyses due to most likely being buried at an intended grave site (Appendix B: Table B1). Thus 19 individuals were excluded for being probable or likely 'archaeological' (Appendix B: Table B4), leaving 22 cases for comparative molecular analyses.

2.4 Discussion

The aim of this studywas to estimate biological sex through morphological assessments of the pelvis, cranium, and mandible, and to evaluate both inter- and intra-rater reliability of these assessments. Skeletal inventories were compiled for the crania, mandibles, and pelves of each

individual whose sex had been morphologically determined. A subset of anthropologically identified female cases was selected for molecular analysis. Further scrutiny of these cases, considering the conditions of interment, led to the exclusion of some cases, resulting in a final set of 22 cases for comparative molecular analysis.

2.4.1 Morphological sex estimation

As previously mentioned, the pelvic bones, particularly the pubis, are the most reliable skeletal indicators of sex. Following this, postcranial measurements and then skull morphology and metrics are most reliable (Spradley & Jantz, 2011; Klales *et al.* 2012). However, in certain cases where these skeletal regions are absent, damaged or fragmented (such as in forensic investigations involving extensive burning or animal scavenging) the ability to assess sex can be affected. In this study, eight individuals (5.5%) could not be sexed due to fragmentation or missing skeletal elements. In such cases, alternative skeletal elements must be assessed. For this reason, research in sex estimation involves almost every region of the human skeleton in different geographical populations with varying accuracy rates.

Sex estimation of neonates or subadult skeletal individuals is a significant challenge for bioarchaeological and forensic work, as the skeletal morphology of juveniles do not exhibit sufficient sex differences in the prepubescent stage (Cunningham *et al*., 2016; Krishan *et al.*, 2016; Ubelaker & DeGaglia, 2017). This makes it difficult to assess the small differences between the sexes in juveniles by methods conventionally used for adult sex estimation, which are based on highly dimorphic traits of the pelvis and cranium. Forensic anthropologists do not attempt to estimate the sex of infants and children unless there genital soft tissue is present (Baliso *et al*., 2019). As a result, eleven individuals (7%) in this study were sub-adult and not sexed. Similarly, of the 75 individuals examined by FACT between 2008-2013, 10% were unsexed due to being infants and children (Baliso *et al*., 2019). To resolve this issue, research into the advancement of juvenile sex estimation methods has been ongoing, as reviewed by Wilson & Humphrey (2017), but they are too unreliable to use. Therefore, DNA analysis is preferred to determine sex. Age-at-death plays an integral role in sex estimation, and it is crucial to develop a reliable method for sex estimation in juveniles to advance bioarchaeological and forensic investigations.

Out of the 126 individuals who were included in the study for anthropological sex estimation, the majority were morphologically identified as male (56%), while females accounted for 33%. Results that were similar to those reported in the FACT repository, which found that most of

the deceased individuals examined between 2006-2018 were male at 67% (116 out of 174), with females accounting for 28% (49 out of 174) (Baliso *et al*., 2023). Moreover, this was also commonly observed by other forensic anthropology laboratories and mortuaries, locally (Steyn *et al*., 1997; Brits, Steyn & Hansmeyer, 2020; Reid *et al.* 2020) and internationally (Yaşar İşcan & Olivera, 2000; Grisbaum & Ubelaker, 2001; Komar, 2003; Vaz & Benfica, 2008; Evison *et al*., 2012; Parsons, 2017; Indra & Lösch, 2021).

2.4.2 Inventory of skeletal elements

The cranium, mandible, and long bones have a high proportion of cortical bone that are generally less susceptible to destruction. In contrast, bones with a higher proportion of spongy bone, such as the sternum, ribs, vertebrae, and the bones of the hands and feet, are more vulnerable to damage. This observation is well-supported by previous studies (Mays, 1992; Stojanowski *et al*., 2002; Manifold, 2012). The cranium was the most frequently recovered skeletal element, found in 89.7% of individuals, a finding consistent with Krüger *et al.*, (2015) , who also identified the cranium as the most commonly recovered element in South Africa. This prevalence is likely due to the cranium's robust structure. The mandible was present in 61.1% of cases, attributed to its dense bone composition and resistance to post-mortem changes, as noted by Steyn & İşcan (1998), Hu, Koh, Han, *et al.* (2006), and Franklin *et al.* (2008). Conversely, the pelvis was only present in 53.2% of individuals, aligning with Stojanowski, Seidemann & Doran's (2002) observation that the pelvis is less well-preserved than other bones with a higher proportion of cortical bone.

The context of bodies found in graves can provide valuable insights into forensic significance. For a death to be considered of medico-legal relevance, it must have occurred under unknown circumstances. Duplicate graves typically indicate formal burials, and such cases lack medico-legal significance, even if referred by SAPS and FPS for biological profiling. Consequently, nineteen individuals were excluded from molecular analyses as they were classified as 'archaeological or historic,' rendering them unrepresentative of the contemporary South African population.

2.5 Summary

Morphological sex estimation was conducted using the crania, mandibles, and pelves. From the study sample of 145 individuals, 11 sub-adult cases were excluded and a further eleven

individuals could not be sexed due to fragmentation or missing skeletal elements. Out of 126 individuals sexed, 56% were male, 33% female, and 11% ambiguous; 36.5% had all three skeletal elements available, with the cranium being the most frequently recovered. The results highlight the limitations of estimating sex through morphological analysis, which can be overcome by employing molecular testing methods. This is especially relevant for sub-adults, cases with heavily fragmented remains, and individuals whose skeletal features used for sex estimation are missing or show ambiguous morphology.

Due to previous discordance found in anthropological sex estimation and molecular testing in the two local forensic cases discussed previously, as presented in Finaughty *et al.*, (2020) and Bennett *et al.*, (2021), purpose of this dissertation was to further investigate the occurrence of males being incorrectly estimated as female in the Western Cape South African population. Forensic identification of human remains is particularly challenging in the Western Cape due to the heterogeneous genetic and ancestral make-up of the population (Tawha *et al.*, 2020). The Western Cape population has shown a high degree of genetic contribution from San and Khoe (de Wit *et al.*, 2010; Chimusa *et al.*, 2014; Hammarén *et al.*, 2022; Pfennig *et al.*, 2023) ancestry who are small bodied and more gracile, these genetic predisposition for smaller body size, likely contribute to the accuracy rate of assessment on morphology alone, as the sexual dimorphic robusticity of males when assessed may be perceived as more female or ambiguous traits. The question is how many skeletons which have been estimated to be female using anthropological methods are male? As such, solely morphologically female cases were selected for molecular sex testing. After the exclusion of the archaeological cases, 22 anthropologically female estimated individuals were taken forward for molecular analysis.

3 Chapter 3: Molecular sex determination

This chapter presents the methods, materials, and results of molecular sex determination. Thereafter, this chapter will detail the discussion and summary of these results. Following the morphological sex estimation (Chapter 2), 22 female estimated cases were selected for subsequent molecular analysis (Appendix D: Table D1).

3.1 Introduction

Molecular methods, particularly DNA analysis, have revolutionized forensic identification by enabling precise and reliable individual identification from biological samples. These techniques are especially valuable when morphological analysis is inconclusive due to poor preservation or fragmentary remains. By analyzing genetic markers, such as short tandem repeats (STRs), molecular methods can establish identity, kinship, or ancestry, playing a crucial role in solving criminal, civil and humanitarian cases, mass disasters, and historical investigations.

3.2 Materials and methods

Molecular sex determination was performed on bone and teeth samples and carried out in 5 general stages: i) sampling of bone and teeth, ii) decontamination of bone and tooth surfaces, iii) DNA extraction using the QIAamp® DNA Investigator Kit (QIAGEN, Germany), iv) DNA assessment using quantitative real-time PCR (qPCR) using the Quantifiler® Trio DNA Quantification Kit (Applied Biosystems, Foster City, USA) and v) DNA profiling by short tandem repeat (STR) analysis using the Investigator® Argus X-12 QS Kit (QIAGEN, Germany).

Biological sex was determined using qPCR and DNA profiling. Sex was assessed by qPCR using the Quantifiler® Trio DNA Quantification Kit to quantify the total amount of amplifiable human DNA and human male DNA in a sample. If the Y-Chromosome marker amplifies, there is reasonable evidence that the biological sex is male; however, if it does not amplify, then this could be due to the DNA belonging to a female, or that the DNA sample was insufficient in quality or quantity to be amplified at that marker. The results from the qPCR assay also assists in determining if the sample contains sufficient human DNA and/or human male DNA to proceed with STR analysis, as well as the amount of sample to use in subsequent STR analysis

applications. The biological sex results were then confirmed using DNA profiling by STR analysis using the Investigator® Argus X-12 QS Kit. Biological sex can be determined using almost any commercial STR profiling kit, and the choice of the use of the Investigator® Argus X-12 QS Kit in this study was purely due to availability in the laboratory at the time of the project. Detailed methodology for all stages of molecular determination is provided below.

3.2.1 Sample collection

A sample from each of the 22 included cases was obtained for molecular analyses. Selection of bone or teeth samples was dictated by the following criteria:

1. Preservation status
2. Availability
3. Minimally invasive collection
4. Sampling selection should not interfere with any known sites used in morphological or metrical analysis
5. Small bones were preferable (≤2 cm in length) so that bones did not need to be cut ahead of the grinding process (process described further on)

The order of priority for skeletal elements targeted was also guided by the International Commission on Missing Persons recommendations (ICMP, 2015). These guidelines prioritise the following elements based on samples most likely to yield suitable DNA: one healthy tooth (first molars); temporal/petrous bone; femur; tibia; pelvis; metatarsal and talus. Moreover, a meta-analysis by Finaughty *et al.*, (2022) reported that bone had a higher profiling success than teeth. When working with older human remains, it is common to find incomplete skeletons. In cases where only the torso was present, studies sampling the ribs and vertebrae have shown the best DNA yield success rates amongst other skeletal elements of the torso (Finaughty *et al.*, 2022).

Only one hard tissue sample was collected from each of the 22 individuals selected for molecular analysis (Appendix D: Table D1). Based on the above criteria 1-5, published guidelines and literature this study prioritised small bones such as metacarpals and metatarsals. A single loose first or second molar tooth per individual were chosen as the second-best sample. If not the case, then a tooth was removed from the jawbone using dental pliers if necessary. For individuals where teeth or small bones were unavailable (n=5), alternative samples were collected: a rib (n=2) or a portion of the petrous bone (n=3). The method of collection of

alternative samples is denoted as the ‘collection of alternative samples’, and further details are described in Appendix D.2. All case samples collected were placed into labelled evidence bags and sealed. Lastly, control samples consisted of fresh human teeth, donated from third molar (wisdom teeth) extractions from a female individual who had provided informed consent.

3.2.2 Sample preparation

Instruments and work surfaces were sterilized between the sampling of individual teeth or bones in 3 steps: 1) wiped with bleach (5% concentrate); 2) wiped with molecular biology grade (MBG) water; 3) wiped with 70% ethanol solution. Utensils such as those in the dissection kit and toothbrushes were soaked in 3.5% commercial bleach solution (No Name Brand, Pick-n-Pay, South Africa) for 5 minutes. Personal protective equipment consisted of a laboratory coat, disposable gloves, safety glasses, mop cap and face mask were worn to minimize the potential for contamination during sampling. Gloves were cleaned in between handling instruments and were changed between individuals’ teeth or bones.

Each hard tissue surface was scrubbed with 3.5% bleach solution, using a sterile toothbrush. The surface was then rinsed with MBG water and dried with a clean paper towel. Scrubbing of the surfaces were repeated using 70% ethanol solution. Subsequently, the samples were rinsed with MBG water and wiped dry with clean paper towel. Once decontaminated, the samples were held firmly with a sterile forceps. Additional decontamination of samples was performed using a Dremel tool with a 13-millimetre (mm) Dremel sanding band attachment to remove the outer layer of the tooth or bone surface while working directly under a fume hood. This was to remove minerals from the surface of the sample that may interfere with the PCR. The Dremel tool was used on a speed setting between 4 and 6, to prevent the generation of too much heat. A respirator was worn to avoid the inhalation of potential hazardous pathogens from bone or tooth dust. Sanding bands were replaced in between the processing of individual samples to prevent cross-contamination. Samples were collected in 15 millilitre (ml) sterile conical centrifuge tubes and kept on ice until they were ground later that same day. Subsequently, each sample was crushed into a fine powder using the TissueLyser II® system, oscillated at 30 Hz for 1 minute in a sterilized, ice-cold grinding jar. This method is denoted as the ‘sample grinding’, and details are described in Appendix D.3.

3.2.3 DNA extraction and optimisation

The study employed the QIAamp® DNA Investigator Kit (QIAGEN, Germany) for DNA extraction. This kit uses a spin-column method and is validated for forensic use. Furthermore,

the kit's protocol has been optimized for hard tissue samples, specifically within the Division of Forensic Medicine and Toxicology, and is used in ad hoc service delivery for the FPS.

Buffer preparations are detailed in Appendix E. For each sample, 0.05 g of the tooth or bone powder samples was measured using an electronic balance scale (Radwag: model AS 220.R2, Poland). The manufacturer's protocol provided guidelines for DNA extraction from bones and teeth, which served as the basis for the subsequent protocol modifications. The manufacturer's protocol was followed and included the following protocol modifications: Addition of a 1100 µl of 0.5M pH 8.0 ethylenediaminetetraacetic acid (EDTA) (Invitrogen, United States), 20 µl (1M) DTT (Tocris Bioscience, United Kingdom) and 0.0121 g SDS (Sigma-Aldrich, Missouri, USA) was added alongside buffer ATL and Proteinase K, overnight lysis took place for 20 hours at 56 °C at 450 rpm. In the final step DNA was eluted in 50 µl Buffer ATE. The manufacturer's protocol included the optional step of adding carrier RNA, however a previous study by (Longden-Thurgood, 2018) showed that the addition of carrier RNA did not produce any notable improvements in DNA yield. Thus, carrier RNA was omitted from the protocol. A DNA extraction blank (negative control) was processed alongside every sampling batch. The non-template control consisted solely of 50 µl of MBG water and buffers and reagents utilized in DNA extraction.

Preliminary data were obtained using the DNA extraction protocol mentioned above on a subset of five case samples. To enhance DNA recovery, the protocol was adjusted by reducing the final elution volume from 50 µl to 35 µl of Buffer ATE and was repeated on the same five cases.

After that, the final stage of optimization was carried out, which involved extending the incubation period from 20 hours to 24 hours. This final DNA extraction protocol was performed on all the case samples except for UCT 650 (which will be discussed in the results section below).

3.2.4 Assessment of DNA recovery and biological sex

The assessment of DNA quantity and quality was performed using a multiplex TaqMan® real-time PCR assay. DNA quantification using the Quantifiler® Trio DNA Quantification Kit on the 7500 Real-Time PCR thermal cycler (Applied Biosystems, Foster City, USA) was employed according to the manufacturer's user guide ("Quantifiler™ HP and Trio DNA Quantification Kits User Guide", p. 27-30). PCR reaction components were prepared using half volume reactions according to an internally optimized and validated protocol. Thermal cycling

was conducted following the manufacturer's user guide on a 7500 Real-Time PCR System. Data collection was carried out using HID Real-Time PCR Analysis software v1.2 (Applied Biosystems, Foster City, USA).

The Quantifiler™ Trio assay targets three regions: a small (80 bp) human autosomal target, a large (214 bp) human autosomal target, and a 75 bp region on the human Y chromosome. DNA quality was assessed in terms of both degradation and PCR inhibition levels. The degradation index (DI) was calculated using the ratio of the concentrations of the small and large autosomal targets, as described by Vernarecci *et al.* (2015). DI values were classified into four categories: 0-1.5 (non-degraded), 1.5-4 (mildly degraded), 4-10 (degraded), and >10 (severely degraded). An internal PCR control (IPC) of synthetic DNA was included in each reaction to detect PCR inhibitors, with IPC CT (threshold cycle) values greater than 28 indicating the presence of inhibitors (Fondevila *et al.*, 2008). The limit of detection for qPCR was 0.005 ng/µl, which refers to the lowest amount of target nucleic acid that can be reliably quantified.

3.2.5 DNA profiling

DNA profiling was performed to confirm biological sex using the Investigator® Argus X-12 QS Kit (QIAGEN, Germany). The Investigator Argus X-12 QS Kit multiplex system contains primers for amelogenin (AM) for gender determination and 12 X-chromosomal STR loci: DXS7132, DXS7423, DXS8378, DXS10074, DXS10079, DXS10101, DXS10103, DXS10134, DXS10135, DXS10146, DXS10148 and HPRTB. Each X-STR locus is a sex determining marker. A single peak would be observed at each X-STR marker if the individual is male (unless the female is homozygous), and two peaks would be expected if the individual is a female. Additionally, the autosomal STR marker D21S11 was included. The kit also includes a Quality Sensor (QS), acting as an internal performance control that determines if the PCR run was successful and/or if there was no DNA present in the sample.

This kit was used to prepare a multiplex PCR following the instructions provided in the technical manual ("Investigator Argus X-12 QS Handbook", p. 13-15). DNA profiling was only performed on samples where amplification on qPCR was detected (n = 12), as well as on one DNA extraction negative control. Due to very low DNA concentrations, the maximum volume (15 µl) of template DNA was added for each case sample. Thermal cycling was performed on a BioRad T100 thermal cycler (California, USA) according to the technical manual mentioned above.

After PCR, each amplified sample was transferred to an Applied Biosystems MicroAmp® optical 96-well plate, which contained DNA size standard 550 (BTO) and Hi-Di™ formamide (Promega, Madison, USA). Capillary electrophoresis was done according to the technical manual ("Investigator Argus X-12 QS Handbook", p.34-45) using the Applied Biosystems Genetic Analyser 3130xl, and the results were analysed using the Applied Biosystems GeneMapper®ID-X v1.5 software (Foster City, USA). An analytical (50 RFU) and stochastic (200 RFU) thresholds were applied.

3.2.6 Data analysis

Data were collated into tables in Excel, Version 2402 (Microsoft Corporation, USA). Graphs were generated in Excel and IBM SPSS Statistics, Version 29.0.0.0 (IBM, SPSS Inc., USA). The following variables were assessed: qPCR concentration in ng/mg, IPC C_T values, DI, the amplification of each STR sex marker and amplification of the QS marker. No statistical analysis was performed due to limited data (discussed below).

3.3 Results

3.3.1 Optimization

Controls as well as five cases were used for optimization. Quantitative real-time PCR was performed on the DNA extracted from these samples using the baseline protocol as well as the adjusted protocols. The first adjustment to the protocol was reducing the final elution volume from 50 µl to 35 µl of Buffer ATE which resulted in improved amplification of both the small (80 bp) and large (214 bp) autosomal markers for three of the five samples (Appendix E: Table E.1). Additionally, the amplification of the 75 bp target on the Y chromosome was quantified for two cases, UCT 364 and UCT 651, both of which were teeth samples. However, all DNA concentrations remained below 0.005 ng/µl. The degradation indices (DI) could be calculated for two case samples (UCT 233 and UCT 632) and were both classified as "degraded". The last DNA sample (UCT 650) consistently failed to amplify due to PCR inhibition (as shown by the non-amplification of the IPC), which unfortunately could not be overcome. The second adjustment was extending the incubation period from 20 hours to 24 hours which resulted in improved amplification of the small (80 bp) and/or large (214 bp) autosomal markers for three of the five samples. (Appendix E: Table E.2).

This final DNA extraction protocol was performed on all remaining case samples selected for molecular-based sex determination and qPCR was used to determine DNA quantity and quality as well as biological sex.

3.3.2 Assessment of DNA recovery and biological sex

While there was an overall increase in DNA concentration following protocol optimisation, low DNA concentrations were observed in all case samples. The amplification of large and/or small autosomal targets was unsuccessful in several samples (Figure 3.1).

Thirteen out of the 22 case samples (59%) were amplified during qPCR, with no amplifiable DNA detected in 9 case samples. Three case samples showed successful amplification of both the large and small autosomal targets and 10 case samples showed amplification of the small autosomal target only.

The Y chromosome marker was amplified in the following six case samples: UCT 102, UCT 350, UCT 354, UCT 631, UCT 651 and UCT 677; however, Y-target concentrations were below the limit of detection (<0.005 ng/µl). Thus, based off the qPCR results, seven cases were suggested to be female and concordant with anthropological sex results while six cases were possibly discordant. Sex results were inconclusive for nine cases based on these qPCR results (Figure 3.1).

The control's (C2) sex results were concordant with the known sex, being female (Appendix E: Table E.2). The control samples also showed concentrations within the dynamic range of the qPCR assay (>0.005 ng/µl) and these concentrations are consistent with the typical findings of our laboratory, which generally exceed 0.5 ng/µl when analyzing similar samples. The signal noted in the DNA extraction blank was 73 times below the limit of detection for DNA and is likely to be a fluorescent artefact within the qPCR assay, particularly as only the large autosomal target amplified and not the short autosomal target. Due to PCR kinetics affects, there is a tendency towards preferential amplification of shorter DNA fragments in PCR (Walsh *et al.*, 1992). No DNA was detected in any of the negative controls from the DNA extraction process, indicating the absence of exogenous contaminating DNA in the buffers or reagents and confirming that no additional sources of DNA were introduced during the extraction process.

DNA quality was assessed by the IPC amplification and its threshold cycle (C_T) in conjunction with the DI. For 18 case samples, the IPC C_T values were not greater than 28 (Appendix E: Table E.2). However, the IPC failed to amplify for four case samples (including UCT 650 seen in Appendix E: Table E.1), indicating PCR inhibition. Degradation indices could be calculated for three case samples, where the large fragment amplified, indicating non-degraded to mildly degraded sample qualities according to (Vernarecci *et al.*, 2015).

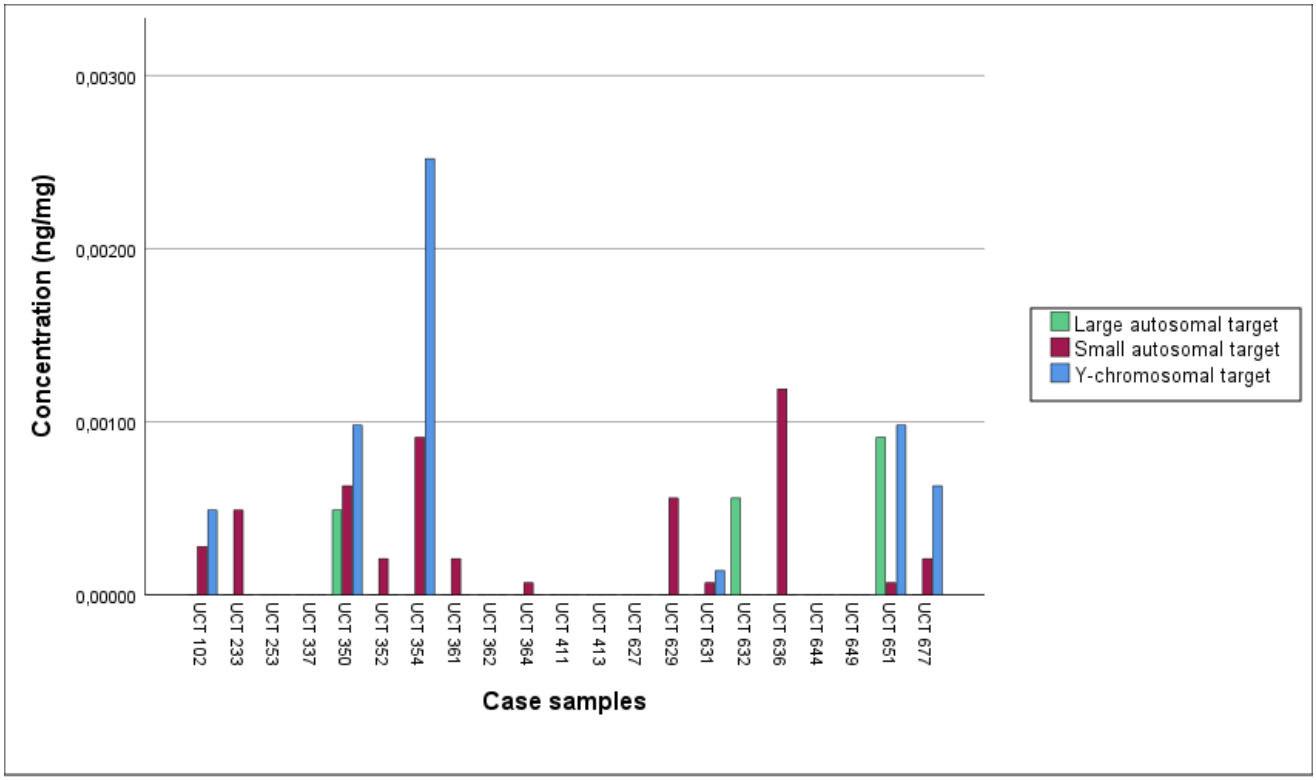

Figure 3.1: DNA concentrations for case samples as determined by qPCR **(**Quantifiler® Trio DNA Quantification Kit (Applied Biosystems, Foster City, USA)) using the optimized protocol for the QIAamp DNA Investigator Kit (QIAGEN, Germany), where there was a 24 hour lysis step and a final elution volume of 35 µl of Buffer ATE.

3.3.3 DNA profiling

DNA profiling was performed to verify biological sex results through the assessment of the amelogenin marker and 12 X-chromosomal STR loci, using the Investigator® Argus X-12 QS Kit (QIAGEN, Germany). DNA profiling was performed on a total of 12 DNA case samples, where amplification was detected in qPCR and one DNA extraction negative control (Table 3.1).

DNA profiling was unsuccessful for six out of the 12 DNA samples, where no STRs nor the QS amplified. Of the remaining six samples that had successful amplification as indicated by the amplification of the QS marker, three DNA samples (UCT 631, UCT 651 and UCT 677) had no STR peaks across the profile, indicating an absence of DNA or a very low amount of template DNA (<0.2 ng), and thus biological sex could not be determined. For the remaining three samples, one case sample has the X,Y genotype at the AMEL marker, and could be confirmed as male (UCT 632). The biological sex of the other two samples was inconclusive due to the height of the peaks not meeting interpretation thresholds. The negative control showed no signs of exogenous DNA contamination.

Table 3.1: DNA profiling results obtained of the Investigator® Argus X-12 QS Kit. Analysis was performed on an Applied Biosystems Genetic Analyser 3130xl using the QIAamp® DNA Investigator Kit. Allele assignment was performed using the GeneMapper® ID-X Software and the Investigator Argus X-12 QS Template File.

	STR loci														
Sample ID	**AMEL**	**DXS10103**	**DXS8378**	**DXS10101**	**DXS10134**	**DXS10074**	**DXS7132**	**DXS10135**	**DXS7423**	**DXS10146**	**DXS10079**	**HPRTB**	**DXS10148**	**D21S11**	**QS**
UCT 102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 233	X,?	18	-	-	-	-	-	-	-	33	-	-	-	-	Pass
UCT 350	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 352	-	15	-	-	-	-	-	-	-	-	-	-	-	-	Pass
UCT 354	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 364	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 629	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 631	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pass
UCT 632	X,Y	-	-	-	-	-	-	-	-	-	-	-	-	-	Pass
UCT 649	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Fail
UCT 651	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pass
UCT 677	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pass
NTC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Pass

QS = Quality sensor acting as an internal performance control that determines if the PCR run was successful or if there was no DNA present in the sample; NTC = Non-template control; - = no alleles called for STR marker; Amelogenin marker is denoted as 'AMEL'.

3.4 Discussion

This section aimed to perform molecular sex determination using bone and tooth samples, following a five-stage process that included sampling, decontamination, DNA extraction, assessment, and DNA profiling. DNA extraction was optimized by adjusting the elution volume and extending incubation periods, leading to improved DNA recovery. DNA quantity and quality were evaluated using quantitative real-time PCR (qPCR), and biological sex was confirmed through short tandem repeat (STR) analysis.

3.4.1 DNA extraction and optimisation

To address poor initial DNA recovery, we explored several DNA extraction protocol adjustments aimed at improving DNA recovery and reducing PCR inhibition. First, we decreased the final elution volume to 35 µl to increase the final concentration of DNA, resulting in some improvement in DNA sample amplification.

Subsequently, we assessed the length and temperature of the cell lysis step, with studies showing a maximum incubation of 24 hours at 56°C (Loreille *et al.*, 2007; Rohland & Hofreiter, 2007a,b; Hasan *et al.*, 2014; Dabney & Meyer, 2019). The lysis buffer contains proteinase K, which digests protein and inactivates nucleases that might cause DNA degradation (Higgins & Austin, 2013; Rohland *et al.*, 2018).

Additionally, EDTA is added to the lysis buffer to prevent the loss of DNA trapped within the mineral matrix of the bone or tooth. EDTA works by chelating mineral ions, such as calcium and magnesium ions, and deactivating DNAases to prevent further DNA degradation (Loreille *et al.*, 2007; Seo *et al.*, 2010; Harrel *et al.*, 2018; Rancourt *et al.*, 2023). Calcium can act as a Taq polymerase inhibitor by competitively binding to the polymerase instead of magnesium during PCR. This binding reduces the efficiency of amplification (Opel *et al*., 2010; Kuffel, Gray & Daeid, 2021). The addition of EDTA in the demineralization step and the increased demineralization duration collectively aim to reduce calcium ions and, consequently, minimize PCR inhibition. The incubation period was increased to 24 hours to minimize undissolved powder containing unextracted DNA, aiming to maximize recovery of PCR-amplifiable DNA from bone and teeth samples while minimizing the co-extraction of inhibitory substances.

3.4.2 DNA recovery

Fifty-six percent (13/22) of the case samples (59%) exhibited amplification during qPCR, as indicated by the presence of the small autosomal marker. However, only three of these samples

showed amplification of the large marker, suggesting significant DNA degradation overall. PCR inhibition has a more pronounced effect on the amplification of larger markers than on smaller ones (Holt *et al.*, 2016; Loftus *et al.*, 2017; Lackey, 2018). When inhibitors are present, they interfere with the polymerase enzyme's ability to function optimally, often by binding to the enzyme or the DNA, or by altering the reaction conditions (such as pH or magnesium ion concentration) (Hedman & Rådström, 2013). Consequently, the efficiency of amplification decreases more dramatically for larger markers, which have more complex structures and longer sequences that are more susceptible to these disruptions. Any inhibition that impairs the polymerase's performance will disproportionately affect the larger markers, leading to their preferential drop-out or underestimation in the qPCR results, while smaller, easier-to-amplify fragments may still be successfully amplified even in the presence of inhibitors.

PCR inhibition compromises the reliability of the Degradation Index (DI) by disproportionately affecting the amplification of larger DNA markers. According to the "QuantifilerTM HP and Trio DNA Quantification Kits User Guide", caution is advised when interpreting the degradation of a sample in the presence of PCR inhibition. A significant limitation of the Quantifiler™ Trio DNA Quantification Kit is that large markers may fail to amplify before the Internal Positive Control (IPC) marker in the presence of inhibition, potentially leading to misinterpretation of the sample's integrity. Additionally, the failure of the IPC to amplify in some samples further suggests PCR inhibition.

The results indicate that PCR inhibition is a key factor influencing the observed DNA concentrations and contributing to their apparent low levels (Hedman & Rådström, 2013; Thompson, Duncan & McCord, 2014). To address this, modifications such as increasing the demineralization incubation time could be explored not solely to increase DNA yield but also to mitigate PCR inhibition. This approach may improve DNA amplification, not by increasing the DNA concentration, but by reducing the presence of inhibitors that interfere with the PCR process. Addressing PCR inhibition is crucial; therefore, future work should incorporate strategies to decrease inhibition, such as adding glycerol, AmpSolution™, or dimethylsulfoxide (DMSO) during PCR. Given the inherent sensitivity of qPCR to PCR inhibition, observed DNA concentrations might be underestimations of the actual quantities, potentially masked by PCR inhibition. To investigate this further, the use of Qubit to quantify dsDNA in samples could be considered, despite its known limitations in terms of detection sensitivity for low-concentration samples. To further combat these challenges the following steps may be implemented: 1) Perform additional cleanup of the sample to remove potential

inhibitors, 3) Use a next generation STR kits for improved performance with inhibited samples, 4) Use an STR assay that includes a high number of miniSTR loci, such as the GlobalFiler™ and MiniFiler™ PCR Amplification Kits or used in conjunction, for maximum data recovery from degraded samples.

It is also important to consider stochastic variation in qPCR results. The Quantifiler™ Trio DNA Quantification Kit can detect DNA concentrations <5 pg/µL; however, at concentrations <5 pg/µL, stochastic effects, or the statistical effect of random sampling of alleles present at a very low copy number, can produce variability in assay results ("Quantifiler™ HP and Trio DNA Quantification Kits User Guide", pp 52.). Thus, DNA quantities in the low picogram range may not always provide reliable results, as samples with minimal or undetectable DNA might still produce successful amplification when using STR kits (Cupples *et al.*, 2009; Butler & Hill, 2010). Future work should include replicate analysis to confirm the absence of DNA in samples. If results indicate that insufficient DNA is present to perform STR analysis, several options are available to improve STR kit performance: 1) Re-extract the DNA and repeat qPCR or 2) Concentrate the sample and repeat qPCR or 3) Use an STR kit which allows for higher volume of DNA input, like the GlobalFiler™ PCR Amplification Kit.

Despite positive amplification results, the recovery of DNA from bones and teeth was poor. LCN DNA samples are generally classified as samples that have less than 100-200 pg of total DNA available for amplification due to either small sample size or other factors such as PCR inhibition or DNA degradation (Word, 2010). Stochastic effects are common with low-template DNA, leading to allele and locus drop-out in DNA profiles (Butler & Hill, 2010), resulting in limited information from the profiles. To enhance assay sensitivity for LCN DNA samples, increasing the number of PCR cycles from 28 to 31 or 34 cycles can be considered. However, Butler & Hill, (2010) found that increasing PCR cycles to just 31 can exacerbate stochastic effects, leading to severe peak imbalance, allelic drop-in and drop-out, and high stutter, particularly when PCR inhibition is present. Alternatively, increasing the injection voltage and time during capillary electrophoresis, a post-PCR adjustment, can be beneficial without exacerbating stochastic effects. Therefore, interpreting LCN profiles should be done with caution, and further analysis is necessary. Future work should focus on optimizing qPCR workflows to overcome PCR inhibition and generating final consensus profiles (Grisedale & van Daal, 2012). Alternatively, utilizing high-sensitivity quantification methods that do not rely on PCR, such as TapeStation, could be considered.

Taphonomic degradation processes such as oxidation and hydrolysis significantly reduce the quality and quantity of DNA available for analysis, leading to challenges in obtaining reliable information from degraded samples.

3.4.3 Biological sex

The Y chromosome marker was amplified in six case samples; however, Y-target concentrations were below the validated dynamic range (<0.005 ng/µl). Although LCN was found in all case samples, six of the cases could be inferred as cases of discordance due to amplification of the Y chromosome marker, albeit below the limit of detection. Due to LCN DNA further investigation into these six cases should be explored and quantification should be replicated to confirm the reliability of the results.

Although little information was provided by DNA profiling due to LCN, one case of discordance was confirmed (UCT 632). This discrepancy prompts further investigation into population anthropological population data.

3.5 Summary

Twenty-two anthropologically estimated female cases were selected for molecular analysis. One hard tissue sample was collected from each individual. A tooth or small bone was prioritised. Alternative samples including a rib or portion of the petrous bone was collected where teeth and small bones were not available. Samples were decontaminated and grinded into a fine powder.

DNA was extracted from hard tissue samples using an optimized workflow utilizing the QIAamp® DNA Investigator Kit. In an attempt to enhance DNA recovery, the protocol was adjusted by reducing the final elution volume from 50 µl to 35 µl of Buffer ATE and extending the cell lysis incubation period from 20 hours to 24 hours.

Sex was assessed by quantitative real-time PCR (qPCR) using the Quantifiler® Trio DNA Quantification Kit, detecting low copy number (LCN) DNA in all samples. Thirteen cases (13/22; 59%) were successfully amplified during qPCR, with six showing evidence of Y-chromosomal DNA and inferring male sex, while seven were suggested as female. Nine remained inconclusive as no DNA was detected. DNA profiling using the Investigator® Argus

X-12 QS Kit did not provide further clarity on the biological sex due to LCN DNA except in one instance, where the case sample was confirmed as male (X,Y).

In summation, these results present another confirmed case report of sex discordance between anthropological and DNA analysis for a sample of decedents from the Western Cape, South Africa. While qPCR suggested 54% (7/13) agreement and 46% (6/13) disagreement, confirmation in most cases was hindered by LCN DNA. This highlights the under-representation in population data for the Western Cape population and the limitations of molecular sex methods, particularly when working with challenging samples of this nature.

4 Chapter 4: Discussion, study limitations and conclusions

The aim of this dissertation was to assess the accuracy rates of anthropological methods of sexing in the forensic Western Cape, South African population. This was done by assessing the agreement of sexing methods in a Western Cape South African forensic sample of skeletonised individuals, who were morphologically estimated to be female.

The following chapter will present four key points related to forensic anthropology and sex estimation in the South African context. Firstly, it will discuss the accuracy comparison between morphological and molecular methods. Secondly, South Africa's population diversity will be described, and its implications for accurate sex estimation will be discussed. After that, the current morphological sex estimation methods will be examined in the context of the South African population. Finally, the chapter will explore the integration of anthropological and molecular sex determination methods. The study's limitations, recommendations for future research, and a conclusion summarizing the study's findings will also be provided.

4.1 Accuracy Comparison Between Morphological and Molecular Methods

Incorrect anthropological sex estimations underscore the need for reliable, population-specific methods in forensics (Stone *et al.*, 1996; Thomas *et al.*, 2016; Inskip *et al.*, 2019). These discrepancies have been observed worldwide. In Cape Town, two cases involved skeletons estimated as female by anthropological methods but identified as male by DNA analyses (Finaughty *et al.*, 2020; Bennett *et al.*, 2021). This study presents another confirmed case report of sex discordance for a sample of decedents from the Western Cape, which further suggests that the anthropological data for the Mixed Ancestry population may be under-represented. While there is plenty of data available for White and Black South Africans, but there is a significant scarcity of data for the Mixed Ancestry population, which constitutes the majority in the Western Cape province (Krüger *et al.*, 2018; Mokoena, Billings, Gibbon & Bidmos, 2019). Consequently, applying standards designed for Bantu-speaking and European populations to individuals of unknown ancestry can be problematic.

This is the first study of its kind to be conducted in South Africa, and it carries important consequences for forensic case work. Although qPCR results indicated 54% (7/13) agreement and 46% (6/13) disagreement, in most cases, confirmation was hindered by LCN DNA. Hence, these findings highlight the need for further research on population-specific anthropological

data for the diverse South African population and the implementation of improved molecular techniques for reliable DNA profiling.

4.2 Re-evaluating morphological sex estimation methods for the South African Population

Forensic anthropologists in South Africa depend on traditional methods of sex estimation, which have historically been based on demographically biased populations. The Phenice (1969) and Buikstra & Ubelaker (1994) and Walker (2008) methods, which rely on the pelvis and cranium, the two best morphological indicators of sex, respectively, have been used to estimate sex. Unfortunately, these methods have limitations, and their accuracy has been questioned when applied to the South African population.

To address this issue, researchers have investigated the accuracy of these morphological sex estimation methods in South Africans. A study by Kenyhercz *et al.* (2017) validated and recalibrated the Klales *et al.* (2012) method expanding the three pelvic traits originally described by (Phenice, 1969). Using a global model with Linear Discriminant Functions (LDF) the study demonstrated that this approach could be confidently applied to South Africans of European and Bantu-speaking ancestry with classification accuracies ranging from 90.6% to 99.2%. Similarly, Krüger *et al.* (2015) tested the reliability and accuracy of the cranial traits described by Walker (2008), based on the foundational work of Buikstra & Ubelaker (1994). Using log regression on South African populations of European and Bantu-speaking ancestry and found that low classification accuracies were achieved, ranging from 31-97%. Furthermore, they found a high frequency of intermediate traits for ‘black’ males and ‘white’ females, demonstrating decreased sexual dimorphism in the South African population. As a result, the study modified the Walker (2008) formulae for use in a contemporary South African population, which increased overall classification accuracies. However, individuals of Mixed Ancestry were not evaluated in either study, highlighting the need for the re-evaluation of sex estimation methods in South Africa.

The methodology for morphological sex estimation has inherent limitations. In this study, 11 cases involved sub-adults (juveniles), rendering conventional adult sex estimation methods, which rely on highly dimorphic traits of the pelvis and cranium, inapplicable. Consequently, further research is imperative to develop reliable morphological sex estimation methods

tailored for juveniles. In such scenarios, DNA analysis emerges as the preferred method for sex determination. Moreover, eight individuals could not be sexed due to severe fragmentation or the absence of skeletal elements. Unidentified remains in South Africa are often incomplete or fragmented due to taphonomic processes such as burning, dismemberment, and animal scavenging (Steyn *et al*., 1997; Asala, 2001; Asala, Bidmos & Dayal, 2004; Barrier & L'Abbé, 2008). In such instances, alternative sex estimation methods could be employed, contingent upon the available bones, as several sex estimation standards for the South African population exist, utilizing different skeletal elements with varying accuracy. Anthropological standards for sex estimation in the South African population, particularly among European and Bantu-speaking ancestries, encompass studies of various bones, including the cranium (Steyn & İşcan, 1998; Krüger *et al.*, 2015), mandible (Franklin *et al.*, 2006; Franklin *et al*., 2008), femur (Steyn & İşcan, 1997; Asala, 2001), foot (Bidmos & Dayal, 2003), humerus (Steyn & İşcan, 1999; Vance *et al*., 2011), radius and ulna (Barrier & L'Abbé, 2008), scapula (Macaluso, 2011), and sternum (Macaluso, 2010). However, very few studies have examined sex estimation in the Mixed Ancestry South African population, which include analysis of the crania (Franklin *et al.*, 2007; Maass & Friedling, 2019; Tawha *et al.*, 2020), clavicle (Scott *et al.*, 2018) and long bones (Fasemore *et al.*, 2018; Maass & Friedling, 2018; Mokoena *et al.*, 2019; Liebenberg *et al.*, 2019).

Another challenge in morphological sex estimation arises from cases where adult skeletons display ambiguous morphological characteristics. In this study, 11% of the individuals examined for sex estimation yielded ambiguous results. While the morphological sex of these individuals remains undetermined, this finding offers valuable insights into the effectiveness of the methods utilized and the extent of sexual dimorphism within the population group (Malek, Sealy & Gibbon, 2023).

Re-evaluating morphological sex estimation methods in the South African population is crucial for enhancing the accuracy and applicability of forensic anthropology in this diverse population. By considering factors such as population-specific variation and the limitations of existing methodologies, researchers can develop more reliable and efficient techniques for sex estimation. This not only improves the accuracy of forensic investigations, alleviating the burden of unidentified individuals, but also ensures that justice is served equitably across different demographic groups.

4.3 Integration of Anthropological and Molecular Methods

Anthropological sex estimation is a crucial aspect of forensic investigations, but numerous studies and case reports have shown that inaccurate estimations are prevalent. Relying solely on anthropological methods to determine sex, given their known rate of inaccuracy and the limited data for certain groups, such as the Mixed Ancestry population of South Africa, presents significant challenges. The occurrence of three documented cases of discordance (the two previous cases in Cape Town and the one from this study), with the potential for more, underscores the severe limitations of morphology-based methods alone. This reliance could lead to the provision of incorrect information to authorities, thereby negatively affecting the process of identification. Therefore, it is imperative to develop population-specific anthropological methods that are reliable in forensic contexts. While traditional methods of sex estimation have limitations, molecular analysis can help address some of these challenges.

Molecular sex analyses offer value in being independent from ancestry and scenarios involving juveniles, highly fragmented remains, and adult skeletons with ambiguous skeletal morphology. However, it is important to note that molecular methods also present their own limitations. Considered the gold standard for sex determination due to its high accuracy, molecular testing nevertheless presents practical challenges.

These challenges were evident in this study, notably with the issues encountered in low copy number (LCN) DNA analysis. Only 59% (13/22) of cases yielded DNA amplification, with six cases exhibiting traces of Y-chromosomal DNA. However, these results remained suggestive, given that DNA concentrations fell below the validated dynamic range. Additionally, while DNA profiling confirmed the male sex in one case, it failed to provide conclusive clarity on the biological sex of the remaining cases due to the presence of LCN DNA. A similar case report of discordance between anthropological assessment and DNA analysis was reported by Bennett *et al.* (2021).

The molecular preservation of skeletal remains is influenced by various complex variables, including both intrinsic factors and extrinsic factors. Intrinsic factors include bone type and density, which may impact DNA decomposition. According to a review by Latham & Miller (2019), reliable DNA profiles can be generated using dense cortical bone regions of the lower limb bones and teeth, as opposed to spongy bone (Edson *et al.,* 2004; Foran, 2006; Leney, 2006; Milos *et al.*, 2007; Mundorff *et al*., 2009; Latham *et al*., 2014). However, a meta-analysis conducted by Finaughty *et al.*, (2022) found that skeletal elements comprising a higher ratio

of cancellous to cortical bone produced significantly more full STR profiles. These elements include tarsals, phalanges, carpals, metacarpals, and metatarsals, which yielded the most DNA per gram (≥50 ng/g). Apart from bone type and density, age of the sample or post-mortem interval (PMI) is another intrinsic factor that affects DNA degradation.

After exposure to environmental conditions, high temperatures, specific traumatic incidents, or prolonged periods following an individual's death, skeletal components are typically the only biological remnants that endure. Over time, the molecules of DNA undergo fragmentation and chemical modification due to hydrolytic and oxidative chemical reactions, leading to their degradation (Latham *et al*., 2014). It should be noted that some of the individuals in this study were discovered as far back as the 1930s, which may have contributed to the significant degradation of the DNA. These remains were distinct from those typically encountered in forensic casework for two key reasons: 1) the remains were older, with a longer PMI than what is commonly seen in cases that enter the lab, and (ii) the remains had not been preserved in a fridge or freezer, which would have helped prevent further cellular degradation. In contrast, forensic cases usually referred for in casework tend to have a much shorter PMI, making the preservation of biological tissues less challenging.

Hence, when collecting biological material for DNA analysis, it is important to consider several extrinsic factors that can affect molecular preservation. These environmental factors include temperature, moisture levels, oxygen levels, soil pH, and soil composition (Latham & Miller, 2019). To slow down the rate of DNA degradation, it is crucial to store tissues in a cool and dry environment that limits the growth and activity of bacteria and fungi. These microorganisms tend to feed on human tissue and can cause rapid degradation. By maintaining low temperatures and low humidity, we can ensure the preservation of biological tissues for accurate and reliable DNA analysis.

However, in forensic cases, the analysis of DNA extracted from bones and teeth can be challenging, especially when the remains are buried in soil. The chemical composition of soil can cause bones and teeth to undergo chemical changes that can affect the rate and degree of DNA degradation. Additionally, soil solutes like tannins and humic acids can also mix with the DNA during extraction and hinder certain genetic analyses (Pate & Hutton, 1988; Collins *et al.*, 2002; Latham *et al*., 2014). Therefore, it is essential to carefully consider the soil composition and take appropriate measures to minimize any negative impact during DNA analysis. PCR inhibitors can negatively affect the amplification of DNA (including sex

markers), and can result in allele dropouts (Dash *et al*., 2020). Degraded samples may also result in ambiguous amelogenin sex results, producing DNA profiles with heterozygous imbalance, making analysis of the sex results challenging (Hansson *et al.*, 2017).

DNA extraction from hard tissues also presents several practical limitations and often requires optimisation of the workflow. When it comes to extracting DNA from skeletal remains, the objective is to optimize the yield of DNA, while minimizing any potential damage to the DNA, and effectively eliminating any inhibitors that may be present and could interfere with subsequent genetic analysis. This process is crucial to ensure accurate and reliable results. Sample preparation requires drilling equipment to cut bones, which leads to a risk of contamination and generation of heat (Zupanc *et al.*, 2021).

While molecular sex analysis can complement morphological sex estimation, it is not without its limitations and should not be solely relied upon. Additionally, with current backlogs at SAPS forensic science laboratories, DNA profiling is being underutilized, making anthropological methods a valuable tool in sex identification. Therefore, this study emphasizes the need for both molecular and anthropological methods to be used in conjunction with one another, to ensure the most accurate results in forensic identification.

4.4 Study limitations

The findings of the study have certain limitations, one of which was the inability to validate the results with further experiments due to time and availability of resources. Next-Generation Sequencing (NGS) could not be performed, and advanced multiplex kits such as GlobalFiler or PowerPlex Fusion 6C, which are highly effective for LCN DNA and PCR inhibition, could not utilized. Additionally, the use of AmpSolution during PCR was not tested. However, even if these techniques had been employed, challenges associated with LCN DNA and the accurate determination of biological sex from these samples would likely have persisted. This limitation is not inherent to the study itself but rather reflects the broader constraints of using DNA as a tool for determining biological sex. We have approached the interpretation of our results with caution, fully acknowledging the limitations associated with drawing conclusions from poor-quality DNA. Despite these limitations, we were able to confirm that one case was biologically male, even though it had been anthropologically estimated to be female. It is important to emphasize that we are not suggesting that either anthropological methods or DNA analysis are

without value. Rather emphasizing the collaboration of both molecular and anthropological methods, to ensure the most accurate results in forensic identification. Furthermore, a power analysis was not performed, as the aim of this study was not to generalize results. Hence, the minimum sample size for adequate statistical power was not of significance for this study.

As previously mentioned, ancestry plays an important role in the level of sexual dimorphism and can vary among different populations (MacLaughlin & Bruce, 1990; Maat *et al*., 1997; Bidmos & Dayal, 2004; Walker, 2005; Spradley & Jantz, 2011; Franklin *et al.*, 2013; Kotěrová *et al.*, 2017; Swift *et al*., 2024). While many skeletal sex traits are correlated with overall body size differences between males and females, certain skeletal elements like the pelvis are specifically linked to functional differences such as childbirth (Klales *et al.* 2012 ; Plavcan, 2012). Therefore, anatomical traits of the pelvis remain consistent across human populations, regardless of ancestry or other specificities (Ubelaker & Volk, 2002). However, the cranium is associated with muscular robusticity and energy requirements (Franklin *et al.*, 2007; Bigoni *et al*., 2010) and may contain population-specific features influenced by genetics and the environment (Sparks & Jantz, 2002; Van Vark *et al*., 2003). As such, the unknown ancestry of the sample group could be a limiting factor in the accuracy of cranial sex estimates in the current study. Known ancestry could also provide more information on which population group in South Africa may be under-represented.

4.5 Recommendations for future research

Further research is needed to unravel the complexities of South Africa's diversity and its implications for accurate sex estimation. To validate the findings of this study, future investigations should be conducted on a larger sample. The main objective of this study was to evaluate the accuracy of sex determination methods in a forensic sample of skeletonised individuals from the Western Cape region of South Africa who were morphologically classified as female. Therefore, additional studies should be carried out to examine the agreement between morphologically classified males and DNA analyses. Furthermore, identifying the most accurate skeletal sex traits for the Western Cape population could be an important area of research. Future studies should also focus on sub-adults, individuals with ambiguous morphological sex, and highly fragmented remains.

It would be worthwhile to explore molecular methods that are more sensitive to low copy number and fragmented DNA. One approach could be to investigate the use of an STR assay that includes a high number of miniSTR loci, such as the GlobalFiler™ PCR Amplification Kits, which allows for maximum data recovery from degraded samples and expanded DNA input volume provides maximum sensitivity for low level DNA samples. Additionally, more sensitive methods such as next-generation sequencing should be investigated.

The question of whether metrical methods would yield different results also warrants further investigation, requiring a study focused on metric analysis for sex estimation from skeletal remains. While extracting DNA from all forensic sample cases was beyond the scope of this study, including all individuals would offer valuable insights into the anthropological population data for the Western Cape.

4.6 Conclusions

The purpose of this study was to evaluate the concordance of sexing methods in a Western Cape South African forensic sample of skeletonised individuals, who were morphologically estimated to be female. Morphological sex estimation involved assessing the crania, mandibles, and pelves. Out of the initial sample of individuals, sub-adult cases were excluded, and an additional group could not be sexed due to skeletal fragmentation or missing elements. Among the sexed individuals, males were observed more frequently than females, while sex determination remained ambiguous for some individuals. Only a subset of individuals had all three skeletal elements available for sexing, with the cranium being the most frequently recovered skeletal element, followed by the mandible and the pelvis.

These findings underscore the challenges inherent in morphological sex estimation, particularly in cases involving sub-adults, heavily fragmented remains, or missing skeletal features that are crucial for accurate determination. Previous discrepancies between anthropological sex estimation and molecular testing in local forensic cases prompted an investigation into the accuracy of anthropological sex estimation, specifically focusing on cases initially classified as female.

A subset of initially morphologically estimated females was excluded from subsequent analyses due to their classification as 'archaeological or historic' cases. Consequently, a subset of 22 cases was selected for further molecular analysis.

One hard tissue sample was collected per individual selected for molecular analysis, with preference given to teeth or small bones. In cases where these were unavailable, alternative samples such as ribs or portions of the petrous bone were collected. Samples were decontaminated and ground into a fine powder before DNA extraction using the QIAamp® DNA Investigator Kit. Adjustments to the protocol were made to enhance DNA recovery by decreasing PCR inhibition.

Sex determination using the Quantifiler® Trio DNA Quantification Kit, detecting apparent low concentrations of DNA due to PCR inhibition. DNA from cases were successfully amplified during qPCR, with six indicating male sex through the presence of Y-chromosomal DNA, while seven were suggested as female. Nine cases remained inconclusive due to undetected DNA. DNA profiling using the Investigator® Argus X-12 QS Kit did not provide further clarity on biological sex due to LCN DNA, except in one case where the sample was confirmed as male (X,Y).

In summary, these results highlight another instance of sex discordance between anthropological and DNA analysis in a sample of decedents from the Western Cape, South Africa. While qPCR suggested additional instances of discordant sex results, confirmation was hindered by LCN DNA and/or PCR inhibition. These results underscore the potential under-representation in anthropological population data for the Western Cape as well as the limitations of molecular sex determination methods, particularly with challenging samples. These findings emphasise the necessity to update anthropological data for the contemporary South African population and implement improved molecular techniques for reliable DNA profiling. Addressing these limitations will aid in the identification of unidentified decedents, thus alleviating a significant burden in South Africa. Furthermore, this study underscores the importance of interdisciplinary collaboration for accurate forensic identification.

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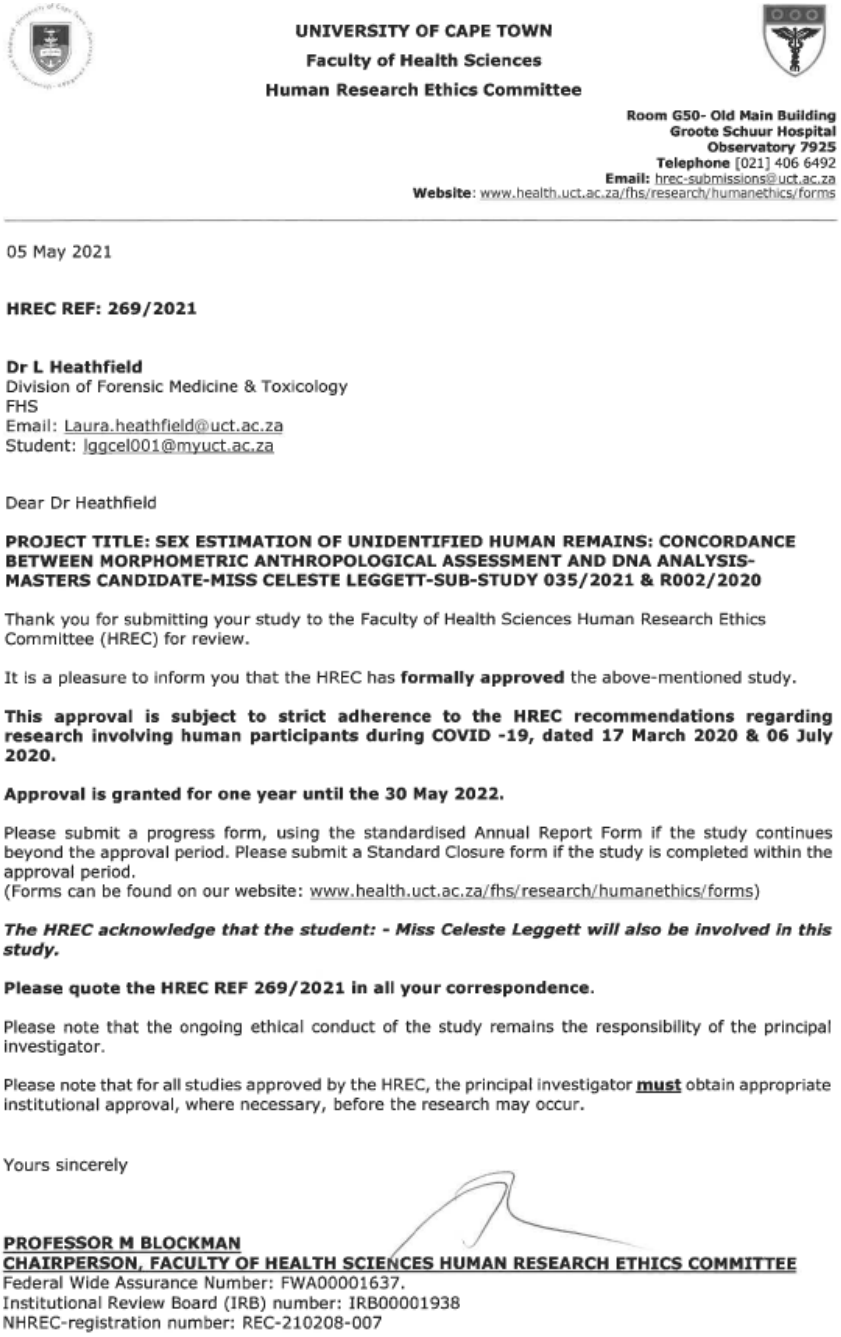
Appendix A: Ethics approval

A.1 Formal ethical approval



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room G50- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-submissions@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

05 May 2021

HREC REF: 269/2021

Dr L Heathfield
Division of Forensic Medicine & Toxicology
FHS
Email: Laura.heathfield@uct.ac.za
Student: lggcel001@myuct.ac.za

Dear Dr Heathfield

PROJECT TITLE: SEX ESTIMATION OF UNIDENTIFIED HUMAN REMAINS: CONCORDANCE BETWEEN MORPHOMETRIC ANTHROPOLOGICAL ASSESSMENT AND DNA ANALYSIS-MASTERS CANDIDATE-MISS CELESTE LEGGETT-SUB-STUDY 035/2021 & R002/2020

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020 & 06 July 2020.

Approval is granted for one year until the 30 May 2022.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: - Miss Celeste Leggett will also be involved in this study.

Please quote the HREC REF 269/2021 in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FACULTY OF HEALTH SCIENCES HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

Figure A1: Formal ethical approval granted by the Human Research Ethics Committee, University of Cape Town, for the present study (2021) (HREC ref: 269/2021).

Appendix B: Anthropological sample information and raw data

B.1 Study sample

Table B1: University of Cape Town's Human Skeletal Repository accession numbers of the total sample.

1.	**UCT 69**	**41.**	**UCT 356**	**81.**	**UCT 574**	**121**	**UCT 639**[+]
2.	UCT 102	**42.**	UCT 357[+]	**82.**	UCT 575	**122.**	UCT 640
3.	UCT 104[#]	**43.**	UCT 358	**83.**	UCT 575	**123**	UCT 641
4.	UCT 104a[#]	**44.**	UCT 359	**84.**	UCT 576	**124.**	UCT 642
5.	UCT 105	**45.**	UCT 360	**85.**	UCT 594	**125.**	UCT 644
6.	UCT 111	**46.**	UCT 361	**86.**	UCT 599	**126.**	UCT 645
7.	UCT 136	**47.**	UCT 362	**87.**	UCT 600 (1)[#]	**127.**	UCT 646
8.	UCT 142	**48.**	UCT 364	**88.**	UCT 600(2)[#]	**128.**	UCT 647a[#]
9.	UCT 143	**49.**	UCT 366	**89.**	UCT 603	**129.**	UCT 647b[#]
10.	UCT 153	**50.**	UCT 367[+]	**90.**	UCT 604	**130.**	UCT 648[+]
11.	UCT 155	**51.**	UCT 368	**91.**	UCT 607(1)[#]	**131.**	UCT 649
12.	UCT 176[+]	**52.**	UCT 369	**92.**	UCT 607(2)[*#]	**132.**	UCT 650
13.	UCT 219	**53.**	UCT 371	**93.**	UCT 607(3)[*#]	**133.**	UCT 651
14.	UCT 221	**54.**	UCT 400	**94.**	UCT 607(4)[*#]	**134.**	UCT 653
15.	UCT 231[+]	**55.**	UCT 401	**95.**	UCT 608(1)[#]	**135.**	UCT 669
16.	UCT 233	**56.**	UCT 402[*]	**96.**	UCT 608(2)[#]	**136.**	UCT 671
17.	UCT 253	**57.**	UCT 403	**97.**	UCT 608(3)[#]	**137.**	UCT 672
18.	UCT 271	**58.**	UCT 404	**98.**	UCT 608(4)[#]	**138.**	UCT 673
19.	UCT 295[+]	**59.**	UCT 405	**99.**	UCT 608(5)[#]	**139.**	UCT 674
20.	UCT 311[#]	**60.**	UCT 406	**100.**	UCT 611[#]	**140.**	UCT 675
21.	UCT 313(1)[#]	**61.**	UCT 407	**101.**	UCT 611(a)[#]	**141.**	UCT 677
22.	UCT 313(2)	**62.**	UCT 409[*]	**102.**	UCT 611(b)[#]	**142.**	UCT 678
23.	UCT 314	**63.**	UCT 410	**103.**	UCT 617	**143.**	UCT 680
24.	UCT 315a#	**64.**	UCT 411	**104.**	UCT 619	**144.**	UCT 682
25.	UCT 315b[*#]	**65.**	UCT 413	**105.**	UCT 621	**145.**	UCT 683
26.	UCT 315c[#]	**66.**	UCT 416	**106.**	UCT 623		
27.	UCT 315d[*#]	**67.**	UCT 438	**107.**	UCT 625		
28.	UCT 316	**68.**	UCT 440	**108.**	UCT 626		
29.	UCT 337	**69.**	UCT 441	**109.**	UCT 627		
30.	UCT 338[+]	**70.**	UCT 442	**110.**	UCT 628		
31.	UCT 339	**71.**	UCT 443	**111.**	UCT 629		
32.	UCT 340	**72.**	UCT 444	**112.**	UCT 630		
33.	UCT 341	**73.**	UCT 452	**113**	UCT 631		
34.	UCT 342	**74.**	UCT 453[+]	**114.**	UCT 632		
35.	UCT 350	**75.**	UCT 454	**115**	UCT 633		
36.	UCT 351	**76.**	UCT 462	**116.**	UCT 634		
37.	UCT 352	**77.**	UCT 464	**117.**	UCT 635[+]		
38.	UCT 353	**78.**	UCT 564	**118.**	UCT 636		
39.	UCT 354	**79.**	UCT 573	**119.**	UCT 637		
40.	UCT 355[+]	**80.**	UCT 574	**120.**	UCT 638[*]		

**Individuals excluded from sex estimation due to severe fragmentation or missing skeletal elements required for morphological sex estimation i.e., cranium, mandible and pelvis..*
+ Sub-adults excluded from morphological sex estimation.
 # Individuals excluded as forensic cases due to being part of duplicate graves.

B.2 Morphological sex estimation raw data

Table B2: Morphological sex estimation data.

Accession no.	Phenice Method			Supplementary pelvic traits		Walker method					Mandibular traits		Final sex estimate
	Subpubic Concavity	*Ischiopubic Ramus*	*Ventral Arc*	*Pre-auricular Sulcus*	*Greater Sciatic Notch*	*Nuchal Crest*	*Mastoid Process*	*Supra-orbital Margin*	*Glabella*	*Mental Eminence*	*Mandibular Shape*	*Gonial Eversion*	
UCT 69	X	X	X	X	X	3	5	4	5	4	5	2	M
UCT 102	X	X	X	X	X	3	1	1	X	X	X	X	F
UCT 104	X	X	X	4	2	4	X	3	X	X	X	X	M
UCT 104a	X	X	X	X	X	3	2	X	X	X	X	X	A
UCT 105	X	X	X	X	X	3	3	5	2	3	2	3	M
UCT 111	X	X	X	X	X	4	4	4	2	X	X	X	M
UCT 136	3	3	4	3	3	4	5	4	3	3	5	3	M
UCT 142	X	X	X	X	X	2	3	4	4	5	5	5	M
UCT 143	X	X	X	X	X	5	5	4	4	4	3	4	M
UCT 153	X	X	X	2	1	X	X	X	X	X	X	X	F
UCT 155	3	2	5	4	4	X	5	5	5	5	3	3	M
UCT 219	5	4	5	4	3	5	1	3	3	4	3	2	M
UCT 221	X	X	4	4	4	2	1	2	1	X	X	X	A
UCT 233	2	3	2	2	3	3	2	2	2	1	3	3	F
UCT 253	X	X	X	X	X	2	1	4	2	4	2	3	F
UCT 271	X	X	X	X	X	4	2	2	4	X	X	X	A
UCT 311	X	X	X	X	X	4	3	4	3	X	X	X	M
UCT 313(1)	X	X	X	X	X	4	4	X	X	4	1	2	M
UCT 313(2)	X	X	X	X	X	X	X	3	X	X	X	X	A
UCT 314	3	3	4	4	4	5	4	4	2	5	5	2	M
UCT 315a	X	X	X	X	X	X	3	3	X	X	X	X	A
UCT 315c	X	X	X	X	X	3	1	X	X	3	3	2	F
UCT 316	X	X	X	X	X	3	3	4	2	5	5	4	M
UCT 337	X	X	X	X	X	2	1	2	X	3	3	X	F
UCT 339	3	2	3	3	4	4	3	5	3	3	2	4	M
UCT 340	4	3	3	4	3	5	3	4	4	3	4	3	M
UCT 341	3	2	4	3	4	5	4	3	4	3	3	3	M
UCT 342	X	X	3	3	5	3	4	3	X	1	5	3	M

UCT 350	X	X	X	X	X	2	1	1	1	X	X	X	F
UCT 351	4	2	3	3	4	3	2	2	5	3	1	3	M
UCT 352	3	2	1	2	2	3	2	3	4	X	X	X	F
UCT 353	4	4	3	3	3	3	3	4	3	3	4	2	M
UCT 354	1	1	1	2	1	2	1	3	1	2	2	2	F
UCT 356	X	X	X	X	X	2	3	3	5	3	3	4	M
UCT 358	X	X	X	X	X	4	3	3	4	5	3	2	M
UCT 359	X	X	X	X	X	4	4	4	3	5	4	3	M
UCT 360	X	X	X	X	X	3	4	4	3	2	2	2	M
UCT 361	X	X	X	X	X	3	2	2	1	X	X	X	F
UCT 362	2	2	4	2	2	3	1	3	2	2	3	2	F
UCT 364	X	X	X	X	X	4	1	1	1	3	3	2	F
UCT 366	4	2	5	4	5	3	4	2	1	X	X	X	M
UCT 368	5	3	4	4	4	3	3	1	1	3	4	2	M
UCT 369	X	X	X	4	4	5	3	1	1	4	5	4	M
UCT 371	5	3	2	3	4	3	3	3	2	4	4	3	M
UCT 400	3	3	4	4	5	4	4	2	4	4	4	4	M
UCT 401	4	2	4	3	4	3	2	1	3	X	X	X	M
UCT 403	3	3	4	4	3	4	5	5	2	3	3	3	M
UCT 404	X	X	X	X	X	3	2	3	2	X	X	X	F
UCT 405	X	X	X	X	X	4	3	3	4	X	X	X	M
UCT 406	X	X	X	X	X	X	3	3	X	X	X	3	A
UCT 407	4	3	4	3	4	5	4	3	4	3	4	5	M
UCT 410	X	3	4	X	X	5	4	4	2	X	X	4	M
UCT 411	2	1	2	1	1	X	X	X	X	X	X	X	F
UCT 413	3	3	2	1	2	3	1	1	1	X	X	2	F
UCT 416	5	2	5	4	4	4	3	5	5	2	3	3	M
UCT 438	X	X	X	X	X	4	4	4	4	X	X	X	M
UCT 440	X	X	X	X	X	4	1	4	5	3	4	4	M
UCT 441	X	X	X	X	X	5	4	3	X	X	X	X	M
UCT 442	5	2	4	4	5	3	3	1	X	3	5	2	M
UCT 443	X	X	X	X	X	X	2	3	3	3	3	3	A
UCT 444	X	X	X	4	2	2	1	1	1	4	3	2	F
UCT 452	4	2	4	4	3	3	4	4	1	2	5	4	M
UCT 454	5	3	4	4	3	4	3	X	3	1	3	3	M
UCT 462	1	1	1	2	1	2	2	2	1	2	3	3	F
UCT 464	X	X	X	4	3	X	X	X	X	X	X	X	M
UCT 564	4	3	5	4	3	4	1	4	3	2	5	3	M

UCT 573	X	X	X	X	X	5	5	2	3	3	3	2	M
UCT 574	X	X	X	X	X	5	5	2	5	X	X	X	M
UCT 574	X	X	X	X	X	5	4	3	5	X	X	X	M
UCT 575	X	X	X	X	X	4	3	2	4	2	4	3	M
UCT 575	3	2	4	4	5	4	3	2	4	3	4	3	M
UCT 576	4	3	4	4	3	X	X	X	X	X	X	X	M
UCT 594	X	X	X	X	4	4	3	1	1	4	4	3	M
UCT 599	4	4	4	4	4	4	3	2	5	2	3	2	M
UCT 600 (1)	X	X	X	X	X	4	2	1	1	3	2	2	F
UCT 600(2)	X	X	X	X	X	X	X	3	X	X	X	2	A
UCT 603	X	X	X	X	X	2	X	3	X	X	X	X	A
UCT 604	X	X	X	X	X	4	4	2	5	3	4	2	M
UCT 607(1)	X	X	X	X	X	X	1	3	X	X	X	2	F
UCT 608(1)	X	X	X	X	X	2	2	3	X	X	X	X	F
UCT 608(2)	X	X	X	X	X	X	2	5	2	X	X	X	F
UCT 608(3)	X	X	X	X	X	X	X	X	X	2	1	3	F
UCT 608(4)	X	X	X	3	1	X	X	X	X	X	X	X	F
UCT 608(5)	X	X	X	3	2	X	X	X	X	X	X	X	F
UCT 611	X	X	X	3	5	X	X	X	X	X	X	X	A
UCT 611(a)	X	X	X	X	X	2	2	2	2	2	1	2	F
UCT 611(b)	X	X	X	X	X	1	1	1	1	X	X	X	F
UCT 617	2	3	X	3	2	X	X	X	X	X	X	X	F
UCT 619	X	X	X	X	X	3	1	1	1	3	3	X	F
UCT 621	X	X	X	4	4	3	X	3	X	X	X	X	M
UCT 623	5	3	4	3	4	4	2	3	4	3	1	4	M
UCT 625	4	2	3	4	4	3	3	3	4	4	5	2	M
UCT 626	5	3	3	4	4	4	4	5	5	4	2	3	M
UCT 627	3	1	X	3	1	X	X	X	X	X	X	X	F
UCT 628	3	3	4	3	3	4	5	5	5	4	4	2	M
UCT 629	3	1	1	3	1	2	2	1	1	2	1	2	F
UCT 630	3	2	5	4	4	4	3	4	2	2	3	3	M
UCT 631	3	1	2	2	1	3	1	3	2	3	2	3	F
UCT 632	5	2	2	3	1	3	4	1	1	3	3	3	F
UCT 633	X	X	X	X	X	4	3	5	5	1	3	3	M
UCT 634	3	3	5	3	4	X	X	X	X	X	X	X	M
UCT 636	3	1	4	3	3	1	1	3	1	1	2	2	F
UCT 637	X	2	1	4	2	5	4	3	1	X	X	X	A
UCT 640	3	2	4	4	3	3	3	3	5	2	4	4	M

UCT 641	5	2	3	4	4	4	5	3	1	4	4	2	M
UCT 642	X	X	X	4	5	3	2	4	3	4	4	2	M
UCT 644	X	X	X	X	X	3	X	2	2	X	X	X	F
UCT 645	X	X	X	X	X	4	4	2	1	X	X	X	A
UCT 646	X	X	X	X	X	1	1	1	1	1	3	2	F
UCT 647a	X	X	X	X	X	X	X	3	1	X	X	X	A
UCT 647b	X	X	X	X	X	2	X	5	4	X	X	X	M
UCT 649	X	X	X	X	X	4	2	2	1	X	X	X	F
UCT 650	X	X	X	X	X	3	2	1	1	3	3	3	F
UCT 651	X	X	X	X	X	1	3	3	1	X	X	X	F
UCT 653	X	X	X	X	X	2	1	5	1	1	3	2	F
UCT 669	X	X	X	X	X	4	3	4	1	X	X	X	M
UCT 671	X	X	X	4	5	4	3	4	3	X	X	X	M
UCT 672	5	2	4	4	5	2	5	5	2	3	5	5	M
UCT 673	5	2	5	X	3	X	X	X	X	X	X	X	M
UCT 674	5	2	3	4		X	X	X	X	X	X	X	M
UCT 675	3	3	X	4	3	4	2	4	5	X	X	X	M
UCT 677	X	X	X	X	X	1	2	2	1	X	X	X	F
UCT 678	X	X	X	X	X	4	5	5	5	X	X	X	M
UCT 680	X	X	X	X	X	3	2	3	X	3	3	3	A
UCT 682	4	4	4	4	5	4	3	2	3	3	3	5	M
UCT 683	X	X	X	4	3	2	2	1	2	3	4	3	F
Final sex estimate is determined from cranial, mandibular**,** and pelvic traits, collectively. M = Male; F = Female; A = Ambiguous. X = trait not assessed.													

B.3 Skeletal element survey

Table B3: Skeletal element survey for individuals from the University of Cape Town (UCT) Skeletal Repository included in morphological sex estimation.

	Accession no.	Skeletal element		
		Cranium	Mandible	Pelvis
	UCT 69	Present	Present	Absent
	UCT 102	Present	Absent	Absent
	UCT 104	Present	Absent	Present
	UCT 104a	Present	Absent	Absent
	UCT 105	Present	Present	Absent
	UCT 111	Present	Absent	Absent
	UCT 136	Present	Present	Present
	UCT 142	Present	Present	Absent
	UCT 143	Present	Present	Absent
	UCT 153	Absent	Absent	Present
	UCT 155	Present	Present	Present
	UCT 219	Present	Present	Present
	UCT 221	Present	Absent	Present
	UCT 233	Present	Present	Present
	UCT 253	Present	Present	Absent
	UCT 271	Present	Absent	Absent
	UCT 311	Present	Absent	Absent
	UCT 313(1)	Present	Present	Absent
	UCT 313(2)	Present	Absent	Absent
	UCT 314	Present	Present	Present
	UCT 315a	Present	Absent	Absent
	UCT 315c	Present	Present	Absent
	UCT 316	Present	Present	Absent
	UCT 337	Present	Present	Absent
	UCT 339	Present	Present	Present
	UCT 340	Present	Present	Present
	UCT 341	Present	Present	Present
	UCT 342	Present	Present	Present
	UCT 350	Present	Absent	Absent
	UCT 351	Present	Present	Present
	UCT 352	Present	Absent	Present
	UCT 353	Present	Present	Present
	UCT 354	Present	Present	Present
	UCT 356	Present	Present	Absent
	UCT 358	Present	Present	Absent
	UCT 359	Present	Present	Absent
	UCT 360	Present	Present	Absent
	UCT 361	Present	Absent	Absent
	UCT 362	Present	Present	Present
	UCT 364	Present	Present	Absent
	UCT 366	Present	Absent	Present
	UCT 368	Present	Present	Present
	UCT 369	Present	Present	Present

	UCT 371	Present	Present	Present
	UCT 400	Present	Present	Present
	UCT 401	Present	Absent	Present
	UCT 403	Present	Present	Present
	UCT 404	Present	Absent	Absent
	UCT 405	Present	Absent	Absent
	UCT 406	Present	Present	Absent
	UCT 407	Present	Present	Present
	UCT 410	Present	Present	Present
	UCT 411	Absent	Absent	Present
	UCT 413	Present	Present	Present
	UCT 416	Present	Present	Present
	UCT 438	Present	Absent	Absent
	UCT 440	Present	Present	Absent
	UCT 441	Present	Absent	Absent
	UCT 442	Present	Present	Present
	UCT 443	Present	Present	Absent
	UCT 444	Present	Present	Present
	UCT 452	Present	Present	Present
	UCT 454	Present	Present	Present
	UCT 462	Present	Present	Present
	UCT 464	Absent	Absent	Present
	UCT 564	Present	Present	Present
	UCT 573	Present	Present	Absent
	UCT 574	Present	Absent	Absent
	UCT 574	Present	Absent	Absent
	UCT 575	Present	Present	Absent
	UCT 575	Present	Present	Present
	UCT 576	Absent	Absent	Present
	UCT 594	Present	Present	Present
	UCT 599	Present	Present	Present
	UCT 600 (1)	Present	Present	Absent
	UCT 600(2)	Present	Present	Absent
	UCT 603	Present	Absent	Absent
	UCT 604	Present	Present	Absent
	UCT 607(1)	Present	Present	Absent
	UCT 608(1)	Present	Absent	Absent
	UCT 608(2)	Present	Absent	Absent
	UCT 608(3)	Absent	Present	Absent
	UCT 608(4)	Absent	Absent	Present
	UCT 608(5)	Absent	Absent	Present
	UCT 611	Absent	Absent	Present
	UCT 611(a)	Present	Present	Absent
	UCT 611(b)	Present	Absent	Absent
	UCT 617	Absent	Absent	Present
	UCT 619	Present	Present	Absent
	UCT 621	Present	Absent	Present

	UCT 623	Present	Present	Present
	UCT 625	Present	Present	Present
	UCT 626	Present	Present	Present
	UCT 627	Absent	Absent	Present
	UCT 628	Present	Present	Present
	UCT 629	Present	Present	Present
	UCT 630	Present	Present	Present
	UCT 631	Present	Present	Present
	UCT 632	Present	Present	Present
	UCT 633	Present	Present	Absent
	UCT 634	Absent	Absent	Present
	UCT 636	Present	Present	Present
	UCT 637	Present	Absent	Present
	UCT 640	Present	Present	Present
	UCT 641	Present	Present	Present
	UCT 642	Present	Present	Present
	UCT 644	Present	Absent	Absent
	UCT 645	Present	Absent	Absent
	UCT 646	Present	Present	Absent
	UCT 647a	Present	Absent	Absent
	UCT 647b	Present	Absent	Absent
	UCT 649	Present	Absent	Absent
	UCT 650	Present	Present	Absent
	UCT 651	Present	Absent	Absent
	UCT 653	Present	Present	Absent
	UCT 669	Present	Absent	Absent
	UCT 671	Present	Absent	Present
	UCT 672	Present	Present	Present
	UCT 673	Absent	Absent	Present
	UCT 674	Absent	Absent	Present
	UCT 675	Present	Absent	Present
	UCT 677	Present	Absent	Absent
	UCT 678	Present	Absent	Absent
	UCT 680	Present	Present	Absent
	UCT 682	Present	Present	Present
	UCT 683	Present	Present	Present

B.4 Morphologically estimated females inclusion-exclusion criteria (forensic vs archaeological)

Table B4: Morphologically estimated females from the UCT Human Skeletal Repository inclusion-exclusion criteria for sex analyses.

	Accession no.	Included/excluded	Forensic or archaeological	Comments
1.	UCT 350	Included	Forensic	
2.	UCT 352	Included	Forensic	
3.	UCT 337	Included	Forensic	Very fragile, fragmented, grainy, root etching. Possibly historic.
4.	UCT 153	Excluded	Archaeological	Covered in glue/waxy substance; taphonomy consistent with archaeological case.
5.	UCT 102	Included	Forensic	
6.	UCT 233	Included	Forensic	Soft tissue present.
7.	UCT 253	Included	Forensic	Burnt remains.
8.	UCT 354	Included	Forensic	Sun bleached; soft tissue and hair present.
9.	UCT 315c	Excluded	Archaeological	Duplicate grave.
10.	UCT 404	Excluded	Archaeological	Taphonomy consistent with archaeological case.
11.	UCT 411	Included	Forensic	
12.	UCT 361	Included	Forensic	
13.	UCT 364	Included	Forensic	
14.	UCT 444	Excluded	Archaeological	Possibly macerated; will not work for DNA extraction
15.	UCT 413	Included	Forensic	Teeth worn, root etching. Possibly archaeological.
16.	UCT 462	Excluded	Archaeological	Teeth worn; cranium stained red; remains covered in sand.
17.	UCT 362	Included	Forensic	
18.	UCT 683	Excluded	Archaeological	Teeth worn, bones fragile, root etching
19.	UCT 650	Included	Forensic	Possibly archaeological
20.	UCT 653	Excluded	Archaeological	Exclude.
21.	UCT 644	Included	Forensic	Remains burnt
22.	UCT 646	Excluded	Archaeological	Teeth worn; taphonomy consistent with archaeological remains, root etching
23.	UCT 649	Included	Forensic	Burnt
24.	UCT 651	Included	Forensic	
25.	UCT 677	Included	Forensic	Burnt

26.	UCT 607(1)	Excluded	Archaeological	Historic, coffin nails present.
27.	UCT 608(1)	Excluded	Archaeological	Duplicate grave.
28.	UCT 608(2)	Excluded	Archaeological	Duplicate grave.
29.	UCT 608(3)	Excluded	Archaeological	Duplicate grave.
30.	UCT 608(4)	Excluded	Archaeological	Duplicate grave.
31.	UCT 608(5)	Excluded	Archaeological	Duplicate grave.
32.	UCT 600 (1)	Excluded	Archaeological	Duplicate grave.
33.	UCT 619	Excluded	Archaeological	Teeth worn; bones fragile, fragmented -Historical period
34.	UCT 611(a)	Excluded	Archaeological	Duplicate grave
35.	UCT 611(b)	Excluded	Archaeological	Duplicate grave
36.	UCT 627	Included	Forensic	
37.	UCT 629	Included	Forensic	
38.	UCT 631	Included	Forensic	
39.	UCT 632	Included	Forensic	
40.	UCT 636	Included	Forensic	Remains fractured.
41.	UCT 617	Excluded	Archaeological	Fragile, fragmented. Artefacts historic

Appendix C: Data analysis - Observer error assessment

C.1 Intra-observer error analysis

Results of Cohen's kappa assessment of morphological sex estimation are summarised in Table C1 and C2 below (where *Cohen's Kappa coefficient formula: K = Po - Pe/1 – Pe, and Po = n(agree)/total; Pe = P(correct) + P(incorrect*)).

Table C.1: Sex estimation intra- observer error analysis – Cohen's kappa assessment.

			Ob 1		Total
			F	M	
Ob2	F	Count	10	2	12
		Expected Count	3,2	8,8	12,0
	M	Count	0	25	25
		Expected Count	6,8	18,2	25,0
Total		Count	10	27	37
		Expected Count	10,0	27,0	37,0
Agreement			10	25	35
By chance					21.5
Kappa	0,871				

C.2 Inter-observer error analysis

Table C.2: Sex estimation inter-observer error analysis – Cohen's kappa assessment.

			Ob1		Total
			F	M	
Ob2	F	Count	8	0	8
		Expected Count	3,0	5,0	8,0
	M	Count	3	18	21
		Expected Count	8,0	13,0	21,0
Total		Count	11	18	29
		Expected Count	11,0	18,0	29,0
Agreement			8	18	26
By chance					16.1
Kappa		0,768			

Appendix D: Molecular sample information

D.1 Morphologically estimated adult female cases selected for molecular sex analyses

Table D1: Description of samples collected from morphologically estimated adult female cases for molecular sex analyses. Information regarding the year the remains was discovered was provided from the UCT Human Skeletal Repository archives.

Accession no.	Sample type	Taphonomy	Year of discovery
UCT 102	Petrous bone (left apex)	-	Found between 1933-1936
UCT 233	Lateral cuneiform (right)	Tissue present	Found in 1970
UCT 253	3 x rib sections	Burnt	-
UCT 337	Carpal/tarsal bone (unidentified)	Very fragile; fragmented; grainy; root etching	Found in1982; Early historic; long term burial.
UCT 350	Upper (maxillary) right first molar	Crown of tooth fractured; roots of teeth brown	Found in 1984
UCT 352	Intermediate cuneiform (right)	-	Found in 1983
UCT 354	Capitate (right)	Sun bleached; tissue present; hair present	Found in 1984
UCT 361	Upper (maxillary) left first molar	-	Found in 1983
UCT 362	1 x rib section	-	Found in 1984
UCT 364	Lower (mandibular) left second molar	Red ochre staining. Small cavity in crown; roots intact; layer of dirt	Found in 1983
UCT 411	Intermediate cuneiform (left)	-	Found in 1984; died 2-3yrs before discovery of skeleton
UCT 413	Lateral cuneiform (left)	Teeth worn, root etching	Found in 1988
UCT 627	Intermediate cuneiform (left)	Relatively modern style clothing	Found in 2004. Time of death: within the last couple of decades
UCT 629	Intermediate cuneiform (left)	-	-
UCT 631	Intermediate cuneiform (left)	-	Found in 1984
UCT 632	Lateral cuneiform (right)	Soil and flesh included	-
UCT 636	Intermediate cuneiform	Remains fractured; light; porous; covered in sand	Found in 2001; Person presumed dead for <5 years
UCT 644	Upper (maxillary) left first molar	Burnt	Found in 1994
UCT 649	Petrous bone (right apex)	Burnt	Found in 1986.

UCT 650	Lower (mandibular) left first molar	Enamel on crown worn; possibly archaeological	-
UCT 651	Upper (maxillary) right first molar	-	Found in 1989
UCT 677	Petrous bone (left apex)	Burnt	Found in 2017; long term skeletonization (*i.e.* >1 year)

D.2 Collection of alternative samples

Alternative samples (rib or a portion of the petrous bone) were collected by cutting the bone using a Dremel tool and attaching Ez Speedclic Mandrel (Dremel: Racine, WI, USA) and thin cutting wheels. Samples were cut at a medium to slow speed by making two small cuts on either side of where the bone was to be cut. Downward pressure was applied in between the two cuts until the bone snapped into two pieces. To ensure complete powdering of the sample by the TissueLyser II (process described further on) the pieces of bone were cut into sections no greater than 2 cm^2. A rib was sampled by cutting a section of the body of the rib. For the petrous bone, the temporal portion of the cranium was targeted. In one case (UCT 102), the cranium had previously been dissected so access to the petrous bone was easy. For the other two cases where the cranium was intact (UCT 649 and UCT 677), the petrous bone was sampled as described by (Pinhasi *et al.*, 2015), to increase chances of obtaining DNA, whilst being as least destructive as possible. In brief, the apex of the petrous pyramid was sampled by vertically cutting it from the lower part of the cranium and then removing the bone via the foramen magnum. For these steps, all equipment was thoroughly decontaminated. Additionally, preparation of samples was done under an extractor hood, and a respirator was worn.

D.3 Sample grinding

Each hard tissue sample was crushed to a fine powder using the TissueLyser II® and Grinding Jar sets (QIAGEN, Hilden, Germany). The jars were cooled at -20°C for at least 24 hours before use. A sample was then placed in a sterilised ice-cold grinding jar. The grinding jars were cooled further by submerging them in liquid nitrogen for 1 minute and were then immediately attached to the TissueLyser instrument and subjected to oscillation at a frequency of 30 Hz for 1 minute. Temperature-resistant gloves were used for the handling of the jars as protection from the cold. The entire sample powder was removed from the grinding jars using a sterile

spatula and placed into labelled 1.5 ml micro-centrifuge tube. The spatula and work area were decontaminated between use with different samples to prevent cross-contamination using bleach (5% concentrate), MBG water and 70% ethanol. The powder was transported on ice and subsequently stored at -20 °C until further processing.

Appendix E: DNA extraction optimisation

E.1 DNA extraction optimisation data

Table E.1: Preliminary optimisation data generated using the QIAamp DNA Investigator Kit: The quantity and quality was assessed for control samples and a subset of five case samples (as determined by qPCR using the Quantifiler® Trio DNA Quantification Kit).

				qPCR				
Sample ID	**Input sample weight (mg)**	**Volume suspended (µl)**	**Lysis time (hrs)**	**IPC (CT)**	**Large target (ng/mg)**	**Small target (ng/mg)**	**DI**	**Y target (ng/mg)**
UCT 233	50	35	20	27.7282	0.00007	0.00042	5.8949	-
UCT 364	50	50	20	27.9862	-	-	-	-
UCT 364	50	35	20	28.2082	-	0.00042	-	0.00112
UCT 632	50	35	20	27.3622	0.00049	0.00273	5.3132	-
UCT 650	50	50	20	27.85747528	-	-	-	-
UCT 650	50	35	20	failed	-	-	-	-
UCT 650	50	35	20	failed	-	-	-	-
UCT 651	50	50	20	28.0329	0.0006	0.0006	0.9840	0,0034
UCT 651	50	35	20	27.8062	-	0.00056	-	0.00077
NTC (31/01/22)	N/A	35	20	27.5652	-	-	-	-
NTC1 (25/02/22)	N/A	35	20	27.5268	-	-	-	-
NTC2 (25/02/22)	N/A	35	20	27.6383	-	-	-	-

IPC: internal positive control; DI: degradation index; CT: threshold cycle.

C = Control tooth; NTC = Non- template control from DNA extraction; DNA concentration was measured by Qubit® Fluorometry (ThermoFisher Scientific, USA). DNA concentration and quality was measured by qPCR using the Quantifiler® Trio DNA Quantification Kit (Applied Biosystems, Foster City, USA); - = DNA concentration of samples were out of range for Qubit® (< 0.5 ng/µl) or no amplification occurred during qPCR analysis.

E.2 DNA extraction data using the optimized protocol

Table E.2: Data generated using the optimized protocol for the QIAamp DNA Investigator Kit: The quantity and quality data generated for all case samples (as determined by qPCR using the Quantifiler® Trio DNA Quantification Kit).

				qPCR				
Sample ID	**Input sample weight (mg)**	**Volume suspended (µl)**	**Lysis time (hrs)**	**IPC (CT)**	**Large target (ng/mg)**	**Small target (ng/mg)**	**DI**	**Y target (ng/mg)**
UCT 102	50	35	24	27.5042	-	0.00028	-	0.00049
UCT 233	50	35	24	27.6651	-	0.00049	-	-
UCT 253	50	35	24	27.6047	-	-	-	-
UCT 337	50	35	24	27.3494	-	-	-	-
UCT 350	50	35	24	27.5820	0.00049	0.00063	1.2711	0.00098
UCT 352	50	35	24	27.6521	-	0.00021	-	-
UCT 354	50	35	24	28.0103	-	0.00091	-	0.00252
UCT 361	50	35	24	failed	-	0.00021	-	-
UCT 362	50	35	24	failed	-	-	-	-
UCT 364	50	35	24	27.9640	-	0.00007	-	-
UCT 411	50	35	24	27.3680	-	-	-	-
UCT 413	50	35	24	27.8029	-	-	-	-
UCT 627	50	35	24	27.5078	-	-	-	-
UCT 629	50	35	24	27.2032	-	0.00056	-	-
UCT 631	50	35	24	27.6566	-	0.00007		0.00014
UCT 632	50	35	24	27.7489	0.00056	0.00119	2.2783	-
UCT 636	50	35	24	failed	-	-	-	-
UCT 644	50	35	24	27.7954	-	-	-	-
UCT 649	50	35	24	27.6977	-	0.00007	-	-
UCT 651	50	35	24	27.7121	0.00091	0.00021	0.2675	0.00098
UCT 677	50	35	24	27.5568	-	0.00063	-	0.00063
C2	50	35	24	28.9497	2.2936	1.3376	0.5832	-
C2	50	35	24	29.0421	1.35801	1.0518	0.7745	-
NTC (16/03/22)	N/A	35	24	27.8852	-	-	-	-
NTC (26/04/22)	N/A	35	24	27.6556	4.78044×10^{-5}	-	-	-
NTC 06/05	N/A	35	24	27.6467	-	-	-	-
IPC: internal positive control; DI: degradation index; CT: threshold cycle.								

C = Control tooth; NTC = Non- template control; DNA concentration and quality was measured qPCR using the Quantifiler® Trio DNA Quantification Kit (Applied Biosystems, Foster City, USA); - = DNA yield was too low to be measured or no amplification occurred during qPCR analysis.

Appendix F: Recipes

D.1 Dithiothreitol

0.077125 g	Tocris Bioscience DL-Dithiothreitol powder
500 µl Add powder to water (1M)	Molecular-grade Biology Water

D.2 Buffer AW1 and AW2 (QIAamp® DNA Investigator kit (QIAGEN. Germany)

AW1: Add 25 ml 99.9% absolute ethanol (Kimix. South Africa) to the bottle containing buffer AW1 concentrate.

AW2: Add 30 ml 99.9% absolute ethanol (Kimix. South Africa) to the bottle containing 13 ml Buffer AW2 concentrate.

D.3 Extraction buffer preparation (1 ml/reaction)

900 µl	Sigma Aldrich Ethylenediaminetetraacetic acid (EDTA) [0.5M]
75 µl	Milli-Q Water
25 µl	Sigma Aldrich Proteinase K [10 mg/ml]

Mix EDTA [0.5M]. Milli Q water and Proteinase K solution