Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope

Z Geldenhuys

orcid.org 0000-0003-1684-1704

Dissertation accepted in partial fulfilment of the requirements for the degree *Master of Science in Biochemistry* at the North-West University

Supervisor:	Dr R van der Sluis
Co-supervisor:	Dr J Aucamp

Graduation July 2020

24328405

PREFACE

I would like to thank the following people for their assistance

My supervisor, Dr R van der Sluis, who provided continual assistance in all aspects during the completion of my M.Sc. I have learned a lot during this time from you and will always be grateful for it. Thank you for all your effort and patience during this time.

My co-supervisor, Dr J Aucamp, who provided continual assistance on cell-free DNA work and teaching me this new field and sharing her expert advice on cell-free DNA. Thank you, for your guidance on an academic level and personal level. I have learned tremendously from you during this time and will always be grateful for it. Thank you for all your effort, patience and understanding during this time.

Thank you, to my friends and family for their love, support, understanding, encouragement through the good and bad and embracing this challenge with me.

In addition, I would like to thank the following institutions:

Lumegen Laboratories for the financial support provided by the THRIP project as well as for providing the use of their facilities during the completion of my dissertation.

North-West University (NWU) and the Focus Area for Human Metabolomics department for accommodating my ambitions and providing me with the means to excel.

Colin Engelbrecht, Bona Bona Game Reserve as well as Wintershoek (Wiaan van der Linde) for their interest in this project and providing samples during this study.

Inqaba Biotec™, for the sequencing of the *SRY* gene and conducting analysis on the MassARRAY® system.

ABSTRACT

The research field of cell free DNA (cfDNA) and cell free fetal DNA (cffDNA) has expanded in the past decade, with the inclusion of animal based cfDNA studies. This has resulted in an increase in research making use of cffDNA in prenatal sex determination studies on animals. This study attempted to be the first study to confirm the presence of cfDNA as well as cffDNA in the maternal plasma of pregnant female sable and roan antelope species. The aim of this study was to develop a non-invasive cffDNA-based quantitative assay for foetal sex determination of the sable and roan antelope prenatally. cfDNA was isolated from pregnant female sable antelope plasma samples to determine the foetal sex of the animal using a non-invasive technique. Due to lockdown regulation imposed by the COVID-19 pandemic, pregnant female roan antelopes blood samples could not be obtained. The *SRY* gene, which is only present in the male species, was used as the target gene. The sex determining region y (*SRY*) gene of the sable and roan antelope was sequenced to determine the level of conservation of the SRY gene between and within species as well as to design primers for the quantification assay methods. Mitochondrial DNA sequences were used to identify antelope sub-species. There were no differences found between the *SRY* gene sequence of the sub-species of sable antelope as well as between the *SRY* gene sequence for the roan antelope sub-species. Two different qPCR assays, SYBR Green qPCR and probe-based qPCR assay, as well as the MassARRAY® system was developed to identify the optimal method to be used for the foetal sex determination using cffDNA. However, due to low cfDNA yields from the blood samples, only the SYBR Green qPCR and the MassARRAY® methods could be evaluated to determine the sex of the calves. The results obtained were compared to the sex of the calves at birth. For the SYBR Green qPCR assay, 60% of the samples gave positive results, while 40% were inconclusive. For the MassARRAY® system only one out of five single nucleotides polymorphisms (SNPs) gave a result for 6 samples and only 20% of these results corresponded to the sex at birth. Based on these results, the isolation of cfDNA from plasma samples needs to be optimised to improve yield. Further testing of these two techniques should be done for future studies to determine which techniques would be more reliable to determine the sex of the calf prenatally using cffDNA.

Keywords: Sable antelope (*Hippotragus niger*), roan antelope (*Hippotragus equinus*), cell free DNA, cell free fetal DNA, sex determining region y gene (*SRY* gene), qPCR, MassARRAY® system.

LIST OF ABBREVIATIONS:

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ssDNA	single stranded DNA
dsDNA	double stranded DNA
cfDNA	cell-free DNA
cffDNA	cell-free fetal DNA
SRY	sex determining region y
PCR	Polymerase chain reaction
qPCR	Real-Time PCR
H. n.	*Hippotragus niger*
H. e.	*Hippotragus equinus*
IUCN	International Union for Conservation of Nature
mtDNA	Mitochondrial DNA
TDF	Testis determination factor
HMG	High mobility group
bp	base pairs
RhD	Rhesus blood group D antigen
AMLY	Amelogenin Y gene
AMLX	Amelogenin X gene
dNTPs	deoxyribonucleotides
gDNA	Genomic DNA

MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
NTC	non-template control
PC	positive control
NC	negative control
C_T	Quantification cycle
HS	High sensitivity
SNPs	Single nucleotide polymorphisms
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
(PPT™)	Plasma preparation tube

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CHAPTER 1. INTRODUCTION

Circulating cell-free DNA (cfDNA) are non-encapsulated small DNA fragments found in the bloodstream, as well as other bodily fluids, that are released from cells via either cell death (e.g., necrosis and apoptosis) or active release mechanisms (Aucamp *et al.*, 2018). The discovery of cfDNA in human maternal blood (Lo *et al.*, 1989) opened a new avenue for the development of non-invasive fetal cfDNA-based prenatal genetic testing, as well as sex determination studies of the foetus. Soon after the initial study, Lo *et al.* (1997) identified that male fetal Y-chromosome sequences are present in the maternal blood. The Y-chromosome contains the *SRY* gene that can be used to design probes for sex determination studies (Akolekar *et al.*, 2010).

Since the discovery of cfDNA in human blood plasma, it prompted the research question whether cfDNA would also be present in animal blood. To date there is still limited studies available on animal cfDNA other than simply detecting its presence. Studies have reported the presence of cfDNA in cattle and a few prenatal sex determination studies have been done on domestic animals (Lemos *et al.*, 2011, da Cruz *et al.*, 2012, Mayer *et al.*, 2013, Son *et al.*, 2013). There have also been studies that have focused on the use of fetal cfDNA in horses (de Leon *et al.*, 2012, Kadivar *et al.*, 2016), sheep (Asadpour *et al.*, 2015), elephants (Vincze *et al.*, 2019) and rhinoceros (Stoops *et al.*, 2018) for prenatal sex determination. In these studies, they investigated strategies where fetal cfDNA can be used for sex determination studies with the use of PCR with acceptable accuracy. In all the studies, they identified in each species a nucleotide variation with in the *SRY* gene in order to develop primers that can be used for PCR.

There is presently no method for non-invasive fetal sex determination in the sable and roan antelope as sex is presently determined using ultrasonography. There is a need to develop a non-invasive fetal sex determination technique to determine the fetal sex of popular and endangered species, such as the sable and roan antelope. Therefore, to establish and validate a molecular based technique for sex determination using small amounts of DNA would be of great value. This method would also be less invasive than using ultrasonography for both the female and for the calf. In this study, the presence of cfDNA in the sable and roan antelope will be determined by attempting to extract cfDNA. Sequencing of the *SRY* gene in both the sable and roan antelope was archived in order to determine the level of conservation of this gene between and within species, to design primers for the chosen assays and then to determine the sex of the male foetus. After confirming the presence of cfDNA, the study investigated the suitability of using the fetal cfDNA to determine the fetal sex prenatally using the developed and optimised qPCR and MassARRAY® assays.

CHAPTER 2. LITERATURE STUDY

2.1 Introduction

In South Africa the total number of private game farms is increasing, with more than 11 600 game farms spread over 21 million hectares of land in 2017 (Benjamin-Fink & Reilly, 2017). The increase started in the 1990s, where agricultural land use for game farming (Cloete *et a*l., 2015), excluding game farms that manage both wildlife and domestic livestock, increased by 5.6%, which was estimated in 2005 to equal 15 000 farms (Cousins *et al.*, 2008). To date, this has expanded to the privatisation of wildlife in 6.1% of provincial protected areas, as well as 16.8% of private game farms, to serve as a main source of revenue for the involved stakeholders (Bothma, 2002, Cousins *et al.*, 2010, Benjamin-Fink & Reilly, 2017). Game farming has a higher rate of return on investment per hectare than any other agriculturally based market, as shown in Table 2-1 (Benjamin-Fink & Reilly, 2017). The annual return on the investment of a game farm is approximately 80%, whereas wildlife enterprises can generate an estimated annual income of 4.7 billion rand (Olivier, 2015). Table 2-1 shows the annual income of the different market segments.

Table 2-1 The annual income of the different market segments of the wildlife trade**.**

Market segments	**Rand (ZAR)//year**	Reference:
Game meat production	42 million	(Benjamin-Fink & Reilly, 2017, Du Toit & van Schalkwyk, 2011)
Taxidermy	200 million	(Du Toit & van Schalkwyk, 2011, Benjamin-Fink & Reilly, 2017)
Game translocation	750 million	(Du Toit & van Schalkwyk, 2011, Benjamin-Fink & Reilly, 2017)
Hunting	3.1 billion	(Du Toit & van Schalkwyk, 2011, Benjamin-Fink & Reilly, 2017)
Trophy hunting	510 million	(Du Toit & van Schalkwyk, 2011, Benjamin-Fink & Reilly, 2017)
Live auctions	1 billion	(Du Toit & van Schalkwyk, 2011, Benjamin-Fink & Reilly, 2017)

South African game farms require fences that prevent natural processes, such as dispersal, emigration and colonisation dynamics, from taking place within the enclosed population of the species (Cousins *et al.*, 2008, Benjamin-Fink & Reilly, 2017). With this in mind, the game farm owners are faced with a great need to intensively manage the population of the species, in order

to prevent reduced biological fitness due to inbreeding and outbreeding depression in small and enclosed populations (Lehmann *et al.*, 2008). In order to enhance the genetic diversity of the species found on the game farm, translocation has to take place for outbreeding (Benjamin-Fink & Reilly, 2017). Translocation is defined in this case as the mediated movement of species from any source, private or wild, with the release into another habitat or region. Translocation of species in the population is done, for example, through the purchase of these species at live auctions. The management decisions of translocating individuals from other populations are complex and there is a need to ease the risk of unwanted consequences, such as outbreeding depression (Benjamin-Fink & Reilly, 2017). Outbreeding depression refers to the mating between individuals from different species or sub-species which can result in a decline in reproductive fitness of the species.

2.2 The translocation of wildlife in South Africa

There is a need for a standardised protocol for translocating wildlife throughout South Africa in order to improve wildlife conservation (Grobler *et al.*, 2011). The standardisation of wildlife translocation has the ability to shape ecosystems and to serve as an effective conservation tool in order to increase scarce wildlife species. The conservation-based goal of translocating species into an existing population is to increase the genetic diversity of the population through the mating of unrelated individuals (Rhymer & Simberloff, 1996). To achieve this conservation based goal, national parks and private game farms play and important role as a source for genetically diverse animals and to re-establish taxonomic pure populations (Benjamin-Fink & Reilly, 2017). An example of successful reintroduction in South Africa is that of the once locally extinct southern white rhinoceros (*Ceratatherium simum*) (Benjamin-Fink & Reilly, 2017).

Conversely, a lack of standardised wildlife translocation protocols has, for example, contributed to the classification of the sable (*Hippotragus niger*) and roan (*H. equinus*) antelope as scarce game species in the early 2000s, due to a dramatic decrease in population size (Van der Merwe & Saayman, 2003, Matthee & Robinson, 1999). During the 1980s and 1990s, breeding programs were not implemented, intensive hunting practices were employed, intensive poaching occurred, loss of habitat from the expansion of agricultural settlements took place and there was a lack of legislation, all of which contributed to this decrease (East, 1999). Consequently, these species became popular at live auctions due to an increase in breeding programs (Van der Merwe & Saayman, 2003).

The body stature and horns of both these antelope species make these species a prised trophy animal for big-game hunters, as well as for the tourism market. It is due to this aesthetic appeal of the sable antelope that the numbers have stabilised, primarily via intensive breeding programs

on private farms. The lifting of certain nature conservation prohibitions has also made the trade of antelope more effective than in the past (Matthee & Robinson, 1999). As a result, the relocation of subspecies between game farm owners across different countries became more practical, further contributing to the success of the breeding programs. By 2017, the International Union for Conservation of Nature (IUCN) Red List classified the sable and roan antelope as least concern, since their population sizes are stable at the moment (IUCN, 2017b). Nevertheless, certain subspecies are still vulnerable to a decrease in numbers, in particular the Giant sable antelope (*H. n. variani*) found in Angola. According to the IUCN Red List, if the roan antelope's current trend in numbers continues to decline, they will soon be classified as near threatened or vulnerable species (IUCN, 2017a), where about 60% of the population occurs in protected areas.

The antelope is popular among wildlife breeders for ecotourism as well as for breeding purposes; however, they are also popular as trophy animals. As a breeding animal, and especially the stud-bull, the value of the antelope has increased unexpectedly during the last decade, especially with the inclusion of the Zambian sable antelope (*H. niger kirkii*). In 2015, a Zambian sable bull was sold for a record price of R27 million at an auction, which is more than double of the previous record prices (Mkentane, 2015). The reason for this record price was due to the Zambian sable bull, named Mopanie, which had 1200 centimetres curved horns, very rare in these species. Since 2015, there was no new record price for a sable bull. Due to their popularity and the risk of being reclassified as endangered/threatened species, focus has been placed on the sable and roan antelope for the remainder of this study.

2.3 The sable and roan antelope

Both the sable and roan antelope are part of the *Bovidae* family (Figure 2-1), which forms part of the *Antilopinae* leg of the *Bovidae* tree. The subfamily of these two species is the *Hippotragini* and the genus is *Hippotragus*. The *Hippotragus* genus consists of three different species, namely the *H. niger, H. equinus* and the *H. leucophaeus*. *H. leucophaeus*, known as the Cape blue buck or blue antelope, was endemic to the coastal region of South Africa and went extinct in the 18th century (Espregueira Themudo & Campos, 2017).

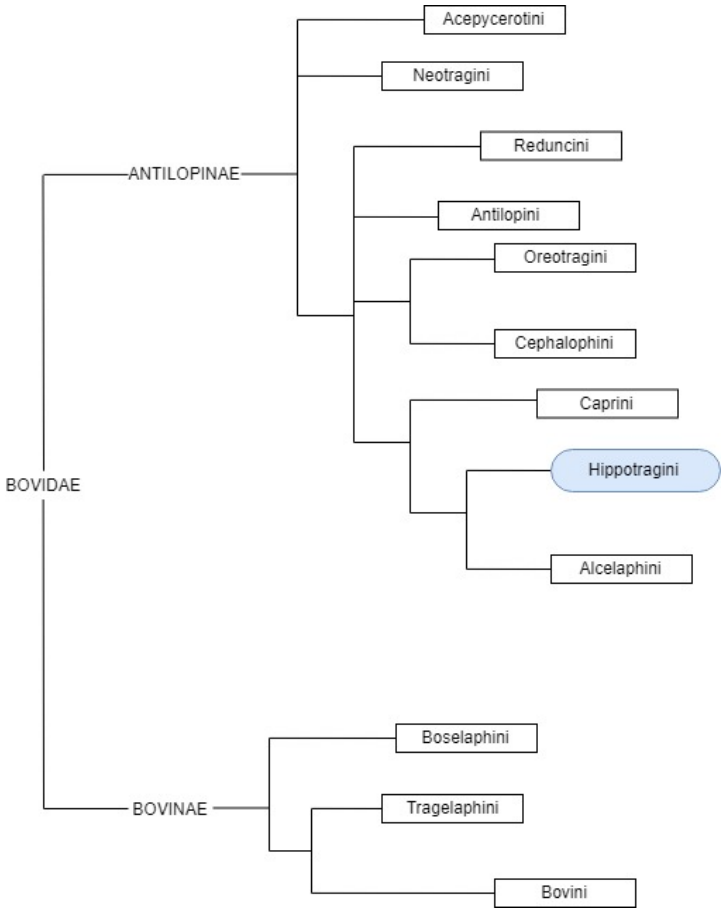


Figure 2-1 Phylogenetic tree of the family *Bovidae* (Castelló, 2016).

2.3.1 Sable antelope (*Hippotragus niger*)

The sable antelope is one of the largest African antelope, with a body mass that rarely exceeds 225 kg and a shoulder height of 135 cm (Wilson & Hirst, 1977). Both sexes have evenly divergent horns that curve backwards, with the mean length of the bull's horns reaching 95 – 105 cm and the cow's horns approximately 80 cm long (Wilson & Hirst, 1977). The general colour of the sable antelope is a glossy black for the bull and a paler black to an almost bright chestnut colour for the cow. Both the bull and cow have black and white face markings and the abdomen, and the hindquarters are pure white in colour (Figure 2-2).

Figure 2-2 Representation of a male *H. niger* (sable antelope). This photo was taken on Wintershoek private game reserve in the Northern Cape.

The sable antelope is one of the largest African plains antelope and is broadly distributed throughout the savanna woodlands of the African continent - from East Africa, Mozambique across to the southern Congo, as well as an isolated population in Angola (Kingdon, 1982) (Figure 2-3). *H. niger* consists of four different sub-species, each sub-species representing a different region throughout the woodlands of the African continent (Ansell, 1971) (*Figure 2-3*): (i) *H. niger variani*, also known as the Giant Sable antelope, is restricted to the northern Angola regions; (ii) *H. niger kirkii* is found in Zambia, Malawi, Katanga province in the Democratic Republic of Congo and eastern Angola; (iii) *H. niger rooseveltii* is found in southern Kenya and eastern Tanzania; (iv) *H. niger niger* is found in the more southern African regions of the continent. More recently, Vaz Pinto (2018) analysed the nuclear DNA of the sable antelope and sub-species to identify their geographic distribution and it has been found that the distribution of sub-species is similar to what was previously reported by Ansell (1971). The sable antelope can occur in a variety of habitats in the savanna biome, but prefers open woodlands with nearby marsh of grasslands and avoids areas with short grass and high tree density (Skinner & Chimimba, 2005, Alpers *et al.*, 2004). A permanent water source must be present as the antelope do not move further than 2 – 4 km from a drinking site (Wilson & Hirst, 1977).

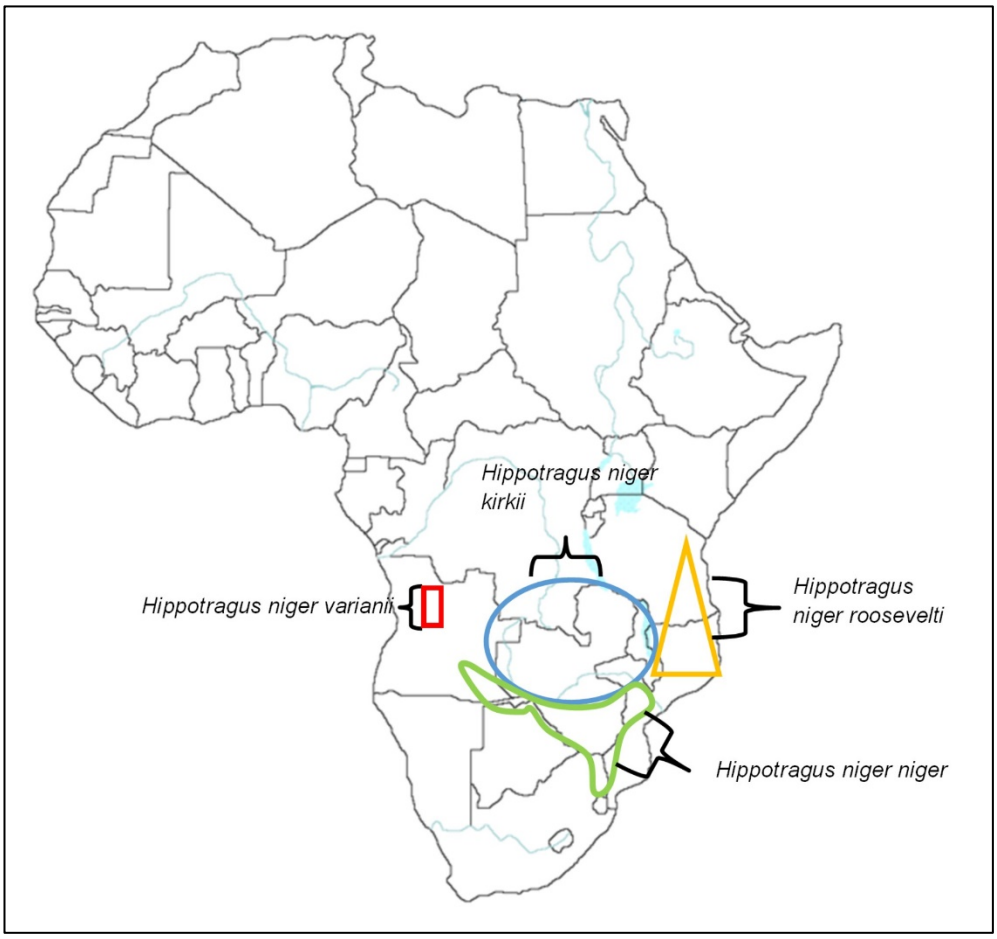


Figure 2-3 The approximate geographic range of the four sub-species of the *H. niger* (Sable antelope) found in the wild in Africa (Ansell, 1971, Themudo *et al.*, 2015, Matthee & Robinson, 1999, Vaz Pinto, 2019).

The reproduction process of the sable antelope is usually described as an annual-seasonal breeder. The duration and timing of the calving season of the sables show variations over their distribution range but coincides with the peak availability of nutritious forage that is needed for the lactating females. The gestation period of the sable antelope is 240 – 248 days (Wilson & Hirst, 1977). The female leaves the herd to calve and the calf is isolated from the herd for the first two weeks (Wilson & Hirst, 1977); however, within a day after parturition the female returns to the herd. It is not unusual for the calf to be left isolated and unattended throughout the day by the female, but the calf is attended to in the early morning and early evening hours (Wilson & Hirst, 1977). The relationship between the mother and the calf appears to be very loose. There are a

few factors that make the isolated calf difficult to locate, such as a very short flight distance, absence of characteristic odour and effective camouflage colouring (Wilson & Hirst, 1977)

2.3.2 Roan antelope (Hippotragus equinus)

The roan antelope is also one of the largest antelope species and is in the same size range as the sable antelope. The average mass of the male roan antelope is 260 – 300 kg and the female is 225 – 275 kg. However, the male roan has a height of 1.5 -1.6 m and the female of 1.4 – 1.5 m. Both sexes carry horns that curve backwards like the sable but are much shorter (Figure 2-4). The male has longer, thicker horns than the female roan. The general body colour is greyish brown with touches of dark rufous to a light reddish brown, which varies among individuals of this species, with white underparts. The legs are a darker brown compared to the rest of the body, with an erected dark-tipped mane. The most distinctive features of the roan antelope are the black and white facial markings, where the largest part of the face is black with white patches around the eyes and mouth. A long tuft of dark brown hair is present on the tips of the ears.

Figure 2-4 Representation of a female *H. equinus* (Roan antelope). This photo was taken on Wintershoek private game reserve in the Northern Cape.

The roan antelope is endemic to the African continent and the typical habitat consists of open savannah woodlands with extensive open areas of medium to tall grasses (Dorst, 1970). They were one of the most widely distributed antelopes, found essentially throughout the African savannas where water was adequate (Kingdon, 1982). The roan antelope originally used to occur in 34 African countries (East, 1988), but now they are only found in 30 countries (IUCN, 2017a). This is due to poaching and loss of habitat from the expansion of settlements in these countries. The overall population of the roan is decreasing in the North, West and Eastern regions of Africa, with the only increase in the population found in the private game reserves of South Africa. As with sables, this species rarely moves further away from a drinking site, since it is an important factor for this species to have permanent water (Wilson & Hirst, 1977). In a previous study, it was noted that the roan antelope avoids woodland areas where the underbrush forms a thick closed stand or where the trees form a closed covering (Joubert, 1976). The ideal area is where the bush growth is up to 1.5 m in the grassland, but they also avoid areas with shorter grasslands.

H. equinus consists of six sub-species and this classification is based on the phenotypic and geographic characteristics of the subspecies (Ansell, 1971): *H. equinus koba* (North-West Africa region), *H. equinus bakeri* (North-East Africa region), *H. equinus charicus* (North Africa region), *H. equinus equinus* (Southern Africa region), *H. equinus cottoni* (West Africa region), and *H. equinus langheldi* (East Africa region). Figure 2-5 is an approximate representation of where these roan antelope subspecies were found in the wild as stated by Ansell (1971). However, this geographic distribution could have changed in the past years, since there is no new literature to state otherwise.

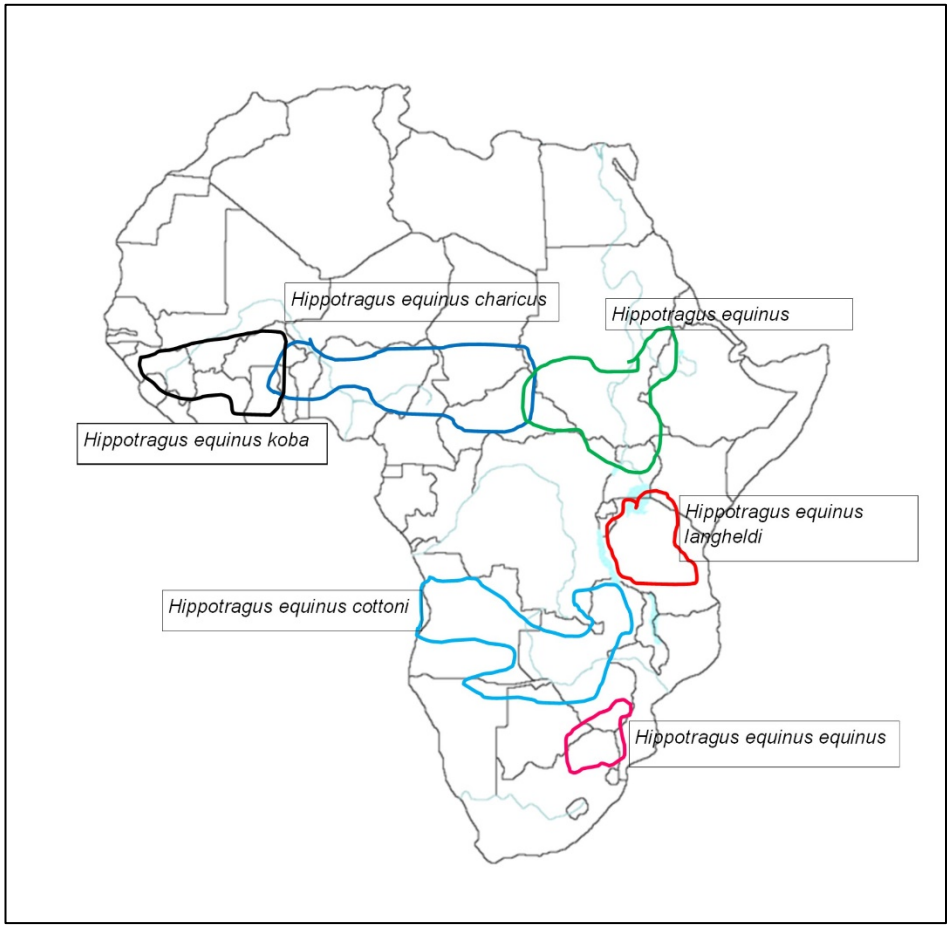


Figure 2-5 Approximate geographic distribution of the six subspecies of the *H. equinus* (Roan antelope) found in the wild in Africa (Ansell, 1971, Matthee & Robinson, 1999, Alpers et al., 2004).

The calving season of the roan antelope is not as well defined as that of the sable antelope, (Ansell, 1960, Child & Wilson, 1964, Smithers, 1966, Fairall, 1968, Blower, 1961). The female of the roan antelope also leaves the herd to calve like the sable antelope and the calf is isolated from the herd for 4 – 6 weeks (Wilson & Hirst, 1977). The female returns to the herd within a day following the parturition and then the calve remains isolated and unattended throughout the day. The mother-calf relationship appears very loose, with the roan antelope cows wondering as far as 2 km from the concealed calves (Wilson & Hirst, 1977). The female conceives shortly after the age of 2 years and calves at the age of 3 years. The gestation period of the roan antelope is approximately 270 days (Wilson & Hirst, 1977, Smithers, 1966, Joubert, 1976).

2.3.3 Conservation of these antelope species via captive breeding

As the sable and roan antelope species are classified as least concern according to the IUCN red list (IUCN, 2017a, IUCN, 2017b), there is still a need to conserve these animals. The main reasons for the decrease in numbers are due to hunting and the reduction of their natural habitats. This leads to a great concern regarding the wildlife conservation of these animals.

Wildlife conservation and ecotourism are growing industries worldwide and especially in South Africa. Some private game farms in South Africa make use of a captive breeding program, where the main strategy is to conserve endangered and threatened wildlife in order to increase their numbers (Stoops *et al.*, 2018). Captive breeding programs within provincial reserves and private game farms often involve the translocation of wildlife over vast geographical distances (Matthee & Robinson, 1999, Alpers *et al.*, 2004). This is done to prevent inbreeding and outbreeding depression from small and enclosed populations and to increase the genetic diversity within the group (Lehmann *et al.*, 2008, Rhymer & Simberloff, 1996).

Genetics can play important roles in promoting successful genetic diversity in captive breeding, for example via monitoring geographic chromosomal variations among the populations of animals used in the breeding program. Genetics can also help guide commercial and management decisions, the most common example being the use of sex selection during breeding programs. The following paragraphs (sections 2.4 and 2.5) explain the use and benefits of genetic monitoring and fetal sex determination in captive breeding programs, as well as provide summaries of such research in relation to the sable and roan antelope.

2.4 Genetic monitoring to prevent breeding depression

It is important to obtain the genetic status of a population and population demographic parameters via genetic monitoring to optimise conservation processes (La Haye *et al.*, 2017). Genetic monitoring provides information on the effective population sizes, population admixture and genetic relations of individuals required for effective breeding and/or reintroduction. It can also be used after reintroduction to maintain sufficient genetic diversity in the managed populations. This is done by identifying critical genetic units and then managing these units in a co-ordinated manner (Alpers *et al.*, 2004). Monitoring geographic chromosomal variation of endangered and threatened wildlife species, for example, helps to identify geographically defined cytotypes that can possibly influence breeding programs (Robinson & Harley, 1995).

2.4.1 Genetic monitoring of antelope species for subspecies delineation

The first empirical evidence of genetic population subdivision of the roan and sable populations in Africa was done by Matthee & Robinson (1999), giving insight to the management, as well as the conservation of these antelope species. In this study, they investigated the difference in the mitochondrial DNA (mtDNA) of the different subspecies and how the mtDNA resembles and differs between the roan and sable antelope. The roan antelope population has been determined to be geographically partitioned and their phylogeography corresponds to the morphological, as stated by Ansell (1971). For the sable antelope it has been determined that the sable population can be divided into two delineated maternal clades according to phylogeography. The *H. niger roosevelti* is strictly present in east Africa, while the second group is geographically diverse, including *H. niger variani* (Angola), *H. niger niger* (South Africa, Zimbabwe) and *H. niger kirkii* (Zambia, Malawi) under one clade.

Since the discovery of mtDNA, follow-up studies have been done to sequence it (Matthee & Davis, 2001, Alpers *et al.*, 2004, Themudo *et al.*, 2015) in order to identify if certain traits, as well as the taxonomic placement of these species, can be found within the mtDNA sequence. It was found that the mtDNA of the juvenile male is inherited from the mother/female and not from the male species. Accordingly, female mtDNA has since been used to investigate taxonomic placements. For example, Themudo *et al.* (2015) investigated the taxonomic placement of the Giant Sable antelope and identified from the maternal lineage that it is a pure Angolan Giant Sable antelope.

2.4.2 Chromosomal variation monitoring for captive breeding

In all mammalian species, the X and Y chromosomes are present, but chromosome numbers may vary between 30 and 60 chromosomes (diploid) among species. This gives each species a unique karyotype and a number of studies have done research regarding the karyotype of a species. Robinson & Harley (1995) investigated the geographic chromosomal variation between the sable and roan antelope. It has been determined that each of the sable and roan antelope species is characterised with a diploid karyotype of 60, as well as an invariant karyotype (Robinson & Harley, 1995). The two species, however, differ with respect to centromeric constitutive heterochromatin and the number of nucleolar organiser regions.

The investigation of Robinson and Harley (1995) resulted in the identification of the sex chromosomes of the antelope as a large X-chromosome and a small Y-chromosome for the male species and two X-chromosomes for the female species. Following the identification of the sex

chromosomes, further elucidation of the relevant genes in these chromosomes could ensue for use in conservation and breeding management. For example, elucidation of the *SRY* gene (see section 2.5.1) proved to be a useful target for prenatal sex determinations.

2.5 Prenatal sex determination to ease critical management decisions

Another important and basic element of wildlife conservation is scientific progress on reproductive technologies (Saberivand & Ahsan, 2016), such as fetal sex determination. Sex determination refers to the developmental decision that directs the orientation of male and female development during the development of the gonads into either ovaries or testes (Wilhelm *et al.*, 2007). Early fetal sex determination for domestic and wildlife animal species is a key practice for wildlife management (Bucca, 2005) as it serves as a valuable decision making tool for commercial strategies and financial gains (Bucca, 2005). The preference of a specific sex of either domestic or wildlife species is evident, mainly due to commercial implications and for inclusive and efficient results in farm management plans (Davoodian & Kadivar, 2016b). Fetal sex determination in the ovine industry is very useful for management decisions, such as culling decisions, eliminating expenses of the progeny test and sex selection in breeding programs (Kadivar *et al.*, 2013). Thus, it is necessary for the development of a cost-effective and reliable method for early fetal sex determination prenatally.

The most common method of choice for early fetal sex determination is by transrectal ultrasonographic visualisation that is based on locating the genital tubercle of the foetus (Son *et al.*, 2013). It was a commonly used method for bovine foetuses, with similar appropriateness for other mammal species (Curran *et al.*, 1989). This embryonic structure differentiates into the penis in males or the clitoris in females. During differentiation, these embryonic structures move from their initial position either towards the tail in females or towards the umbilical cord in males. In bovine species, these structures are visible between 55- and 60-days post-ovulation and can thereby be used to determine the fetal sex (Ali, 2004).

However, ultrasonography has its limitations: (a) it requires a technician (Kadivar *et al.*, 2016); (b) due to the positioning of the transducer on the foetus, it is sometimes difficult to obtain an image; (c) the visualisation of its structures are hampered by the large amount of allantoic fluid and high fetal mobility due to long umbilical cord (Kadivar *et al.*, 2016); (d) ultrasonography can only be used during a certain time of gestation in order to determine fetal sex (Asadpour *et al.*, 2015); (e) in larger mammals, it is hard to identify as the foetus is lying between the internal organs, which is larger and it's not reachable with the transducer (Vincze *et al.*, 2019). Based on all of these

limitations of ultrasonography, a more reliable and affordable technique is required for sex determination in animal species.

The use of molecular sex determination-based analysis of fetal DNA can be a reliable, practical, and sensitive alternative to traditionally used ultrasonography. This involves the use of a molecular-based test that targets a certain gene found only in either the male or female. The most commonly targeted gene for molecular sex determination is the *SRY* gene situated in the Y chromosome, which is used to determine if the foetus is male.

2.5.1 Molecular-based fetal sex determination – the *SRY* gene

The genes of the mammalian Y chromosome play a particularly important role in sex determination during the development of the embryo, as they are unique to only the male. Originally it was thought that male development was caused by the testis determination factor (*TDF*) gene (Jost *et al.*, 1973, Goodfellow & Darling, 1988). The *TDF* gene is the main gene for male development found on the Y chromosome, as well as the hormonal consequence of the testis (Jost *et al.*, 1973). However, various other genes have later been identified as essential for male sex determination. Thus, it is essential to understand every mode of action of the *TDF* and any other gene that participate in order for male sex development.

Sinclair *et al.* (1990) later identified a probe, named pY53.3, that corresponds to a gene found on the Y chromosome of human, murine and bovine genomic DNA. The location of the pY53.3 sequence on the Y chromosome has a functional role and is consistent with male sex determination. Sequence analysis of pY53.3 showed that the sequence corresponded to a coding sequence of a gene found on the Y chromosome. A 35kb region of Y specific sequence, proximately adjacent to the pseudoautosomal margin, was also identified and subsequent Southern blot analysis of the Y specific sequence revealed that the pY53.3 is found in this 35 kb region in a wide range of eutherian mammal (Sinclair *et al.*, 1990). Accordingly, this Y-located gene was termed the sex determining region Y (*SRY*) gene.

The *SRY* gene encodes a member of a large family of nuclear proteins characterised by a DNA binding domain, known as a high mobility group (HMG) box, according to its identification in a high mobility class of non-histone proteins that associate with DNA (Payen & Cotinot, 1993). The HMG box is divided into two classes of HMG proteins, HMG1 and HMG2, that bind to DNA in a sequence-independent manner. It is unclear whether *SRY* has only one gene that carries out all functions necessary for initiating male sex development or multiple target genes (Akolekar *et al.*, 2010). In mammals it is known that the Y chromosomal gene *SRY* is sufficient and necessary for

male sex differentiation and many other genes have since been implicated in testis development (Akolekar *et al.*, 2010). It is still unknown how *SRY* gene expression is regulated, what patterns may interact with the *SRY* gene and which other genes it may regulate.

2.5.2 Antelope *SRY* gene

There is very little known regarding the genome of both antelope species, but a small study has been done on the *SRY* gene (Vaz Pinto *et al.*, 2016). In this study the occurrence of hybridisation between the giant sable antelope and roan antelope was investigated by camera-tapping and molecular surveys. Their results found that introgressive hybridisation accrued between the roan antelope and giant sable antelope in one of the two remnant giant sable antelope populations found in Cangandala (Vaz Pinto *et al.*, 2016). This was caused by severe wartime poaching of both these species, which followed population depletion. It was also found that the male sable population had decreased dramatically, and this resulted in female sable antelopes mating with male roan antelopes found in the area. Due to the heterospecific mating between male roan antelope and female sable antelope, hybrids formed over time, but it was noticed that the hybrid male offspring were sterile (Vaz Pinto *et al.*, 2016).

Ancestry analysis for the paternal lineage was done on the *SRY* gene and the maternal lineage was based on the mtDNA for both the sable and roan antelope, as well as the hybrids found (Vaz Pinto *et al.*, 2016). The *SRY* gene region of both these species have been identified using specific primers. However, this was only a partial sequence of the *SRY* gene, consisting of 655 base pairs (bp) (GenBank accession number: KU146571.1 (Sable antelope) and KU146572.1 (Roan antelope)). Vaz Pinto *et al.* (2016) used these primers to successfully amplify the *SRY* gene sequence for both roan and sable antelopes. The analysis determined that all of the hybrids had the sable mitochondrial haplotype and that the hybrid males presented with a Y chromosome typical of roan antelope. Paternity analysis identified that a single roan bull sired all of the hybrids.

Figure 2-6 is a representation of the alignment of the *SRY* nucleotide sequence of the *Bos taurus* (cattle) (AB039748.1) *Hippotragus niger* (sable antelope) (KU146571.1), *Hippotragus equinus* (roan antelope) (KU146572.1) as well as the *Gazella dorcas* (gazelle) (AJ003127.1). As shown, the *SRY* gene sequence of the sable and roan antelope is only a partial sequence of 655 bp. There are only small differences which are 7 bp of the total 655 bp within the *SRY* gene alignment of the sable and roan antelope as would be expected of the species from the same genus. The cattle *SRY* gene can be used as the reference sequence for the sable and roan antelope even though there are 41 bp differences found within the 655 bp of the *SRY* gene sequence of the

sable and roan antelope. Different sequences were searched within the same family to be used as a reference for the antelope species but due to the large number of differences within the base pairs of the sequence the cattle *SRY* gene was the closest found due to that they are in the same family and that the complete *SRY* gene sequence of *Bos taurus* is available. The research of the *SRY* gene within this genus or family of mammals is still limited and only a few species *SRY* gene has been identified. Research on the genetics of the *Antilopinae* family and *Hippotragus* genus is still significantly limited. The only other available sequence data in GenBank within *Antilopinae* is that of *Gazella dorcas.* The sequence of the gazelle identified on GenBank consists of only 606 bp. The *SRY* gene for all mammals normally consists of 690 bp and from this sequence there are 18 bp differences within this sequence to the other species mentioned.

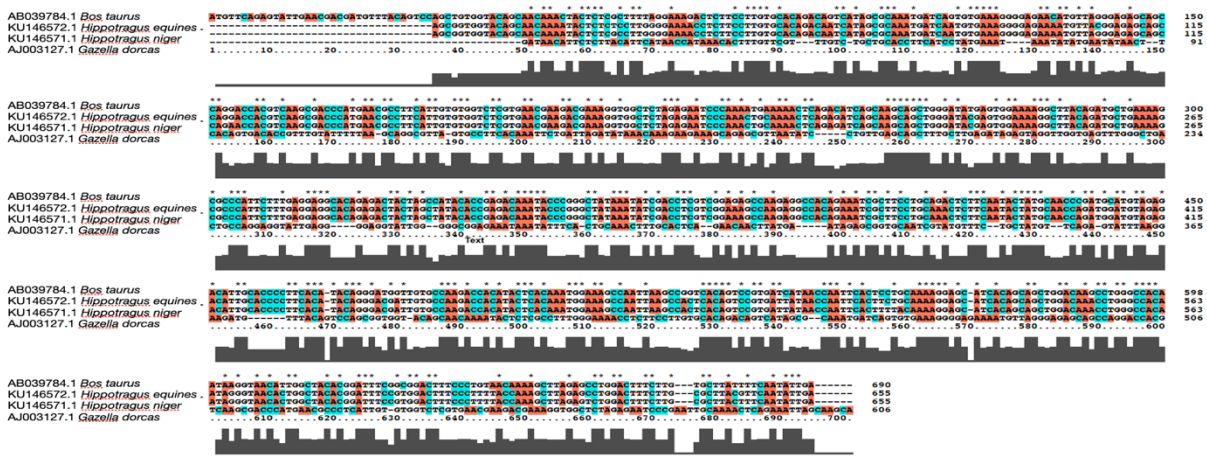


Figure 2-6 The alignment of the SRY gene region between *Bos taurus*, *H. niger*, *H. equinus* and *Gazella dorcas*.

2.5.3 Sources of fetal DNA for molecular-based sex determination

Numerous invasive and non-invasive techniques have been developed for fetal sex determination in domestic animals as well as in wildlife animals (Saberivand & Ahsan, 2016). Some of these tests that were developed consisted of embryo sexing techniques where the embryo's sex was determined before it was transferred. But this technique is time sensitive, technical and there is a great risk of damaging the embryo.

Alternatively, collecting fetal or placental cells during pregnancy for molecular-based sex determination is less technical and more successful. However, the collection of fetal or placental cells via the aspiration of amniotic fluid or chorionic villus sampling is invasive (Papp *et al.*, 1970, Walknowska *et al.*, 1969). It can also only be used after 16-17 weeks of gestation (Alfirevic *et al.*,

2017), whereas earlier detection would more ideal when managing breeding costs. The use of fetal cells circulating in human maternal blood is the first form of non-invasive cell collection for molecular-based sex determination. However, it can only be successfully used after the 14th week of gestation (Walknowska *et al.*, 1969) and false positives are common when using highly sensitive DNA detection techniques, due to the persistence of fetal cells in the maternal organs after pregnancy (Lo *et al.*, 1999, Lissauer *et al.*, 2007).

The following paragraphs introduce another form of fetal information that circulates through the maternal blood during pregnancy, namely fetal cell-free DNA (cffDNA). As cffDNA can be collected in a non-invasive manner, can be detected as early as the 7th week of gestation (Bianchi, 2004), and does not persist in the maternal blood after pregnancy (Yu *et al.*, 2013), it can serve as the ideal replacement target for circulating fetal cells in molecular-based prenatal sex determination.

2.6 Cell-free DNA (cfDNA)

In 1948 the presence of circulating cell-free DNA (cfDNA) was reported for the first time in human blood plasma by Mandel & Metais (1948). The significance of this discovery of extracellular circulating DNA was not initially realised, as well as the clinical potential it would have as a non-invasive screening tool for medical science today. The clinical potential was only realised after a number of studies indicated clear differences between the qualitative and quantitative characteristics of cfDNA from diseased and healthy individuals (Bronkhorst *et al.*, 2019). Table 2-2 shows the prominent discoveries regarding circulating cfDNA. In the following decades after the initial discovery, numerous studies demonstrated that cfDNA levels increase due to cancer and inflammation-related physiological conditions, such as aging, fatigue, traumatic injuries, smoking, diabetes, infections and organ transplant rejection (Van Der Vaart & Pretorius, 2008), and these levels often correlate with the severity, progression, treatment and recovery of these conditions (Schwarzenbach *et al.*, 2011).

Table 2-2 Timeline of the main discoveries and applications of cfDNA

Year	cfDNA analysis application and function	Researchers
1948	cfDNA molecules in human blood plasma.	Mandel & Metais (1948)
1963 – 1970	The active cellular release of circulating cfDNA as a messenger.	Stroun *et al.* (1967)
1972 – 1984	Circulating cfDNA involved in the humoral immune response.	Anker *et al.* (1975), Jachertz *et al.* (1979), Anker *et al.* (1976), Stroun *et al.* (1978)
1977	Higher concentrations of circulating cfDNA found in the blood of cancer patients	Leon *et al.* (1977)
1989	Circulating cfDNA derives from a tumour in cancer patients.	Stroun *et al.* (1989)
1994	RAS mutations detected by circulating cfDNA analysis.	Vasioukhin *et al.* (1994), Sorenson *et al.* (1994)
1994	Extracellular DNA from tumour cell line acts as an oncogenic mediator.	Anker *et al.* (1994)
1997	Foetal DNA circulates in the blood of pregnant women.	Lo *et al.* (1997)
2001	Circulating nucleosomes.	Holdenrieder *et al.* (2001)
2003	Identification of cfDNA in gravid rhesus monkeys	Jimenez & Tarantal (2003)
2005	First clinical study on the detection of point mutations by circulating DNA analysis.	Diehl *et al.* (2005)
2010	Geno metastasis (cancer metastasis via cfDNA).	García-Olmo *et al.* (2010)
2010	Circulating mitochondrial DNA as damage-associated molecular patterns (DAMP).	Zhang *et al.* (2010)
2012	Tracking tumour resistance by circulating cfDNA analysis.	Diaz Jr *et al.* (2012)
2014	First clinical validation of circulating cfDNA analysis.	Thierry *et al.* (2014)
2015	Circulating cfDNA induces apoptosis and DNA damage in recipient cells.	Mittra *et al.* (2015)
2019	cfDNA quantification in dogs with various tumours	(Tagawa *et al.*, 2019)

Studies have shown that both malignant and healthy cells do in fact release detectable amounts of cfDNA into the blood circulation and other body fluids. However, there are two contenders for the release of cfDNA in all body fluids, active DNA release and cellular breakdown mechanisms (Aucamp *et al.*, 2018). The term cellular breakdown mechanism is a collective term used for necrosis, pyroptosis, apoptosis, mitotic catastrophe and autophagy (Aucamp *et al.*, 2018). However, all these processes disclose a common mechanism by which cfDNA is released into body fluids. These cfDNA fragments contain unique genetic and epigenetic alterations that can

be traced back to the different cells from which they originate (Wan *et al.*, 2017). Kinetic analysis of cfDNA can be used as a means for predicting the clinical outcome of health disorders that cause tissue destruction as well as other cell damage. cfDNA also has the potential as comprehensive and highly specific non-invasive diagnostic, theranostic as well as prognostic indicators for a variety of pathologies. Based on personal and precision medicine, especially in the form of non-invasive medicine, cfDNA analysis is an ideal candidate that can be used in the application of molecular and genomic techniques for comprehensive clinical tests. The increased attention in cfDNA by the scientific community has steered the development of ultrasensitive technologies, as well as the improvement of most analytical techniques for the potential applications of cfDNA.

2.6.1 Cell-free fetal DNA (cffDNA) and fetal sex determination

The most successful clinical application of cfDNA studies to date is the utilisation thereof in prenatal screening and sex determination. Lo *et al.* (1989) were the first to discover a high admixture of fetal-derived cfDNA and maternal cfDNA in the human maternal blood. In a later study by this same group of researchers, it was determined that in 70% of human subjects bearing male foetuses, fetal Y-chromosome sequences could be detected in the maternal blood plasma (Lo *et al.*, 1997). This discovery has opened a new possibility for the development of fetal cfDNA based prenatal genetic testing. Prenatal cfDNA screening can be used for fetal sex determination, early detection of fetal RhD (Ramos, 2006) as well as other chromosomal aneuploidies (Swarup & Rajeswari, 2007). Therefore, fetal cfDNA can be used as a tool for non-invasive diagnosis of pregnancy associated complications, prenatally inherited diseases as well as sex-linked disorders (Swarup & Rajeswari, 2007).

A significant amount of research has been done on prenatal cfDNA screening in humans; however, there is a lack of research with regards to the prenatal cfDNA screening in animals. There have been a few studies that focused on the use of fetal cfDNA in cattle (Lemos *et al.*, 2011, da Cruz *et al.*, 2012, Mayer *et al.*, 2013, Son *et al.*, 2013), horses (de Leon *et al.*, 2012, Kadivar *et al.*, 2016), sheep (Asadpour *et al.*, 2015), rhinoceros (Stoops *et al.*, 2018) and elephants (Vincze *et al.*, 2019) for fetal sex determination. Specifically, PCR techniques have been investigated and a generalised PCR method with acceptable accuracy has been identified. Each species tested required a different region in the *SRY* gene in order to develop primers that can be used during the PCR method. *Table 2-3* shows the different primers that were used during these studies as mentioned for the five different species.

Table 2-3 The primers used for the sex determination studies of five different animal species.

Species	Primers used	Number of samples	% Accuracy	Reference :
Mixed bred cattle	5'-CCTCTTCCCGTTCAAACGCCCGGAATCATT-3' and 5'-TGCGTTGCAGGGACTGAGACCAGGTTTGGG-3'	35	95%	(da Cruz *et al.*, 2012)
Sheep	5′-CAGCCAAACCTCCCTCTGC-3' and 5′-CCCGCTTGGTCTTGTCTGTTGC-3′	32	95%	(Asadpour *et al.*, 2015)
Horses	5'-CGCCAGCATAGATCACAGAA-3' and 5'- CGCAAGGTAGCTGAAAGACC-3'	20	95%	(de Leon *et al.*, 2012)
Rhinoceros	5'-TCATGGTGTGGTCTCGTGAT-3' and 5'-CCGGGTATTTCTCTTCATGC-3'	14	100%	(Stoops *et al.*, 2018)
Elephants	5'-AGCAAGCTGCTGGGATACCAGTG-3' and 5'-TATAGTCCGGGTTTGGTTTG-3'	4	100%	(Vincze *et al.*, 2019)

The *SRY* gene has its limitations when it's exclusively used in sex determination studies by PCR. It only amplifies a specific gene region on the Y-chromosome and if amplification does not occur, a positive control is needed to verify if the sample has any genetic material present or not. However, amelogenin Y and amelogenin X genes (AMLY/X) are other genes that can be used as a positive control marker for sex determination in mammals (Asadpour *et al.*, 2015). The amelogenin gene is a genomic sequence found in the X- and Y-chromosomes of every mammal species and this gene sequence encodes for tooth enamel (Nakahori *et al.*, 1991). This AMLX/Y gene is thus present in both male and female mammalian species and can be used as a gene sequence during sex determination studies to act as a positive control. In a study by Divar *et al.* (2012), the sequence length polymorphisms between the AMLX and AMLY genes were used as markers for sexing bovine foetuses. Different sets of amelogenin gene primers could be used as a reliable molecular marker for sex determination of multiple domesticated animals, such as sheep, cattle, pigs, goats, and dogs. However, using this AMLX/Y gene as markers for sex

determination is unsatisfactory for serum samples, as no fetal originated Y-chromosome DNA was amplified from male or female pregnancies (Divar *et al.*, 2012). In a study by Zaffalon *et al.* (2019), a gametologue gene has been identified on both the Y- and X-chromosome, which is described as NGLN4Y and NGLN4X, respectively. The NGLN4Y gene can also be used as a positive control or on its own to determine the sex of the calf. Zaffalon *et al.* (2019) investigated the use of the NGLN4Y and NGLN4X gene variants in fetal sex determination and, from the 29 tested horse samples, they found acceptable results. This AMLX/Y gene and NGLN4Y/X gene can be used as a positive control to identify if there are Y chromosome sequences present in the sample and can be used with the *SRY* gene to identify the sex of the calf (de Leon *et al.*, 2012, Zaffalon *et al.*, 2019).

2.7 Problem statement

The need to develop a non-invasive fetal sex determination technique to effectively determine the fetal sex of sable and roan antelope at an early gestation period, would be beneficial for management decisions and it will provide the opportunity to develop similar techniques for other species. Several studies have demonstrated that cfDNA can be used as an application for early fetal sex determination and it appears to be a promising technique for domestic and wildlife species. Therefore, establishing and validating a molecular sex determination technique that uses very small quantities of DNA would be of great value. This can also be done during routine veterinary check-ups or when animals are moved or auctioned. In wildlife animals, sedatives are administered by darting the animals, during this time veterinary tests can be performed, and an extra tube of blood can be collected for prenatal sex determination from a female animal with no added stress.

Only a partial sequence of the *SRY* gene from the sable and roan antelope is currently available in GeneBank. Therefore, the first objective of this study was to determine the full-length sequence of both species. As previously shown by Vaz Pinto *et al.* (2016), hybridisation between these two species does occur. The second objective was to determine the subspecies by following the method as stated in an article by Pitra *et al.* (2006). Thirdly cfDNA isolation was conducted and lastly a qPCR as well as a MassARRAY® protocol was optimised for fetal sex determination.

2.8 Aims and objectives

The aim of this study is to develop a quantitative method for determining the fetal sex of sable and roan antelope prenatally.

Objectives:

1. Collection of samples for the sable and roan antelope:
   1.1. Hair samples from both sable and roan antelope will be collected and used for sequencing.
   1.2. Blood samples from sable and roan antelope will be collected to determine the fetal sex of the calve and male blood samples will be used as a positive control.
2. To sequence the complete *SRY* gene of the sable and roan antelope using Sanger sequencing.
3. Subspecies identification of both sable and roan antelope using mtDNA for sequencing the hypervariable D-loop region.
4. Isolation of cfDNA from sable and roan antelope blood samples and determine if cfDNA is present.
5. Design primers to amplify the SRY gene of the sable and roan antelope.
6. Optimisation of qPCR and the MassARRAY® system for fetal sex determination.
7. To compare the quantitative assays for optimal determination of foetal sex.

CHAPTER 3. METHODOLOGY

3.1 Introduction

The existing methods for non-invasive fetal sex determination in animals are still limited in a variety of animal species. In this study, we investigated the use of the *SRY* gene in the prenatal sex determination of roan and sable antelope foetuses. The *SRY* gene of the roan and sable antelope was sequenced from hair samples to identify variations between the species, as well as the subspecies. Blood samples were collected from pregnant sable antelopes in order to isolate cfDNA and to determine whether fetal cfDNA was present in these antelope species. Two methods, qPCR and MassARRAY®, were then used to determine the fetal sex from the isolated fetal cfDNA.

The following paragraphs discuss the methods used during this study, including previous results of similar studies and the changes that were used to improve on these previous studies.

3.1.1 Sanger sequencing

The major breakthrough of DNA sequencing (the determination of the nucleic acid sequence/order of a DNA strand) was in 1977 by Fred Sanger and colleagues (Sanger *et al.*, 1977), with the development of the chain-termination technique. The chain-termination technique uses chemical analogues of deoxyribonucleotides (dNTPs), the monomers of DNA, which can be incorporated into DNA strands during DNA replication using DNA polymerase. By the mixing of radiolabelled dNTPs into a DNA extension reaction in low concentrations, the dNTPs result in the production of a DNA strand. Initially, four parallel reactions containing each individual dNTP base are preformed and the results are then used for polyacrylamide gel runs to determine the nucleotide sequence of the original template strand using autoradiography (Heather & Chain, 2016) (Figure 3-1). Due to its accuracy and accessibility, this reaction has led to the creation of the dideoxy chain-termination method, or rather known as Sanger sequencing, the most common technique used for DNA sequencing. The Sanger sequencing technique was later further improved by replacing the radiolabelling with fluorometric-based detection, as well as improving detection through capillary based electrophoresis. The change to fluorometric based detection allowed that the reaction could occur in one reaction tube instead of using four different reaction tubes according to the dNTPs. These further improvements on the Sanger sequencing technique

has ultimately led to the development of automated DNA sequencing machines (Hunkapiller *et al.*, 1991).

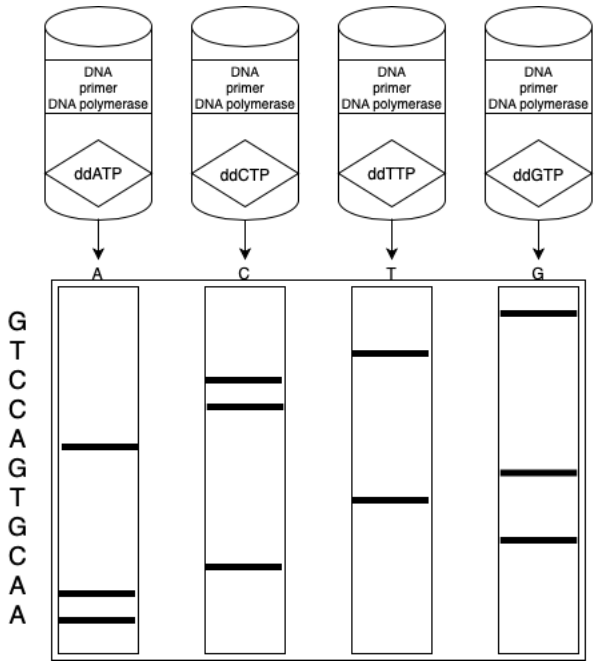


Figure 3-1 Schematic representation of the Sanger sequencing method. The four separate DNA extension reactions are performed, where each contains a single stranded DNA template, primer, DNA polymerase and all four dNTPs to synthesise the new DNA strand.

As mentioned in chapter 2, the *SRY* gene region of both antelope species have been sequenced and related *SRY* gene sequences are available on GenBank and they had positive amplification using this sequence (Vaz Pinto *et al.*, 2016). However, this relates to only a partial sequence of the *SRY* gene. The previous study of De Villiers (2020) that used qPCR of cffDNA for prenatal testing in cattle and buffalo, experienced sensitivity issues that lead to false negative results. The study used a commercial, validated qPCR kit for the bovine qPCR and GenBank data for the primers of the buffalo *SRY* gene, including only one gene target for each animal. To improve on the outcomes of this previous study, Sanger sequencing was used during this study to determine the complete nucleotide sequence of the *SRY* gene of sable and roan antelope. Antelope hairs were used as a source of gDNA for the sequencing. Hair samples were chosen because it is a minimal invasive sampling method and since some breeders had hair samples available that were sampled and stored for previous tests. The resulting data was then used to assess variations between the antelope species and subspecies and to develop primers for several different target

areas of the *SRY* gene. This was done to increase the success rate by using these multiple different primers for gene amplification of cffDNA during the qPCR assay and by using the different SNPs for the MassARRAY® system for the identification of male fetal DNA.

3.1.2 Blood sample collection and processing for cfDNA extraction

De Villiers (2020) investigated fetal sex determination in cattle and African buffalo and developed a cfDNA blood collection protocol for wildlife and domesticated animals. BD vacutainer® plasma preparation K2E tubes (PTT™) were identified as not being sufficient blood collection tubes for cfDNA isolation. gDNA release has been observed in these tubes over time, indicating that these tubes do not prevent cell damage. The use of specific cfDNA blood collection tubes, for example Streck cfDNA blood collection tubes, was recommended (De Villiers, 2020).

Indeed, Streck cfDNA blood collection tubes have been shown to stabilise whole blood over time, indicating that cell lysis does not take place within this tube and prevents the release of gDNA. Fernando *et al.* (2010) investigated the stability of K_3EDTA (BD vacutainer®) and Streck cfDNA tubes when the whole blood is stored at room temperature for 14 days before plasma isolation occurs. Their results have shown that the Streck cfDNA tubes remain stable over time for cfDNA and that the cffDNA can also be preserved in these tubes for whole blood samples (Fernando *et al.*, 2010). Another study compared BD vacutainer®, Streck cfDNA tubes and PAXgene Blood ccfDNA tubes, where it was shown that the Streck and PAXgene tubes stabilise the plasma within the tube, as well as the cfDNA (Warton *et al.*, 2017). This is attributed to the tubes containing a preservative and stabilising agent to prevent cell lysis and stabilise the plasma within the tube. These specific cfDNA tubes are also more stable during transportation at ambient temperature or when transported on ice packs. According to the manufacturer's instructions, of the PAXgene® Blood ccfDNA tube, the whole blood collected remains stable for up to 10 days at temperatures up to 25°C before processing. This is an important factor for this study due to the fact that blood samples would be retrieved on farms where the samples cannot be processed immediately or placed in a fridge. Thus, these PAXgene® Blood ccfDNA tube makes it possible for the transportation of the blood samples that are received on the farm to be transported at ambient temperatures to the laboratory for processing.

Accordingly, for this study, PAXgene Blood ccfDNA tubes were used according to the PAXgene Blood ccfDNA tube protocol, where the optimal transportation temperatures are up to 25°C and the optimal storage for the isolated plasma sample at -80°C after sample processing.

3.1.3 cfDNA isolation

The second critical step after sample collection and processing is the isolation of the cfDNA. Over the previous couple of years, isolation protocols for cfDNA have increased, as well as the development of specific cfDNA isolation kits. There are currently 40 commercial cfDNA extraction methods available and these kits are either automated or manual isolation kits (Bronkhorst *et al.*, 2020). The most common methods used for isolation of cfDNA include salting-out, phenol-chloroform (Asadpour *et al.*, 2015), column-based kits adapted for smaller fragments (de Leon *et al.*, 2012) and magnetic bead-based extraction (De Villiers, 2020, Bronkhorst *et al.*, 2020). This, in turn, has led to the development of a variety of different isolation protocols from different manufacturers. All the commercially available kits share a similar route of extraction, consisting of a lysis step, followed by a nucleic acid binding step, where the contaminants are washed with a wash buffer before being eluted with elution buffer. It can be assumed that all these commercial isolation kits have higher salt concentrations for the isolation of small fragments of nucleic acids.

In a study by Bronkhorst *et al.* (2020), six different commercially available cfDNA isolation kits were compared, including three spin-column kits, two magnetic bead-based manual kits and one automated magnetic bead-based isolation kit. They determined that the NucleoSpin® Gel and PCR Clean-up kit showed the fourth highest cfDNA yield and highest reproducibility (Bronkhorst *et al.*, 2020). It was also found that magnetic bead-based isolation is best for short cfDNA fragments (50-250 bp) and spin-column isolation is suitable for the recovery of larger cfDNA fragments (250-10,000 bp). From all the recent studies of cfDNA isolation there is still no consistent isolation method that can produce overall high yield of short and long cfDNA fragments.

As mentioned, the study of De Villiers (2020) experienced assay sensitivity issues, producing false negative qPCR results when *SRY* gene amplification was attempted in cffDNA. Apart from the potential impact of the type and number of gene targets used (3.1.2), the cffDNA extraction method may also have an impact due to the above-mentioned differences in extraction kit yield, fragment targets and reproducibility. Accordingly, a different extraction kit was used during this study to try and improve assay success. The NucleoSpin® Gel and PCR Clean-up kit was used due to its moderate cfDNA yield and high reproducibility (Bronkhorst *et al.*, 2020).

3.1.4 DNA quantification

The quantification of DNA is an important step to determine the concentration of DNA present within a sample, before preforming different techniques. The most used method for the

quantification of DNA is spectrophotometry, where the amount of the ultraviolet light absorbed by the sample is measured. For DNA and RNA, the absorbance is at a wavelength of 260 nm and this can be used with the Beer-Lambert law to determine the concentration of the sample. With the advancement of technology, a wide variety of Spectrophotometers and Fluorometers were developed to determine the DNA concentration of a given sample.

In this study, the NanoDrop™ one/oneC Microvolume UV Spectrophotometer (Thermo Fisher Scientific) was used to quantify gDNA samples. For the cfDNA samples, however, the chaotropic salt contents of the extraction kit binding buffer poses the risk of interfering with spectrophotometric quantification of DNA (see NucleoSpin® Gel and PCR Clean-up kit manufacturer's instructions Appendix). Instead, both the Qubit fluorometer and capillary electrophoresis were used for quantification due to the low concentration of cffDNA found in maternal plasma. The cffDNA concentration range between 3,4 and 6,2% of the total DNA found in maternal plasma of animals (Davoodian & Kadivar, 2016a).

The Qubit fluorometer provides a sensitive and accurate quantification of dsDNA, ssDNA, RNA and protein through quantitation assays. On the other hand, one of the most sensitive methods used to quantify DNA is the use of capillary electrophoresis via the BioAnalyzer 2100 of Agilent Technologies. The BioAnalyzer is a chip-based electrophoresis system for the analysis of DNA, RNA and protein via capillary electrophoresis. The benefit of using capillary electrophoresis to quantify cfDNA samples, is the small sample volume needed (1 µL of minimum 500 pg DNA). For each application between DNA, RNA and protein, there are specific chips and reagents needed for quantification. For a DNA assay, a high sensitivity DNA chip and reagents were used to identify DNA with a base pair length of 50 to 10 000 bp.

3.1.5 Real-Time PCR versus the MassARRAY® system

PCR is one of the most frequently used methods in molecular biology. The PCR method is an enzymatic reaction that amplifies the initial nucleic acid molecules exponentially to form a measurable amount of replicates of the initial sequence (Singh & Kumar, 2001). For the PCR reaction to take place, a polymerase enzyme is needed to synthesise a complementary strand after the binding of a sequence specific primer, (Singh & Kumar, 2001). The primer that binds at the starting point is known as the forward primer and that at the end point is the reverse primer. However, this enzymatic reaction is also temperature dependant, where high temperatures are required for the denaturation of the DNA strand and lower temperatures are for the annealing of the primers on the DNA strand. After this reaction, one copy of the desired sequence has formed,

and this process is known as a PCR cycle. After each cycle, the desired sequence is double the original amount, thus after each cycle the amount of nucleotide sequences increases exponentially. After 40 cycles, theoretically, millions of copies are formed from the initial nucleotide sequence. These products can then be visualised with the use of electrophoresis.

Real-time PCR or qPCR uses the same principals as PCR, but for each successful polymerase reaction fluorescence is emitted and is measured in real time (Smith & Osborn, 2009). This fluorescence is then detected by a detector that monitors the reaction and when more sequence copies are formed after each cycle, an increased fluorescence is detected. The advantage of qPCR is the confirmation of the analytes by the use of the melting curve analysis. The melting curve analysis indicates how many amplicons were generated and how many primer-dimers or non-specific products were formed during the reaction. The efficiency of the reaction can be measured during qPCR and quantitative analysis of the target gene sequence can be performed. During this, two popular methods are available for qPCR assays namely SYBR Green and probe based. SYBR Green is a cost effective and simple to use method, which is based on the binding of the fluorescent dye to the dsDNA (Tajadini *et al.*, 2014) but the disadvantage of this is primer dimer formation (Bustin *et al.*, 2009). Thus, during the amplification of the sequence fluorescence is emitted and detected by the qPCR. The probe-based approach is more expensive and it is based on the dual labelling of the oligonucleotide and exonuclease activity (Tajadini *et al.*, 2014) this is known as the reporter and quencher. During the amplification of the sequence the reporter is detached by DNA polymerase from the probe and fluorescence is emitted for detection by the qPCR.

Another method or technique that can be used for the analysis of cffDNA is MassARRAY® integrated system (Akolekar *et al.*, 2010). The MassARRAY®- system refers to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Akolekar *et al.*, 2010), which measures the mass of the DNA molecules. The different nucleotides are distinguished by their time-of-flight after ionization in the vacuum chamber and the mass of the nucleotide is measured. The MassARRAY® system from Agena® Bioscience (San Diego, USA), combines accuracy, flexibility, automated analysis, and high throughput data generation (Ding & Cantor, 2003, Jurinke *et al.*, 2004, Li *et al.*, 2006). The MassARRAY® system is an integrated system that combines PCR amplification with MALDI-TOF MS to obtain the results of the amplified gene sequence. The results obtained are represented in the form of a mass spectrum for the specific target nucleotide within the amplified gene sequence that is investigated. The advantage of using the MassARRAY® system is it allows targeting of 10s – 100s of genetic markers per sample per run. The MassARRAY® system is highly targeted and sensitive for specific gene markers that are

investigated using only a small amount of DNA. There is no need for using fluorescence or labelling due to that the individual mass of the product is measured.

The most frequently used method for fetal sex determination using cfDNA in animals, including the bovine, ovine, equine, rhinoceros and elephants, relies on the basis of a PCR method together with the amplification of the *SRY* gene (da Cruz *et al.*, 2012, de Leon *et al.*, 2012, Kadivar *et al.*, 2013, Asadpour *et al.*, 2015, Stoops *et al.*, 2018, Vincze *et al.*, 2019). Another possible method or technique that can be used for the analysis of cffDNA is the MassARRAY® integrated system (Akolekar *et al.*, 2010). In this study we investigated an approach to determine the fetal sex using cfDNA for a SYBR Green qPCR assay, probe-based qPCR assay and then lastly the MassARRAY® system. The MassARRAY® method was then compared to a SYBR Green qPCR method to determine which method was the most successful in determining antelope fetal sex using cffDNA.

The following paragraphs detail the methodology used during this study to extract genomic and cfDNA from antelope samples, sequence the *SRY* gene, and detect male cffDNA using the primers developed from the gene data in qPCR and MassARRAY®.

3.2 Sequencing of the *SRY* gene

3.2.1 Ethical clearance

Blood and hair samples from the sable antelope and only hair samples from the roan antelope were used in this study. The sample collection and handling procedures were the same for both species.

Ethical clearance was obtained from AnimCare, North-West University – ethics reference number: NWU-00595-19-A5 (see Appendix A).

Informed consent forms were signed by the owners from which samples were collected (see Appendix B).

3.2.2 Sample collection and processing

Hair samples of both sable and roan antelopes were received from different farmers. No formal sample size calculation was performed for the hair samples, samples size was based on sample

availability. A total of 45 sable antelope hair samples and 10 roan antelope hair samples were received. The age of all the hair samples is unknown. For each species, about 30 to 50 hairs, with the follicle still attached, were sorted, and placed in a 15 ml Falcon tube and the sample list can be found in Appendix C.

PAXgene® Blood ccfDNA tubes (Qiagen, Germany) were used to collect blood samples. The blood collection was done by venepuncture of the jugular vein with a 18G vacutainer needle. The samples were taken during the farm's routine health screening processes, during which the pregnancy of the females was confirmed via ultrasonography. Samples from ten pregnant females and one male sable antelope were obtained. Two 10 ml PAXgene® blood ccfDNA tubes were collected per animal. Unfortunately, obtaining blood samples from roan antelope was not possible, due to the restrictions of the Stage 4-5 Covid-19 lockdown regulations in South Africa during the pregnancy period of these species. The blood of the male antelope was used to obtain additional gDNA, for sequencing, and to serve as a positive control gDNA sample for the qPCR and MassARRAY® assays. The blood of the female antelopes was used to obtain the cfDNA required as test material for the qPCR and MassARRAY® assays.

The collected blood tubes were handled as prescribed by the manufacturer during and post blood collection of the samples. Samples were placed in a cooler box with gel ice packs after sample collection for transport at ambient temperature. The blood was transported to the laboratory, where it was immediately after arrival used for plasma isolation which was 6 hours after collection. The blood was centrifuged at 1900 x g for 15 minutes and the plasma was extracted from each sample, making sure not to disturb the buffy coat and the cellular fraction, and placed in 5 mL Eppendorf tubes. The plasma was then further centrifuged at 1900 x g for 10 minutes to remove any remaining proteins. After the final centrifugation step, the plasma was stored at -80°C until cfDNA extraction. Previous studies showed that plasma samples prior to cfDNA isolation can be stored for up to 3 months at -20°C, for up to 9 months at -80°C, (El Messaoudi *et al.*, 2013). The male blood samples were stored without prior processing at -80°C until gDNA extraction.

3.2.3 Genomic DNA extraction

gDNA was extracted from the hair samples using the Zymo Quick-DNA™ Miniprep Plus kit (Zymo Research, USA), according to manufacturer's instructions. This extracted gDNA was then used for the sequencing of the SRY gene and the identification of sub-species.

gDNA extraction from male sable antelope whole blood was done using the NucleoSpin® Blood, gDNA from blood (Macherey-Nagel, Germany) according to the manufacturer's instructions. The

extracted gDNA concentration was determined using the NanoDrop™ one/oneC Microvolume UV Spectrophotometer (Thermo Fisher Scientific). This gDNA was used as positive control during the qPCR reactions as well as for the optimisation of the qPCR and MassARRAY® protocols.

Two aliquots of gDNA were fragmented by the use of the Soniprep 150 (MSE Centrifuges) ultrasonic machine. During the fragmentation step a 100 µL tip was placed on the tip probe of the Soniprep 150, the sample was added on ice and the samples were sonicated. The sonification of the sample was done for 1 minute at full power and a rest period of 30 seconds was used, this was continued for 30 minutes. This fragmented gDNA was then used for the optimisation of the qPCR and MassARRAY® system to represent smaller fragments of the DNA strand. This was also used during the BioAnalyser quantification step of the cfDNA.

3.2.4 Phylogenetic analysis

Mitochondrial DNA (mtDNA) was also isolated from the hair samples and sequenced (using ABI 3500XL Genetic Analyzer) at Inqaba Biotec™ to identify the D-loop fragment of these species. The sequenced data was used for phylogenetic analysis, to determine the different sub-species of the given samples. A mtDNA GenBank search for both sable and roan antelope was done to identify reference sequences and controls during the phylogenetic analysis. Various studies have identified these sequences and their findings can be found on GenBank for both the sable and roan antelope (GenBank reference numbers available in Appendix C) (Matthee & Robinson, 1999, Pitra *et al.*, 2002, Alpers *et al.*, 2004, Pitra *et al.*, 2006, Themudo *et al.*, 2015). The inclusion of the D-loop fragment sequence was done as described by Themudo *et al.* (2015) for the phylogenetic reconstruction. The identified sequences of the sable and roan antelope samples were aligned with these reference sequences using Clustal W (Larkin *et al.*, 2007) and the phylogenetic tree was constructed using MegaX (Kumar *et al.*, 2018) under maximum likelihood and bootstrap analysis settings with a 1000 bootstrap replications.

3.2.5 Sanger sequencing of the *SRY* gene

Sanger sequencing was performed at Inqaba Biotec™ to determine the complete sequence of the *SRY* gene for both sable and roan antelope species. An alignment of these sequences was done with the bovine *SRY* gene (accession number: AB039748) as a reference sequence to identify primers that can be used during Sanger sequencing. **Table 3-1** shows the primers that

were used for sequencing the *SRY* gene of both the antelope species and these primers were designed by Inqaba Biotec™.

Table 3-1 Primers for Sanger sequencing of the SRY gene.

Primer	Sequence (5' to 3')
SRY-F	CAGTGCAGTCGTATGCTTCTGC
SRY-R	GCGAGAGTAAGGAAGTCAATATTG

3.3 Assay and primer design using sequence data

For this study, we investigated two different qPCR assays as well as the MassARRAY® system. This was done to identify the most optimal method to be used for the foetus sex determination from sable antelope samples and for this specific *SRY* gene. The two qPCR assays that were investigated were the SYBR Green qPCR and a probe-based qPCR assay.

3.3.1 SYBR green qPCR assay design and optimisation

In the first assay, five target primers across the *SRY* gene were designed from the *SRY* gene sequence obtained from Sanger sequencing. (summarised in Table 3-2). These five target primers produce PCR products between 70 and 100 bp. A schematic representation of the five different binding sites is shown in Figure 3-2 and this shows that the primers were distributed throughout the *SRY* gene sequence.

Table 3-2 Primers used in the SYBR green qPCR assay to amplify fragments from the SRY gene

Primer		Sequence (5' – 3')	Fragment length of amplicons (bp)
SRY -1	Forward primer	AAGACGATGTTTACAGTCCAGC	96 bp
	Reverse Primer	CATTTGCGCTATGATTGTCTGTG	
SRY -2	Forward primer	TTCATTGTGTGGTCTCGTGAAC	70 bp
	Reverse Primer	TCTCTGAGTTTTGCAGTTTGGG	
SRY -3	Forward primer	TTTGAGGAGGCACAGAGACTAC	98 bp
	Reverse Primer	AAGCGATTTCTGTGGCCTCTTG	
SRY -4	Forward primer	CGATTGTGCCAAGACCACATAC	104 bp
	Reverse Primer	AGCTGCTGTGATGCTCCTTTTG	
SRY -5	Forward primer	ATAGGGTAACACTGGCTACACG	83 bp
	Reverse Primer	AAGTAAGCGCAAGAAAGTCCAG	

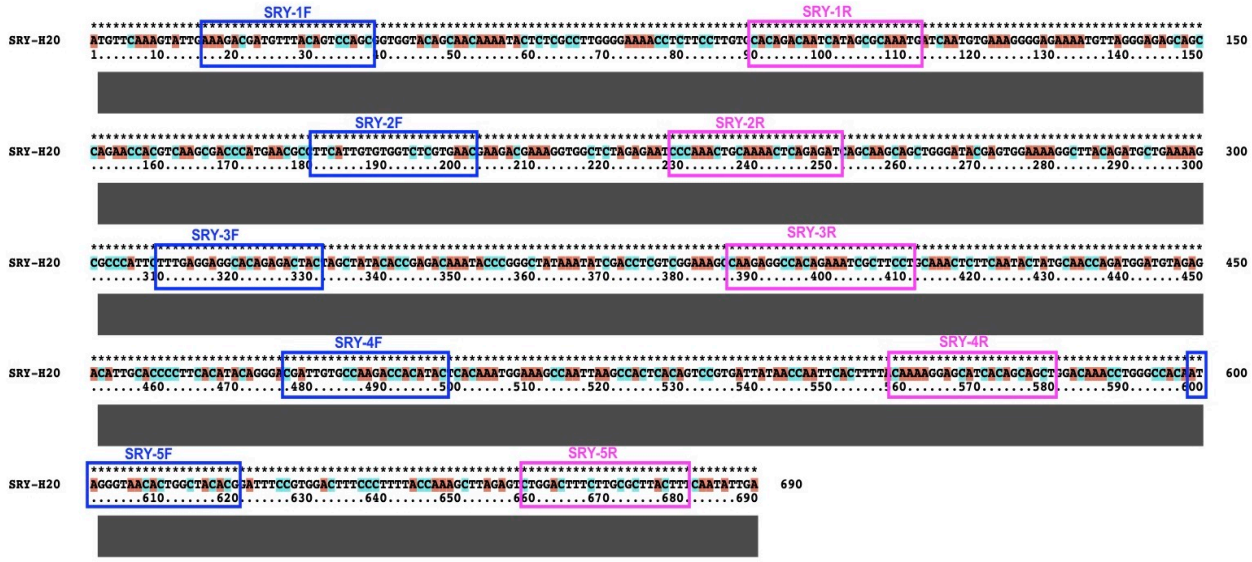


Figure 3-2 Schematic representation of the different binding sites of the five identified primers on the *SRY* gene sequence.

The optimisation of the qPCR method was done by setting up a 10-fold serial dilution (20 ng/µL, 2 ng/µL, 0.2 ng/µL, 0.02 ng/µLand 0.002 ng/µL) of the gDNA from the male sable for each primer set. This was done to test the amplification efficiency of the primer sets and to determine the lowest concentration of DNA used that still gave reliable results. Primer dimer formation was tested by adding a melting curve.

In the optimisation of the qPCR assay for the amplification of the *SRY* gene, reactions were performed in triplicate in a 20 µL qPCR reaction: Luna MasterMix, forward primers (10µM), reverse primers (10 µM), serial dilution ranging from 20 ng/µL to 0.002 ng/µL gDNA and PCR grade H_2O to 20 µL. The reactions were run on the Applied Biosystems QuantStudio 5 using the following reaction conditions: an initial step at 95°C for 60 sec, followed by a two-step phase at 95°C for 15 sec and extension at 60°C for 30 sec this was done for 45 cycles. The melting curve analysis was performed after the qPCR run was completed.

Fragmented male gDNA with a concertation of 20ng/µL was diluted to 2 ng/µL and 0.2 ng/µL for a serial dilution experiment and was used to simulate the cfDNA samples. A 20 µL qPCR reaction was performed using: Luna MasterMix, forward primers (10µM), reverse primers (10 µM), the serial dilution ranging from 20 ng/µL to 0.002 ng/µL fragmented gDNA and PCR grade H_2O to 20 µL. The reactions were run on the Applied Biosystems QuantStudio 5 using the following reaction

conditions: an initial step at 95°C for 60 sec, followed by a two-step phase at 95°C for 15 sec and extension at 60°C for 30 sec this was done for 45 cycles.

The PCR products were separated and analysed by electrophoresis. The Flashgel™ system (Lonza Rockland, Inc.) was used for the electrophoresis of the qPCR products. The qPCR products were loaded, along with Loading Dye Solution (10 mM Tris-HCl pH7.6, 0.03% bromophenol blue, 0.03% xylene cyanole FF, 60% glycerol and 60 mM EDTA), into the 2% agarose gel in TAE buffer and the agarose gel was stained with 0.5 µg/mL ethidium bromide. For the Flashgel™ system the samples were run at 250V for 10 minutes and the gel was photographed using the Flashgel™ system.

3.3.2 Probe based qPCR assay design and optimisation

The probe-based primers were designed from the *SRY* gene sequence of the sable antelope. Three different targets were identified and are shown in Table 3-3. Three different probes or fluorescent tags were used namely FAM, HEX and Cal Fluor 610. The FAM probe was used for primer set SRYP-1, Cal Fluor 610 for SRYP-2 and HEX for primer SRYP-3. A schematic representation of the different binding sites of the primers as well as the probe is shown in Figure 3-3.

Table 3-3 Probe-based primers to amplify the SRY gene of the sable antelope.

Primer		Sequence (5' – 3')	Fragment length of amplicons (bp)
SRYP -1	Forward primer	AGCGCAAATGATCAATGTGAAAGG	79 bp
	Reverse Primer	AGGCGTTCATGGGTCGCTTG	
	Probe (FAM)	TGTTAGGGAGAGCAGCCAGAACCA	
SRYP -2	Forward primer	CAGCTGGGATACGAGTGGAAA	100 bp
	Reverse Primer	CCGGGTATTTGTCTCGGTGTATA	
	Probe (Cal Fluor 610)	TTACAGATGCTGAAAAGCGCCCA	
SRYP -3	Forward primer	CGCTTCCTGCAAACTCTTCA	83 bp
	Reverse Primer	GGCACAATCGTCCCTGTATG	
	Probe (HEX)	TGCAACCAGATGGATGTAGAGACATTGCA	

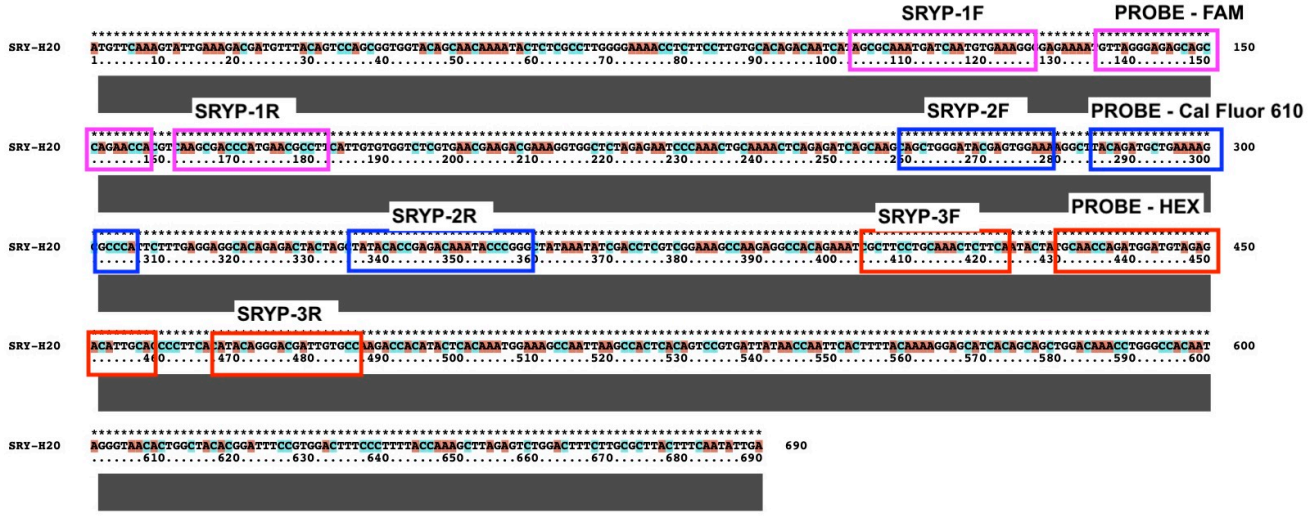


Figure 3-3 Schematic representation of the different binding sites for the probe-based primers on the *SRY* gene sequence.

The optimisation for the qPCR was done with the use of a 10-fold serial dilution (20 ng/µL, 2 ng/µL, 0.2 ng/µL, 0.02 ng/µLand 0.002 ng/µL) of the gDNA for each primer set. A qPCR assay for the different primer sets was designed and optimised for the specific probe.

In the optimisation of the qPCR assay for the amplification of the *SRY* gene, reactions were performed in triplicate in a 20µL qPCR reaction: Luna® Universal Probe qPCR MasterMix, forward primers (10µM), reverse primers (10µM), Probe (10µM), serial dilution ranging from 20 ng/µL to 0.002 ng/µL of gDNA and PCR grade H_2O to 20 µL. The reactions were run on the Applied Biosystems QuantStudio 5 using the following reaction conditions: an initial denaturation step at 95°C for 60 sec, followed by a two-step phase of denaturation at 95°C for 15 sec and extension at 60°C for 30 sec, this was done for 45 cycles.

Fragmented male gDNA with a concentration of 20 ng/µL was diluted to 2 ng/µL and 0.2 ng/µL for the serial dilution experiment that was used to stimulate cfDNA samples. A 20 µL qPCR reaction was performed as described previously. The PCR products were then separated and analysed by electrophoreses according to section 3.3.1.

3.3.3 MassARRAY® assay design and optimisation

The optimisation of the MassARRAY® system was conducted by Inqaba Biotec™. A 10-fold serial dilution of gDNA was done from 20 ng, 2 ng and 0.2 ng and were amplified by PCR. Single base extension reactions were performed on the different single nucleotide polymorphisms (SNPs) or targets. The single base extension for the sequences was as follows: SRY-1 A/G, SRY-2 G/A, SRY-3 T/C, SRY-4 G/A and SRY-5 C/T (Figure 3-4) and these represent the different targets or SNPs that were used during the MassARRAY® assay. After the single base extension, the samples were run on the MassARRAY® system from Agena® Bioscience (San Diego, USA).

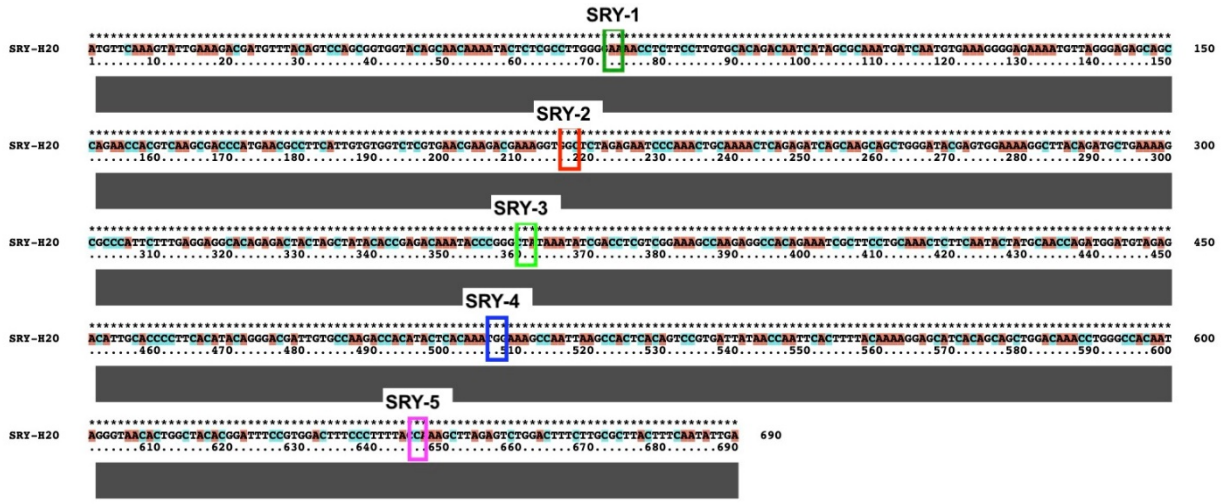


Figure 3-4 Schematic representation of the five different SNPs genotyped for the MassARRAY® system.

The MassARRAY® assay was also optimised using the five different SNPs for the fragmented gDNA. gDNA was fragmented for 30 and 35 minutes and the concentrations were measured for both fragmented samples used. For the 30-minute digested sample, a serial dilution was done from 10 ng to 1ng and 0.1 ng. For the 35-minute digested sample, a serial dilution was done from 5 ng to 0.5 ng and 0.005 ng. The five different targets were then tested on both the fragmented samples, as well as an unfragmented gDNA (20 ng/µL, 2 ng/µL and 0.2 ng/µL) sample used as control, the samples were run on MassARRAY® system from Agena® Bioscience (San Diego, USA). This optimisation step was done to identify the detection limit as well as the DNA concentration that would be optimal for the MassARRAY® system.

3.4 cfDNA extraction and analysis

3.4.1 Isolation of cfDNA

The cfDNA of the stored plasma from the 10 blood samples of the sable antelope species were isolated using the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany) with a vacuum manifold. The 10 sable plasma samples were thawed at 37°C and centrifuged at 500 x g for 5 minutes, after which the plasma was transferred to a new centrifuge tube. The total volume of available plasma sample was used for the extraction at 1 mL of plasma per isolation step. For the isolation step, a 1:3 ratio of plasma to NTB Binding Buffer (Macherey-Nagel, Germany) was

used, where 1 mL plasma was added to 3 mL of NTB Binding Buffer. Due to the increased volume size the vacuum manifold and column extenders were used with the NucleoSpin® Gel and PCR Clean-up Column. Each plasma-NTB buffer mixture was divided into four of the NucleoSpin® Gel and PCR Clean-up Columns to avoid loss of bound DNA due to large volumes flowing through the column. After the mixture was vacuumed through the column, the columns were centrifuged at 11000 x g for 30 seconds in order to dry the silica membrane of the columns. The sample was thereafter washed with 700 µL NT3 Wash Buffer and centrifuged for 30 seconds at 11000 x g. The wash step was repeated twice in order to minimise chaotropic salt carry-over and to improve the purity of the sample. After each centrifugation step, the flow-through was discarded and the column was placed back into the collection tube. The silica membrane is dried with an extra centrifugation step for 1 minute at 11000 x g to remove any remaining NT3 Wash buffer. The column was then placed into a 1.5 mL microcentrifuge tube and 20 µL Nuclease free water was added as elution medium to the membrane of the column and incubated for 1 minute at room temperature. After the 1-minute incubation period the column was centrifuged at 11000 x g for 1 minute to elute the cfDNA, which consists of both the maternal and fetal cfDNA). The eluted cfDNA of each sample was pooled from all four tubes and placed in one microcentrifuge tube. The isolation process was repeated until all of the plasma was used. The pooled cfDNA samples were concentrated with the SpeedVac system (Thermo Fisher Scientific, USA) until the final volume of each sample was 20 µL.

3.4.2 Quantification and characterisation of cfDNA

Fluorometric quantification, using the Qubit 3.0 Fluorometer, as well as capillary electrophoresis, using the BioAnalyser 2100, were used for the quantification of the isolated cfDNA. The Qubit Fluorometer was used for the quantification of the total dsDNA, while capillary electrophoresis confirms the presence of shorter fragments between 100 – 400bp of DNA by characterising the DNA fragment contents of the extracted cfDNA.

3.4.2.1 Qubit 3.0 Fluorometer

The Qubit Fluorometer uses a working solution that contains 200µL Qubit dsDNA HS buffer and 1 µL Qubit dsDNA HS reagent dye (Thermo Fisher Scientific, USA). A stock solution of the working solution was prepared for each sample and for two standards. The two standards were used to create a calibration curve wherein the different sample concentrations could be detected.

The two standards were prepared with 190 µL of working solution and 10 µL of both Standard 1 and 2 respectively. Standard 1 has a DNA concentration of 0 ng/µL and standard 2 has a DNA concentration of 10 ng/µL. For each sample, 199 µL of working solution and 1 µL of each sample were used per tube. The samples were incubated for 2 minutes at room temperature and thereafter mixed to remove bubbles. The samples were analysed on the Qubit 3.0 following the dsDNA High Sensitivity assay protocol.

3.4.2.2 BioAnalyser 2100

After the isolation of the cfDNA from the samples, cfDNA fragment size and cfDNA levels were determined with the Agilent 2100 BioAnalyser using the Agilent High sensitivity DNA kit (Agilent Technologies, USA) according to manufactures instructions. During this procedure, only 1µL of the cfDNA samples was used, which is useful for when small volume of cfDNA is retrieved during the isolation procedure. The cfDNA levels and fragment sizes were determined using the Agilent 2100 BioAnalyser software. The fragment size of the cfDNA is defined as the mode of the peak within the electropherogram and the cfDNA level is calculated using the area under the main peak of the electropherogram.

3.5 Sex determination using cfDNA

3.5.1 SYBR Green qPCR assay of sable antelope cfDNA

The amplification of the SRY gene fragments was performed in a 20µL qPCR reaction: Luna MasterMix, forward SRY-1 primer (10 µM), reverse SRY-1 primer (10 µM), 5 µL of cfDNA (2 ng) were added to each reaction and PCR grade H_2O was added to volume. A positive control (PC) of 2 ng male gDNA, a negative control (NC) of 2 ng female cell line and non-template control (NTC) were added to each run. For the NTC PCR grade H_2O was added. The extracted cfDNA samples were run on the Applied Biosystems QuantStudio 5 with the same cycling conditions as described in section 3.3.1. The PCR products were then separated and analysed by electrophoreses. The same electrophoreses protocol was followed as described in section 3.3.1.

3.5.2 MassARRAY® of sable antelope cfDNA

The sex determination of the foetuses using cffDNA present in the isolated cfDNA from the maternal blood sample was also conducted on the MassARRAY® system by Inqaba Biotec™. The 10 cfDNA samples were amplified using PCR, whereafter the samples were run on the MassARRAY® system from Agena® Bioscience (San Diego, USA). As describe in section 3.3.3.

CHAPTER 4. RESULTS AND DISCUSSION

In the optimisation of the sex determination methods for both the sable and roan antelope, hair and blood samples were collected from 45 male sable and only hair samples were collected from 10 male roan antelope. Genomic DNA was extracted from the hair samples to sequence the *SRY* gene of both species and for the design of the primers to be used during qPCR. In addition, the sub-species of these animals were determined, since it was unknown for certain samples. The sub-species identification was needed to evaluate if there are any nucleotide differences between the sub-species *SRY* gene as this would affect the binding of primers designed for the different assays. A comparison of the SRY gene between the sub-species was made to identify whether the *SRY* gene is conserved between sub-species. With the *SRY* gene sequence elucidated for both these species, prenatal sex determination of the cfDNA could be done to identify the sex of the calf. From the collected sable blood samples, cfDNA was extracted to be used for qPCR as well as MassARRAY®. Unfortunately, the sex of the roan antelope calves could not be determined during this study, due to the lack of opportunities for blood collection from pregnant roan antelopes. This chapter discusses the results obtained during this study.

4.1 Sub-species identification of the sable and roan antelope

Information regarding the sub-species of the sable and roan antelope was not available for all of the samples received thus it was important to identify the different sub-species. This was needed to identify if there are any differences in the nucleotide sequence of the different sub-species *SRY* gene as this could affect the binding of primers used for the different assays. Thus, the same sample was used for the sub-species identification was also used for the sequencing of the *SRY* gene and from these results it was determined whether there is a difference between the *SRY* gene of the sub-species. Phylogenetic analysis of the samples was required to identify the different sub-species of sable and roan antelope that can be found on the wildlife farms from which the samples were obtained. Previous studies (Matthee & Robinson, 1999, Pitra *et al.*, 2002, Alpers *et al.*, 2004, Pitra *et al.*, 2006, Themudo *et al.*, 2015), used mtDNA to identify the different sub-species and investigated the geographical range where these sub-species can be found. This was done by sequencing the hypervariable D-loop region of the mtDNA (Pitra *et al.*, 2006) and comparing these sequences to reference sequences to construct a phylogenetic lineage.

The sub-species of all 46 sable antelope samples and 10 roan antelope samples could be identified successfully using hair samples of the sable and roan antelopes and one blood sample

from a sable antelope. The hair samples were collected from two different wildlife farms situated in South Africa, mainly in the North-West province (see Appendix C and Appendix D for sample list). The sable antelope hair samples were collected from both farms and the roan antelope from only one of the farms. The male sable blood sample was collected on a wildlife farm in the Northern Cape province.

4.1.1 Sub-species identification of the sable antelope

As stated in the initial study by Ansell (1971), there are four distinct sub-species of *H. niger* or sable antelope (Chapter 2 section 2.2.1). These four sub-species are *H. n. kirkii*, *H. n. roosevelti*, *H. n. variani* and *H. n. niger*. From the 46 sable antelope samples received, only three sub-species were identified. The hypervariable D-loop region of the mtDNA tree (Figure 4-1 the enlarged Figure is in Appendix E) shows the evaluation of genetic diversity within the species and provides an indication of geographical boundaries of the sub-species currently recognised. Table 4-1 shows the different sub-species for the sable antelope that were identified. According to the results, 57% (26/46) of the sub-species that were tested originated from Zambia (*H. n. kirkii*), 37% (17/46) was from the Matetsi region in Zimbabwe, south of Zambia (*H. n. niger*) and 7% (3/46) was from Tanzania (*H. n. rooseveltii*) (Ansell, 1971, Matthee & Robinson, 1999, Themudo *et al.*, 2015, Vaz Pinto, 2019).

The phylogenetic tree (Figure 4-1) was derived from using the hypervariable D-loop region of the mtDNA and shows the genetic diversity within the species. From the results there was relatively high bootstrap support that divided into two clades. The one clade was sub divided into two different nodes which corresponds to *H. n. niger* (54%) and *H. n. rooseveltii* (70%). The other clade that was divided into another node was identified as the *H. n. kirkii* (23%). Thus, this bootstrap support for the *H. n. kirkii* of 23% is a very poor bootstrap support (Felsenstein & Felenstein, 2004). From these results it was evident that *H. n. niger*, *H. n. kirkii* and *H. n. rooseveltii* were the only sub-species found in this data set from the given hair samples and the results excludes the *H. n. variani* sub-species. This can be attributed to the fact as seen in Figure 2-3 that these sub-species are more closely geographically positioned to the southern region of Africa and is the sub-species that are potentially bought at wildlife auctions.

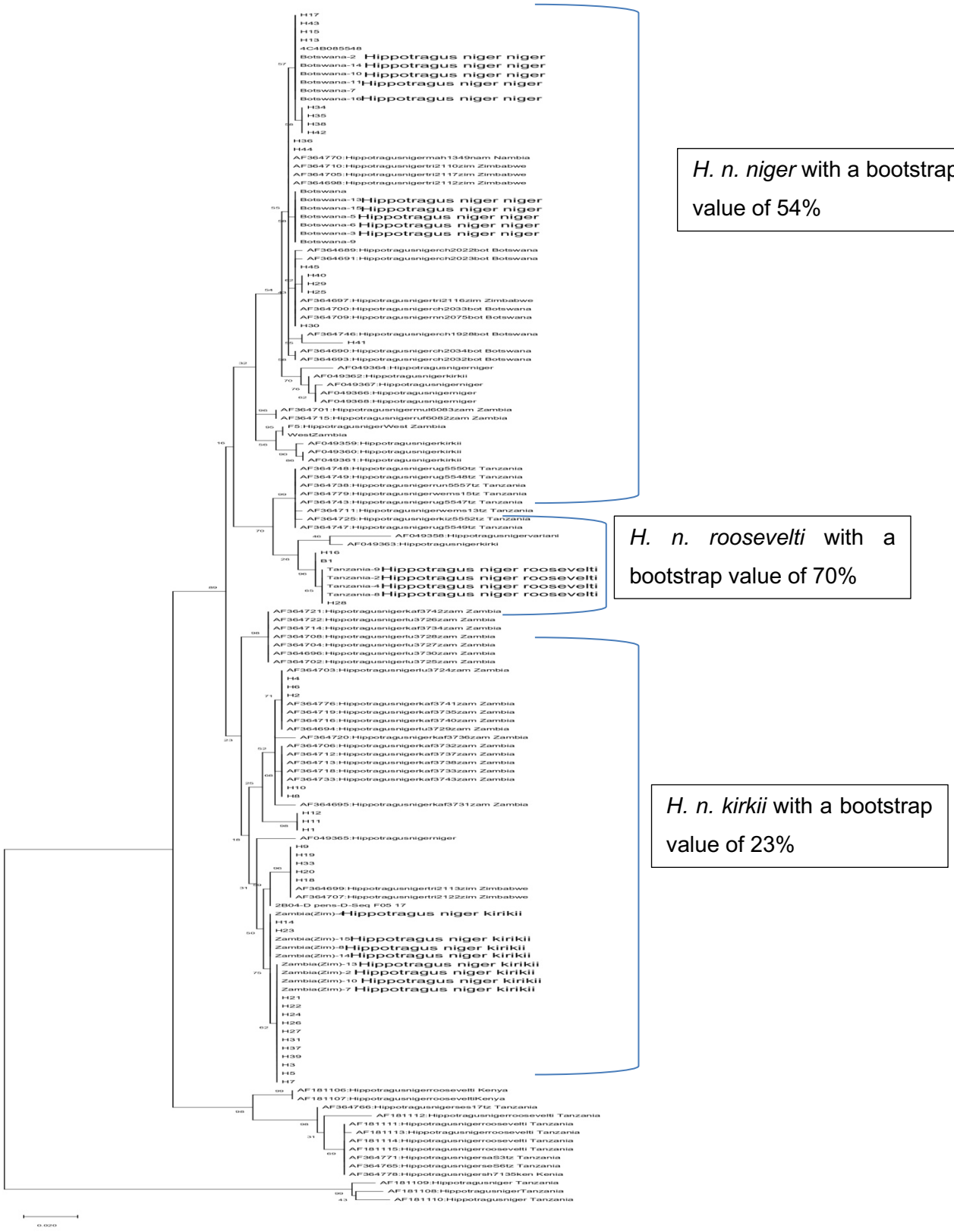


Figure 4-1 Phylogenetic analyses of the *Hippotragus niger* sub-species using the hypervariable D-loop region. The samples in this study are indicated by H followed by a number (H1-H45 and B1) (see Appendix E).

Table 4-1. The identification of sub-species of the sable antelope samples that were used in this study.

Sable antelope		
Lab ID	Male/Female	Sub-species as identified by Sanger sequencing
H1	Male	*H. n. kirkii*
H2	Male	*H. n. kirkii*
H3	Male	*H. n. kirkii*
H4	Male	*H. n. kirkii*
H5	Male	*H. n. kirkii*
H6	Male	*H. n. kirkii*
H7	Male	*H. n. kirkii*
H8	Male	*H. n. kirkii*
H9	Male	*H. n. kirkii*
H10	Male	*H. n. kirkii*
H11	Male	*H. n. kirkii*
H12	Male	*H. n. kirkii*
H13	Male	*H. n. niger*
H14	Male	*H. n. kirkii*
H15	Male	*H. n. niger*
H16	Male	*H. n. rooseveltii*
H17	Male	*H. n. niger*
H18	Male	*H. n. kirkii*
H19	Male	*H. n. kirkii*
H20	Male	*H. n. kirkii*
H21	Male	*H. n. kirkii*
H22	Male	*H. n. kirkii*
H23	Male	*H. n. kirkii*
H24	Male	*H. n. kirkii*
H25	Male	*H. n. niger*
H26	Male	*H. n. kirkii*
H27	Male	*H. n. kirkii*

H28	Male	*H. n. rooseveltii*
H29	Male	*H. n. niger*
H30	Male	*H. n. niger*
H31	Male	*H. n. kirkii*
H32	Male	*H. n. niger*
H33	Male	*H. n. kirkii*
H34	Male	*H. n. niger*
H35	Male	*H. n. niger*
H36	Male	*H. n. niger*
H37	Male	*H. n. kirkii*
H38	Male	*H. n. niger*
H39	Male	*H. n. kirkii*
H40	Male	*H. n. niger*
H41	Male	*H. n. niger*
H42	Male	*H. n. niger*
H43	Male	*H. n. niger*
H44	Male	*H. n. niger*
H45	Male	*H. n. niger*
B1	Male	*H. n. rooseveltii*

4.1.2 Sub-species identification of the roan antelope

For the roan antelope, there are six different sub-species identified by Ansell (1971), namely *H. e. koba*, *H. e. bakeri*, *H. e. charicus*, *H. e. cottoni*, *H. e. langheldi* and *H. e. equinus.* The geographic distribution of these sub-species can be seen in section 2.2.3. From the 10 roan antelope samples that were collected, only two sub-species were identified. From the phylogenetic tree, the genetic diversity within the sub-species was also determined for the roan antelope and is shown in Figure 4-2. The two sub-species were identified from the roan antelope samples and these two sub-species that were identified is the *H. e. cottoni* (3/10) and the *H. e. equinus* (7/10). The *H. e. equinus* can be found in the Southern region of Africa and the *H. e. cottoni* can found in the Western region of Africa (Ansell, 1971, Matthee & Robinson, 1999, Alpers *et al.*, 2004). From the phylogenetic tree results, using the hypervariable D-loop region of the mtDNA (Figure 4-2), of the roan antelope there was a high bootstrap support with two nodes. In the one node from this phylogenetic tree the *H. e. equinus* was found with a bootstrap value of 87% and in the other node the *H. e. cottoni* was found with a bootstrap value of 64%.

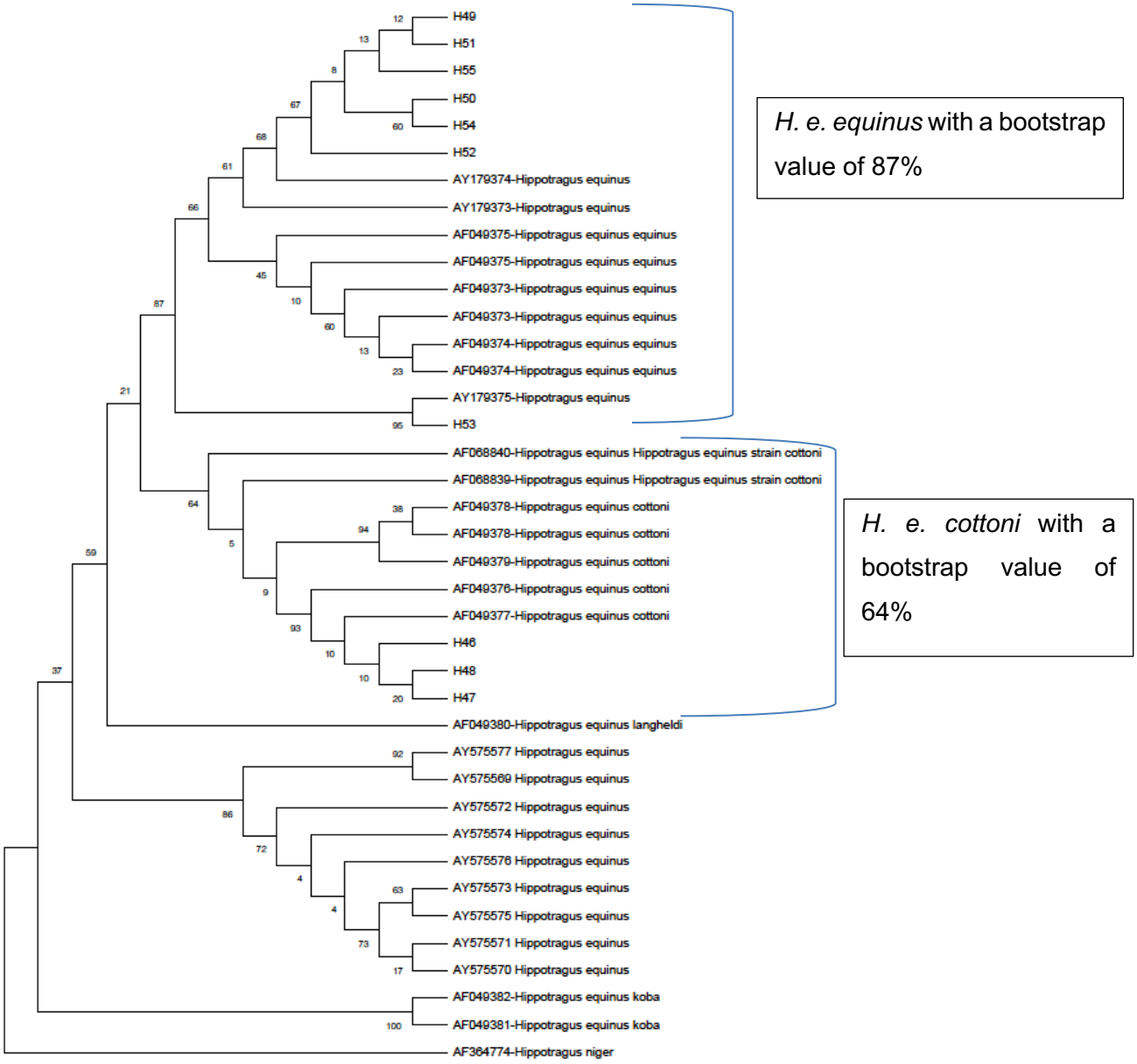


Figure 4-2 Phylogenetic analyses of the *Hippotragus equinus* sub-species using the hypervariable D-loop region. The samples in this study are indicated by H followed by a number (H46-H55).

Table 4-2. The identification of sub-species of the roan antelope samples used in this study.

Roan antelope		
Lab ID	Male/Female	Sub-species as identified by Sanger sequencing
H46	Male	*H. e. cottoni*
H47	Male	*H. e. cottoni*
H48	Male	*H. e. cottoni*
H49	Male	*H. e. equinus*
H50	Male	*H. e. equinus*
H51	Male	*H. e. equinus*
H52	Male	*H. e. equinus*
H53	Male	*H. e. equinus*
H54	Male	*H. e. equinus*
H55	Male	*H. e. equinus*

4.2 Sequencing of the *SRY* gene

The *SRY* gene from the hair samples was successfully sequenced by Inqaba biotech™ for both sable and roan antelope. The alignment of the *SRY* gene sequence was done and the results for sable and roan antelope can be found in Appendix F and G, respectively. The *SRY* gene sequence for both sable and roan antelopes is comprised of 690 bp and 229 amino acid residues. As multiple sub-species were involved in this study, it was necessary to investigate whether the *SRY* gene is conserved within these sub-species. The *SRY* gene sequence alignment data of the sable antelope (Appendix F) as well as for the roan antelope (Appendix G) was investigated, no differences were visible between the *SRY* gene sequence for the sable antelope samples as well as for the roan antelope samples. After the sub-species of the sable and roan antelope samples were confirmed, the multiple sequence alignment was used to identify differences in *SRY* gene between the sub-species. According to the sequence alignment results there were no differences within the sequence were found among the sub-species for the sable antelope as well as the roan antelope. From this data one can confirm that the *SRY* gene is conserved between the sub-species. These complete *SRY* gene sequences that were found during this study contributes to the study of Vaz Pinto *et al.* (2016) where only the partial sequence of the *SRY* gene with 655 bp were sequenced (see section 2.5.2).

4.3 Assay and primer design using sequence date

4.3.1 Optimisation of SYBR Green qPCR assay

The optimisation of the qPCR assay was performed for all five SRY primers: SRY-1, SRY-2, SRY-3, SRY-4 and SRY-5 as described in section 3.3.1 in Table 3-2. Standard curves were generated for the five different *SRY* primers assays by plotting the average C_T values against the log of the input DNA (20 ng/µL, 2 ng/µL, 0.2 ng/µL, 0.02 ng/µL and 0.002 ng/µL). The lower DNA concentration dilutions had no amplification and therefore only the data from the first three dilutions were used. The amplification efficiency percentage of these different primer sets were determined from the slope of each curve (Figure 4-3). The amplification efficiency of the five primer sets were 88.9% for SRY-1, 84.78% for SRY-2, 86.01% for SRY-3, 90.25% for SRY-4 and 96.84% for SRY-5. Theoretically, the amplification efficiency percentage should be between 90% and 110% (Svec *et al.*, 2015) as this is regarded as adequate for the optimisation of the qPCR assay. Since the amplification efficiency percentage were below 90%, they were still close enough to be considered for this qPCR assay. It was found in the results that the SRY-3 and SRY-5 primers had signals in the NTC with an average C_T value of 35 for SRY-3 and 33 for SRY-5 due to primer-dimer formation (Bustin *et al.*, 2009). For SRY-1, SRY-2 and SRY-4 no signals were visible for the NTC which did not indicate any primer dimer formation.

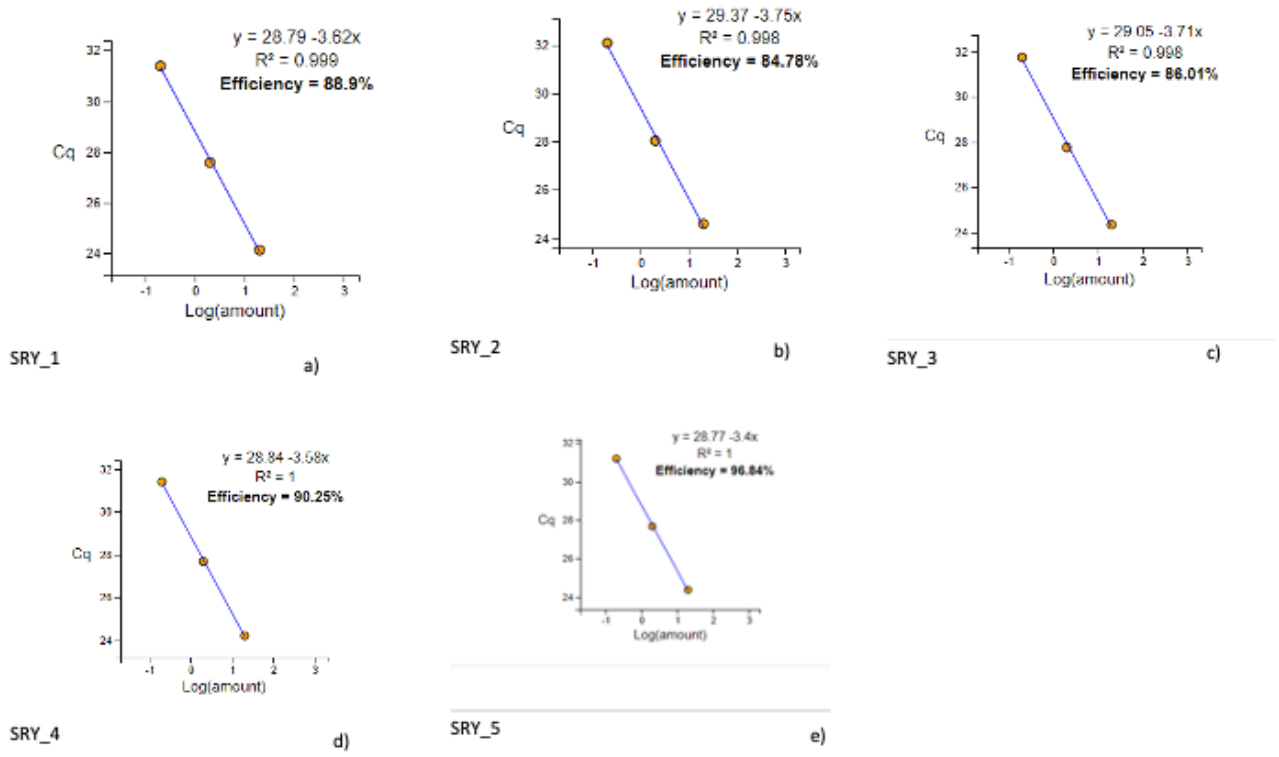


Figure 4-3 The standard curves for the efficiency percentage determination of the SYBR Green SRY gene expression of all five different primers SRY-1 (a); SRY-2 (b); SRY-3 (c); SRY-4 (d) and SRY-5 (e).

During the optimisation of the qPCR assay, intact and different concentrations of diluted fragmented male gDNA (see section 3.7) of sable antelope were used. The fragmented DNA was added as an attempt to mimic the short-length and fragmented nature of cfDNA when compared to gDNA. Diluted concentrations of this fragmented gDNA were also used in an attempt to mimic the low concentrations of cfDNA normally obtained in plasma samples. For the fragmented gDNA, the DNA was sonicated, and the fragment sizes were observed using gel electrophoreses. The fragmented gDNA was loaded in duplicate on a 2% (w/v) agarose gel in TAE buffer (Figure 4.3). The DNA fragments found range between under 100 bp to above 1000 bp and definite bands between this range are visible. This fragmentation that was found and shown is not as would be expected from literature (Elsner & Lindblad, 1989, Mann & Krull, 2004, Larguinho *et al.*, 2010, Grokhovsky *et al.*, 2011, Tseng *et al.*, 2012, Garafutdinov *et al.*, 2016) were no definite band is present and is more illustrated as a smear on the electropherograms. The fragmented gDNA sample with 20 ng/µL concentration was then used during the optimisation of the qPCR assays.

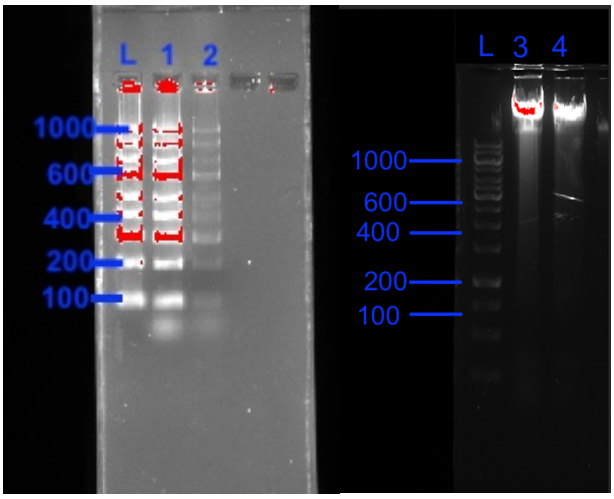


Figure 4-4 Electrophoreses results of the fragmented gDNA. The DNA ladder was loaded into the lane marked L and the ladder size are indicated on the left. well 1 and 2 are the fragmented gDNA that were loaded into these lanes in well 3 and 4 are unfragmented gDNA as control. (L – Ladder, 1 – fragmented gDNA, 2- fragmented gDNA 3 - unfragmented gDNA and 4 – unfragmented gDNA)

Primers sets SRY-1, SRY-2 and SRY-4 were used for the optimisation of the qPCR method. Figure 4-5, Figure 4-6 and Figure 4-7 are representations of the amplification results for the gDNA and the dilution of the fragmented gDNA for primers SRY-1, SRY-2 and SRY-4, respectively and this was done in triplicate. Table 4-3 shows the average C_T value of the amplification results for the three different primers (SRY-1, SRY-2 and SRY-4) used during the qPCR optimisation. Higher amounts of DNA amplified first at lower cycles and lower concentrations at higher cycles. For this primer set the C_T values of the higher concentration DNA was in the range of 23 cycles and is acceptable due to any cycle value which is below 35 cycles is seen as acceptable amplification (Bustin *et al.*, 2009). According to the amplification results of SRY-1, the 0.2 ng/µL amplification is too close to the NTC amplification results thus this concentration of fragmented gDNA could not be used. At primer sets SRY-1 and SRY-2, amplification was observed in the NTC and this may be due to primer dimers that formed during the amplification of the DNA. No amplification occurred in the case of the SRY-4 target in the NTC. Based on the triplicate amplification results on the gDNA (20 ng/µL), fragmented gDNA (20 ng/µL and 2 ng/µL) for primer sets SRY-1 and SRY-2 reliable amplification was found for these samples. However, for the 0.2 ng/µL fragmented gDNA showed a small difference between the C_T values for the SRY-1 and SRY-2 primer sets which is not reliable due to the close amplification of the NTC. For the SRY-4 primers set the triplicate amplification results for the gDNA (20 ng/µL) and fragmented gDNA (20 ng/µL, 2 ng/µL and 0.2 ng/µL) reliable amplification was found and no amplification was observed for the NTC.

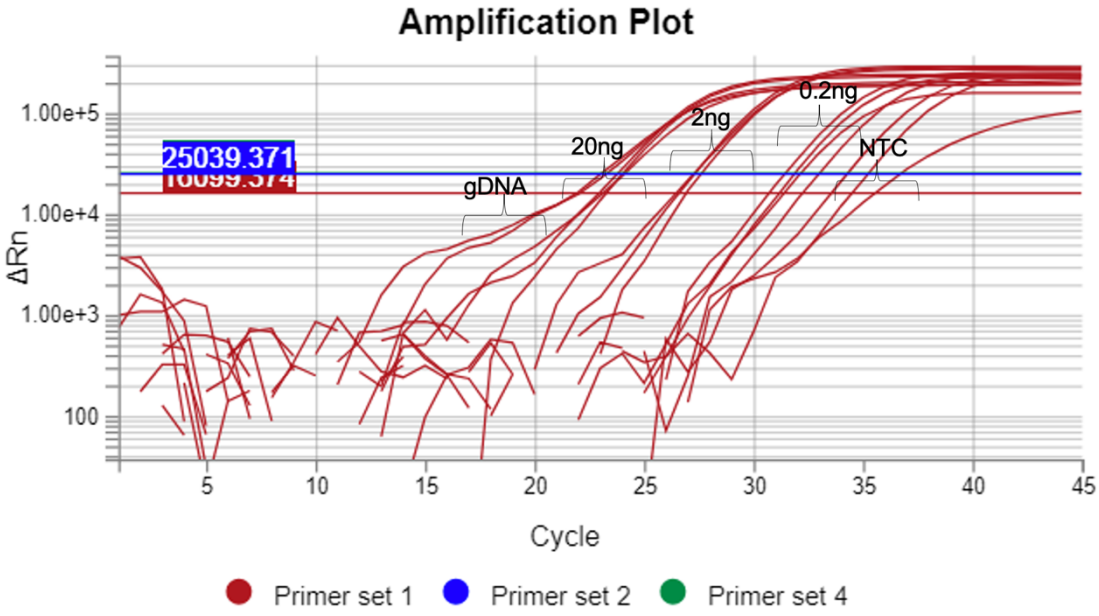


Figure 4-5 qPCR results for primer set SRY-1 which was run in triplicate for gDNA (20 ng/µL), the serial dilution of fragmented gDNA 20 ng/µL, 2 ng/µL, 0.2 ng/µL and the NTC.

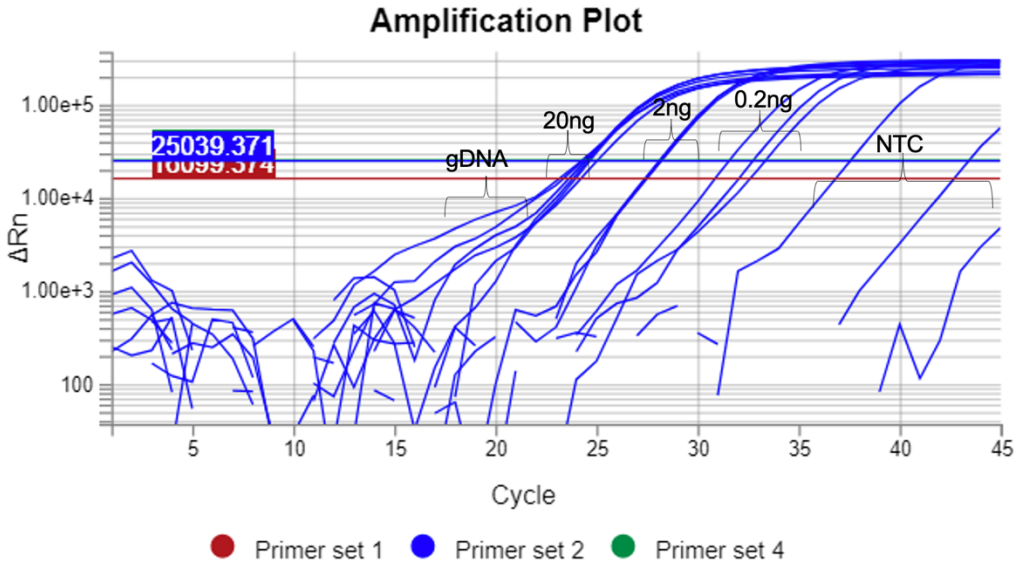


Figure 4-6 qPCR results for primer set SRY-2 which was run in triplicate for gDNA (20 ng/µL), the serial dilution of fragmented gDNA 20 ng/µL, 2 ng/µL, 0.2 ng/µL and the NTC.

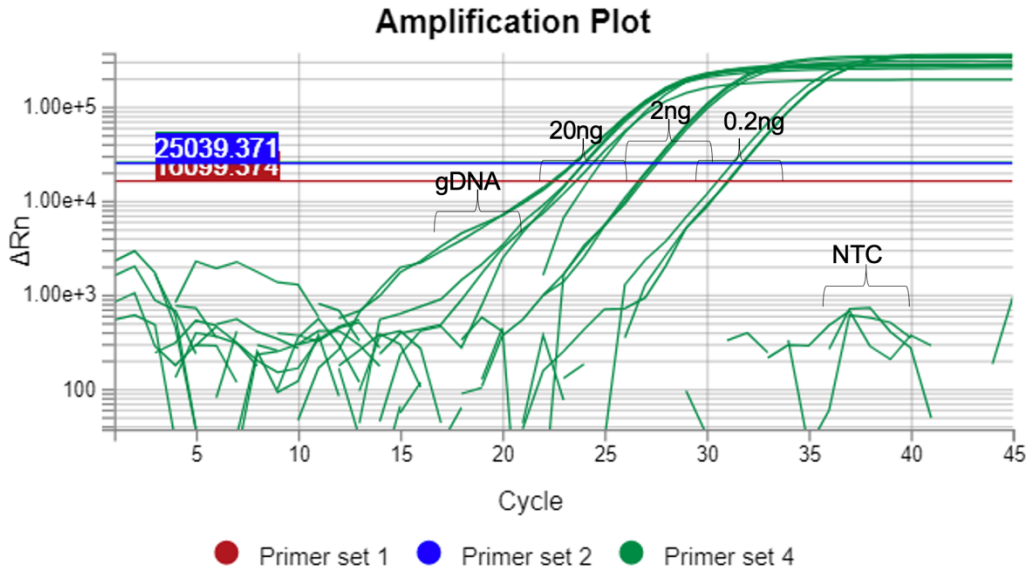


Figure 4-7 qPCR results for primer set SRY-4 which was run in triplicate for gDNA (20 ng/μL), the serial dilution of fragmented gDNA 20 ng/μL, 2 ng/μL, 0.2 ng/μL and the NTC.

Table 4-3. C_T values obtained during optimisation of the qPCR assay for the different primer sets used.

Sample	**SRY-1**	**SRY-2**	**SRY-4**
	C_T Value	C_T Value	C_T Value
gDNA	22	24	23
	22	24	23
	23	24	24
20ng	35	24	24
	23	24	24
	23	24	5
2ng	26	28	27
	26	28	28
	26	28	27
0.2ng	31	32	31
	31	33	31
	32	32	32
NTC	32	43	-
	34	38	-
	22	-	-

The PCR products after the qPCR step was then used for gel electrophoreses for all samples to determine whether the PCR products of the primers have formed, and the gel electrophoreses results are shown in Figure 4-8. The PCR product for the SRY-1 gene primer was 96 bp and when analysing wells 1 to 15, bands were formed at a close proximity of 100 bp as shown by the ladder, indicating that positive results was obtained during the qPCR step. As stated above, amplification was found for the NTC for the SRY-1 primer, and this could be attributed to primer dimer formation. Thus, these results correspond to the PCR products (which is 96 bp for SRY-1, 70 bp for SRY-2 and 104 bp for SRY-4) and not primer dimers due to primer dimers would be between 20 and 50 bp (Brownie *et al.*, 1997). For the PCR product of the SRY-2 primers, the electrophoreses results (well 16 to 30) shows a band at about 100 bp and due to the PCR products of this SRY-2 primer set is 70 bp these bands indicate positive PCR results. For the PCR products of the SRY-2 primers, electrophoreses results were shown in wells 16 to 30 and positive band formation was visible, indicating that positive amplification of the SRY-2 primer was achieved, and these bands correspond to the estimated PCR products. The PCR products of the SRY-4 primers were also used for electrophoreses, shown in wells 31-45, and the results shown corresponded to the base pair length of 104 bp for the SRY-4 primer set (see section 3.3.1). As shown in Figure 4-7, no amplification of the NTC was visible and the electrophoreses results also showed that no PCR products were in these samples, thus no primer dimers have formed for the SRY-4 primer.

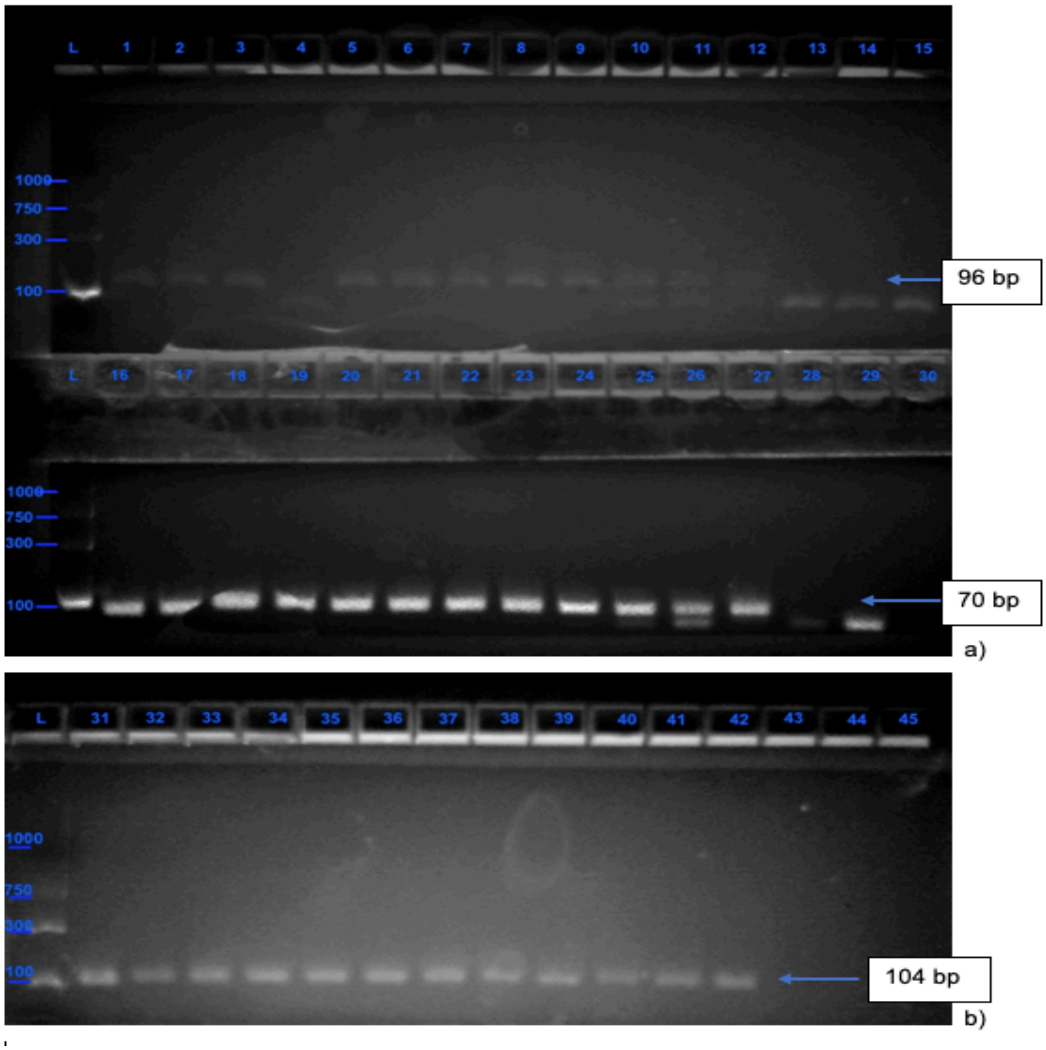


Figure 4-8 Electrophoreses results of the qPCR products of all the samples, which was performed in triplicate. [L- Ladder; SRY-1 (Wells 1-3 gDNA, wells 4-6 fragmented gDNA 20 ng, wells 7-9 fragmented - gDNA 2 ng, wells 10-12 - fragmented gDNA 0.2 ng, wells 13-15 - NTC); SRY-2 (Wells 16-18 - gDNA, wells 19-21 - fragmented gDNA 20 ng, wells 22-24 - fragmented gDNA 2 ng, wells 25-27 - fragmented gDNA 0.2 ng, wells 28-30 - NTC) and SRY-4 (wells 31-33 – gDNA, wells 34-36 - fragmented gDNA 20 ng, wells 37-39- fragmented gDNA 2 ng, wells 40-42 - fragmented gDNA 0.2 ng, wells 43-45 - NTC)]

4.3.2 Optimisation of probe-based qPCR assay

The probe qPCR assay was designed using all three SRY primers identified: SRYP-1, SRYP-2 and SRYP-3. The amplification efficiency was also determined for the three different probe-base primers and this was done in single plex as well as in multiplex. The amplification efficiency values

for the single plex reactions were significantly lower than 80% and for the multiplex reaction they were significantly higher than 80% (Figure 4-9). Even though these efficiency percentage values are lower than the theoretically values they are still sufficient to be used for qPCR due to the linearity of the efficiency values.

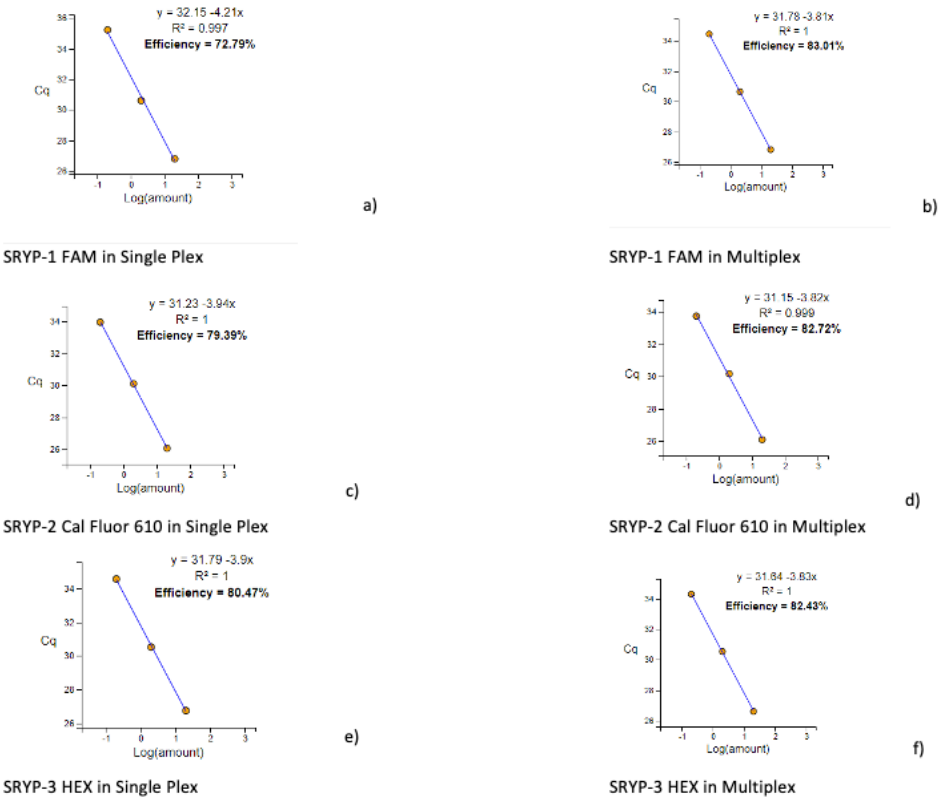


Figure 4-9 The standard curves for the efficiency percentage determination of the probe-based primer of all three different primers in single plex as well as in multiplex SRYP-1in single plex (a); SRYP-1 in multiplex (b); SRYP-2 in single plex (c); SRYP-2 in multiplex (d); SRYP-3 in single plex and SRYP-3 in multiplex (f) .

During the optimisation of the qPCR assay, intact and fragmented gDNA of sable antelope was used as described in 4.3.1. Figure 4-10, Figure 4-11 and Figure 4-12 is a representation of the amplification results for the gDNA and the dilution of the fragmented gDNA for primers SRYP-1, SRYP-2 and SRYP-3, respectively. The C_T values of the amplification results for the different primers is shown in Table 4-4. Higher amounts of DNA amplified first at lower cycles which were on average at 28 cycles and lower concentrations at higher cycles which were on average of 34 cycles. No amplification occurred for all three different primer targets of the NTC. In all instances of the gDNA and the fragmented gDNA with concentration of 20 ng, the amplification starting sites

for all three primer targets were quite similar with a higher C_T value. As the concentration of fragmented gDNA decreased, the C_T values of each primer target increased. Usable results were obtained at even the lowest concentration of 0.2 ng of fragmented gDNA.

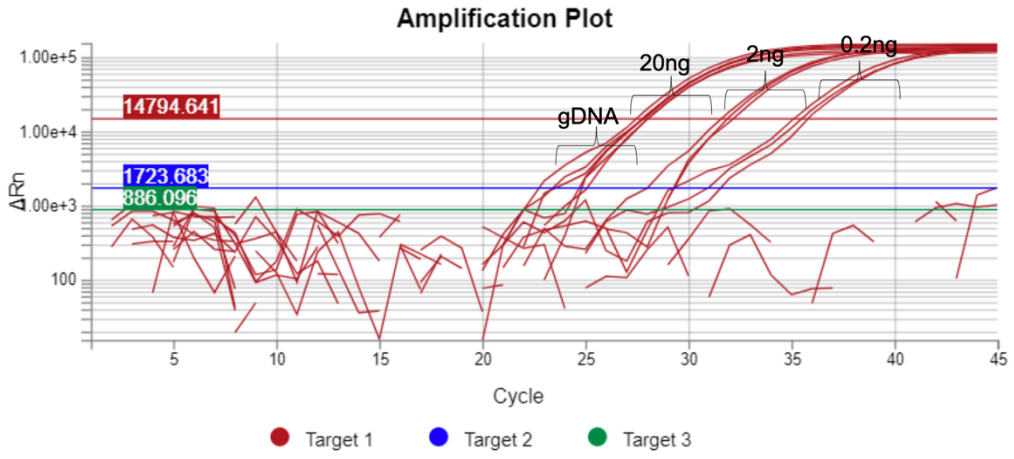


Figure 4-10 qPCR results for primer SRYP-1, which was run in triplicate for gDNA (20 ng/µL), the serial dilution of fragmented gDNA 20 ng/µL, 2 ng/µL, 0.2 ng/µL and the NTC.

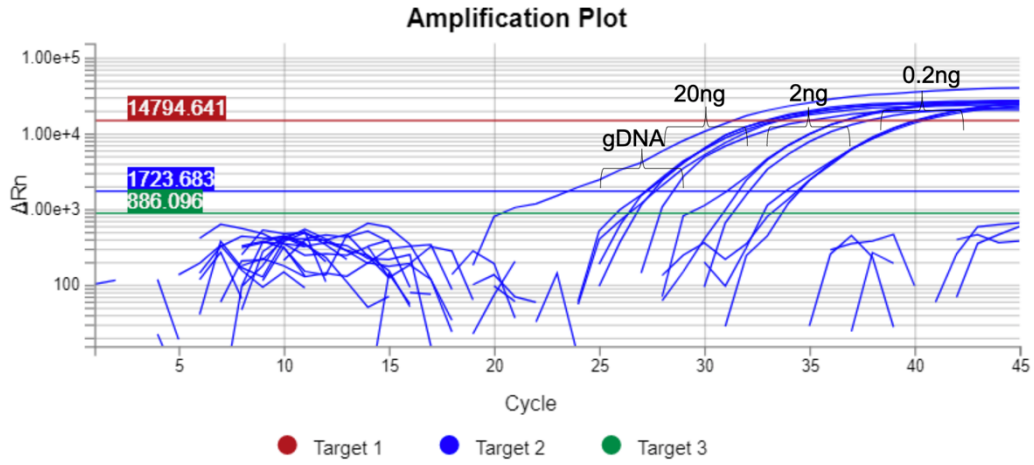


Figure 4-11 qPCR results for primer SRYP-2, which was run in triplicate for gDNA (20 ng/µL), the serial dilution of fragmented gDNA 20 ng/µL, 2 ng/µL, 0.2 ng/µL and the NTC.

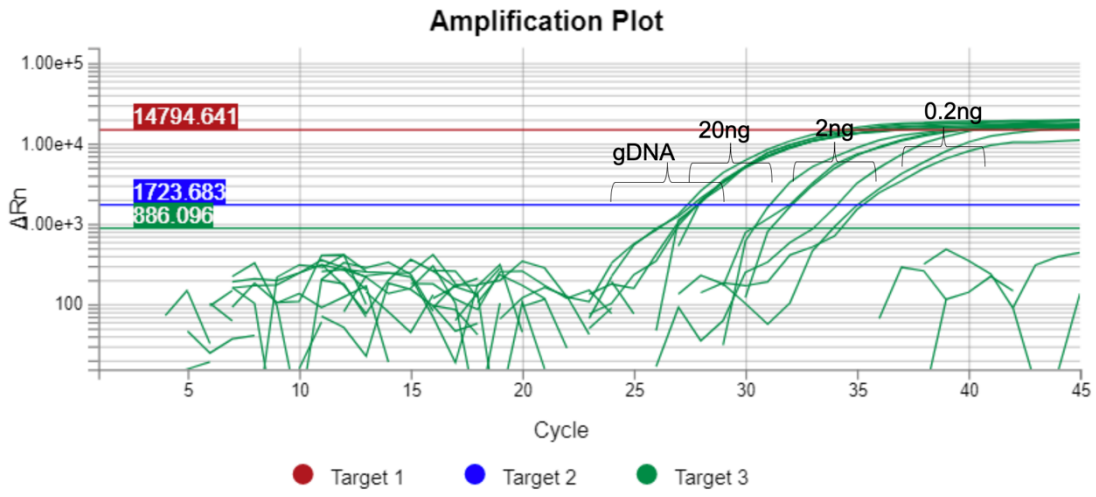


Figure 4-12 qPCR results for primer SRYP-3, which was run in triplicate for gDNA (20 ng/µL), the serial dilution of fragmented gDNA 20 ng/µL, 2 ng/µL, 0.2 ng/µL and the NTC.

Table 4-4 C_T values obtained during optimisation of the qPCR assay for the different primer sets used.

Sample	**SRYP-1**	**SRYP-2**	**SRYP-3**
	C_T Value	C_T Value	C_T Value
gDNA	28	28	27
	28	28	27
	28	27	26
20ng	27	24	27
	27	28	26
	28	27	26
2ng	32	32	31
	32	31	30
	31	32	32
0.2ng	35	34	33
	35	33	34
	36	35	33
NTC	-	-	-
	-	-	-
	-	-	-

The qPCR product was then used for gel electrophoreses for all the sample and these results are shown in Figure 4-13. This step was done in order to state that the amplification of the samples was positive and that reliable results were obtained during qPCR. As can be seen for samples 1 to 12, a band formed at around 100 bp, indicating that positive PCR products formed during amplification. No bands were visible at 13 to 15, which also indicates that no primer dimers formed during the amplification of SRYP-1 target. For the SRYP-1 target, the PCR products consisted of a base pair length of 79 bp. For the SRYP-2 target, a base pair length of 100 bp resulted and from samples 16 to 27, a band was visible, indicating that positive PCR products formed during amplification. The SRYP-3 target resulted in a base pair length of 83 bp and for these samples the appropriate bands were also visible. Thus, from the electrophoreses results one can confirm that all the samples were positively amplified during qPCR and that no primer dimers formed. In well 40 and 43 which is part of the SRY-3 primer set, the bands that formed are significantly lower than the other results and this could correspond to only the PCR products that have formed.

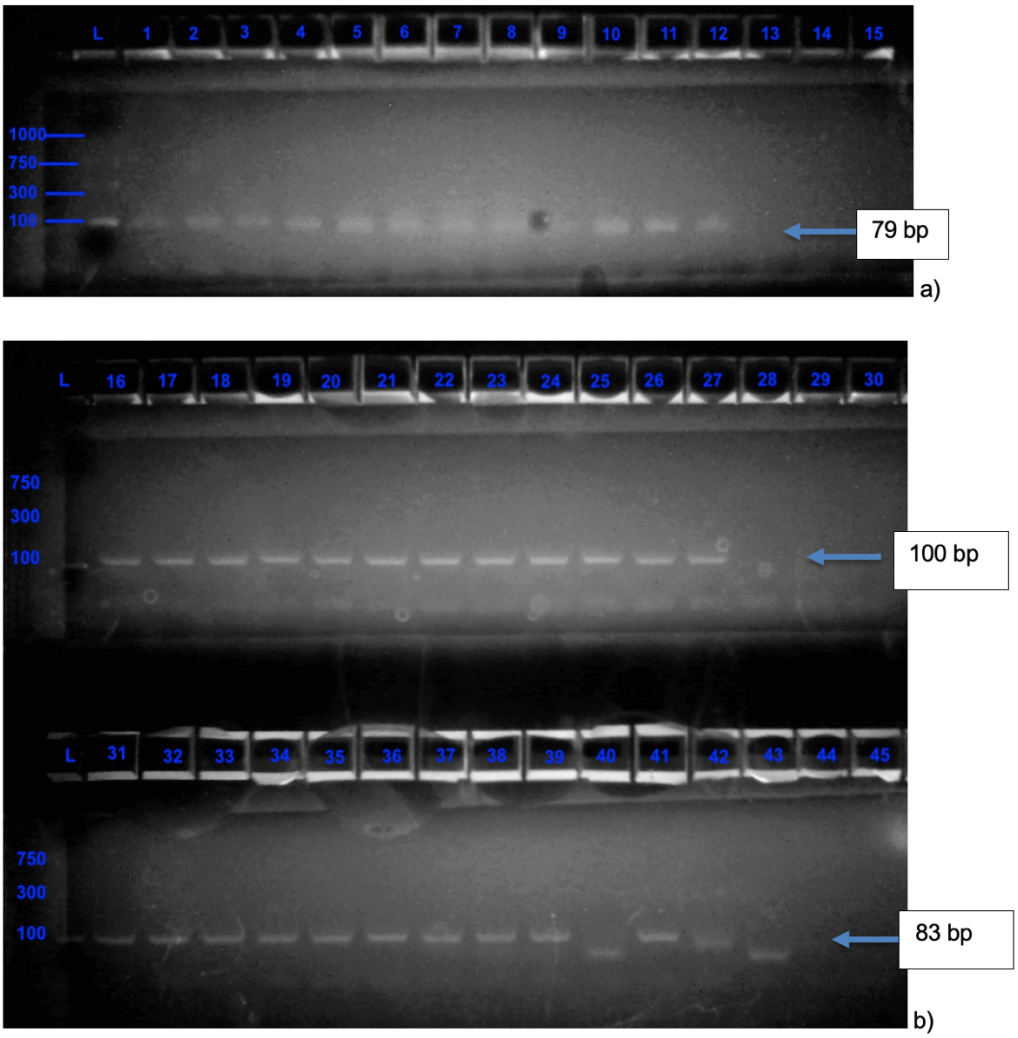
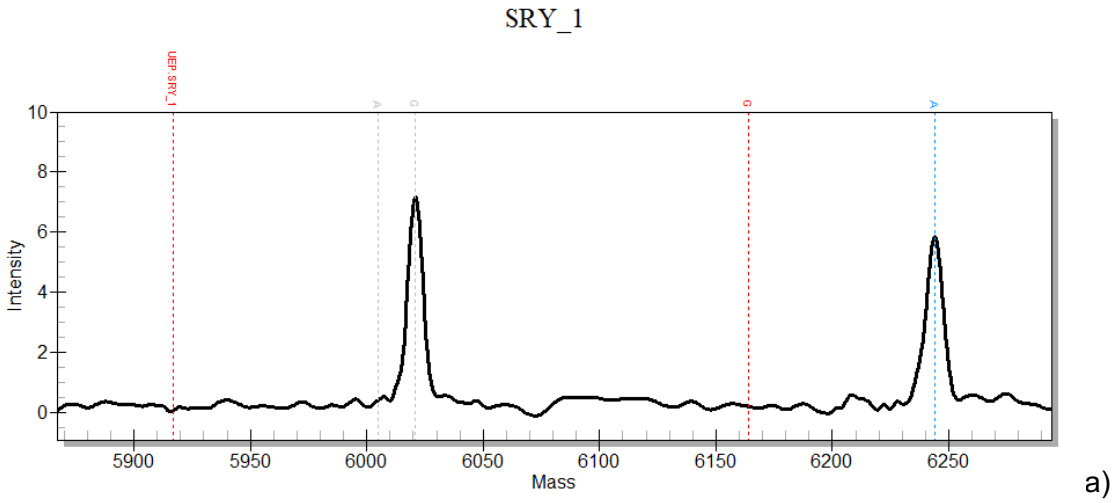


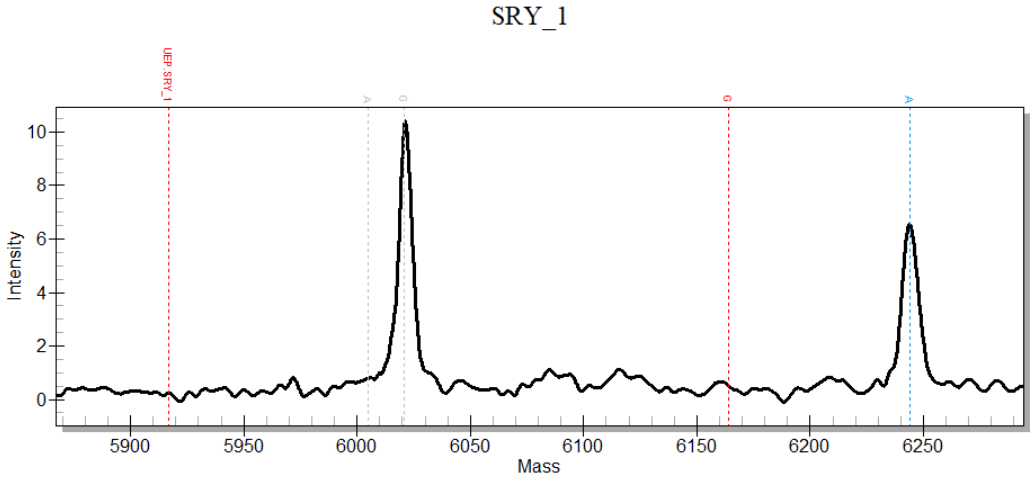
Figure 4-13 Electrophoreses results of the qPCR products of all the samples which was in triplicate. [L- Ladder; SRYP-1 (1-3 gDNA, 4-6 fragmented gDNA 20 ng, 7-9 fragmented gDNA 2 ng, 10-12 fragmented gDNA 0.2 ng, 13-15 NTC); SRYP-2 (16-18 gDNA, 19-21 fragmented gDNA 20 ng, 22-24 fragmented gDNA 2 ng, 25-27 fragmented gDNA 0.2 ng, 28-30 NTC) and SRYP-3 (31-33 gDNA, 34-36 fragmented gDNA 20 ng, 37-39 fragmented gDNA 2 ng, 40-42 fragmented gDNA 0.2 ng, 43-45 NTC)].

4.3.3 Optimisation of the SNPs on the MassARRAY® system

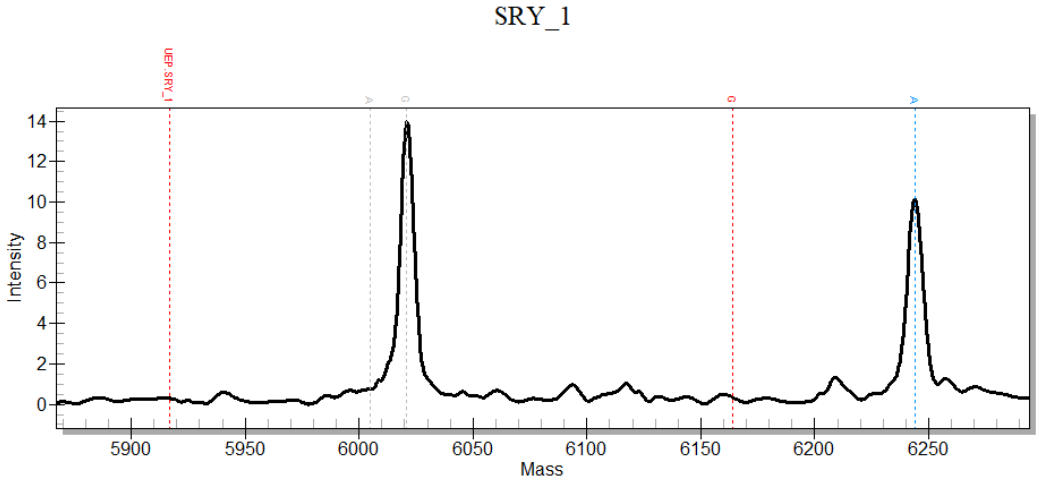
Five SNPs were identified from the *SRY* gene sequence and were optimised for the MassARRAY® system using three different gDNA concentrations: 20 ng, 2 ng and 0,2 ng. The five SNP targets consisted out of five different regions within the *SRY* gene sequence, and a single base extension reaction was performed in these five regions. The single base extension for the SNPs sequences was as follows: SRY-1 A/G, SRY-2 G/A, SRY-3 T/C, SRY-4 G/A and SRY-5 C/T. Thus, during the MassARRAY® analysis of the specific SRY target sequences, the detector searches for the first nucleotide that was identified for the single base extension. These single base extensions are the peaks that indicate the mass of the single base extension and they are shown in the following figures. (Figure 4-14, Figure 4-15, Figure 4-16, Figure 4-17 and Figure 4-18). In these figures two peaks are visible, which represents either one of the single base extensions, however the peak with the blue line indicates the SNP that should be present. Thus, for the SRY-1 target the single base extension is A/G where A is found in the *SRY* gene sequence and is the positive identified result for this sequence. The nucleotides could be genotyped at each of the tested concentrations of the SRY target sequences.



a)



b)



c)

Figure 4-14 Optimisation of the MassARRAY® system for SNPs SRY-1 for the three different concentrations: a) 20 ng, b) 2 ng and c) 0,2 ng. SRY-1 sequence and that shown in [N/N] are the single base extension.

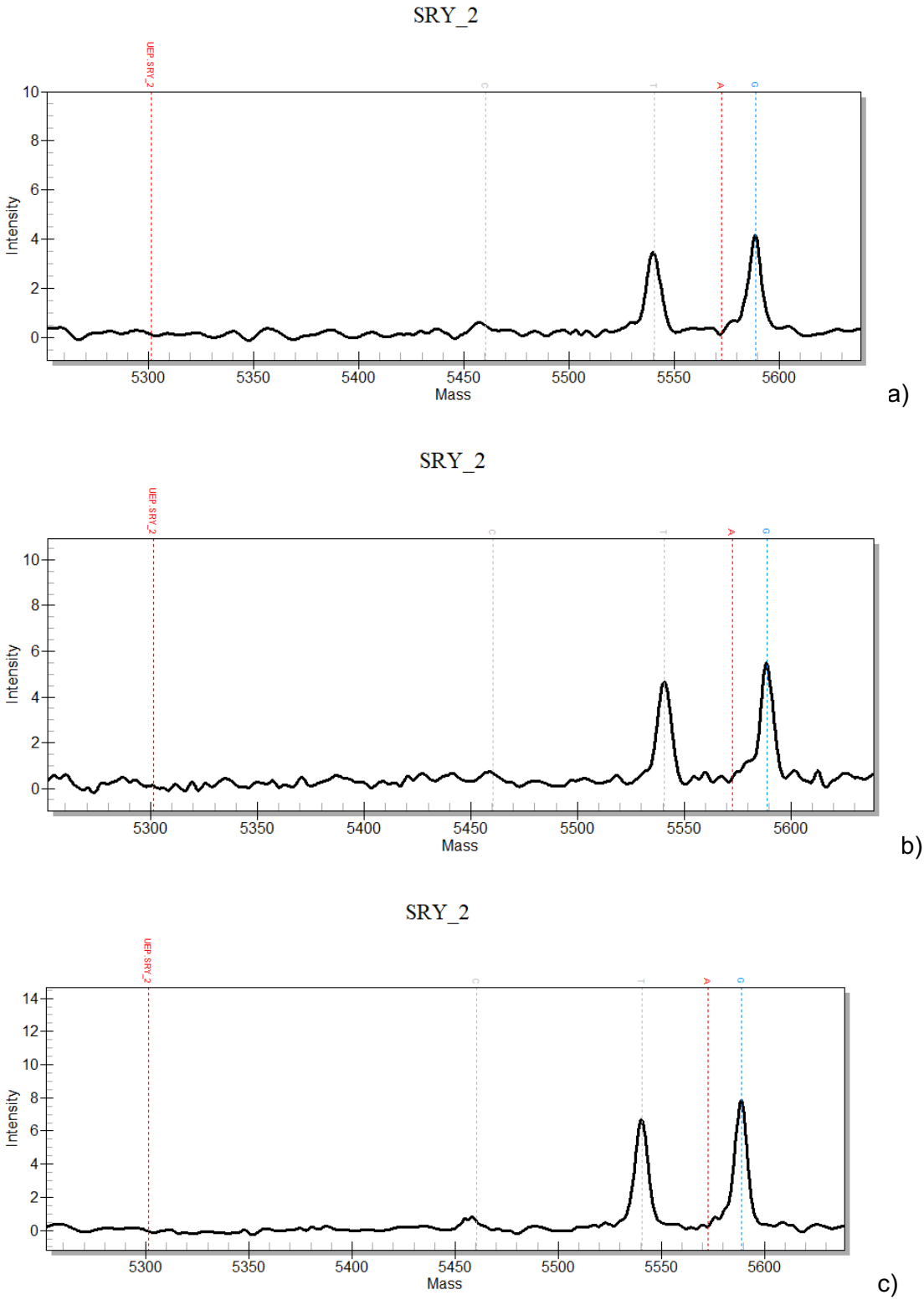


Figure 4-15 Optimisation of the MassARRAY® system for SNPs SRY-2 for the three different concentrations: a) 20 ng, b) 2 ng and c) 0,2 ng. SRY-2 sequence and that shown in [N/N] are the single base extension.

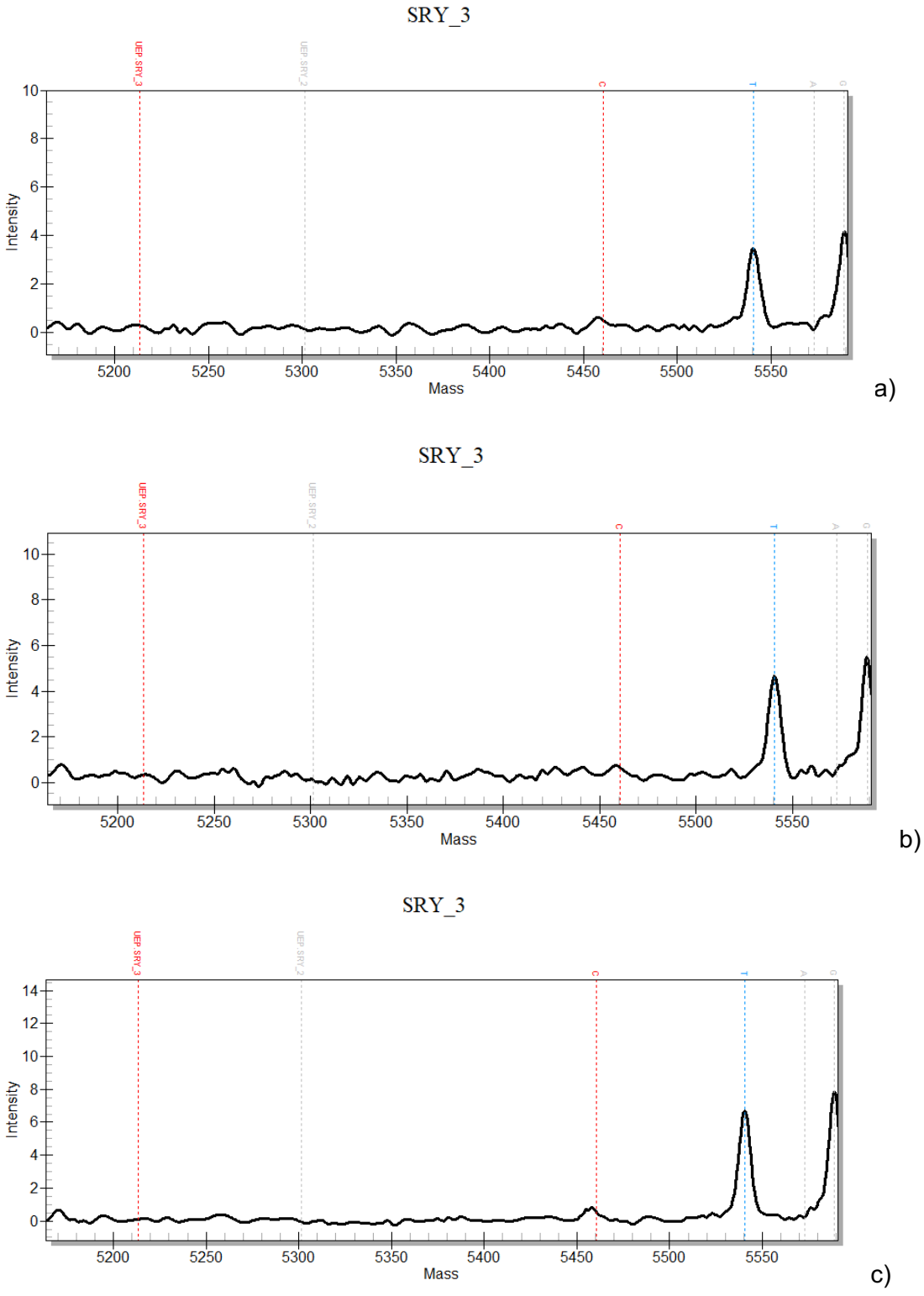
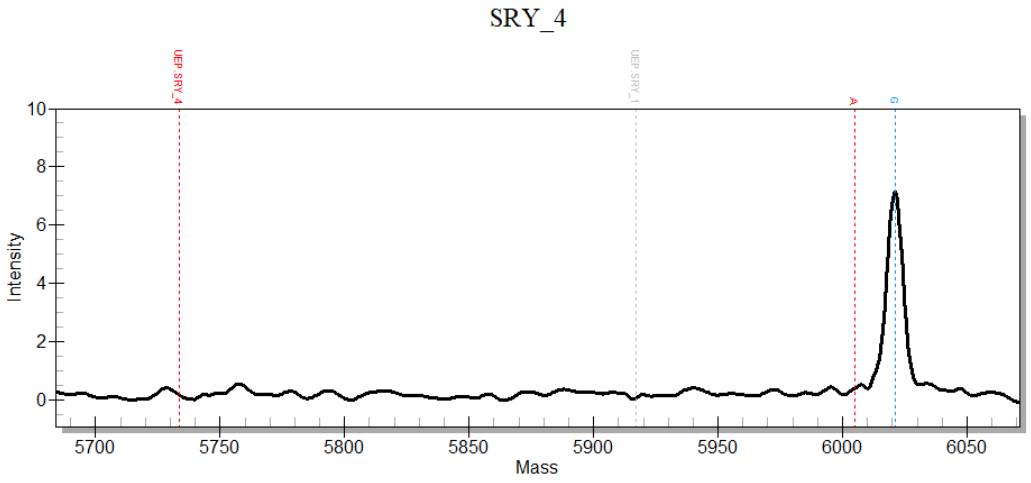
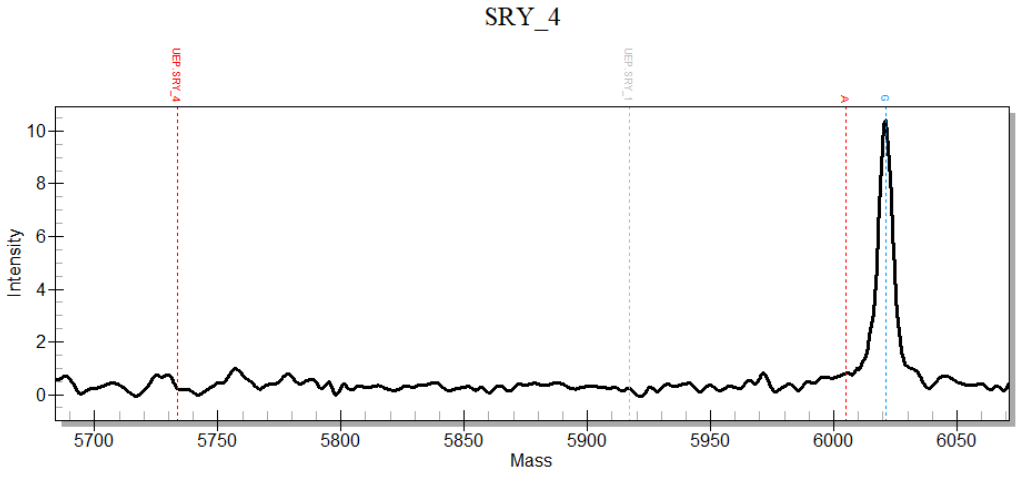


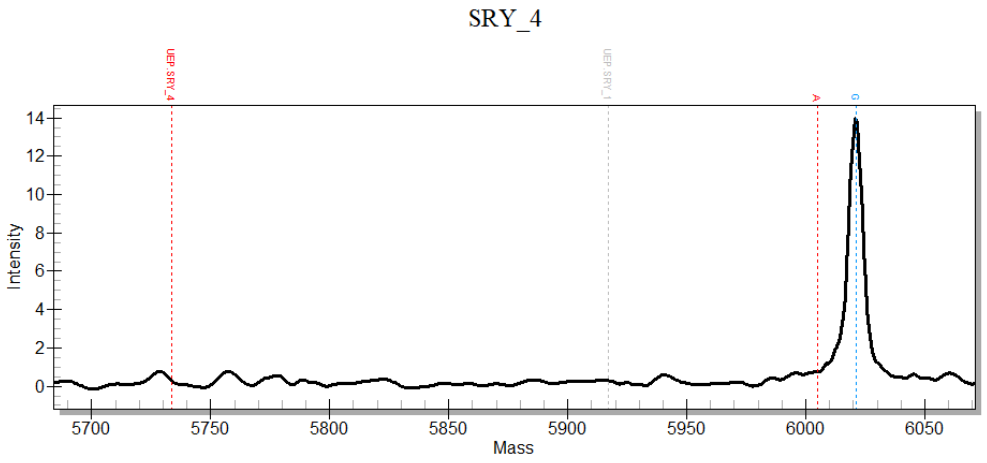
Figure 4-16 Optimisation of the MassARRAY® system for SNPs SRY-3 for the three different concentrations: a) 20 ng, b) 2 ng and c) 0,2 ng. SRY-3 sequence and that shown in [N/N] are the single base extension.



a)

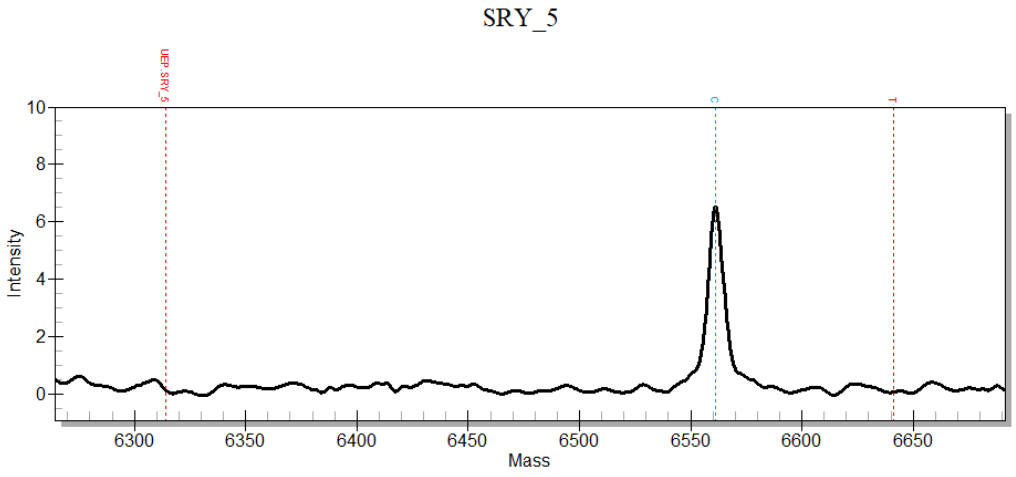


b)

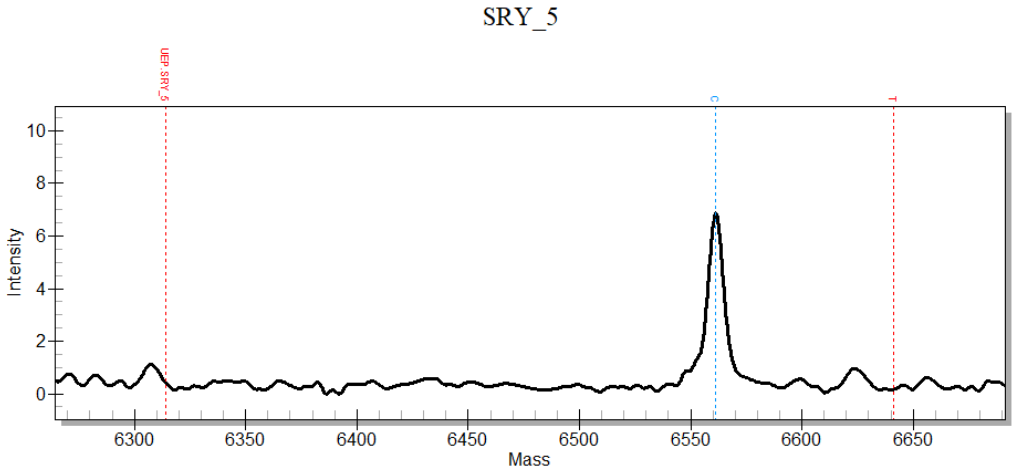


c)

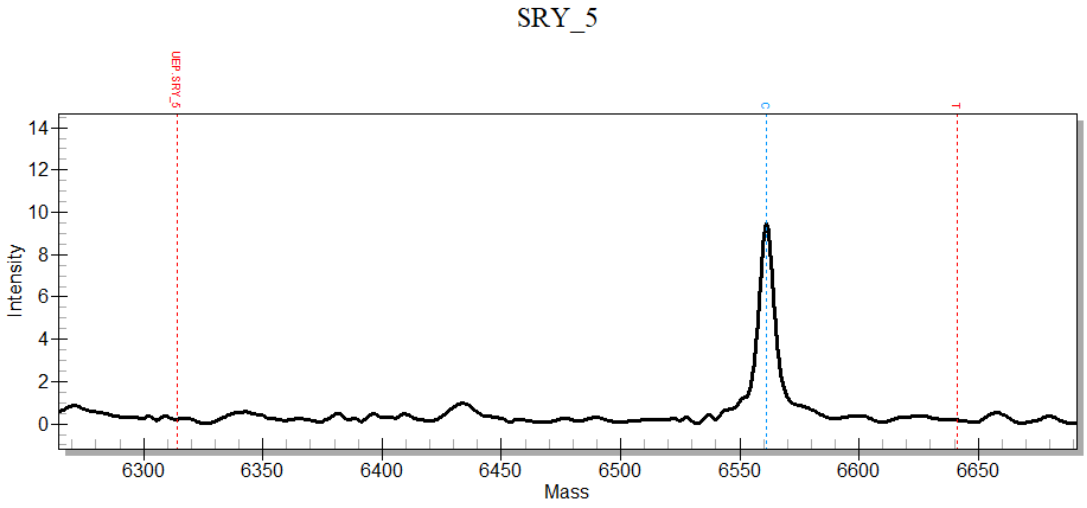
Figure 4-17 Optimisation of the MassARRAY® system for SNPs SRY-4 for the three different concentrations: a) 20 ng, b) 2 ng and c) 0,2 ng. SRY-4 sequence and that shown in [N/N] are the single base extension.



a)



b)



c)

Figure 4-18 Optimisation of the MassARRAY® system for SNPs SRY-5 for the three different concentrations: a) 20 ng, b) 2 ng and c) 0,2 ng. SRY-5 sequence and that shown in [N/N] are the single base extension.

The MassARRAY® system was also optimised using these five different targets, during which fragmented gDNA was used where one sample was fragmented for 30 minutes and the other for 35 minutes. Both these fragmented gDNA samples were then used, at a serial dilution of 20 ng/µL, 2 ng/µL and 0.2 ng/µL, to test for the five different targets across the *SRY* gene (Table 4.5). The 1:10 diluted gDNA showed positive results for all five targets and was, therefore, used further as a positive control. For the 30-minute fragmented gDNA dilution, all the targets were positively identified, except for target SRY-1 at dilution 1:100. The same was observed for the 35-minute fragmented gDNA dilution, where target SRY-4 and SRY-5 did not show any results for the specific target at dilution 1:100. The absence of these targets can be attributed to that the DNA was fragmented on this specific region of the SRY gene and due to the low amount of DNA present within the sample.

Table 4-5 The MassARRAY® results for the five different targets of the SRY gene of the gDNA, 30-minute fragmented gDNA and 35-minute fragmented gDNA

Sample		**SRY-1**	**SRY-2**	**SRY-3**	**SRY-4**	**SRY-5**
	Dilution factor					
gDNA	1:1	A	G	T	G	C
	1:10	A	G	T	G	C
	1:100	A	G	T	G	C
30 min Fragmented gDNA	1:1	A	G	T	G	C
	1:10	A	G	T	G	C
	1:100	-	G	T	G	C
35 min Fragmented gDNA	1:1	A	G	T	G	C
	1:10	A	G	T	G	C
	1:100	A	G	T	-	-

4.4 Isolation and characterisation of cfDNA

4.4.1 Isolation and quantification of cfDNA

Table 4-6 shows the results of the cfDNA isolation concentrations. As per the method that was used during the isolation of cfDNA (see section 3.9), 1mL of sample was used during the isolation, however, 4mL of plasma was obtained from each PAXgene® Blood ccfDNA tube. Thus, the isolation of cfDNA was repeated four times to achieve total volume cfDNA that was used in this

study. All isolated cfDNA samples were concentrated using a SpeedVac™ system of Thermo Fisher Scientific to concentrate the total volume eluted cfDNA. As shown in Table 4-6, the concentration varied from the different isolations that were done, even though the same isolation procedure was followed. It is also shown that these cfDNA concentrations of the samples are generally lower concentration and this could be attributed to DNA degradation due to the long storage of the plasma sample and these samples were stored for 2 years before cfDNA isolation was done. From these concentrations of cfDNA, only 3,4% - 6,2% are estimated to be fetal DNA (Davoodian & Kadivar, 2016a), indicating that a significantly low concentration of fetal DNA would be expected from these samples.

Table 4-6 cfDNA concentration of all samples.

Sample Name	Isolation A Qubit Concentration (ng/µL)	Isolation B Qubit Concentration (ng/µL)	Isolation C Qubit Concentration (ng/µL)	Isolation D Qubit Concentration (ng/µL)
1- M29	0,136	0,106	0,108	0,146
2- M27	0,118	0,14	0,134	0,164
3- J5	2,44	5,08	1,57	1,6
4 - K11	0,302	0,21	0,11	0,192
5 R104	0,13	0,158	0,122	0,102
6- 13/17	0,178	0,174	0,11	0,154
7- M21	0,174	0,122	1,36	0,248
8 -R103	0,11	0,16	0,12	0,246
9 - K9	0,1	0,16	0,12	0,186
10 - R122	0,1	0,152	0,114	0,228

4.4.2 Characterisation of cfDNA

Figure 4-19 shows the capillary electrophoresis results of cfDNA samples of isolation D, which was selected due to the fact that the overall concentrations of these samples were higher than the other isolation samples. The high sensitivity DNA chip used can identify DNA fragments of 50 to 10 000 bp. Due to the facts that capillary electrophoresis has a very high detection limit of small amounts of DNA and that cfDNA consists out of small amounts of DNA of multiple fragment sizes (Bronkhorst *et al.*, 2019, Bronkhorst *et al.*, 2020), the BioAnalyser was used to determine the base pair length of the cfDNA. In the resulting electropherograms, two marker peaks form at 35 bp and 10380 bp, which is used by the software to correctly identify fragment sizes.

From the BioAnalyser results, one can see that only sample 3 - J5 gave any usable peaks to identify the average amount of DNA between 100 – 400 bp. For the positive control of the experiment, fragmented male gDNA was used and this was represented by sample 11. However, for this sample fragmented sizes higher than that of the 10380 bp marker were observed. Similar peaks of large fragment sizes were observed in the cfDNA samples, possibly confirming both gDNA contamination and the degradation thereof during plasma storage. For all the samples except sample 3 – J5, the BioAnalyser results could not definitely show that there were cfDNA (< 2 000 bp) present in the samples and this can be attributed to the low cfDNA concentrations. The BioAnalyser detection limit is a minimum of 500 pg/μL of DNA within a sample, bearing in mind that “sample” in this context more commonly refers to a PCR product consisting of one DNA fragment size, not a pool of multiple fragment sizes. Most of the tested samples were tested at DNA concentrations less than 500 pg/μL. Thus, from the Qubit fluorometer results lower concentration of DNA within the samples were obtained, and no DNA fragments were found during the BioAnalyser analysis. These results found on the BioAnalyser could be attributed to the lower concentration values of the cfDNA samples and which indicated that not enough cfDNA was present during the capillary electrophoreses step.

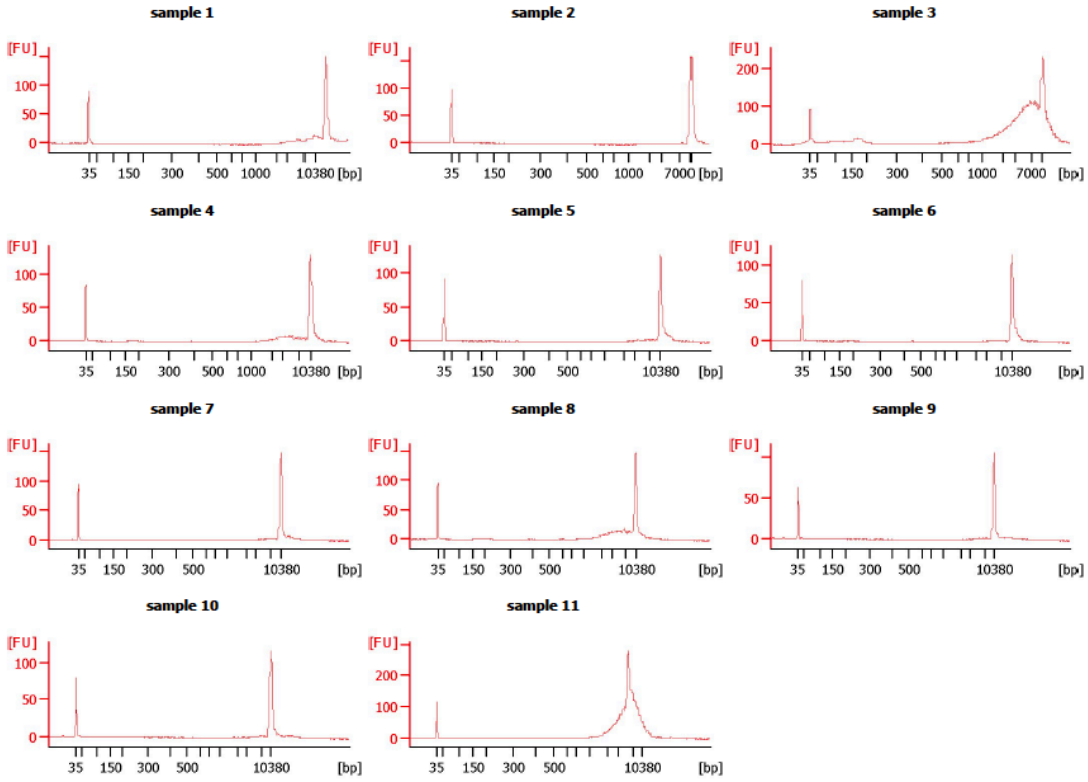


Figure 4-19 The capillary electrophoresis results for all 10 cfDNA samples, where the peaks at 35bp and 10380bp are internal ladders (sample 1- M29; sample 2 – M27; sample 3 – J5; sample 4 – K11; sample 5 – R104; sample 6 – 13/1; sample 7 – M21; sample 8 – R103; sample 9 – K9; sample 10 – R122 and sample 11 – fragmented male gDNA)

4.5 Sex determination using cfDNA

As mentioned in section 2.5.1, a wide variety of research has been done for prenatal sex determination on animals using cfDNA. From all these studies a qPCR tests was developed for cattle (da Cruz *et al.*, 2012), sheep (Asadpour *et al.*, 2015), horses (de Leon *et al.*, 2012), rhinoceros (Stoops *et al.*, 2018) and elephants (Vincze *et al.*, 2019) to determine the sex of the calf prenatally. These qPCR tests made use of cfDNA that was isolated from female blood samples and where the SRY gene was amplified to determine whether a male calf is present. For this study, we also investigated two different techniques to determine the sex of the calf prenatally using cfDNA. The two techniques used were qPCR using the SYBR Green primers and the MassARRAY® system and this was due to the small amount of cfDNA that was isolated and available.

4.5.1 Identification of fetal sex using SYBR Green qPCR assay

Firstly, in Table 4-7 the sex of the calves at birth is stated and this was used as a positive match during the qPCR assay. Table 4-7 shows the identified sex of the sable calves using qPCR and the amplification of the SRY-1 primer. During the qPCR, a positive control was used and consisted of male gDNA, as well as a negative control from a female cell line (CHO cells). From the 10 samples, 5 results corresponded to the sex at birth. False positive results were also found during the qPCR assay, which showed that 4 out of the 10 were male, but at birth they were identified as female. As shown in Figure 4-20 the bands of the PCR products are just below 100 bp this represents the products as would be found for the SRY-1 primers which are 96 bp. Based on the electrophoreses results shown in Figure 4-20 PCR products was visible for all the samples lower than 100 bp which corresponds to the PCR products of the SRY-1 primer. But when these PCR products results are compared to the sex at birth of the calves, 6 out of the 10 samples should not have any PCR products or bands present in the electrophoreses results due to the fact that they were female. This can be attributed to contamination during the qPCR assay. Electrophoreses of the qPCR results was done to identify whether primer dimers formed or if the SRY gene product had formed during this step. The results found was also attributed to the low concentration of the cffDNA and that the fragments of the cffDNA that were tested by primer SRY-1 was not present within the samples. It can also be attributed to the degradation of the DNA during the long storing period of the plasma samples and the freeze-thaw cycles. The false positives were, therefore, likely due to the formation of primer dimers. Due to the fact that the total amount of cfDNA that were isolated was so little and that the concentration of the cfDNA was so low, the other primer sets of the SRY gene could not be tested during this study.

Higher levels of accuracy were found for other PCR based assays for sex determination from cfDNA for cattle (da Cruz *et al.*, 2012, Lemos *et al.*, 2011), sheep (Kadivar *et al.*, 2013, Asadpour *et al.*, 2015), horses (de Leon *et al.*, 2012, Kadivar *et al.*, 2016), rhinoceros (Stoops *et al.*, 2018) and elephants (Vincze *et al.*, 2019). In these studies, they have used the *SRY* gene sequence to determine the sex of the calf and cfDNA with a PCR-based assay. Unfortunately, we were unable to establish the accuracy of the newly developed sable qPCR method based on the amount false positives results that were found. However, one can confirm that cfDNA is present in the sable antelope species and that when fresh plasma samples would be used, more accurate results would be identified using this qPCR method.

Table 4-7 Identification of the fetal sex using qPCR and primer SRY-1of the sable antelope samples.

Sample number	Sex at birth	Male according to qPCR (Yes/No)	False positive results obtained (Yes/Not applicable)
1- M29	None	None	N/A
2- M27	Female	Yes	Yes
3- J5	Female	Yes	Yes
4 - K11	Female	Yes	Yes
5 R104	Male	Yes	N/A
6- 13/17	Male	Yes	N/A
7- M21	Male	Yes	N/A
8 -R103	Female	Yes	Yes
9 - K9	Male	Yes	N/A
10 - R122	Female	No	N/A

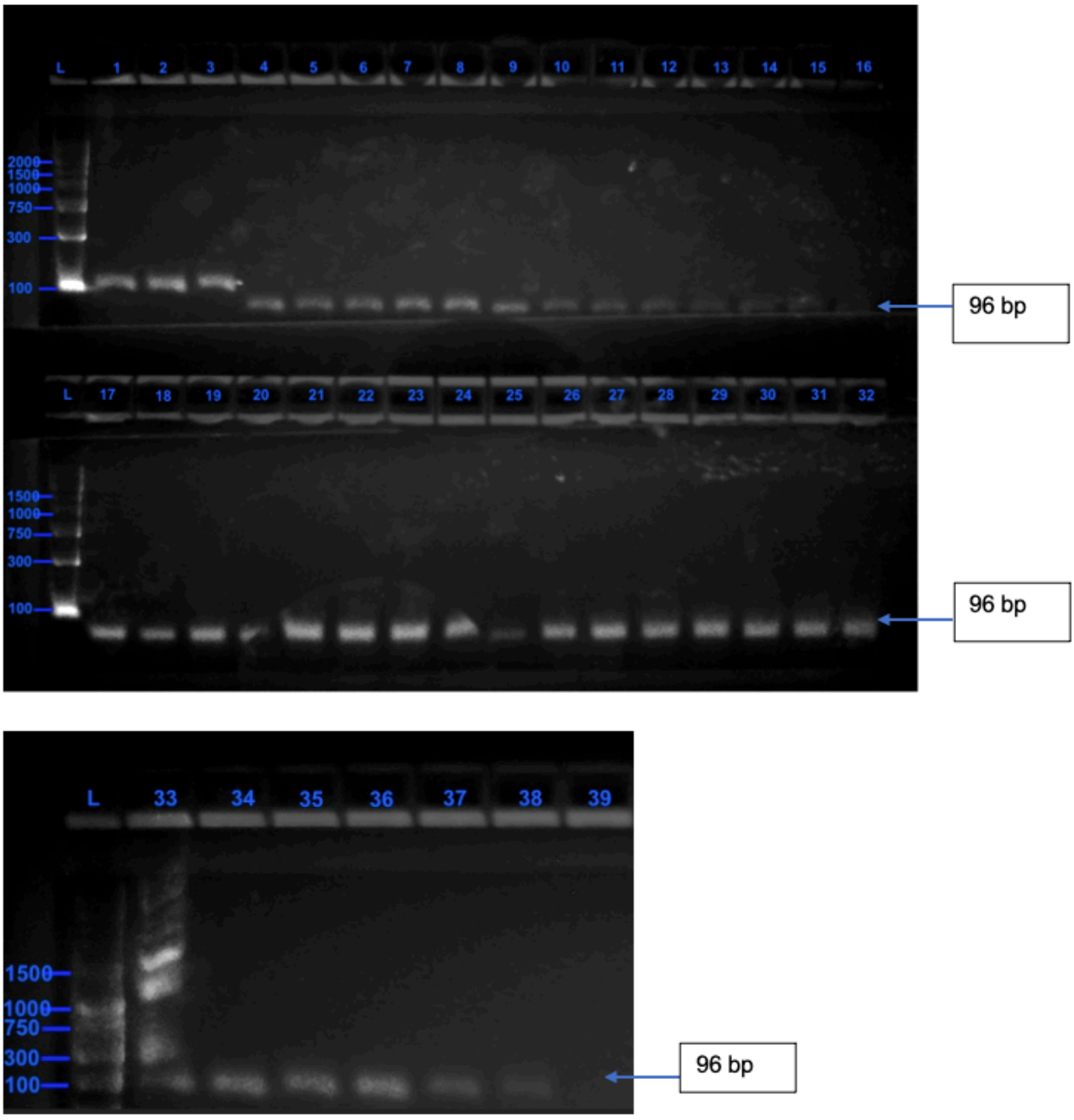


Figure 4-20 Electrophoreses results of the qPCR products and the samples were done in triplicate. (1-3 gDNA; 4-6 Female cell line; 7-9 M29; 10-12 M27; 13-15 J5; 16-18 K11; 19-21 R104; 22-24 13/17; 25-27 M21; 29-31 R103; 32-34 K9; 35-37 R122 and 38-39 NTC)

4.5.2 Identification of fetal sex using MassARRAY® system

The isolated cfDNA samples of the sable antelope was sent to Inqaba Biotec™ for MassARRAY® analysis of the five identified targets. Table 4-8 shows the results found from the MassARRY® system using the cfDNA together with two positive controls, one gDNA (2 ng/µL) and one fragmented gDNA (2 ng/µL) samples. Only one target or SNPs was identified during the MassARRAY® analysis of the cfDNA samples and this was for target SRY-1. Six samples showed positive results for the SRY-1 target and, from these samples, only two of the six samples were born as male (7-M29 and 9-K9), the other three samples were born as female (2-M27, 3-J5 and 10-R122). The other sample (1 – M29) that showed positive results for target SRY-1 did not have a calf at birth, it is possible that the female sable had lost the calf during parturition after the sample was taken.

Table 4-8 Identification of the fetal sex using the MassARRAY® system of the sable antelope cfDNA samples as well as positive control samples.

Sample number	**Sex at birth**	**MassARRAY® results**					**Male (Yes/No) based on MassARRAY® results**	**False positive results obtained (Yes/Not applicable)**
		SRY-1	SRY-2	SRY-3	SRY-4	SRY-5		
1- M29	None	A	-	-	-	-	Yes	Yes
2- M27	Female	A	-	-	-	-	Yes	Yes
3- J5	Female	A	-	-	-	-	Yes	Yes
4 - K11	Female	-	-	-	-	-	-	-
5 R104	Male	-	-	-	-	-	-	-
6- 13/17	Male	-	-	-	-	-	-	-
7- M21	Male	A	-	-	-	-	Yes	N/A
8 -R103	Female	-	-	-	-	-	-	-
9 - K9	Male	A	-	-	-	-	Yes	N/A
10 - R122	Female	A	-	-	-	-	Yes	Yes
Positive controls during MassARRAY®								
11 – gDNA	Male	A	G	T	G	C	Yes	N/A

12 – fragmented gDNA (35 minutes)	Male	A	G	T	G	C	Yes	N/A

Table 4-9 is a representation of the cfDNA concentration of the samples used for the MassARRAY® analysis. The cfDNA samples that was used and sent to Inqaba Biotec® was from isolation C and isolation D of Table 4-6, pooled together and concentrated to smaller amounts. Thus, the cfDNA concentrations of Table 4-9 was the pooled cfDNA of both these isolation procedures. It was expected from the results in Table 4-6 that the total concentration of cfDNA would be higher than that found in Table 4-9. The reason for the lower concentration can be attributed to that the samples were degraded during the freeze thaw cycles before it was used.

Table 4-9 The cfDNA concentrations of the samples used for the MassARRAY® system.

Sample number	cfDNA concentration (ng/µL)
1- M29	0.633
2- M27	0.000
3- J5	1.544
4 - K11	0.034
5 R104	0.000
6- 13/17	0.000
7- M21	0.034
8 -R103	0.086
9 - K9	0.000
10 - R122	0,034

Since only one target showed positive results for some of the samples and the concentration of the cfDNA were extremely low, the results found are unreliable for the sex determination of these given samples. Based on the fact that a positive result was found for target SRY-1 at three samples where the calf was female, further studies has to be done. It would be recommended that fresh cfDNA would be isolated from fresh maternal plasma then to be used for the

MassARRAY® system. Since the positive control shows positive results for all five targets identified, one can interpret that the MassARRAY® analysis was successful even though no results were found for some of the cfDNA samples.

CHAPTER 5. CONCLUSION AND FUTURE PROSPECTS

Since Lo *et al.* (1997) had discovered cffDNA in the maternal plasma of pregnant women, significantly more research has been done on non-invasive prenatal sex determination of fetal sex. This has prompted further research, using cffDNA in the diagnosis of fetal aneuploidies. Attributable to the discovery of cffDNA and cfDNA, it has become a growing field of research, with the inclusion of animal based cfDNA studies. However, the research on animals is still limited where cfDNA is used for fetal sex determination of the animal foetus and whether cfDNA is present in all mammalian species. cfDNA has been identified and used for prenatal sex determination studies in animals which were done in bovine (Lemos *et al.*, 2011, da Cruz *et al.*, 2012), ovine (Kadivar *et al.*, 2013, Asadpour *et al.*, 2015), equine (de Leon *et al.*, 2012, Kadivar *et al.*, 2016), rhinoceros (Stoops *et al.*, 2018), elephants (Vincze *et al.*, 2019) and buffalo species (De Villiers, 2020). The development of a sex determination assay has the potential to guide future management decisions for any wildlife species if the *SRY* gene of the species is known.

In this study, the presence of cfDNA as well as cffDNA was investigated in the sable antelope species. The *SRY* gene of the sable and roan antelope species was sequenced to determine the level of conservation between the species as well as sub-species. As part of the sex determination of the calf using cfDNA, two different techniques were used, namely qPCR and the MassARRAY® system. Due to the nature and extremely low concentrations of cffDNA, the suitability of the chosen techniques to determine fetal sex could not be verified. With this in mind, the results obtained during this study provides valuable insight for future sex determination studies.

5.1 Evaluation and Future prospects

5.1.1 Sub-species identification of the sable and roan antelope

Firstly, mtDNA was used to identify the different sub-species of both sable and roan antelopes due to the fact that some of the samples sub-species were unknown. The mtDNA was used to sequence the D-loop of these samples to be used for phylogenetic tree analysis together with selected reference sequences. With the phylogenetic analysis the different sub-species within the samples were identified. For the sable antelope out of the 45 hair samples three different sub-species were identified namely *H. n. niger*, *H. n. roosevelti* and *H. n. kirkii*. For the roan antelope 2 different sub-species were identified namely *H. e. equinus* and *H. e. cottoni*. The sub-species that were identified are not all the sub-species found in Africa, however, these sub-species are

located, as shown in **Figure 2-3** and **Figure 2-5**, in the southern region of Africa. Based on this, these sub-species are potentially bought at wildlife auctions.

5.1.2 Sequencing of the SRY gene

Hair samples were received from two different farms found in South Africa and used to sequence the *SRY* gene for both sable and roan antelope species. Sinclair *et al.* (1990) has identified the location where the *SRY* gene can be found on the Y chromosome. Since the *SRY* gene is linked to the testes determining factor (TDF) in male sex development it is frequently used for sex determination studies. The *SRY* gene was successfully sequenced for the sable and roan antelope species during this study and the *SRY* gene sequence is shown in section 4.2. This is the first complete *SRY* sequence information for these species. Since the *SRY* gene of all these samples were known and the different sub-species identified, a comparison was done regarding the SRY gene sequence. From these results it was found that the *SRY* gene sequence is conserved between species and sub-species. Thus, there were no differences in the *SRY* gene sequences between the three identified sub-species of sable antelope as well as for the two identified roan antelope sub-species.

5.1.3 Isolation of cfDNA

In a previous study by De Villiers (2020), it was determined that cfDNA is more stable in blood samples when Streck tubes are used during blood collection and storage. However, it was recommended that Qiagen blood tubes might be more stable over a period of time than the Streck tubes. Furthermore, for the isolation of cfDNA, a study by Bronkhorst *et al.* (2020) found that using the NucleoSpin® Gel and PCR Clean-up kit delivers the highest reproducibility and cfDNA yield. Accordingly, in this study all these recommendations were taken into consideration and the Qiagen blood tubes and NucleoSpin® Gel and PCR Clean-up kit were used. However, the concentration of the cfDNA that was isolated was lower than expected (ranging from 0.100 ng/µL to 2.440 ng/µL), even though all recommendations were met. The concentration of the cfDNA was determined using the Qubit Fluorometer, which is a more sensitive spectrophotometer to determine cfDNA concentrations. The concentration results of the cfDNA showed extremely low concentration. It has been found that the cffDNA concentration can range between 3.4% and 6.2% (25.4 genome equivalents/mL to 69.4 genome equivalents/mL) (Kadivar *et al.*, 2016, Bischoff *et al.*, 2005) of the total cfDNA found in maternal plasma. Thus, from this knowledge and

the low concentrations found, the possibility decreased whether cffDNA were present in these samples. Capillary electrophoresis was also used as a quantification method, due to that it is highly sensitive for shorter fragments of DNA. The electropherograms showed significant results for only one sample J5 and not the others, this can be attributed to the low concentrations of the cfDNA, although, this does not mean that cfDNA were not present within the samples. Due to these low concentrations and from the capillary electrophoresis results, there is possibility of DNA degradation during storage of the samples. The samples were stored at -80°C for a long period of time before cfDNA isolation was done and this could also have been a reason for the lower cfDNA concentration of these samples. Due to the suspected degradation due to long-term storage, one cannot confidently determine whether these antelope species have lower amounts of cfDNA present in their blood circulatory system compared to other mammals. The research regarding whether cfDNA is present in the sable antelope is still limited and, to our knowledge, this was the first study where these animals have been used. It is recommended that fresh blood samples from pregnant female sable antelopes must be retrieved to eliminate the potential of DNA degradation via storage. If fresh blood samples can be retrieved, processed immediately and the isolation of cfDNA can be done, one can identify whether the concentration would be higher than that was found during this study. It is also recommended to include an incubation step with proteinase K during the isolation of cfDNA to determine whether plasma proteins interfered with the extraction process.

5.1.4 qPCR and MassARRAY® system

The aim of this study was to develop a quantitative assay for fetal sex determination of the sable and roan antelopes prenatally. Three different methods were designed and optimised for the prenatal sex determination of sable and roan antelope. Due to certain constrains during this study the methods were only investigated for the sable antelope species, because roan antelope blood samples could not be obtained due to restraints and the national lock down. Only two different methods were used for the sex determination of the fetal sex due to low amount of cfDNA that were isolated and used to concentrate the cfDNA in order to increase the cfDNA concentration.

The two methods that were designed and optimised for this study were the qPCR and the MassARRAY® system. For the qPCR method of the study, two different approaches were used, SYBR Green, which contained five different primer pairs, and three probe-based primer pairs. A SYBR Green qPCR assay is a qPCR assay where SYBR Green dye is used during the qPCR method to obtain the fluorescent emission during the amplification of the DNA. During a probe-based qPCR assay a probe is used for the fluorescent emission during the amplification and this

is achieved when the reporter is detached from the probe. All the different primers sets were designed to bind at different binding sites on the *SRY* gene of the sable antelope. This was needed due to the fact that the *SRY* gene consist out of 690 bp, while cffDNA consists out of smaller fragments of DNA. By using more primers, the probability of the primers binding to the cffDNA fragments found in the maternal plasma increases. For the SYBR Green primers, two of the primers were not ideal to use due to primer dimer formation (SRY-3 and SRY-5). However, primer pairs SRY-1, SRY-2 and SRY-4 showed considerably better results during the optimisation of the qPCR. For fetal sex determination of the SYBR Green assay, the SRY-1 primer pair was used, and amplification of a 96 bp fragment was obtained. However, a significant amount of false positives was seen. This could be attributed to primer dimer formation and this was also seen in one of the two NTC during qPCR. Due to the low amount of cfDNA available, only the SRY-1 primer was used. For the probe-based primers three different primers were designed and during the optimisation of the qPCR, these primers showed good amplification results when compared to the primers used for the SYBR Green qPCR assay. No primer dimer formation was observed. Unfortunately, these probe-based primers could not be used for the fetal sex determination due to the low amount of cfDNA that were available after isolation (see section 4.3).

The second method that was designed and optimised was the MassARRAY® system. Five different targets were identified from the SRY gene sequence and were optimised for the MassARRAY® system. Fragmented and intact gDNA was used during the optimisation of the MassARRAY® system and positive results were obtained for all five targets. For the fetal sex determination using the MassARRAY® system, only one target showed positive results for four samples. However, only two of these samples were positive when compared to the sex at birth and none of the other targets showed results for these cfDNA samples. It is thus recommended that fresh blood samples from pregnant female sable antelopes must be obtained and cfDNA isolation should take place immediately. This should lead to higher cfDNA concentrations to be used for the qPCR, as well as for the MassARRAY® system. In the study by Stoops *et al.* (2018), they used fresh blood samples as well as samples that were frozen and the fresh plasma samples had more optimal results.

The low accuracy of the qPCR amplification and the MassARRAY® system when cfDNA was used may be attributed to two possible reasons: (1) The extremely low cfDNA concentrations suggested that the cffDNA might be too degraded for amplification; (2) An excessive percentage of the DNA within the cffDNA was cut (degraded) in areas where the binding of primers or where amplification should have taken place. Blood samples should also be obtained from pregnant sable antelopes with a higher gestation period due to the fact that the amount of fetal DNA increases in later of gestation periods (da Cruz *et al.*, 2012). Thus, the higher the ratio of cffDNA

to cfDNA obtained for the optimisation of the two methods, the more reliable and accurate results should be once the methods are functional. Another limitation during this study was that a female gDNA sample was not available to ascertain the non-specific binding of the assay in the female sable antelope genome.

5.2 Summary of future objectives

I. Obtain fresh blood samples from pregnant female sable and roan antelopes and use more than one blood tube during collection, if possible, to obtain these samples.
II. After the blood collection from the animals, limit the time from the collection to processing of the sample to obtain the plasma of the sample.
III. During the transportation of the sample add extra ice to the cold box and gel ice packs to obtain a temperature of 4°C inside the cold box.
IV. It would be recommended to use fresh plasma for the isolation of cfDNA in order to identify whether the cfDNA concentration would be higher.
V. Include a proteinase K incubation step during the cfDNA isolation procedure for more optimal cell lysis if fetal DNA might still be encapsulated.
VI. Increasing the amount of plasma used during the isolation procedure than that was used during this study.
VII. It would be recommended to use fresh isolated cfDNA for both qPCR and MassARRAY® system to determine the fetal sex.
VIII. Determine whether the other primers that were designed for both SYBR and probe-based primers can be used for fetal sex determination if higher amounts of cfDNA with higher concentration is available.

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APPENDIX A. – ETHICAL CLEARENCE LETTER

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

3 June 2020

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 03/06/2020, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope
Principal Investigator/Study Supervisor/Researcher: Dr R van der Sluis
Student: Z Geldenhuys – 24328405

Ethics number:

N	W	U	-	0	0	5	9	5	-	1	9	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 03/06/2020
Expiry date: 30/06/2021

Risk: Category 3

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of an annual monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of June annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*

- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Special in process conditions of the research for approval (if applicable):

a. Please provide the NWU-AnimCareREC with a copy of the proof of ethics training once they become available from the Health Sciences Ethics Office for Research, Training and Support.

As the study progresses the aforementioned conditions should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating "Outstanding documents for approval: NWU-XXXXX-XX-XX." The letter should include the title of the approved study, the names of the researchers involved, that the documents are being submitted as part of the conditions of the approval set by the NWU-AnimCareREC, the nature of the document i.e. which condition is being fulfilled and any further explanation to clarify the submission.

The *e-mail*, to which you attach the documents that you send, should have a *specific subject line* indicating the nature of the submission e.g. "Outstanding documents for approval: NWU-XXXXX-XX-XX". The e-mail should indicate the nature of the document being sent. This submission will be handled via the expedited process.

Special COVID-19 arrangements:

There may be certain changes that you may need to make to your study, after it has been approved, due to the impact of the COVID-19 pandemic or the lockdown e.g.:

a. Changing the ages of the animals to be used in the research study
b. Changing the approved methodology to use data collection methods that ensure physical distancing from farmers and owners
c. The implementation of risk-mitigating strategies to prevent infection during data collection
d. Changes in time schedules etc.

Please note that any 1) amendments, 2) request for extensions or 3) any other kind of modification to the proposal or other associated documentation must be submitted for review by the NWU-AnimCareREC, Faculty of Health Sciences, and must be approved, prior to these changes being implemented.

These amendment requests will be handled via the *expedited process*.

Process to be followed:

a. Ensure all your amendments are indicated in yellow highlight in the amended documents.
b. Submit your request to Ethics-AnimCare@nwu.ac.za with a *cover letter* with a specific subject title indicating, "Amendment request (COVID-19): NWU-XXXXX-XX-XX". The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted.
c. Name the emails, to which you attach the documents that you send, with a *specific subject line* indicating that it is an amendment request e.g. "Amendment request (COVID-19): NWU-XXXXX-XX-XX". This e-mail should indicate the nature of the amendment.
d. Send a short email to Prof Wayne Towers and Prof Tiaan Brink indicating to them that you have submitted an amendment request due to the COVID-19 impact.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Chairperson: NWU-AnimCareREC

Current details:(22654704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
11 May 2020

File Reference: 9.1.5.4.2

APPENDIX B. – INFORMED CONSENT FORMS

NWU®

NWU Animal Care, Health and Safety in Research
Ethics Committee
(NWU Animal ethics)
Faculty of Health Sciences
2020 -06- 30
NWU

TITLE OF THE RESEARCH STUDY:
Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

ETHICS REFERENCE NUMBERS:

PRINCIPAL INVESTIGATOR:
Dr Rencia van der Sluis

POST GRADUATE STUDENT:
Mr Zarco Geldenhuys

ADDRESS:
108, Building F3, Biochemistry NWU, Potchefstroom, 2531

CONTACT NUMBER:
018-2992068

You are being invited to take part in a research study that forms part of an MSc research project. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research is about and how you might be involved. Also, your participation is entirely voluntary, and you are free to say no at any time. If you say no, this will not have any negative affect on you in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part in the study now.

This study has been approved by the NWU-AnimCareREC Committee of the North-West University (NWU) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of

animals for scientific purposes". It might be necessary for the research ethics committee members or other relevant people to inspect the research records.

What is this research study all about?

In this project we aim to develop a method to determine the sex of unborn Sable and Roan antelopes in a non-invasive manner. We will receive two blood collection tubes (10ml) from pregnant females (10 Sable antelopes and 10 Roan antelopes) as well as two blood collection tubes from one male Roan and one male Sable antelope. These two samples will serve as a positive control for the analyses. We will also receive previously collected hair samples from male sable and roan antelopes to use to determine the identity of the DNA that will be used to design markers to determine the male sex. These markers will be developed to bind to the unborn calves' DNA present in the mother's blood sample, to determine the sex of the animal. This process will then be validated to the extent that a commercial test could be available.

Why have you been invited to participate?

You have been invited to be part of this research because you are an owner of either a Sable antelope or Roan antelope.

What will be expected of you?

When animals are sold or relocated in South Africa it is required by the Department of Agriculture, Forestry and Fisheries (DAFF) that the animals undergo testing for certain controlled diseases. These animals have to be immobilised whereby blood is collected. The blood samples are collected for routine (veterinary check-ups and otherwise) analyses. Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. We, therefore, require permission from you to access the farm at the time of the routine analyses to receive the blood samples from the veterinarian. We will receive two blood collection tubes for the project.

Previously collected hair samples will be received for the subspecies found in Southern Africa of the Sable (Hippotragus niger niger and H. niger kirkii) and Roan antelope (Hippotragus equinus equinus). These hair samples were collected by farmers to send to Ecological Genomics and Wildlife conservation. They offer a service to determine the genetic provenance of animals.

Will you gain anything from taking part in this research?

The gains for taking part in this study will be feedback on whether the development of a diagnostic test was successful. The results of the study (i.e. the sex of the unborn calf) will also be sent to you.

Are there risks involved in you taking part in this research and what will be done to prevent them?

Blood collection: Serum and plasma are the least invasive known sources of DNA.

Anaesthesia: Sable and Roan antelope are not domesticated, and anaesthesia is the only way to collect the samples, the animals will receive anaesthesia for other required testing that will be taking place. We will utilize this opportunity to collect samples for our research. Samples will be taken by a qualified veterinarian. The blood and hair samples will be collected as per routine (veterinary check-ups and otherwise). Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. There are more gains for joining this study than there are risks involved.

What will happen with the findings or samples?

The owners of the animals will be informed of the results and a scientific publication will also be written.

Is there anything else that you should know or do?

- You can contact Dr Rencia van der Sluis at 0182992067 if you have any further questions or have any problems.
- You can also contact the NWU-AnimCareREC at 0182991208 or Ethics-AnimCare@nwu.ac.za if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Declaration by participant

By signing below, I [signature] agree to take part in the research study titled: Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

Signed at (place) Klerksdorp on (date) 20 September 2020.

Signature of participant

Signature of witness

Declaration by person obtaining consent

I (name) Zarca Liebenberg declare that:

- I clearly and in detail explained the information in this document to

.....

- I did/did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (place) NWU - Potchefstroom on (date) 11 November 2020

Liebenberg

Signature of person obtaining consent

Declaration by researcher

I Dr Rencia van der Sluis declare that:

- I had it explained by Mr Zarco Geldenhuys who I trained for this purpose.
- I did/did not use an interpreter
- I was available should he/she want to ask any further questions.
- The informed consent was obtained by an independent person.
- I am satisfied that he/she adequately understands all aspects of the research, as described above.
- I am satisfied that he/she had time to discuss it with others if he/she wished to do so.
-

Signed at (*place*) Potchefstroom on (*date*) 30 Sept 20 20

.....

Signature of researcher

NWU®

TITLE OF THE RESEARCH STUDY:

Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

ETHICS REFERENCE NUMBERS:

PRINCIPAL INVESTIGATOR:
Dr Rencia van der Sluis

POST GRADUATE STUDENT:
Mr Zarco Geldenhuys

ADDRESS:
108, Building F3, Biochemistry NWU, Potchefstroom, 2531

CONTACT NUMBER:
018-2992068

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This study has been approved by the NWU-AnimCareREC Committee of the North-West University (NWU) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of

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Why have you been invited to participate?

You have been invited to be part of this research because you are an owner of either a Sable antelope or Roan antelope.

What will be expected of you?

When animals are sold or relocated in South Africa it is required by the Department of Agriculture, Forestry and Fisheries (DAFF) that the animals undergo testing for certain controlled diseases. These animals have to be immobilised whereby blood is collected. The blood samples are collected for routine (veterinary check-ups and otherwise) analyses. Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. We, therefore, require permission from you to access the farm at the time of the routine analyses to receive the blood samples from the veterinarian. We will receive two blood collection tubes for the project.

Previously collected hair samples will be received for the subspecies found in Southern Africa of the Sable (Hippotragus niger niger and H. niger kirkii) and Roan antelope (Hippotragus equinus equinus). These hair samples were collected by farmers to send to Ecological Genomics and Wildlife conservation. They offer a service to determine the genetic provenance of animals.

Will you gain anything from taking part in this research?

The gains for taking part in this study will be feedback on whether the development of a diagnostic test was successful. The results of the study (i.e. the sex of the unborn calf) will also be sent to you.

Are there risks involved in you taking part in this research and what will be done to prevent them?

Blood collection: Serum and plasma are the least invasive known sources of DNA.

Anaesthesia: Sable and Roan antelope are not domesticated, and anaesthesia is the only way to collect the samples, the animals will receive anaesthesia for other required testing that will be taking place. We will utilize this opportunity to collect samples for our research. Samples will be taken by a qualified veterinarian. The blood and hair samples will be collected as per routine (veterinary check-ups and otherwise). Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. There are more gains for joining this study than there are risks involved.

What will happen with the findings or samples?

The owners of the animals will be informed of the results and a scientific publication will also be written.

Is there anything else that you should know or do?

- You can contact Dr Rencia van der Sluis at 0182992067 if you have any further questions or have any problems.
- You can also contact the NWU-AnimCareREC at 0182991208 or Ethics-AnimCare@nwu.ac.za if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Declaration by participant

By signing below, I ...Bona Bona Ga/Kgabo... agree to take part in the research study titled: Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is voluntary and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

Signed at (place) Bona Bona game lodge on (date) 16/10/ 2020.

Signature of participant

Signature of witness

Declaration by person obtaining consent

I (name) Johan declare that:

- I clearly and in detail explained the information in this document to

- I did/did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (place) Bona Bona Game lodge on (date) 16/10/ 2020.

Signature of person obtaining consent

Declaration by researcher

I Dr Rencia van der Sluis declare that:

- I had it explained by Mr Zarco Geldenhuys who I trained for this purpose.
- I did/did not use an interpreter
- I was available should he/she want to ask any further questions.
- The informed consent was obtained by an independent person.
- I am satisfied that he/she adequately understands all aspects of the research, as described above.
- I am satisfied that he/she had time to discuss it with others if he/she wished to do so.
-

Signed at (*place*) ..... on (*date*) ..... 20..

.....

Signature of researcher

NWU®
NORTH-WEST UNIVERSITY
NOORDWES-UNIVERSITEIT
YUNIBESITI YA BOKONE-BOPHIRIMA

TITLE OF THE RESEARCH STUDY:
Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

ETHICS REFERENCE NUMBERS:

PRINCIPAL INVESTIGATOR:
Dr Rencia van der Sluis

POST GRADUATE STUDENT:
Mr Zarco Geldenhuys

ADDRESS:
108, Building F3, Biochemistry NWU, Potchefstroom, 2531

CONTACT NUMBER:
018-2992068

You are being invited to take part in a research study that forms part of an MSc research project. Please take some time to read the information presented here, which will explain the details of this study. Please ask the researcher or person explaining the research to you any questions about any part of this study that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research is about and how you might be involved. Also, your participation is entirely voluntary, and you are free to say no at any time. If you say no, this will not have any negative affect on you in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part in the study now.

This study has been approved by the NWU-AnimCareREC Committee of the North-West University (NWU) and will be conducted according to the ethical guidelines and principles of Ethics in Health Research: Principles, Processes and Structures (DoH, 2015) and the South African National Standard (SANS) document 10386:2008 entitled, "The care and use of

animals for scientific purposes". It might be necessary for the research ethics committee members or other relevant people to inspect the research records.

What is this research study all about?

In this project we aim to develop a method to determine the sex of unborn Sable and Roan antelopes in a non-invasive manner. We will receive two blood collection tubes (10ml) from pregnant females (10 Sable antelopes and 10 Roan antelopes) as well as two blood collection tubes from one male Roan and one male Sable antelope. These two samples will serve as a positive control for the analyses. We will also receive previously collected hair samples from male sable and roan antelopes to use to determine the identity of the DNA that will be used to design markers to determine the male sex. These markers will be developed to bind to the unborn calves' DNA present in the mother's blood sample, to determine the sex of the animal. This process will then be validated to the extent that a commercial test could be available.

Why have you been invited to participate?

You have been invited to be part of this research because you are an owner of either a Sable antelope or Roan antelope.

What will be expected of you?

When animals are sold or relocated in South Africa it is required by the Department of Agriculture, Forestry and Fisheries (DAFF) that the animals undergo testing for certain controlled diseases. These animals have to be immobilised whereby blood is collected. The blood samples are collected for routine (veterinary check-ups and otherwise) analyses. Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. We, therefore, require permission from you to access the farm at the time of the routine analyses to receive the blood samples from the veterinarian. We will receive two blood collection tubes for the project.

Previously collected hair samples will be received for the subspecies found in Southern Africa of the Sable (Hippotragus niger niger and H. niger kirkii) and Roan antelope (Hippotragus equinus equinus). These hair samples were collected by farmers to send to Ecological Genomics and Wildlife conservation. They offer a service to determine the genetic provenance of animals.

Will you gain anything from taking part in this research?

The gains for taking part in this study will be feedback on whether the development of a diagnostic test was successful. The results of the study (i.e. the sex of the unborn calf) will also be sent to you.

Are there risks involved in you taking part in this research and what will be done to prevent them?

Blood collection: Serum and plasma are the least invasive known sources of DNA.

Anaesthesia: Sable and Roan antelope are not domesticated, and anaesthesia is the only way to collect the samples, the animals will receive anaesthesia for other required testing that will be taking place. We will utilize this opportunity to collect samples for our research. Samples will be taken by a qualified veterinarian. The blood and hair samples will be collected as per routine (veterinary check-ups and otherwise). Hence samples are used that will be collected even without this study taking place and therefore this study is not causing additional impact through collection from animals that otherwise would not have been captured or sampled. There are more gains for joining this study than there are risks involved.

What will happen with the findings or samples?

The owners of the animals will be informed of the results and a scientific publication will also be written.

Is there anything else that you should know or do?

- You can contact Dr Rencia van der Sluis at 0182992067 if you have any further questions or have any problems.
- You can also contact the NWU-AnimCareREC at 0182991208 or Ethics-AnimCare@nwu.ac.za if you have any concerns that were not answered about the research or if you have complaints about the research.
- You will receive a copy of this information and consent form for your own purposes.

Declaration by participant

By signing below, I Wiaan v. d. Linde agree to take part in the research study titled: Development of a quantitative assay for the non-invasive determination of fetal sex in Sable and Roan antelope.

I declare that:

- I have read this information/it was explained to me by a trusted person in a language with which I am fluent and comfortable.
- The research was clearly explained to me.
- I have had a chance to ask questions to both the person getting the consent from me, as well as the researcher and all my questions have been answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be handled in a negative way if I do so.
- I may be asked to leave the study before it has finished, if the researcher feels it is in the best interest, or if I do not follow the study plan, as agreed to.

Signed at (*place*) Winterhoek on (*date*) 15/10/2020

Signature of participant

Signature of witness

Declaration by person obtaining consent

I (*name*) Zara Feldenhuys declare that:

- I clearly and in detail explained the information in this document to

.....

- I did/did not use an interpreter.
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I gave him/her time to discuss it with others if he/she wished to do so.

Signed at (*place*) Nieu-Bethesda on (*date*) 11/11/2020

Feldenhuys.

Signature of person obtaining consent

Declaration by researcher

I Dr Rencia van der Sluis declare that:

- I had it explained by Mr Zarco Geldenhuys who I trained for this purpose.
- I did/did not use an interpreter
- I was available should he/she want to ask any further questions.
- The informed consent was obtained by an independent person.
- I am satisfied that he/she adequately understands all aspects of the research, as described above.
- I am satisfied that he/she had time to discuss it with others if he/she wished to do so.
-

Signed at (*place*) ..... on (*date*) ..... 20.20.

.....

Signature of researcher

APPENDIX C – HAIR SAMPLE LIST

Sable antelope Hair samples					
Bona Bona Game reserve					
Sable antelope	Sub-species	ID 1	ID 2	Male (Y/N)	Hair samples ID
Niger Kirkii (12 male samples)	Zambian	43909	43897	y	H1
	Zambian	43401	43393	y	H2
	Zambian	43400	43392	y	H3
	Zambian	39887	39884	y	H4
	Zambian	36841	36809	y	H5
	Zambian	38566	38556	y	H6
	Zambian	43902	43890	y	H7
	Zambian	43905	43893	y	H8
	Zambian	48504	48627	y	H9
	Zambian	43898	43886	y	H10
	Zambian	43900	43888	y	H11
	Zambian	43901	43889	y	H12
Niger Kirkii (Matetsi rivier)	Matetsi	48443	48612	y	H13
11 male samples	Mixed	R13		Y	H14
	Mixed	S30		Y	H15
	Mixed	N2		Y	H16
	Mixed	E1		Y	H17
	Mixed	R12		Y	H18
	Mixed	R18		Y	H19
	Mixed	R14		Y	H20
	Mixed	R17		Y	H21
	Mixed	R10		Y	H22
	Mixed	R11		Y	H23
	Mixed	R15		Y	H24
Owner: Collin Engelbrecht of Klein Buisfontuin Ranch					
Group 1 unknown sub-species	Unknown	Beige 1		Y	H25
		Beige 2		Y	H26
		Beige 3		Y	H27
		Beige 4		Y	H28
		Beige 5		Y	H29
		Beige 6		Y	H30
		Beige 7		Y	H31
		Beige 8		Y	H32
		Beige 9		Y	H33
		Beige 10		Y	H34
		Beige 11		Y	H35
Group 2 unknown sub-species	Unknown	Purple 2/16		Y	H36
		Purple 3/16		Y	H37
		Purple 6/16		Y	H38
		Red 13		Y	H39
		Red 14		Y	H40
		Red 15		Y	H41
		Orange 14		Y	H42
		Orange 15		Y	H43
		Orange 16		Y	H44
		Orange 17		Y	H45
Owner: Wiaan van der Linde of Wintershoek					
Sable antelope	Unknown	W29		Y	B1
ID 1 is the ID which is used on the farm					
ID 2 is the ID which is used on the farm					
Hair sample ID is the ID used during the study					

Roan Antelope Hair samples				
Bona Bona Game reserve				
Roan Antelope	ID 1	ID 2	Male (Y/N)	Hair sample ID
10 samples	T94	7110322B03	Y	H46
	T95	900250000705640	Y	H47
	T92	7110322341	Y	H48
	T286	900252003058631	Y	H49
	T263	900252003058636	Y	H50
	T265	900252003058622	Y	H51
	T126	900252003058640	Y	H52
	T270	900252003058630	Y	H53
	T267	900252003058629	Y	H54
	T226	900252003058632	Y	H55

APPENDIX D. – GENBANK REFERENCE NUMBERS OF REFERENCE SEQUENCES USED FOR PHYLOGENETIC ANALYSES

Species	Subspecies	GenBank accession number	Reference
Hippotragus niger	niger	AF364689	Pitra et al 2002
Hippotragus niger	niger	AF364691	Pitra et al 2002
Hippotragus niger	niger	AF364693	Pitra et al 2002
Hippotragus niger	niger	AF364695	Pitra et al 2002
Hippotragus niger	niger	AF364698	Pitra et al 2002
Hippotragus niger	niger	AF364699	Pitra et al 2002
Hippotragus niger	niger	AF364700	Pitra et al 2002
Hippotragus niger	niger	AF364702	Pitra et al 2002
Hippotragus niger	niger	AF364703	Pitra et al 2002
Hippotragus niger	niger	AF364706	Pitra et al 2002
Hippotragus niger	niger	AF364710	Pitra et al 2002
Hippotragus niger	niger	AF364711	Pitra et al 2002
Hippotragus niger	niger	AF364715	Pitra et al 2002
Hippotragus niger	niger	AF364720	Pitra et al 2002
Hippotragus niger	niger	AF364725	Pitra et al 2002
Hippotragus niger	niger	AF364743	Pitra et al 2002
Hippotragus niger	niger	AF364746	Pitra et al 2002
Hippotragus niger	niger	AF364749	Pitra et al 2002
Hippotragus niger	niger	AF364770	Pitra et al 2002
Hippotragus niger	niger	AF364779	Pitra et al 2002
Hippotragus niger	niger	AF364689	Pitra et al 2002
Hippotragus niger	kirkii	AF364692	Pitra et al 2002
Hippotragus niger	kirkii	AF364724	Pitra et al 2002
Hippotragus niger	kirkii	AF364726	Pitra et al 2002
Hippotragus niger	kirkii	AF364727	Pitra et al 2002
Hippotragus niger	kirkii	AF364728	Pitra et al 2002
Hippotragus niger	kirkii	AF364732	Pitra et al 2002
Hippotragus niger	kirkii	AF364734	Pitra et al 2002
Hippotragus niger	kirkii	AF364735	Pitra et al 2002
Hippotragus niger	kirkii	AF364741	Pitra et al 2002
Hippotragus niger	kirkii	AF364742	Pitra et al 2002
Hippotragus niger	kirkii	AF364744	Pitra et al 2002
Hippotragus niger	kirkii	AF364745	Pitra et al 2002
Hippotragus niger	kirkii	AF364750	Pitra et al 2002
Hippotragus niger	kirkii	AF364752	Pitra et al 2002
Hippotragus niger	kirkii	AF364753	Pitra et al 2002
Hippotragus niger	kirkii	AF364754	Pitra et al 2002
Hippotragus niger	kirkii	AF364756	Pitra et al 2002
Hippotragus niger	kirkii	AF364758	Pitra et al 2002
Hippotragus niger	kirkii	AF364762	Pitra et al 2002
Hippotragus niger	kirkii	AF364763	Pitra et al 2002
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Hippotragus niger	rooseveltii	AF364686	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364717	Pitra et al 2002

Hippotragus niger	rooseveltii	AF364759	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364766	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364767	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364769	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364771	Pitra et al 2002
Hippotragus niger	rooseveltii	AF364772	Pitra et al 2002
Hippotragus niger	variani	AF049358	Mathee and Robinson 1999
Hippotragus niger	variani	AY875643	Pitra et al 2006
Hippotragus niger	variani	AY970694	Pitra et al 2006
Hippotragus niger	variani	AY970695	Pitra et al 2006
Hippotragus niger	variani	AY970696	Pitra et al 2006
Hippotragus niger	variani	AY970697	Pitra et al 2006
Hippotragus niger	variani	AY970698	Pitra et al 2006
Hippotragus niger	variani	AY970699	Pitra et al 2006
Hippotragus niger	variani	AY971589	Pitra et al 2006
Hippotragus niger	variani	AY971590	Pitra et al 2006
Hippotragus niger	variani	AY971591	Pitra et al 2006
Hippotragus niger	variani	AY971592	Pitra et al 2006
Hippotragus niger	n/a (Not Available)	AF364687	Pitra et al 2002
Hippotragus niger	n/a	AF364688	Pitra et al 2002
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Hippotragus niger	n/a	AF364696	Pitra et al 2002
Hippotragus niger	n/a	AF364697	Pitra et al 2002
Hippotragus niger	n/a	AF364701	Pitra et al 2002
Hippotragus niger	n/a	AF364704	Pitra et al 2002
Hippotragus niger	n/a	AF364705	Pitra et al 2002
Hippotragus niger	n/a	AF364707	Pitra et al 2002
Hippotragus niger	n/a	AF364708	Pitra et al 2002
Hippotragus niger	n/a	AF364709	Pitra et al 2002
Hippotragus niger	n/a	AF364712	Pitra et al 2002
Hippotragus niger	n/a	AF364713	Pitra et al 2002
Hippotragus niger	n/a	AF364714	Pitra et al 2002
Hippotragus niger	n/a	AF364716	Pitra et al 2002
Hippotragus niger	n/a	AF364718	Pitra et al 2002
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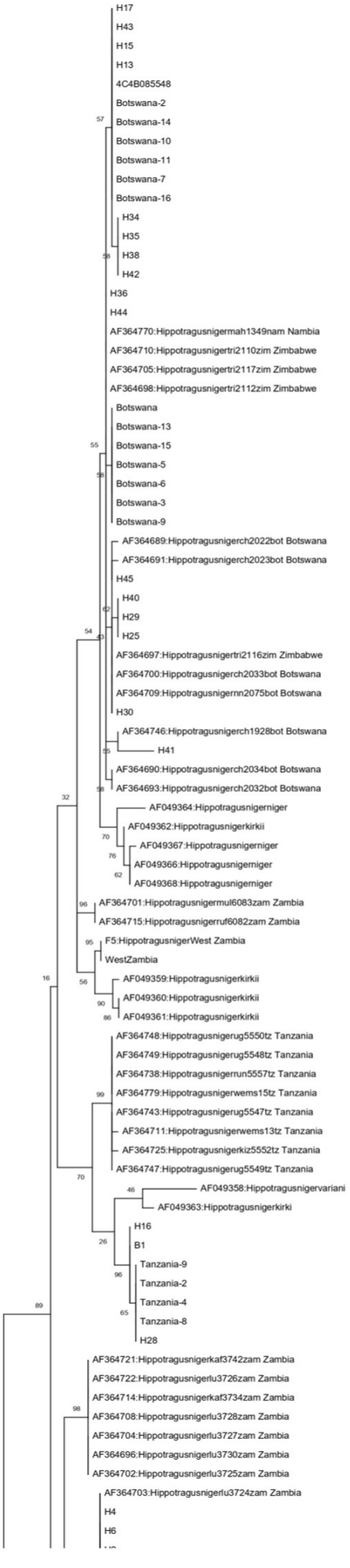
Hippotragus niger	n/a	AF364721	Pitra et al 2002
Hippotragus niger	n/a	AF364722	Pitra et al 2002
Hippotragus niger	n/a	AF364723	Pitra et al 2002
Hippotragus niger	n/a	AF364729	Pitra et al 2002
Hippotragus niger	n/a	AF364730	Pitra et al 2002
Hippotragus niger	n/a	AF364731	Pitra et al 2002
Hippotragus niger	n/a	AF364733	Pitra et al 2002
Hippotragus niger	n/a	AF364736	Pitra et al 2002
Hippotragus niger	n/a	AF364737	Pitra et al 2002
Hippotragus niger	n/a	AF364738	Pitra et al 2002
Hippotragus niger	n/a	AF364739	Pitra et al 2002
Hippotragus niger	n/a	AF364740	Pitra et al 2002
Hippotragus niger	n/a	AF364747	Pitra et al 2002
Hippotragus niger	n/a	AF364748	Pitra et al 2002

Hippotragus niger	n/a	AF364751	Pitra et al 2002
Hippotragus niger	n/a	AF364755	Pitra et al 2002
Hippotragus niger	n/a	AF364757	Pitra et al 2002
Hippotragus niger	n/a	AF364760	Pitra et al 2002
Hippotragus niger	n/a	AF364761	Pitra et al 2002
Hippotragus niger	n/a	AF364764	Pitra et al 2002
Hippotragus niger	n/a	AF364765	Pitra et al 2002
Hippotragus niger	n/a	AF364768	Pitra et al 2002
Hippotragus niger	n/a	AF364773	Pitra et al 2002
Hippotragus niger	n/a	AF364774	Pitra et al 2002
Hippotragus niger	n/a	AF364775	Pitra et al 2002
Hippotragus niger	n/a	AF364776	Pitra et al 2002
Hippotragus niger	n/a	AF364777	Pitra et al 2002
Hippotragus niger	n/a	AF364778	Pitra et al 2002

Species	Subspecies	GenBank accession number	Reference
Hippotragus equinus	equinus	AF049374	Mathee and Robinson 1999
Hippotragus equinus	equinus	AF049375	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF049376	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF049377	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF049378	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF049379	Mathee and Robinson 1999
Hippotragus equinus	langheldi	AF049380	Mathee and Robinson 1999
Hippotragus equinus	koba	AF049381	Mathee and Robinson 1999
Hippotragus equinus	koba	AF049382	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF068839	Mathee and Robinson 1999
Hippotragus equinus	cottoni	AF068840	Mathee and Robinson 1999
Hippotragus equinus	langheldi	AF068841	Mathee and Robinson 1999
Hippotragus equinus	n/a	AJ235321	Pitra et al 2006
Hippotragus equinus	n/a	AY179371	Alpers et al 2004
Hippotragus equinus	n/a	AY179372	Alpers et al 2004
Hippotragus equinus	n/a	AY179373	Alpers et al 2004
Hippotragus equinus	n/a	AY179374	Alpers et al 2004
Hippotragus equinus	n/a	AY179375	Alpers et al 2004
Hippotragus equinus	n/a	AY179376	Alpers et al 2004
Hippotragus equinus	n/a	AY179377	Alpers et al 2004
Hippotragus equinus	n/a	AY179378	Alpers et al 2004
Hippotragus equinus	n/a	AY179379	Alpers et al 2004
Hippotragus equinus	n/a	AY179380	Alpers et al 2004
Hippotragus equinus	n/a	AY179381	Alpers et al 2004
Hippotragus equinus	n/a	AY179382	Alpers et al 2004

APPENDIX E. - ENLARGED FIGURE OF FIGURE 4.1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H43
H15
H13
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Botswana-16
H34
H35
H38
H42
H36
H44
AF364770:Hippotragusnigermah1349nam Nambia
AF364710:Hippotragusnigertri2110zim Zimbabwe
AF364705:Hippotragusnigertri2117zim Zimbabwe
AF364698:Hippotragusnigertri2112zim Zimbabwe
Botswana
Botswana-13
Botswana-15
Botswana-5
Botswana-6
Botswana-3
Botswana-9
AF364689:Hippotragusnigerch2022bot Botswana
AF364691:Hippotragusnigerch2023bot Botswana
H45
H40
H29
H25
AF364697:Hippotragusnigertri2116zim Zimbabwe
AF364700:Hippotragusnigerch2033bot Botswana
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H30
AF364746:Hippotragusnigerch1928bot Botswana
H41
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AF364693:Hippotragusnigerch2032bot Botswana
AF049364:Hippotragusnigerniger
AF049362:Hippotragusnigerkirkii
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AF049368:Hippotragusnigerniger
AF364701:Hippotragusnigermul6083zam Zambia
AF364715:Hippotragusnigerruf6082zam Zambia
F5:HippotragusnigerWest Zambia
WestZambia
AF049359:Hippotragusnigerkirkii
AF049360:Hippotragusnigerkirkii
AF049361:Hippotragusnigerkirkii
AF364748:Hippotragusnigerug5550tz Tanzania
AF364749:Hippotragusnigerug5548tz Tanzania
AF364738:Hippotragusnigerrun5557tz Tanzania
AF364779:Hippotragusnigerwems15tz Tanzania
AF364743:Hippotragusnigerug5547tz Tanzania
AF364711:Hippotragusnigerwems13tz Tanzania
AF364725:Hippotragusnigerkiz5552tz Tanzania
AF364747:Hippotragusnigerug5549tz Tanzania
AF049358:Hippotragusnigervariani
AF049363:Hippotragusnigerkirki
H16
B1
Tanzania-9
Tanzania-2
Tanzania-4
Tanzania-8
H28
AF364721:Hippotragusnigerkaf3742zam Zambia
AF364722:Hippotragusnigerlu3726zam Zambia
AF364714:Hippotragusnigerkaf3734zam Zambia
AF364708:Hippotragusnigerlu3728zam Zambia
AF364704:Hippotragusnigerlu3727zam Zambia
AF364696:Hippotragusnigerlu3730zam Zambia
AF364702:Hippotragusnigerlu3725zam Zambia
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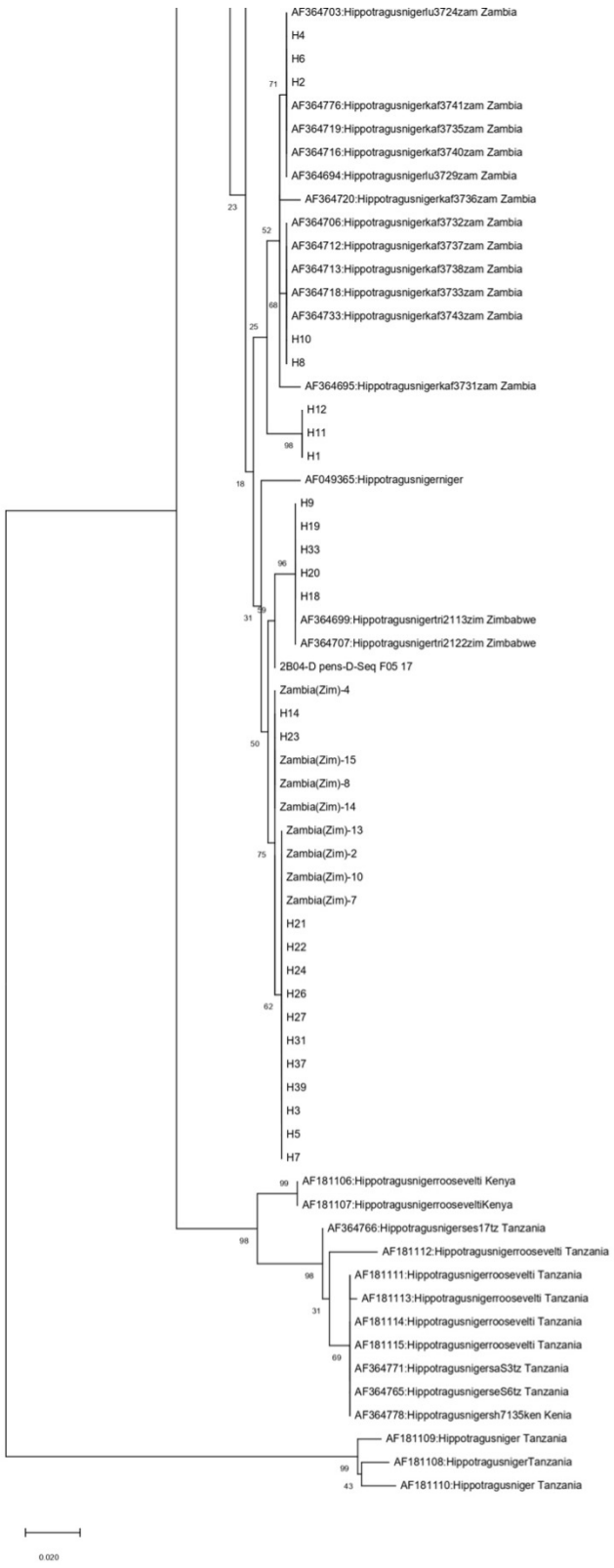
H4
H6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H4
H6
H2
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H10
H8
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H12
H11
H1
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H9
H19
H33
H20
H18
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2B04-D pens-D-Seq F05 17
Zambia(Zim)-4
H14
H23
Zambia(Zim)-15
Zambia(Zim)-8
Zambia(Zim)-14
Zambia(Zim)-13
Zambia(Zim)-2
Zambia(Zim)-10
Zambia(Zim)-7
H21
H22
H24
H26
H27
H31
H37
H39
H3
H5
H7
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AF364766:Hippotragusnigerses17tz Tanzania
AF181112:Hippotragusnigerroosevelti Tanzania
AF181111:Hippotragusnigerroosevelti Tanzania
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AF364765:HippotragusnigerseS6tz Tanzania
AF364778:Hippotragusnigersh7135ken Kenia
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AF181108:HippotragusnigerTanzania
AF181110:Hippotragusniger Tanzania
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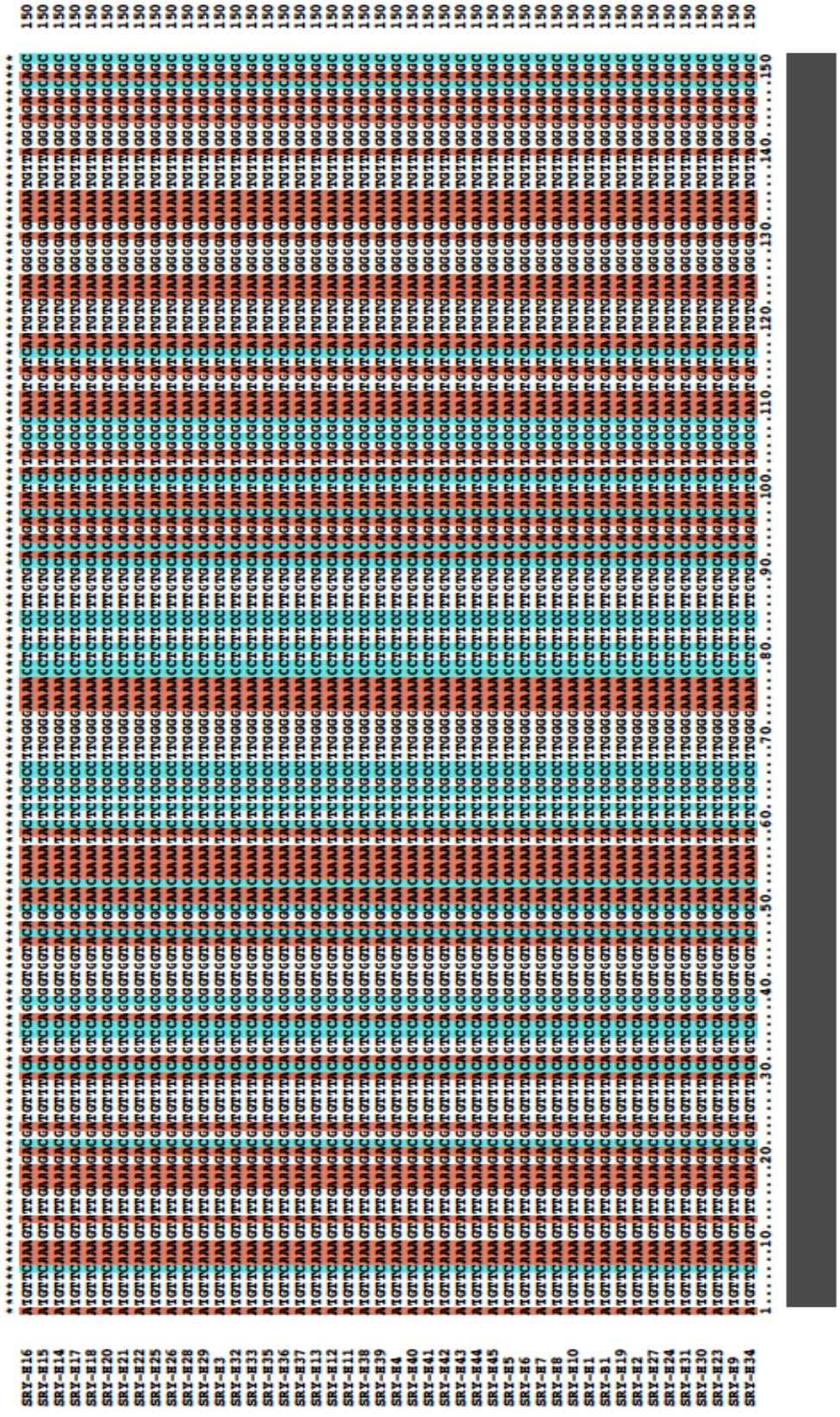
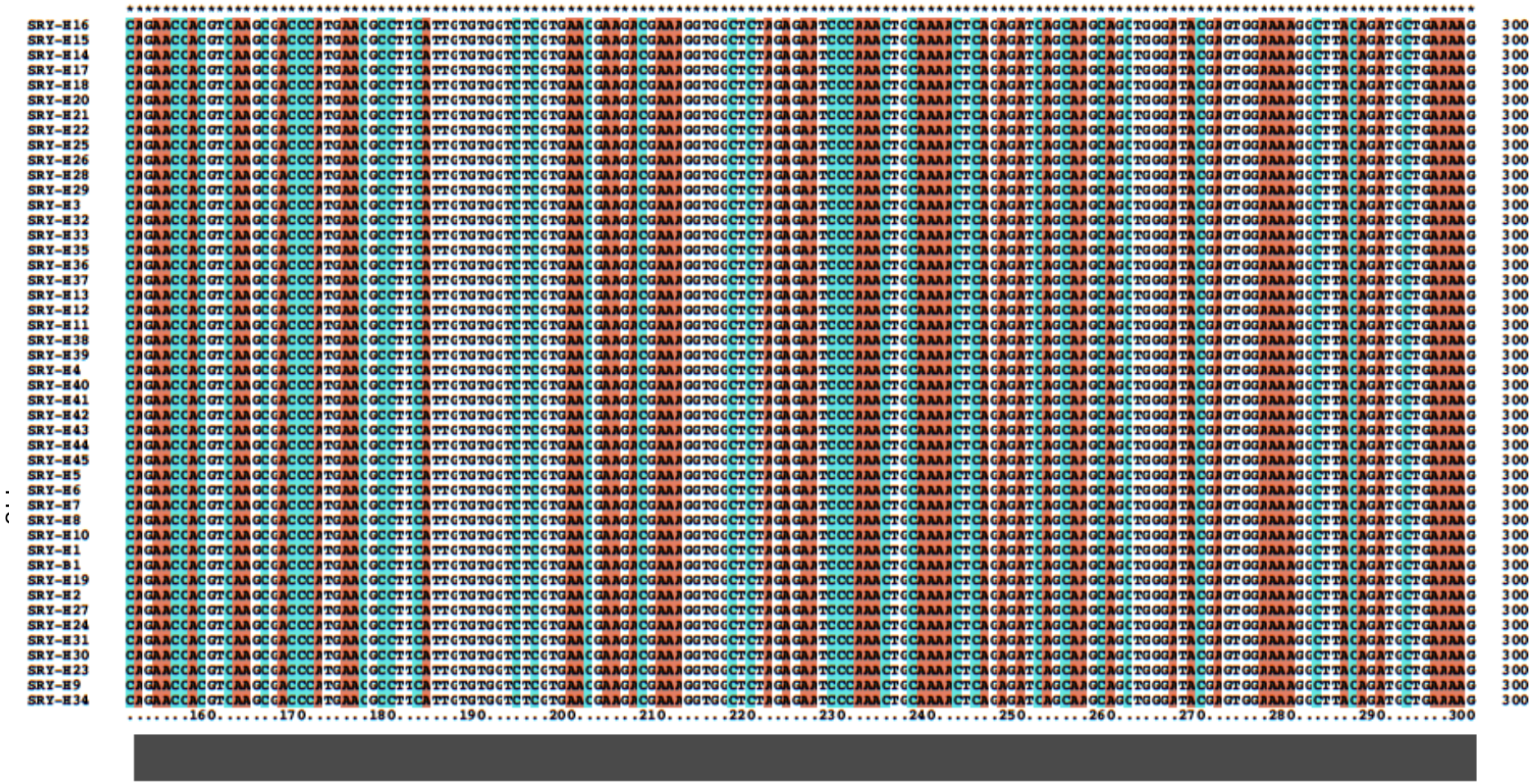
APPENDIX F. – SRY GENE MULTIPLE SEQUENCE ALIGNMENT OF SABLE ANTELOPES H1 – H45 & B1

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SRY-H15
SRY-H14
SRY-H17
SRY-H18
SRY-H20
SRY-H21
SRY-H22
SRY-H25
SRY-H26
SRY-H28
SRY-H29
SRY-H3
SRY-H32
SRY-H33
SRY-H35
SRY-H36
SRY-H37
SRY-H13
SRY-H12
SRY-H11
SRY-H38
SRY-H39
SRY-H4
SRY-H40
SRY-H41
SRY-H42
SRY-H43
SRY-H44
SRY-H45
SRY-H5
SRY-H6
SRY-H7
SRY-H8
SRY-H10
SRY-H1
SRY-B1
SRY-H19
SRY-H2
SRY-H27
SRY-H24
SRY-H31
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SRY-H34
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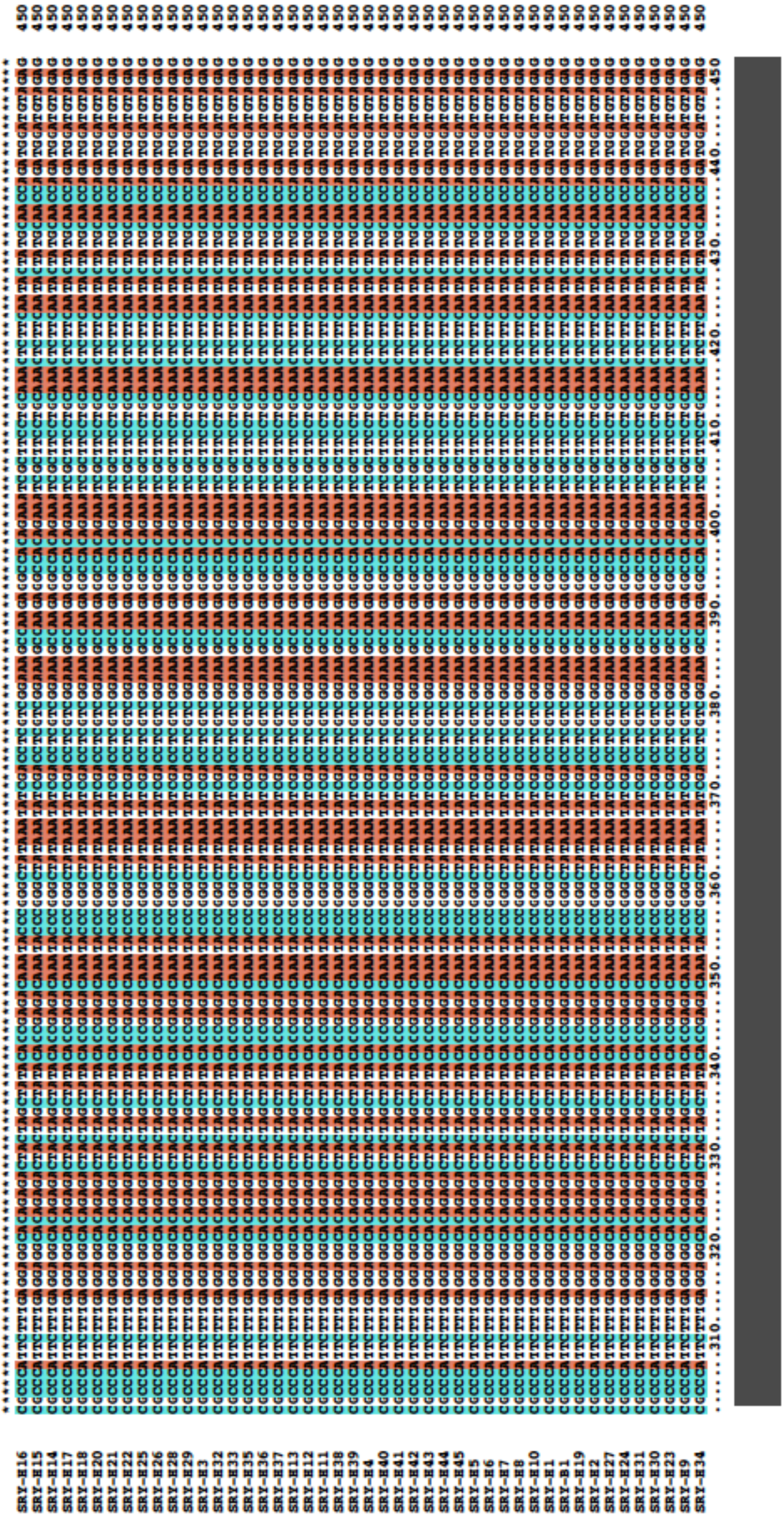

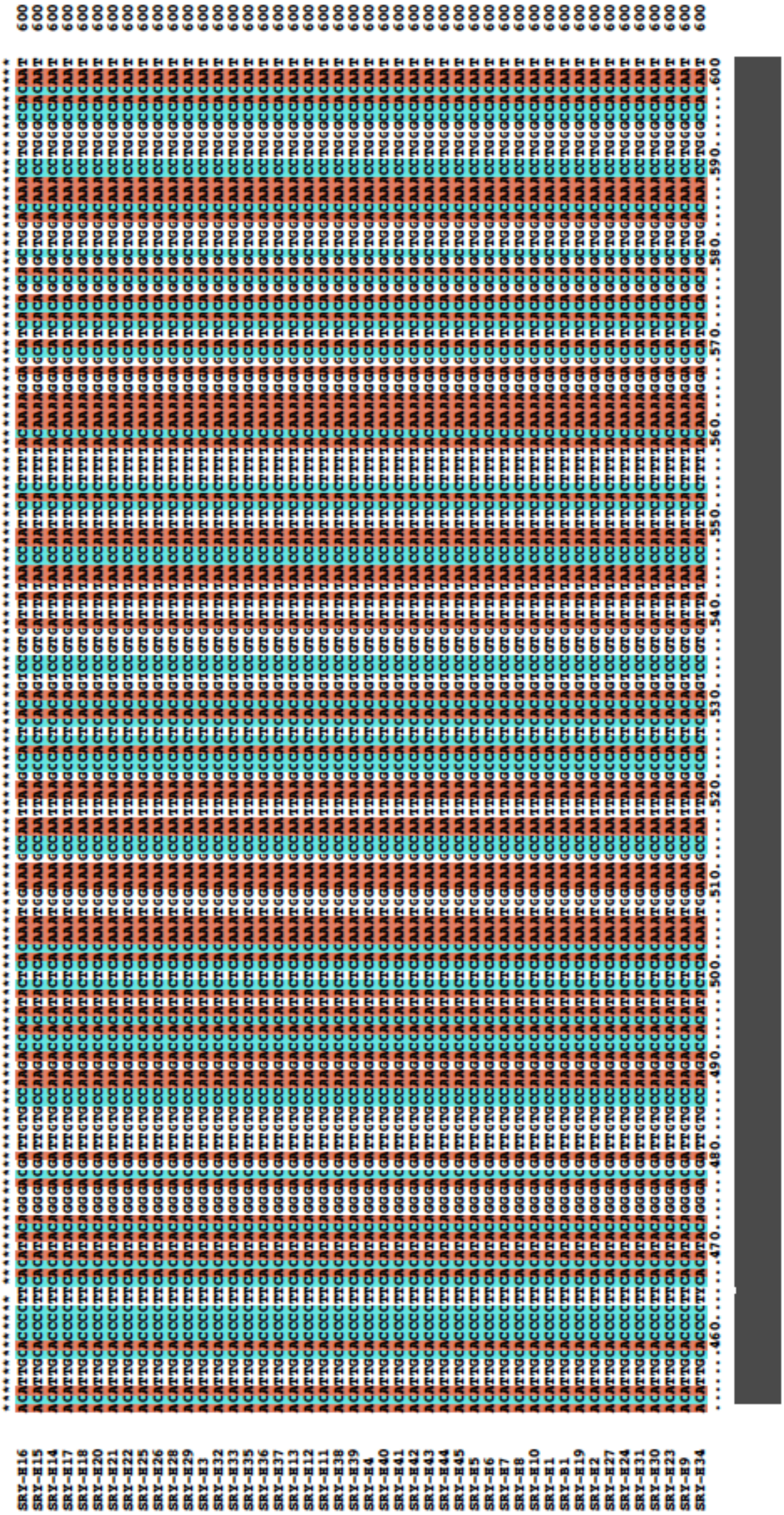

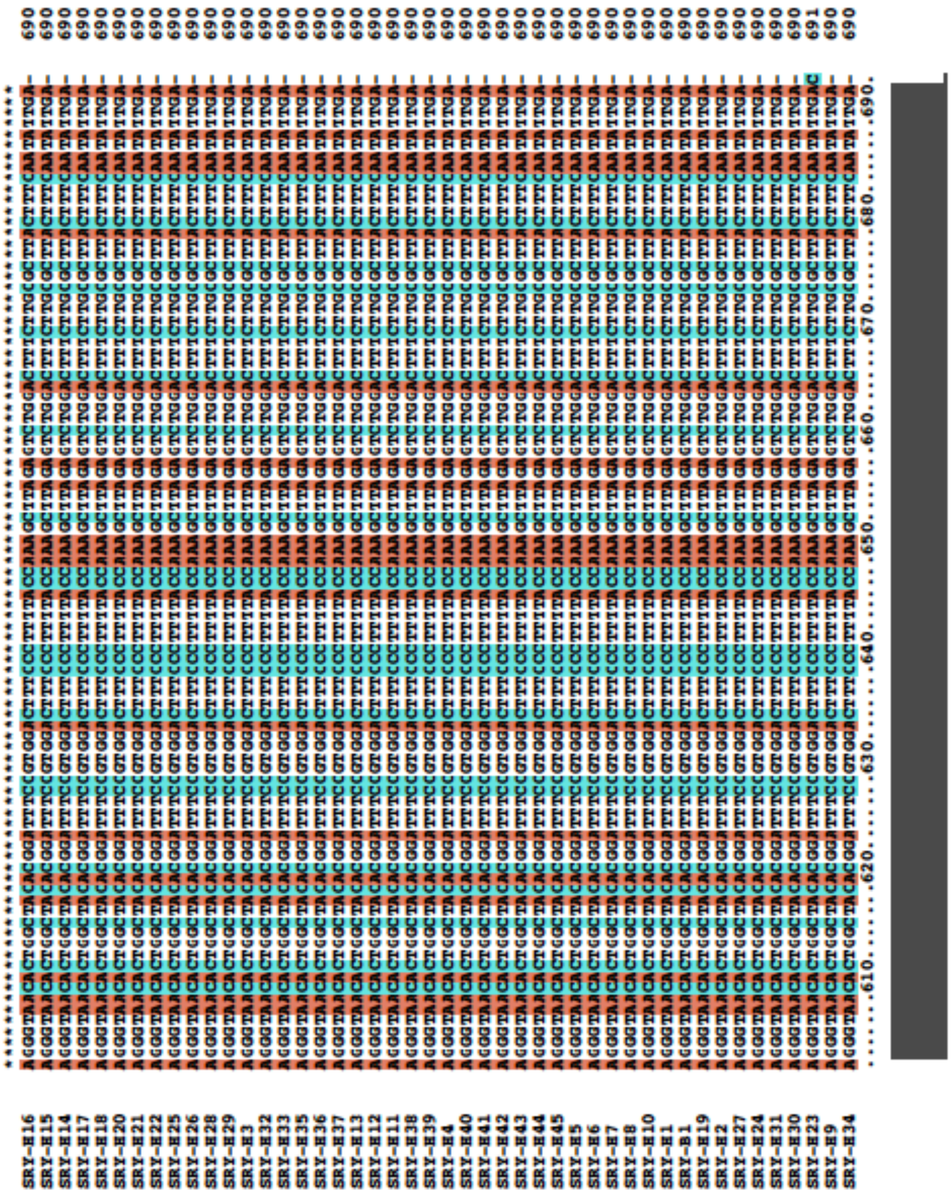

APPENDIX G. SRY GENE MULTIPLE SEQUENCE ALIGNMENT OF ROAN ANTELOPES: H46 – H55