

CROSS-SPECIES MICROSATELLITE  
MARKERS FOR THE DETECTION OF  
HYBRIDS IN THE GENUS *CONNOCHAETES*

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# DECLARATION

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I certify that the dissertation hereby submitted for the degree *M.Sc. Genetics* at the University of the Free State is my independent effort and had not previously been submitted for a degree at another University/Faculty. I furthermore waive copyright of the dissertation in favour of the University of the Free State

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**L. Wessels**

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# LIST OF ABBREVIATIONS AND SYMBOLS

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## Symbols:

°C	Degrees Celsius
%	Percentage
μ	Micro: 10 <sup>-6</sup>
™	Trademark
®	Registered Trademark

## Abbreviations:

A	Average number of alleles per locus
A <sub>260</sub> /A <sub>280</sub>	Ratio of absorbency measured at 260 nm and 280 nm
ABI	Applied Biosystems
AMOVA	Analysis of Molecular Variance
B.P.	Before Present
BP <sub>1</sub>	First generation backcrossed with parental species 1
BP <sub>2</sub>	First generation backcrossed with parental species 2
DETEA	Department of Economic Development, Tourism and Environmental Affairs
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediamine tetra-acetic acid
F	Forward primer
F <sub>1</sub>	First generation
F <sub>2</sub>	Second generation
F <sub>ST</sub>	Genetic differentiation among populations
<i>g</i>	Gravitational Force
H <sub>o</sub>	Observed heterozygosity
H <sub>z</sub>	Unbiased heterozygosity
HWE	Hardy-Weinberg Equilibrium
IUCN	International Union for the Conservation of Nature and Natural Resources

## LIST OF ABBREVIATIONS AND SYMBOLS

<i>K</i>	Number of clusters/populations
km	kilometres
MCMC	Markov Chain Monte Carlo
mg	Milligram
Mg <sup>2+</sup>	Magnesium ion
MgCl <sub>2</sub>	Magnesium chloride
min	Minutes
mM	Millimolar
mtDNA	Mitochondrial Deoxyribonucleic Acid
N	Number of individuals
N/A	Not applicable
ng	Nanogram
ng/μl	Nanogram per micro litre
nm	nanometers
Nm	Gene flow
No.	Number
NR	Nature Reserve
P <sub>1</sub>	Parental species 1
P <sub>2</sub>	Parental species 2
PCR	Polymerase Chain Reaction
R	Reverse primer
SD	Standard deviation
Sec	seconds
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
T <sub>a</sub>	Annealing temperature
T <sub>m</sub>	Melting temperature
U	Units
μl	Micro litre
μM	Micromolar
U.S.	United States
U.S.A.	United States of America

# LIST OF EQUATIONS

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# CHAPTER ONE: LITERATURE STUDY

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## 1.1 Introduction

The blue wildebeest, *Connochaetes taurinus* (Burchell, 1823), and the black wildebeest, *C. gnou* (Zimmerman, 1780), belong to the family Bovidae and are further classified into the subfamily Alcelaphinae. Fossil evidence suggests that the black wildebeest diverged from a blue wildebeest like ancestor in the early Pleistocene, approximately two million years ago (Gentry, 1978; Vrba, 1979). During these historic times the migratory blue wildebeest were widely distributed throughout eastern and southern Africa. In more recent times the blue wildebeest, an important animal in the game farming industry, has been extensively translocated among farms and nature reserves within South Africa (Corbet and Robinson, 1991). Historically the distribution of black wildebeest has been restricted to southern Africa where large numbers were found in the temperate savannas, until the early 1900s, when this species was hunted to the brink of extinction (Smithers, 1983).

It is reported that the number of black wildebeest decreased to 300 animals in 1938 (Kirkman, 1938). These animals were restricted to a small number of protected herds, in which the animals were probably highly related. It is not unrealistic to assume that this would have led to a decrease in genetic diversity in the black wildebeest (Corbet and Robinson, 1991). The black wildebeest has however been reintroduced to several nature reserves and many game farms, and is no longer seen as an endangered species (Smithers, 1983).

Black and blue wildebeest share the same chromosome number,  $2n = 58$  (Corbet and Robinson, 1991) as well as many morphological similarities. Although considerable differences in body and horn shape are present (Smithers, 1983), these two species are capable of hybridizing and the offspring of such hybridization events are fertile (Fabricius *et al.*, 1988). The study by Fabricius *et al.* (1988) raised serious concerns over the status of black wildebeest populations in South Africa. Several studies aimed to identify molecular markers for the identification of wildebeest hybrids, with little success (Corbet and Robinson, 1991). However, in 2005 Grobler *et al.* found that the use of microsatellite markers for

hybrid identification in black wildebeest was successful. These studies emphasized the importance of developing molecular markers or techniques for hybrid identification in black wildebeest populations.

Hybrid identification is extremely important for the management and conservation of species affected by hybridization. Several diverse methods for hybrid identification have been developed. These methods include morphological characterization and more reliable molecular methods. The molecular techniques used for hybrid identification are often accompanied by statistical analyses such as assignment tests and more recently, simulation tests. These simulation approaches can be implemented for a case-by-case evaluation of the statistical power for correctly identifying the status of an individual as purebred or hybrid (Nielsen *et al.*, 2006). The use of simulations has been applied to numerous studies involving hybridization between fish species (Nielsen *et al.*, 2003; Schwartz and Beheregaray, 2008; Sanz *et al.*, 2009) and can be used to determine the long term effect of introgression on black wildebeest populations.

## 1.2 Wildebeest distribution and habitat

### *Connochaetes gnou* – Black wildebeest

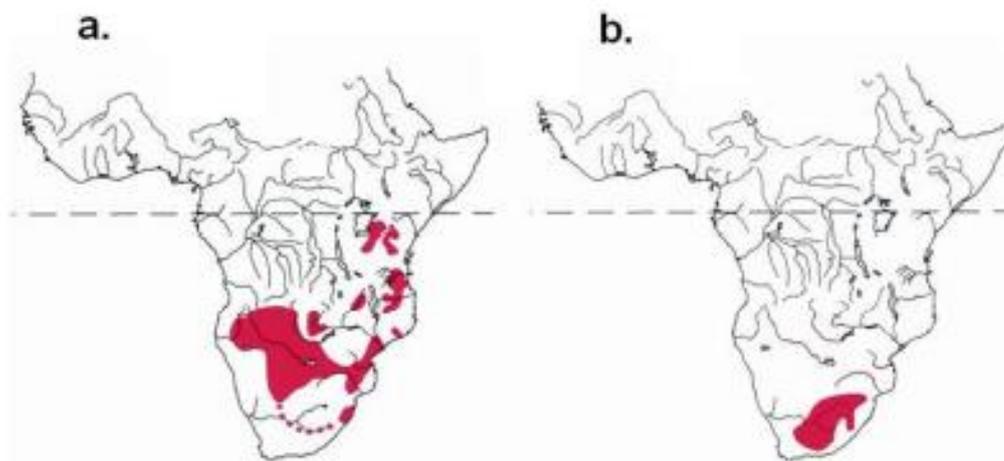
Black wildebeest are endemic to South Africa and historically the distribution of this species has been restricted to southern Africa (Smithers, 1983). Their former range included the Northern Cape, the Western Cape and the Eastern Cape, throughout the Free State, north into the North West Province, Gauteng and Mpumalanga, extending into western KwaZulu-Natal. After their decline in numbers, which brought them to the point of extinction, the species was reintroduced to its former range, mainly in the Free State, Gauteng and KwaZulu-Natal. This was made possible through the efforts of the South African National Parks Board, provincial nature conservation departments and private individuals. Reserves in these three provinces where the animals were reintroduced included the Willem Pretorius Game Reserve and Tussen-die- Riviere Nature Reserve in the Free State, the Suikerbosrand Nature Reserve in Gauteng and the Spioenkop Nature Reserve in KwaZulu-Natal. They can also be found in the Vaalbos, Mountain Zebra and Golden Gate National Parks. Black wildebeest were also later reintroduced outside of South Africa, to the Malolotja Nature Reserve in Swaziland and the Sehlabathebe National Park in Lesotho, where they formerly occurred. Black wildebeest did not historically occur in Namibia but have been introduced to private farms in this region (Skinner and Chimimba, 2005).

### *Connochaetes taurinus* – Blue wildebeest

During historic times the migratory blue wildebeest were widely distributed (Smithers, 1983). In more recent times this species has been extensively translocated. Outside South Africa blue wildebeest occur in Kenya, Tanzania, Angola, Zambia, Mozambique, northern and north-eastern parts of Namibia, Botswana, north-west and southern Zimbabwe, and north-eastern Swaziland. In South Africa the species has historically been recorded in the Northern Cape, Limpopo, North West, Mpumalanga and north-eastern KwaZulu-Natal Provinces. Presently this species is still found throughout their historical range in Nature Reserves such as the Kruger National Park and adjoining reserves in Limpopo and Mpumalanga, in the Pilanesberg National Park and the Madikwe National Park, in the North West Province, and in the Hluhluwe-iMfolozi Park and the Mkhuze Game Reserve in north-eastern KwaZulu-

Natal. Large numbers of blue wildebeest are found on private land, predominantly in the North West, Limpopo and KwaZulu-Natal Provinces (Skinner and Chimimba, 2005).

Historic geographical ranges of the blue and black wildebeest overlapped in parts of the Gauteng, Free State, and Northern and Western Cape provinces (see figure 1.1 a and b). Both species of wildebeest are grazers but they have slightly different habitat preference (Smithers, 1983). In the areas where overlap of their ranges occurred difference in habitat preference kept these species separate, since black wildebeest prefer short open grassland and were formerly associated with the grassland and Karoo scrub, whereas blue wildebeest prefer short grass and are usually associated with open savannah woodland (Smithers, 1983).



**Figure 1.1: Maps of sub-Saharan Africa showing the historic distribution of (a) blue wildebeest and (b) black wildebeest. Historic and fossil records indicated by the broken line in (a) suggests a substantial overlap with black wildebeest (after Brink, 2005; adapted from Kingdon, 1997)**

### **1.3 Past and present population status of the black wildebeest**

During the late 1930s and 1940s, hunting and human settlement caused a serious decline in black wildebeest numbers, so much so that black wildebeest were on the brink of extinction. It is reported that the number of black wildebeest decreased to 300 animals in 1938 (Kirkman, 1938). These animals were restricted to a small number of protected herds, in which the animals were probably highly related.

A survey conducted by Bigalke (1947) found that the total number of black wildebeest in South Africa was 1,048 (Table 1.1). These animals were distributed throughout the former Cape, Free State and the former Transvaal Provinces, with a few animals in Natal.

**Table 1.1: Number of black wildebeest in South Africa in 1945 (Bigalke, 1947)**

<b>Province</b>	<b>Number of black wildebeest</b>
Former Cape Province	215
Free State Province	755
Former Transvaal Province	61
Natal Province	17
<b>Estimated total</b>	<b>1,048</b>

In 1965 Brand (1965) released results (Table 1.2) indicating an increase in the numbers of black wildebeest throughout the country.

**Table 1.2: Number of black wildebeest in South Africa and the former South-West Africa, 1965 (Brand, 1965)**

<b>Province</b>	<b>Number of black wildebeest</b>
Former Cape Province	311
Free State Province	1,216
Former Transvaal Province	177
Natal Province	75
Former South-West Africa	12
Pretoria Zoo	6
Bloemfontein Zoo	4
Johannesburg Zoo	7
<b>Estimated total</b>	<b>1,808</b>

Brand (1965) also stated that although the species was listed as threatened with extinction by the International Union for the Conservation of Nature and Natural Resources (IUCN), with

the help of South African nature reserves and farmers, the conservation of the species has become fairly assured.

Another four surveys were later done and revealed the following results: in 1970 the Free State Directorate of Nature and Environmental Conservation indicated an increase in numbers to 3,220 animals. In 1979 the Transvaal alone boasted numbers of 1,532 black wildebeest, in 1981 a population size of 6,493 were recorded and in 1988 the total number of black wildebeest in South Africa stood at 6,685 (Kay, 1992). Records indicated that the number of black wildebeest in South Africa were just below the 1,000 mark at the beginning of the twentieth century (Fabricius and Oates, 1985). In 1997 Mills and Hes indicated that the numbers of wildebeest in South Africa has increased to approximately 12,000 animals and presently the estimated number is more than 18,000 black wildebeest, 80% of which can be found on private land and 20% in protected areas (IUCN, 2008).

After the earlier decline in numbers black wildebeest were reintroduced to much of their former range (Skinner and Chimimba, 2005) and also to other parts of the country and to neighbouring countries (Mills and Hes, 1997) such as Namibia, where the estimated number of black wildebeest increased from 150 in 1982 to more than 7,000 in 1992 (East, 1998). Following the reestablishment of black wildebeest across South Africa, more emphasis has been placed on the conservation of this indigenous species as well as maintaining its genetic integrity. A different possible threat to the genetic integrity of the black wildebeest was discovered during the 1960's when hybridization with the congeneric blue wildebeest was observed.

#### **1.4 Hybridization**

Hybridization forms a natural part of evolution (Allendorf *et al.*, 2001). Along with the introgression of genetic material, hybridization can increase genetic diversity through the production of new recombinant genotypes. Increased levels of variability could allow organisms to better adapt to environmental change and this can lead to increased rates of evolution. Hybrid breeding is a commonly used and accepted tool in agricultural practices, however, humans influence the balance of natural hybridization, by the introduction of exotic species and habitat disturbance which can lead to extensive introgressive hybridization (Dowling and Secor, 1997).

Allendorf *et al.*, (2001) explains the differences between natural and anthropogenic hybridization by categorizing these events into six different types:

- Type 1: Natural hybrid taxon
- Type 2: Natural introgression
- Type 3: Natural hybrid zone
- Type 4: Hybridization without introgression
- Type 5: Widespread introgression
- Type 6: Complete admixture

Hybrid taxa that have arisen through natural genetic admixture are grouped into the type one category. Natural introgression that does not lead to the creation of a new taxon is type two hybridization and hybrid zones are classified as type three hybridization. In the last three types of hybridization human activities play a major role. A situation where only F<sub>1</sub> hybrids have been detected is type four hybridization. In this category genetic mixing through hybridization does not pose such a serious threat, the problem is rather wasted reproductive effort (Allendorf *et al.*, 2001).

In types five and six hybridization, the existence of hybrid swarm makes conservation and recovery of threatened species very difficult. Type five hybridization indicates a situation where hybridization is still recent or geographically restricted and parental taxa does still exist. If swift conservation action is not taken in this case it could lead to hybrid swarms, which is type 6 hybridization (Allendorf *et al.*, 2001).

If hybrids are fertile and mate among themselves as well as with parental individuals, it is difficult to stop and after a couple of generations this results in hybrid swarms. After continuous generations of hybridization the proportion of hybrids in a population increases as the proportion of pure parental individuals decrease gradually (Allendorf *et al.*, 2001). This could lead to loss of genotypically distinct populations, which is one of the main concerns regarding hybridization. Small populations are more at risk even if hybridization is not accompanied by introgression (Rhymer and Simberloff, 1996). Unfortunately in the case of the blue and black wildebeest, human intervention has played a major role in hybridization events and it is crucial to precisely determine what effect introgression through hybridization will have on this species.

### 1.5 Hybridization between blue and black wildebeest

Hybridization between blue and black wildebeest is not a recent occurrence and dates back to the 1960's. In 1988 Fabricius *et al.* studied the characteristics of wildebeest hybrids encountered on a private farm in the Northern Cape. All the original black and blue wildebeest had died out and it was reported that the entire population consisted of hybrids. The aim of their study was to test the hypothesis that these hybrids are fertile. After a surveillance of the wildebeest population, 46 hybrid wildebeest were counted. Hybrid females were accompanied by neonates and yearlings, providing strong evidence that these hybrid animals are in fact able to reproduce. All the hybrids were easily distinguishable from pure blue and black wildebeest based on morphological characteristics (Fabricius *et al.*, 1988).

The most striking morphological characteristic of a first generation hybrid wildebeest (Figure 1.2) is the horns, which turn downward for the first third of their length, similar to black wildebeest, and then curl outwards, like that of the blue wildebeest. The colour of the hybrid animal is dark brown like that of the black wildebeest. The tail of the hybrid is white to cream coloured on the lower third, whereas the mane is black with a white lower part, resembling black wildebeest. Brindled streaks are evident on its neck similar to those of the blue wildebeest (Fabricius *et al.*, 1988). The hybrid animals show the same social organization as black wildebeest (Von Richter, 1971). Fabricius *et al.* (1988) concluded that the inadequate habitat and the disruption of social organization of these two species was possibly the cause of hybridization.



**Figure 1.2: F<sub>1</sub> hybrid animal (Photo: KwaZulu-Natal wildlife)**

Corbet and Robinson (1991) conducted one of the first molecular studies on wildebeest. The aims of her study were firstly to determine the evolutionary relationship between these two species and secondly to determine whether wildebeest hybrids can be distinguished from pure bred animals using genetic analysis. The second part of the study would then potentially provide molecular proof that hybridization does occur and could provide a diagnostic test for hybrids which would be extremely useful in wildlife management. Corbet and Robinson (1991) found that the karyotypes of *C. taurinus* and *C. gnou* were invariant and would probably offer no structural barrier to interspecific hybridization. This provided further proof of cross-breeding potential in wildebeest.

In the second part of the study different tests were used to examine genetically pure black and blue wildebeest and their putative hybrids in an attempt to identify genetic markers which would differentiate between them. The study specifically set out to utilize more than one test due to the fact that a single test might not identify all hybrids examined, whereas a number of parameters increase the chances. Analysis of the G- and C-banded preparation for the two species already revealed that there was no variation between the two species and these could therefore not be used as a method for hybrid identification. Protein analysis as well as DNA fingerprinting also failed to provide any species specific markers. The only technique which yielded species specific markers was the mitochondrial analysis, but due to the mitochondrial

genome's maternal mode of inheritance, this method cannot be used alone to test for hybrids, due to the nature of initial hybridization events. The mating between blue and black wildebeest is almost always unidirectional, with blue bulls introgressing into black herds (Vrahimis pers. obs.). The other techniques tested did not positively identify species specific markers but could be used in some instances to positively identify hybrids (Corbet and Robinson, 1991).

A significant breakthrough in wildebeest hybrid identification came in 2005, when Grobler *et al.* (2005) used microsatellite (also known as short tandem repeats) markers to assess the genetic purity of a black wildebeest population at the Abe Bailey Nature Reserve in the Gauteng Province. In this instance, cross-species amplification of microsatellites from domestic members of the Bovidae family was used. Their approach was to screen for introgressed alleles, assuming that some of the markers used would reveal alleles that are fixed in alternative species. The two wildebeest species were analyzed as separate groups, each containing possible unique alleles, with an expected influx of blue wildebeest alleles into black wildebeest populations due to hybridization. A large number of alleles shared between these two species were expected due to their relatively recent divergence. Eight out of 39 alleles were found to be unique to black wildebeest, 22 to blue wildebeest, and nine alleles were shared between these species. An allele found to be absent from control black wildebeest populations but shared between the black wildebeest from the Abe Bailey Nature Reserve and blue wildebeest population, indicated introgression in the former population. Statistical analysis of the results, which included assignment test and coefficients of population divergence, did however not support a hypothesis of persistent introgression of blue wildebeest alleles into the black wildebeest population (Grobler *et al.*, 2005).

Grobler *et al.* (2005) utilized five microsatellite markers, all of these markers identified alleles specific to each of the two species. Two of the markers were however more diagnostic than the rest, these two markers ETH 10 and BM 1824 were therefore chosen for the current study.

The studies described above highlighted the need for the development of more accurate molecular or morphological techniques, or even a combination of different techniques for wildebeest hybrid identification.

## 1.6 A summary of the methods available for hybrid identification

Several diverse methods for hybrid identification have been developed. These methods include external morphological characterization, osteology and molecular methods. Molecular methods for hybrid identification are often accompanied by dedicated statistical approaches such as assignment tests. The accuracy and sensitivity of the various methods for identification differ significantly.

### 1.6.1. External morphological characterization

Detection of hybridized individuals relied on morphological characteristics until the mid-1960s (Allendorf *et al.*, 2001). This method is not reliable, since not all morphological variation has a genetic basis and because a greater amount of morphological variation exists among and within populations than is commonly recognized (Campton, 1987). When using morphological characteristics as a method for hybrid identifications, it is assumed that the hybrid will be phenotypically intermediate to parental individuals (Smith, 1992). Leary *et al.*, (1996) found that individuals from hybrid swarms with most of their genes from one parental taxa are often indistinguishable from that specific parental taxa on a morphological basis. Although using several characteristics simultaneously permits a reasonably successful identification of hybrids, there could be some bias introduced due to geographical variability and differences in techniques among researchers (Väli *et al.*, 2010).

It is also important to be able to distinguish between first generation ( $F_1$ ) hybrids, backcrossed individuals and later generation hybrids, for conservation purposes such as the recovery of parental individuals from hybrid swarms by removing hybrids. This can however not be done on the basis of morphological variation alone (Allendorf *et al.*, 2001). This is unfortunately the case with hybrid wildebeest, where first generation hybrids are easily distinguishable based on external morphological characteristics, but where this is no longer possible after generations of backcrossing (Vrahimis pers. comm.).

### 1.6.2 Osteology

Osteological studies on wildebeest skulls have successfully indicated morphological differences between known hybrid skulls and known pure black wildebeest. A preliminary report by Ackermann *et al.*, (2010) showed certain hybrid features in the crania of wildebeest. Dental and sutural morphological anomalies were found in 13 hybrid wildebeest analysed. In addition to these morphological anomalies, three individuals had abnormal horn sheath morphology, as well as pronounced horn asymmetry. The study also proofed the potential to identify hybrid wildebeest based on these morphological characteristics and in conjunction with molecular data it could play an important role in the effort to conserve the endemic black wildebeest of South Africa (Ackermann *et al.*, 2010).

### 1.6.3. Molecular markers

Researchers are faced with a wide variety of molecular markers to study population structure. An important consideration during these studies is to choose the correct marker as well as determining the amount of markers needed to resolve the question at hand (Morin *et al.*, 2009).

Molecular markers are powerful tools with which the extent of hybridization processes can be established (Linder *et al.*, 1998). These markers can also provide the relevant information needed for the implementation of genetic conservation programs. If hybridization is studied, as it is in this case, different factors can influence the ability to detect introgression between two species, including the type of marker system, the length of time since hybridization, whether advanced backcrosses exist within a population as well as the distinctness of a parental species (Halbert *et al.*, 2005).

The possible markers or molecular techniques include mitochondrial DNA sequencing, Y-chromosome markers, single nucleotide polymorphisms and microsatellite markers.

### 1.6.3.1. Mitochondrial DNA sequencing

Vilá *et al.*, (2003) made use of maternal, paternal and bi-parental genetic markers to identify hybrids between wolves and dogs in Scandinavia. The identification of hybrids with mtDNA and Y-chromosome markers relies on the identification of haplotypes or alleles specific to each species. Since mtDNA is a highly diagnostic marker it was powerful in distinguishing between wolf populations and domestic dogs. Wolves and dogs typed so far do not share any haplotypes (Vilá *et al.*, 1997). The study was able to successfully identify a hybridization event between a dog and a wolf in an endangered Scandinavian wolf population. The combined use of autosomal markers and maternally inherited markers is often recommended; this can allow for the determination of the direction of hybridization events (Vilá *et al.*, 2003) and give a more complete assessment of the impact of introgression (Ward *et al.*, 1999).

Unfortunately the use of mtDNA for hybrid identification in wildebeest is not possible due to the suspected unidirectional nature of matings between blue wildebeest bulls and black wildebeest cows in the initial hybridization events. The maternal mode of inheritance of mtDNA dictates that identification of hybrids will not be possible under these circumstances. Hybrids will contain mtDNA haplotypes of black wildebeest and introgression will not be detectable using markers in the mitochondrial genome. Valuable information regarding the history of the two species could however be obtained by studying the mtDNA variation, which could contribute greatly to conservation and management strategies (Grobler *et al.*, 2011). An example of this is previous mitochondrial DNA analysis done on wildebeest to estimate genetic divergence time between the two species. The results obtained were in concordance with estimates of divergence times obtained from the fossil record, indicating an evolutionary divergence time of slightly over one million years ago. Low nucleotide diversity found within the mitochondrial DNA region could also have some implications for future management strategies (Corbet and Robinson, 1991).

### 1.6.3.2 Y-chromosome markers

Verkaar *et al.*, (2003) stated that Y-chromosome markers are especially relevant because hybridization in herds mostly occur via male introgression. In the study by these authors, a test was designed to determine paternal lineages in bovine populations using sequence variation in the Y-chromosomal *SRY* (the sex-determining region Y-chromosome) gene.

Previous studies made use of Y-chromosomal microsatellites, which are only informative if species-specific alleles have been identified (Edwards *et al.*, 2000; Vila *et al.*, 2003). This study confirmed earlier studies by Ward *et al.* (2001) that uniparentally inherited markers may be able to detect the origin of a population, even after many generations of breeding which has obscured the original species composition.

Ward *et al.*, (2001) also found that there could be discordance in the levels of introgression indicated by uniparentally inherited markers. This can be explained by the observation that (for example) first generation (F<sub>1</sub>) male hybrids between domestic cattle and bison (*Bison bison*) have very low viabilities and are generally sterile. Hybridization events in these two species favour mating between male bison and female cattle, which suggests that male cattle do not contribute significantly to the composition of bison populations with hybrid ancestries (Ward *et al.*, 2001). A similar situation is most likely encountered in wildebeest hybridization events.

With the evidence suggesting that initial hybridization events involve blue wildebeest males mating with black wildebeest cows, Y-chromosome markers can be applied for hybrid identification in wildebeest. Unfortunately, Y-chromosome markers specifically developed for wildebeest does not exist. However cross-species application of microsatellite markers is well accepted, therefore studies are underway at the University of the Free State to test the use of these cross-species Y-chromosome markers for hybrid identification in wildebeest.

### **1.6.3.3 Single nucleotide polymorphisms**

Growing attention is being given to single nucleotide polymorphisms (SNP) to address a wide range of evolutionary questions (Brumfield *et al.*, 2003; Morin *et al.*, 2004). Individual SNP loci have fewer alleles per locus than most microsatellite markers. However, SNPs have a higher density and are more evenly distributed throughout the genome. Studies have also indicated that SNPs produce a lower error rate during genotyping, when compared to microsatellite markers. An additional advantage of SNPs is more readily comparable results between different laboratories than those derived from microsatellites (Coates *et al.*, 2009).

In the simplest scenarios, a single SNP with two fixed alleles is sufficient to assign individuals to a specific species, as well as to identify hybrid individuals. This is especially

possible for species that have been separated for a long time. If the species have diverged only recently or introgression has taken place over a long period of time, fixed differences are more difficult to identify. In these situations a larger amount of markers with species-specific alleles would be necessary (Väli *et al.*, 2010). Although the power of an individual SNP is not the same as an individual microsatellite marker, multi-locus SNP data sets have the ability to detect structure at recent divergence times (Haasl and Payseur, 2011). A research project on the identification of hybrids in bird species found that the simultaneous use of short tandem repeats (STRs) and SNPs gave the best results, if a small number of each marker type is to be used (Väli *et al.*, 2010).

#### **1.6.3.4. Microsatellite markers**

The identification of hybrid populations has been greatly simplified by the use of molecular genetic markers (Allendorf *et al.*, 2001). These markers allow the characterization of animals as purebred or hybrid when studying hybridization. It has also proven useful in the identification of F<sub>1</sub>, F<sub>2</sub> and backcross individuals which is important for tracking gene exchange and introgression. This method for identifying hybrids makes use of alleles that are unique to each species (Anderson and Thompson, 2002).

The number of markers necessary to determine the taxonomic status of individuals will vary, when attempting to separate F<sub>1</sub> hybrids from parental taxa fewer markers will be needed, but when trying to discriminate between backcross individuals, much more markers will be needed. Four or five markers will provide sufficient power when coarsely classifying individuals into parental, F<sub>1</sub> and simple backcross categories. Larger numbers of markers will be needed for discriminating between advanced backcrosses (Boecklen and Howard, 1997). This was confirmed by Vähä and Primmer (2006) who found that up to 42 loci were needed for the accurate differentiation between pure animals and hybrid backcrosses after only a few generations.

The successful application of microsatellite markers for hybrid identification has been proven in several studies. An example of this was the use of 22 microsatellite markers to determine the extent of hybridization between red (*Cervus elaphus*) and sika deer (*C. nippon*). Senn and Pemberton (2009) revisited the Kintyre peninsula population previously identified as a hybrid population by Goodman and colleagues (1999). The 22 cross-species microsatellite

markers were chosen for having no shared alleles between test panels of red and sika deer from diverse geographical origins. A large number of animals were screened with these loci and an appreciable proportion of hybrids (6.9%) were identified. These authors were also able to conclude that the extent of gene flow between the two deer species was extremely variable across different locations and that the total number of hybridization events was likely to be low (Senn and Pemberton, 2009).

As mentioned previously Grobler *et al.* (2005) also successfully utilized cross-species microsatellite markers from the Bovidae family to assess the genetic purity of black wildebeest populations, albeit in a limited scenario. Cross-species application of such primers is well accepted between taxonomically close groups (Wilson *et al.*, 1997). A large number of markers have already been developed for domestic cattle, and due to the relatively close relationship between wildebeest and cattle the use of these markers was particularly appropriate (Grobler *et al.*, 2011). This method of hybrid identification has proven to be very successful across a wide range of different species such as birds, aquatic species and wolflike canids including a number of bovine species such as the hybridization between domestic cattle and domestic yak as well as domestic cattle and bison.

A set of polymorphic species specific markers for East African blue wildebeest have been developed for population studies to reduce potential problems that can arise when cross-species loci are amplified (Røed *et al.*, 2011). Unfortunately no markers specific to black wildebeest have thus far been identified or characterized

#### **1.6.4. Statistical analysis**

The advent of faster sequencing and genotyping technologies has made the collection of large data sets in various organisms a reality. This advancement is linked to increasing variety in computational methods to aid in the analysis and interpretation of the data. Comparison of the previously used statistical techniques versus newer developments gives a clear indication of extensive possibilities of these techniques to analyse large and complex data sets that are being collected with various amounts and types of marker systems (Marjoram and Tavaré, 2006).

The population information necessary to use these statistical techniques also vary, for example some approaches rely on the use of alleles that are unique to each species, whilst others do not need any prior genetic information about the species (Anderson and Thompson, 2002).

The potential of current statistical software programs to assign individuals to a given population is outstanding. Estimated allele frequencies can be used to compute the likelihood that a given genotype originated in a specific population. This method can be applied to various types of markers if the loci are unlinked and at linkage equilibrium (Pritchard *et al.*, 2000). The occurrence and extent of introgression can also be inferred by identifying backcross individuals (Nason and Ellstrand, 1993). These methods can be especially useful in situations like that of the black wildebeest, where possible introgression of genetic material from another species has occurred over a period of time.

#### **1.6.4.1 Maximum likelihood methods**

Maximum likelihood methods are used to determine the estimated frequencies of different classes of hybrid individuals from observed molecular genotype frequency data. Co-dominant molecular markers can be used to estimate the frequencies of parental species as well as first- ( $F_1$ ) and second ( $F_2$ ) generation hybrid individuals. The method is used to determine the frequencies of six genealogical classes that could occur when hybridization between two species is encountered. These six classes consists of the two parental species ( $P_1$  and  $P_2$ ) as well as the four possible first and second generation hybrids that could originate when they hybridize,  $F_1$  (cross between  $P_1$  and  $P_2$ ),  $F_2$  ( $F_1$  cross with  $F_1$ ), and  $BP_1$  and  $BP_2$  ( $F_1$  backcrossing with  $P_1$  and  $P_2$  respectively) (Nason and Ellstrand, 1993).

Several assumptions about the populations are made when using this method, for example that mating is random within and between genealogical classes and that the markers are selectively neutral. Allele frequencies are then determined for the parental species as well as for the hybridizing populations. Each individual can then be classified into one of the categories according to the number of unique alleles present (Nason and Ellstrand, 1993).

#### 1.6.4.2 Bayesian methods

When it is not possible to sample the different parent populations separately the classification of hybrids can become very complicated. Newer techniques such as Bayesian methods calculate the probability that an individual in a population belongs to each of the various hybrid categories. This method presented by Anderson and Thompson (2002) has several other useful features, it is based on a genetic model and diagnostic loci are not needed. The method can identify hybrids based on allele frequency differences. To test the abilities of this method, two simulated data sets were created; one set had many relatively uninformative markers while the other set had diagnostic markers. Genotype results of two closely related trout species, the steelhead trout (*Oncorhynchus mykiss*) and the cutthroat trout (*O. clarki clarki*) was used for the simulated data sets and even with uninformative markers the method was still able to identify individuals with a high posterior probability of being hybrids. As can be expected, greater certainty was obtained when 20 nearly diagnostic loci were used. This approach can be used with various genetic marker types and special sampling scenarios (Anderson and Thompson, 2002).

Over the years various Bayesian methods have been developed for hybrid identification making use of different approaches (Vähä and Primmer, 2006). Simulation approaches can be used to investigate the efficiency of Bayesian methods. These Bayesian methods are tailored for the identification of hybrid individuals, the different approaches implemented in software packages such as STRUCTURE (Pritchard *et al.*, 2000; Falush *et al.*, 2003), which assigns probabilities for an individual to have ancestry in a certain population. Other software programs include for example NEWHYBRIDS (Anderson and Thompson, 2002) which estimates the probability of an individual belonging to a distinct hybrid or purebred class.

The method developed by Pritchard *et al.* (2000) infers population structure using genotypic data. A number of populations ( $K$ ) are chosen which is characterized by allele frequencies at each locus. Individuals can then be probabilistically assigned to a population or to joint parental populations in the case of admixed ancestry. A second method described by Anderson and Thompson (2002) infer each individual's genotype frequency class, this

provides a posterior probability of the level of certainty that an individual belongs to a certain hybrid group.

Vähä and Primmer (2006) tested the performance of these methods to detect  $F_1$  hybrids and backcross individuals with varying levels of genetic divergence in the parental populations, while also applying varying locus numbers and different hybridization levels. To ensure that the maximum performance of each of these methods was achieved, reference samples were included for some of the test performed. A very encouraging result of this was that it did not make a significant difference when comparing it to tests conducted without reference samples, proving the effectiveness of this method for hybrid identification in natural systems. Using the program STRUCTURE, these authors found that an average of 95% identification was achieved using 12 loci with  $F_{ST} = 0.21$ . However when the pairwise  $F_{ST}$  value was lowered to  $F_{ST} = 0.12$ , 24 loci was needed to give the same level of identification. Both methods work well even with low levels of genetic divergence ( $F_{ST} = 0.03-0.06$ ) between the parental populations. First generation ( $F_1$ ) could be distinguished with high level of certainty and efficiency with both methods, when a relatively large number of loci were used. The comparison of these methods identified an additional factor that should be considered when estimating the efficiency of hybrid identification: this is the expected proportion of hybrids in the sample. When the proportion of hybrids in the populations is less than 1% there was a definite decrease in the efficiency of hybrid identification (Vähä and Primmer, 2006).

After reviewing various methods available for analysis of data, whether it be for hybrid identification or for other conservation purposes, researchers suggest the simultaneous use of two or more methods, especially when the number of markers used is limited (Väli *et al.*, 2010).

#### **1.6.5. Case studies of hybrid detection**

The following section highlights the potential application of the statistical methods discussed, together with varying amounts of microsatellite loci and other marker sets for hybrid identification in various scenarios.

*Hybridization of the Farm fox and wild arctic foxes (Alopex lagopus)*

Evidence of hybridization between divergent species has been found in a variety of carnivore species (Fergus, 1991; Lehman *et al.*, 1991; Rozhnov, 1993; Reich *et al.*, 1999). One of the aspects of hybridization involves domesticated animals escaping from captivity and hybridizing with their wild progenitors. These domesticated populations are often subjected to breeding programs which involve intensive selection which leads to a reduction in genetic variation (Arnold, 2004).

Selective breeding processes, different origins, and even possible inbreeding indicate that farm-bred arctic foxes are genetically distinct from wild arctic foxes in certain parts of Scandinavia. A study by Norén *et al.*, (2005) aimed to find a genetic marker specific for farm foxes to identify escaped animals and possible hybrids in the wild. The successful identification of genetic markers would make it possible to detect genetic mixture with the wild population. These markers would also make it possible to identify wild individuals outside their natural ranges and they can thus be returned to their population of origin if necessary. A strong genetic differentiation between the farm foxes and the wild arctic foxes were found. Microsatellite analysis revealed that a large number of alleles were unique to either the wild or the farmed foxes, with no fixed loci detected. Although microsatellite analysis can distinguish between farmed and wild foxes, the use of several loci would be needed, which could be very time consuming. A clear difference was however found between farmed and wild foxes when comparing mitochondrial DNA results. Statistical analysis of the genotypic data was done using Structure software (Pritchard *et al.*, 2000).

A specific haplotype was found in farm foxes, which does not exist among wild Scandinavian arctic foxes. This provided an excellent method for the identification of farm foxes in the wild. Four of the samples collected in the wild had this farm fox specific haplotype, of which two animals could be identified as pure farm foxes that escaped and another indicated a high likelihood of being an escaped farm fox. The last sample could not be identified as a pure farm fox, but had a high probability of having a single parent from the farm fox population. However the possibility of the animal being a pure farm fox could not be excluded and it was therefore classified as a possible hybrid individual. Fortunately the high level of genetic differentiation ( $F_{ST} = 0.254$ ) between farmed and wild arctic foxes suggest that the amount of

gene flow between these populations have been limited and that this is a relatively recent event (Norén *et al.*, 2005).

*Hybridization of wild and domestic cats (Felis silvestris)*

*Felis silvestris* is the only member of the family Felidae to survive in Britain today. This species experienced a reduction in its range due to hunting and habitat destruction. Hybridization with domestic cats, increased as a result of the recolonization of Scotland (Beaumont *et al.*, 2001). The Scottish wildcat was awarded full legal protection, since 1988, under the Wildlife and Countryside Act and is also protected by the European Directive on the conservation of natural habitats and of wild fauna and flora. Unfortunately due to the hybridization with feral domestic cats this legislation is ineffective because of the inability to distinguish between wildcats and hybrids (Balharry and Daniels, 1998). The impact of this hybridization was still largely unknown, but behavioural similarities between these cats indicated that it could have a substantial impact on the genetic composition of the wildcats in Scotland (Beaumont *et al.*, 2001).

It is very important to compare purebred wildcats with purebred domestic cats, when selecting reliable molecular markers for wildcat identification. Hybrids cannot be identified on morphology alone because in this case the wildcats and the domestic cats are very closely related taxa and there is some controversy over the identification of distinctive discriminatory characters (Beaumont *et al.*, 2001). Nine microsatellite markers were identified for use in this study. These markers were originally isolated in domestic cats. Statistical analysis included genetic admixture analysis using the software developed by Pritchard *et al.* (2000). It was assumed that there were two parental populations contributing to the gene pool with an unknown gene frequency distribution at each locus. The study presented evidence that the wildcats of Scotland consists of individuals with a mixture of domestic and wildcat genes. There is strong evidence of a unique population of wildcats that differ from domestic cats and may need legal protection. A diagnostic test could however not be developed to indicate pure wildcats that contain no ancestry from the domestic cat (Beaumont *et al.*, 2001).

In a later study by Oliveira *et al.* (2008) the issue of wild and domestic cat hybridization was also raised in Portugal. This study employed the use of highly polymorphic loci combined with Bayesian statistical approaches to investigate the genetic variation in Portuguese cat

populations and to evaluate the introgression of domestic alleles into the wildcat population. The population structure, individual assignments and admixture proportions were calculated by using different Bayesian statistical techniques implemented in STRUCTURE (Pritchard *et al.*, 2000) and HYBRIDLAB (Nielsen *et al.*, 2006). HYBRIDLAB software was used to assess the power of admixture analysis to detect parental and backcross hybrid individuals by simulating parental and hybrid genotypes. The simulated sample sets were then analyzed using STRUCTURE to evaluate the efficiency of admixture analysis. Results obtained from the simulated study revealed that the 12 microsatellite loci used in this study were able to identify 100% of the parental and F<sub>1</sub> hybrid individuals. Only 88% and 80% of the F<sub>2</sub> and backcrosses were detected, respectively. These findings supported the previous detection of four admixed cats in the Portuguese population. However the true number of existing hybrids might be underestimated since some F<sub>2</sub> and backcrosses can remain undetected. These uncertainties in the detection of admixture highlight the difficulty of identifying hybrids when dealing with closely related species. It also explains why strong genetic differentiation and an increased number of loci, and even different marker types is so crucial (Oliveira *et al.*, 2008).

The hybridization rates identified in various parts of Europe will increase if the wildcat population continue to decline as a result of further habitat destruction. Coexistence of parts of forests and villages in the same agricultural landscape could favour contact between wildcats and domestic cats. The results of various studies on wildcat populations confirmed that the hybridization with domestic cats is a priority threat to the conservation of wildcat populations in Europe (Pierpaoli *et al.*, 2003).

*Hybridization between native red deer (Cervus elaphus) and invasive sika deer (C. nippon) in European countries*

Sika deer (*C. nippon*) were first introduced to the British Isles in 1860. Breeding programs was established and sika deer was distributed to parks throughout Ireland, England, and Scotland. Either these animals were deliberately released or escaped and became part of the natural fauna. Sika deer are congeneric with the native red deer (*C. elaphus*). At first hybridization between these two species were thought to be unlikely due to differences in body size. However, there were reports of phenotypic introgression in populations of red and sika deer (Goodman *et al.*, 1999). The occurrence of hybridization which leads to changes in

the appearance of red deer could have serious consequences, such as a decrease in trophy value or it is possible that sika could completely out-compete red deer, causing a hybrid swarm (Senn *et al.*, 2010).

Abernethy (1994) used various marker systems to investigate the problem and reported that alleles typical of the opposite species had introgressed in both species and that sika alleles could be detected at high frequencies to the north of the studied region. This suggested that there was a high dispersion of the sika stags. Goodman *et al.* (1999) further elaborated on the study by Abernethy (1994) by screening the same populations with additional microsatellite loci as well as a mitochondrial marker. A quantitative analysis of the genetic interaction between the introduced sika deer species and the native red deer species in Scotland, were performed. New methods were introduced to separate contributions of ancestral polymorphism (the possible presence of alleles shared between the populations prior to contact) from current hybridization; these methods are more appropriate when hybridization is still a rare occurrence (Goodman *et al.*, 1999).

Results of the 11 microsatellite loci and the mitochondrial marker indicated that all 246 deer sampled fall into two classes, this correspond to their sika- or red-like phenotype. However individuals cannot be classified as containing introgressed alleles based on phenotype alone. Many individuals carried alleles typical of the opposite population at one or more loci. This might either be due to hybridization between red and sika deer or to polymorphism within the ancestral populations. Three individuals had five possibly introgressed alleles out of 23 sampled per individual, this could be an indication that these individuals were first- or second-generation backcrosses. The results of this study indicate a low rate of hybridization (Goodman *et al.*, 1999).

The original population tested by Abernethy (1994) was again revisited 15 years later by Senn and Pemberton (2009). This time a larger sample set of 735 red and sika deer were collected and a new panel of 22 microsatellite loci and one mtDNA marker were used for the analysis. The Bayesian clustering method implemented in the software Structure (Pritchard *et al.*, 2000) were used to infer the extent of hybridization in this region. Senn and Pemberton (2009) also set out to assess whether the direction of hybridization (sika stag with red deer hind and *vice versa*) will have an effect on the gene flow. Results of the analysis revealed that there were a slight proportion of hybrids (6.9%) in the study sample set. These

individual animals did not show any phenotypic characteristics indicating that they were hybrid individuals. An absence of introgression was found in certain regions where both species were present. Mitochondrial DNA introgression also showed the same spatially clumped pattern. It was found that most introgression from mtDNA occurred from red deer into the sika deer populations (Senn and Pemberton, 2009).

Previous findings that the rate of hybridization between these two species is low (Goodman *et al.*, 1999) were again supported by the failure to detect any F<sub>1</sub> hybrids in the sample of 735 animals. Introgression, although varied across sites, did however have a noticeable effect on the genetic structure of the population. An explanation for the relative small proportion of introgression in the genomes of certain individuals could be that due to the absence of selection against hybrids, a large number of individuals could potentially be generated over time, that contain a small proportion of introgressed alleles. Mitochondrial DNA haplotypes that are discordant with nuclear results, which indicated a genome free from introgression, can be an indication of older hybridization events. It can generally be accepted that with a panel of 22 microsatellite markers one would no longer expect to find introgressed alleles after six generations of backcrossing. However the discordant mtDNA results in otherwise non-introgressed individuals could also be an indication that this marker is not truly diagnostic and that the pattern was caused by ancestral polymorphism. On the other hand this scenario is unlikely due to the spatial distribution pattern of mtDNA discordance. Mitochondrial DNA data obtained from this study were also able to reveal the direction of hybridization taking place: in the majority of situations a sika stag mated with a red deer hind (Senn and Pemberton, 2009).

The increasing phenotypic similarity of the two populations caused by hybridization between red and sika deer was also later studied by Senn *et al.* (2010). These authors hypothesized that since hybridization results in this increased similarity it can also be expected to lead to further hybridization since the two species become more similar. Various phenotypic measurements were taken from animals culled for the study, such as carcass weight, kidney fat weight, jaw length, incisor arcade breadth and pregnancy. It was found that pure red deer had significantly higher weight, jaw length and incisor arcade breadth than pure sika deer sampled from the same area. On the other hand sika had significantly higher kidney fat weight and higher pregnancy rates than red deer. No evidence was found that hybridization led to changes in the weight of the female kidney fat or pregnancy rate within females, but

evidence was found that hybridization increased the carcass weight of sika-like males and females. Increases in the incisor arcade breadth and jaw length of sika-like females were also observed. Hybridization is also causing a decrease in the weight and incisor arcade breadth of red deer-like females (Senn *et al.*, 2010).

The study predicts that in the long run, the phenotypic and genetic outcome of hybridization will be determined by selection. In the short term, predictions were made that red and sika deer will become more phenotypically similar through hybridization and this is expected to continue in parts of Britain if hybridization occurs (Senn *et al.*, 2010). The management and conservation of red deer in large parts of Europe is a very important issue and the development of stable meta-population networks by providing corridors and habitat connectivity will be crucial to the viability of red deer populations (Zachos and Hartl, 2011).

*Hybridization between North American Wolflike canids such as the gray wolf (Canis lupus), coyote (C. latrans) and the red wolf (C. rufus)*

Wolflike canids such as the gray wolf, coyote and red wolf disperse over long distance in search of mates and territories. These animals can survive in a variety of habitats. Coyotes expanded their range over the last couple of years by several thousand square kilometres, into the territories once occupied by gray wolves. Gray wolves on the other hand suffered from isolation due to habitat fragmentation. This disturbance of habitat and the abundance of coyotes over the past few hundred years have resulted in hybridization between the two species (Wayne *et al.*, 1992).

Previous studies of allozyme polymorphisms revealed very little differentiation among the three canid species in North America which can be expected because these three species diverged only 1-2 million years ago (Kennedy *et al.*, 1991; Wayne *et al.*, 1991). In 1994, Roy *et al.* set out to determine, with the use of 10 microsatellite loci, the genetic differentiation among populations of wolflike canids as well as estimate the effect of interspecific matings on allele frequencies in these hybridizing populations. These authors analysed populations where only gray wolves or coyotes are found, populations where both co-exist but do not hybridize, and lastly populations where previous studies done with mtDNA analyses indicated hybridization. Finally the study also set out to determine whether microsatellite data can support the possibility of a recent origin of the red wolf through

hybridization of coyotes and gray wolves. The microsatellite loci used were highly polymorphic in gray wolves and coyotes, with approximately 4-20 alleles per locus. It was found that hybridization between wolves and coyotes has significantly affected the allele frequencies of gray wolves, but has had little effect on coyote populations. This could be due to mating asymmetry caused by male wolves mating with female coyotes and then by the resultant offspring backcrossing with gray wolves. The microsatellite analyses also support the hypothesis that the intermediate phenotype of the red wolf is due to historic hybridization between gray wolves and coyotes. Interspecific hybridization is threatening the genetic integrity of eastern gray-wolf populations and continued habitat changes favour an increase in coyotes at the expense of gray wolves (Roy *et al.*, 1994).

A similar situation to that of the gray wolves in North America is found in the European wolf population where the genetic integrity of the wolves is threatened by free ranging dogs. Randi and Lucchini (2002) set out to establish, with the use of 18 canine microsatellite markers, the extent of genetic differentiation between wolves and dogs and to determine if genetically differentiated clusters exist by means of various techniques, including Bayesian clustering. Structure software was used with different modelling approaches. In the first approach wolves and dogs were pooled into a single population for analysis, while in the second approach it was assumed that a sample should belong to one of four pre-defined groups. These groups included wild-living Italian wolves, dogs, hybrids, and captive-reared wolves of unknown origin. Structure then assigned the individuals to one of the pre-defined groups. Many private alleles were identified in both the wolf and dog populations, suggesting that there has been very little gene flow between the two groups during recent generations. Morphological characterization identified some of the animals as putative hybrids based on external characteristics. Genetic analysis and assignment of these individuals classified them into more than one cluster, suggesting that they had admixed ancestry. These findings provided evidence of the occurrence of rare hybridization and backcrossing between wolves and dogs in the Italian population. Fortunately, the availability of molecular markers for the identification of hybrid animals could help to map distribution of pure wolf populations and locate areas of introgression where conservation authorities can control the population of free ranging dogs (Randi and Lucchini, 2002).

As mentioned previously, wolflike canids disperse over long distances which could create potential problems when studying the effect of hybridization. A possible solution to this

problem was the use of molecular techniques coupled with noninvasive sampling to screen vast areas for the presence of hybrid individuals. Adams *et al.* (2003) developed a technique that uses faecal material (scats) to address the problem of hybrid identification in the red wolf population. More than a 1,000 scat samples were collected over a period of two years. Mitochondrial DNA analysis was conducted on the scat samples and provided a useful method for differentiating between red wolves and coyotes or hybrid animals with maternal coyote ancestors. However, further analysis of the results indicated that the genetic test used in this study would potentially miss 35% of the hybrids. Nevertheless, the study did provide proof that noninvasive sampling techniques can be an important tool in identifying hybridization. This technique also allows conservation authorities to screen a large section of the experimental population area. The method can successfully identify all coyotes and approximately 65% of the hybrids in a very cost-effective manner. These authors recommend the addition of nuclear DNA analysis to increase the proportion of hybrids identified (Adams *et al.*, 2003).

*Hybridization between domestic cattle (*Bos taurus*) and North American bison (*Bison bison*)*

The situation of hybridization between the United States Federal Bison herds and domestic cattle is very similar to that of the black and blue wildebeest in South Africa. Bison (*Bison bison*) are endemic to North America and first entered the continent approximately 500,000 – 250,000 BP (Guthrie, 1970; McDonald, 1981). The first domestic cattle (*Bos taurus*) only arrived much later on that continent, in the early 1500s (Rouse, 1973). The two species diverged from one another between 1.0 and 1.5 million years ago and they share the same chromosome number ( $2n = 60$ ) (Hartl *et al.*, 1988). It has been found that the two species do not readily produce hybrids and will preferentially mate with their own species (Halbert *et al.*, 2005).

In the late 1800s, the number of North American bison declined rapidly to the point of near extinction. Fortunately a small number of private ranchers saved the species from extinction through the establishment of small foundation herds. These herds were used to either experimentally create bison-domestic cattle hybrids or were supplemented from bison herds involved in such experiments. Protected U.S. and Canadian federal and state bison populations were also later stocked with animals from these small herds; the surplus later supplied virtually all extant public and private bison herds (Coder, 1975). This effort was

ultimately successful and in 2001 the number of bison in the USA and Canada was estimated to be more than 200,000. Most of these animals should however be considered as domestic animals, since they exist in production settings (Ward *et al.*, 2001).

Evidence of domestic cattle maternal introgression has been identified in several public bison populations through analysis of mitochondrial DNA (Polziehn *et al.*, 1995; Ward *et al.*, 1999). In the study by Ward *et al.* (1999), domestic cattle introgression in a sample of 572 bison from fourteen public and one private herd were investigated with the use of mtDNA analysis. Also demonstrated in this study, was the ability of the analysis to discriminate between domestic cattle haplotypes and the haplotypes of European bison, yak and guar. The results obtained from this analysis indicated that independent hybridization events did take place between domestic cattle and North American bison as well as between European bison and yak. Phylogenetic analysis was also consistent with interspecific hybridization. These authors did however suggest that a full assessment be done to determine the impact of domestic cattle introgression into bison. This will require information from multiple nuclear loci, in addition to mtDNA results (Ward *et al.*, 1999).

Due to the uniparental inheritance of the mitochondrial genome it is possible for a bison herd, with a history of hybridization to contain no mtDNA evidence of introgression. Therefore in 2001, Ward and co-workers investigated the pattern of introgression of the Y chromosome in these species. None of the animals analysed showed any cattle Y chromosome introgression. This discordance in levels of introgression exhibited by uniparentally inherited markers can partially be explained by the fact that F<sub>1</sub> male hybrids between cattle and bison have low viability and are generally sterile. This has also been found in situations where hybridization was purposefully induced between these two species. Bison males would breed easily with female cattle whereas the reverse was nearly impossible to achieve (Ward *et al.*, 2001). Halbert *et al.* (2005) therefore set out to rather assess the levels of nuclear introgression in bison to determine the significance of introgressive hybridization and the potential impact thereof on the conservation of bison species. This was done with the use of microsatellite markers which has a good potential for assessing cross-species introgression across a range of mammals (Goodman *et al.*, 1999; Miller *et al.*, 2003; Vilá *et al.*, 2003). For the identification of domestic cattle introgression in North American bison, fourteen microsatellite markers were identified with no shared alleles between domestic cattle or the populations of bison tested (Yellowstone National Park and Wood Buffalo National Park) (Halbert *et al.*, 2005).

Results from this study indicated that historic hybridization and introgression took place between bison and domestic cattle, posing a threat to the genetic integrity and therefore conservation of this indigenous wildlife species. The populations studied have served as founders and supplements for many public and private bison populations around the world over the past 100 years. Fortunately there are still closed bison populations with no evidence of introgressed domestic cattle alleles. Halbert *et al.*, (2005) concluded their study with the important observation that accurate identification of hybrid animals and proper management of both hybrid and pure bison is crucial to conserving the genetic integrity of bison populations.

A follow-up study on hybridization in bison was conducted by Halbert and Derr (2007) who set out to do a comprehensive examination of the bison from the US federal populations for both mitochondrial and nuclear domestic cattle introgression. The study also aimed to determine the prevalence of domestic cattle introgression in several of the federal bison populations for the first time. A total of 3,301 animals from eleven federal populations were surveyed using both mitochondrial and nuclear loci (Halbert and Derr, 2007).

This study identified three federal bison populations with domestic cattle introgression. The introgression found in some of the population has been maintained for 15-20 generations after the initial hybridization events. Unfortunately, these authors were unable to accurately assess the involvement of natural selection on the maintenance of this introgression in these specific regions (Halbert and Derr, 2007). Even though introgression was only identified in three populations, it is important to keep in mind that the ability to detect hybridization is dependent on various factors. This includes the proportion of introgression in the original hybrid founders of some populations, the proportion of introgression in each individual, the number of animals sampled and the number of independent diagnostic markers used for detection (Halbert *et al.*, 2005).

Even though it does not seem that the domestic cattle introgression poses a serious threat, due to low levels of introgression found, it is still very important to try to further reduce this frequency over time. Many recommendations have been made to protect the genetic integrity of the bison in North America. Hedrick (2009) recommended that bison populations with evidence of cattle ancestry should not be introduced in pure populations, but that the introduction of pure bison into populations with cattle ancestry could prove to be beneficial,

if surplus animals are available. This type of introduction could result in an increase of genetic variation as well as a possible genetic swamping of cattle ancestry. Culling of known hybrid individuals (with known cattle mtDNA) could also help eliminate cattle mtDNA from the herds (Hedrick, 2009).

As mentioned previously, the situation of hybridization between domestic cattle and North American bison is very similar to the situation found in South Africa, where introgression from blue wildebeest is found within the black wildebeest population. Determining the extent of this hybridization is crucial for the implication of management and conservation guidelines.

## 1.7 Aims

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The aims of the current study were:

1. To investigate the effectiveness of previously identified cross-species markers for the identification of hybrid herds and individuals
2. To screen for hybrid wildebeest on a number of nature reserves controlled by the Free State Tourism, Economic Development and Environmental Affairs (DETEA) as well as private properties
3. To investigate the efficiency of different statistical approaches and available software to identify hybrids
4. To track the progress of introgression through the simulation of hybrid events
5. To contribute to the bigger program to find diagnostic markers for hybrid black wildebeest in South Africa using a range of markers

The rest of this dissertation is presented as four independent yet interrelated sections. In Chapter 2 the power of resolution of two possibly diagnostic loci, previously identified by Grobler *et al.* (2005), is investigated in a large sample of 607 blue, black and possibly hybrid wildebeest. In Chapter 3, the nature of these same markers in a population of confirmed hybrid animals is described. Chapter 4 presents a simulation on the persistence of introgression under various management regimes. Finally, a re-analysis of a wider panel of markers previously described by Grobler *et al.* (2005), to reflect more recent developments in assignment testing, is presented in Chapter 5.

# CHAPTER TWO: SCREENING BLACK WILDEBEEST POPULATIONS FOR PUTATIVE HYBRIDS

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## 2.1 Introduction

The first published studies on *C. gnou* and *C. taurinus* aimed to identify molecular differences between these congeneric species. In the early 1990s, Corbet and Robinson (1991) used mitotic chromosomes and mitochondrial DNA (mtDNA) to try to differentiate between these two species. No differences in the G- and C-banding patterns could however be found. Following this initial study, both Corbet *et al.* (1994) and Grobler and van der Bank (1995) focussed their attention on allozyme divergence between these two species. Corbet *et al.* (1994) found very low genetic distances whereas Grobler and van der Bank (1995) were able to detect allele frequency differences between these two species. However, no fixed diagnostic species specific alleles could be identified.

A significant breakthrough in hybrid identification came in 2005 when Grobler *et al.* first used microsatellite markers for hybrid detection between blue and black wildebeest. These authors used cross-species application of five bovid microsatellite markers and were able to successfully identify a number of potentially species-specific alleles. The current study is aimed at testing the power of resolution of a number of potentially diagnostic alleles identified by Grobler *et al.* (2005). Only two of the original five microsatellite markers were chosen (BM1824 and ETH10), based on the presence of possible species-specific alleles at these loci. The sample size was however increased dramatically compared to that first used by Grobler *et al.* (2005). Additional reference material was added to confirm the species-specific nature of alleles previously identified and a large number of black wildebeest populations (with diverse management histories) were screened to determine if introgression from blue wildebeest occurred in any of these populations.

Table 2.1 shows the results obtained in the study by Grobler *et al.* (2005), these authors were able to successfully identify alleles specific to pure blue and pure black wildebeest. Comparison of a pure blue wildebeest population with a pure black wildebeest population gave a clear indication that alleles specific to each of the two species do exist. For the marker BM1824, alleles ranging from 202-218 were only identified in blue wildebeest populations whereas alleles ranging from 194-200 were only found in pure black wildebeest populations. Analysis of the locus ETH10 revealed that alleles ranging from 209 and 211 were only found in the blue wildebeest populations and alleles 203 and 205 in pure black wildebeest populations. The presence of alleles previously identified as unique to blue wildebeest, in a black wildebeest population, is an indication that hybridization could have occurred in the given population.

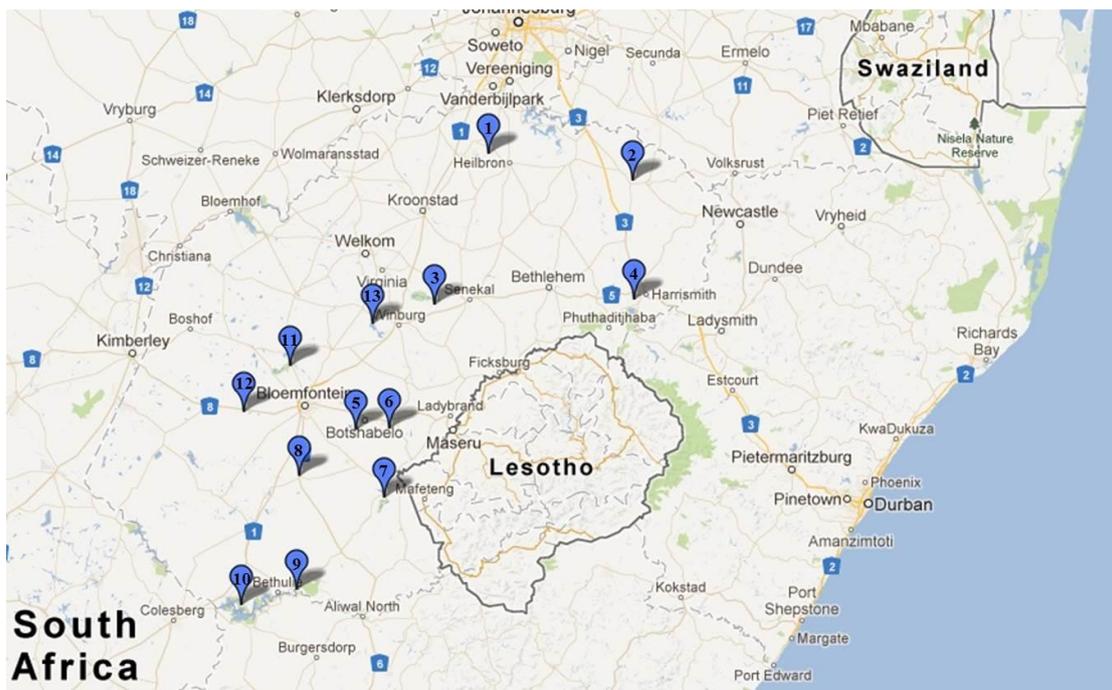
**Table 2.1: Allele frequencies of the blue wildebeest and black wildebeest reference populations used by Grobler and colleagues (2005), modified to include only the two microsatellite markers that was used in the present study**

Locus	Allele	Blue wildebeest	Black wildebeest
<b>BM1824</b>	180	0.43	-
	194	-	0.03
	196	-	0.35
	200	-	0.62
	202	0.18	-
	204	0.03	-
	206	0.18	-
	210	0.05	-
	212	0.13	-
	218	0.03	-
<b>ETH10</b>	203	-	0.97
	205	-	0.03
	209	0.81	-
	211	0.19	-

## 2.2 Study Populations

### 2.2.1 Black wildebeest populations

The study focussed on the genetic status of black wildebeest populations in the Free State Province. Black wildebeest populations on 13 Nature Reserves managed by the Department of Economic development, Tourism and Environmental affairs in this province were studied (Figure 2.1). At least one of these (Maria Maroka) has previously been identified as a putative hybrid population (Kotze, unpublished results).



**Figure 2.1: Sampling locations of black wildebeest herds in the Free State Province. These localities are: (1) Koppiesdam Nature Reserve in the Northern Free State; (2) Seekoeivlei Nature Reserve stretching from Memel to Villiers; (3) Willem Pretorius Nature Reserve approximately 150 km north-east of Bloemfontein; (4) Sterkfonteindam Nature Reserve near Harrismith; (5) Rustfonteindam Nature Reserve east of Bloemfontein; (6) Maria Moroka Nature Reserve at the foot of the Thaba’Nchu mountains; (7) Caledon Nature Reserve located between Wepener and Smithfield; (8) Reddersburg; (9) Tussen-die-Riviere Nature Reserve the southernmost reserve in the Free State Province; (10) Gariepdam Nature Reserve near Colesburg; (11) Soetdoring Nature Reserve and (12) De Brug just outside Bloemfontein and (13) Erfenisdam Nature Reserve located between Theunissen and Windburg (Image: <http://maps.google.com>)**

There have been extensive translocations of animals between the nature reserves in the Free State Province (see Figure 2.2) and it was therefore crucial to determine the possible effect, if any, of introgression in these populations. Two of the nature reserves were identified as high priority:

- Maria Moroka Nature Reserve

The last introduction of black wildebeest into the reserve occurred in 1985 when 50 animals were introduced here from Springfontein game farmers (Vrahimis pers. comm.). Animals from this reserve have been used as founders for new populations, including a large number exported to Namibia. Due to previous controversy regarding the hybrid status of the black wildebeest on the Maria Moroka Nature Reserve no animals have been translocated from the reserve in recent years.

- Willem Pretorius Nature Reserve

Introductions of black wildebeest into this reserve were made in 1956 when 47 animals were relocated from Somerville to Willem Pretorius. The population on Somerville originated from several private game farms. This nature reserve is the source population for numerous other reserves, as can be seen by the number of translocations (see Figure 2.2). Willem Pretorius has thus served as the source population for various nature reserves including, Erfenisdam, Caledon, Soetdoring, Koppiesdam, Tussen-die-Riviere, and Sterkfonteindam (Vrahimis pers. comm).

Figure 2.2 illustrates the extensive translocation processes that took place up to 2005. If hybridization took place at Willem Pretorius hybrid animals will subsequently have been transferred to numerous other localities. It is therefore crucial to determine the status of this population.

Three private game farms were also sampled for this study. The private game farms sampled were Geluk, Florida and Langkuil game farm (localities not indicated on the map); two of these game farms, Geluk and Langkuil are not located within the Free State province.

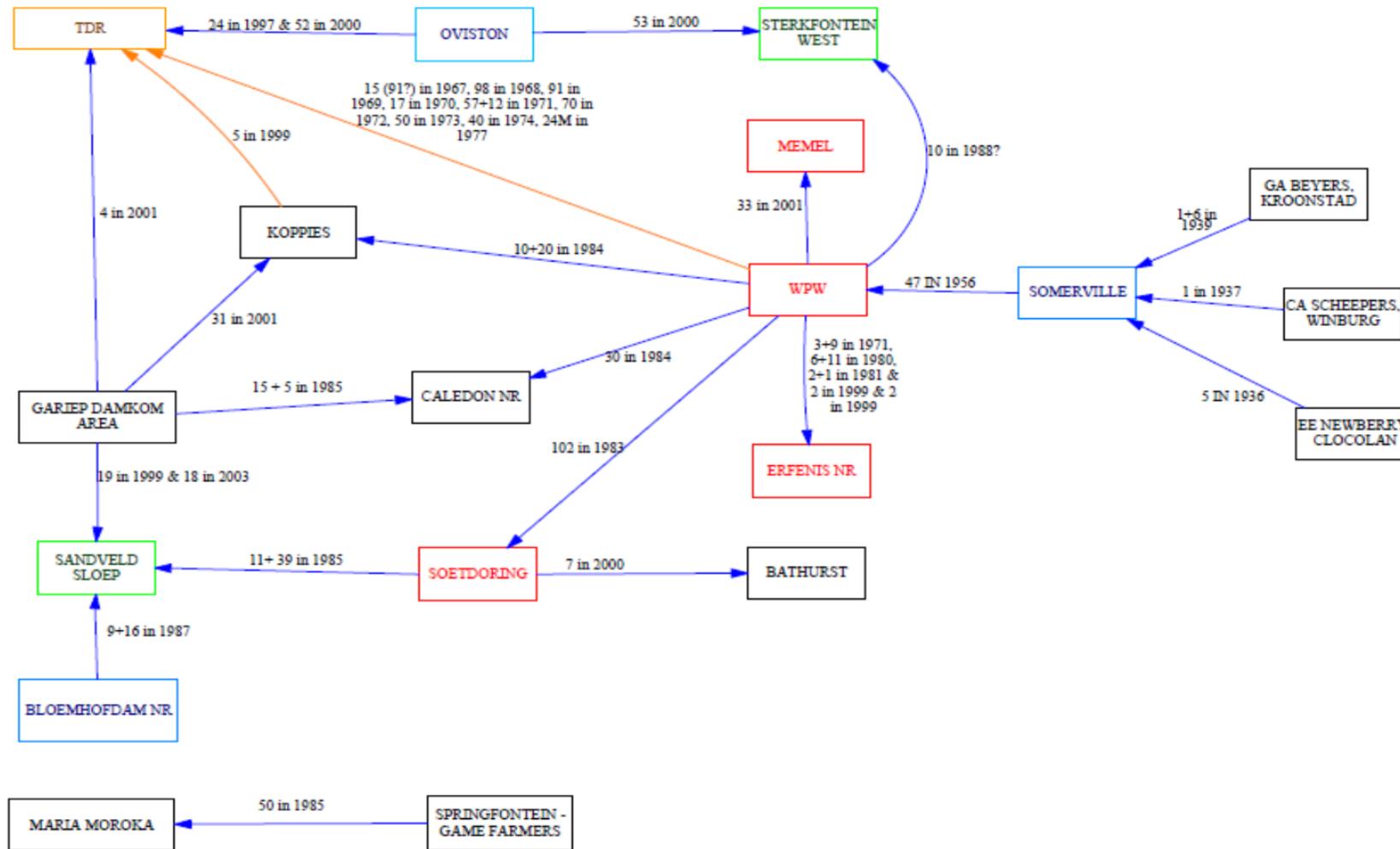
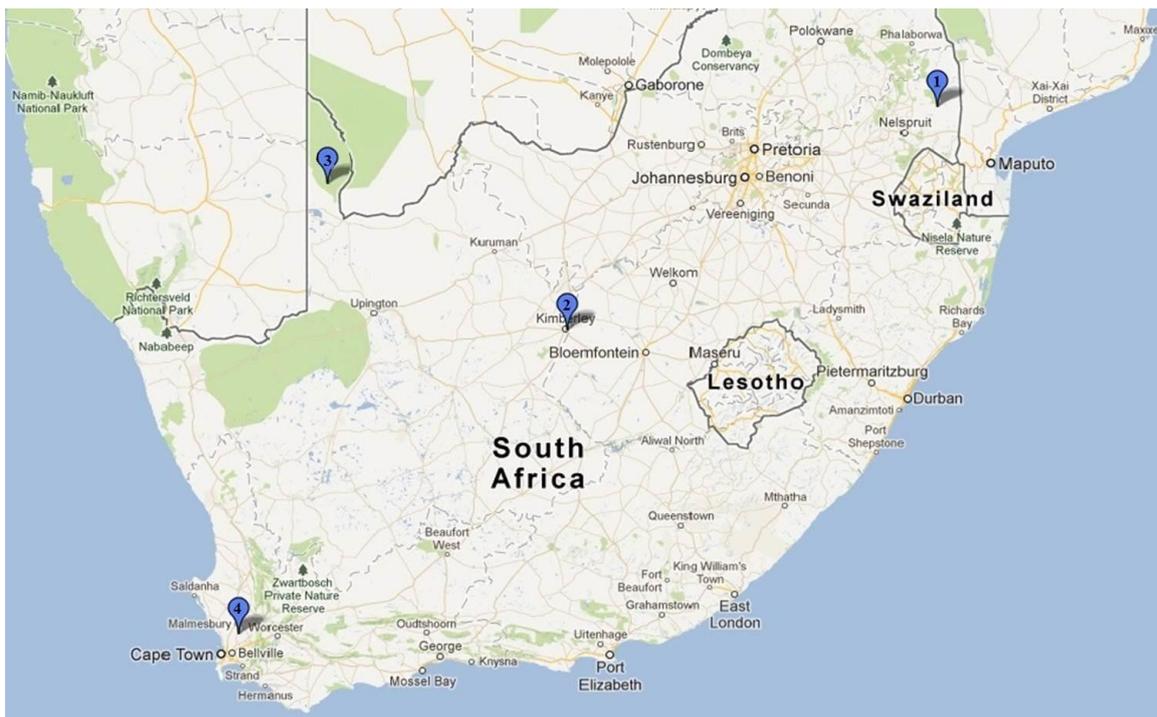


Figure 2.2: Wildebeest translocations among Free State Nature Reserves until 2005 (TDR – Tussen-die-Riviere, WPW – Willem Pretorius, NR – Nature Reserve)

### 2.2.2 Reference Populations

Populations with a detailed history of all introductions were chosen to serve as reference for the current study. New reference populations for black wildebeest were sampled from Benfontein, a game farm near Kimberley in the Northern Cape as well as Grootte Schuur Game farm in the Western Cape. In the case of the blue wildebeest, new reference samples were obtained from Kgalagadi Transfrontier Nature Reserve and the Kruger National Park (see Figure 2.3).

The reference material used in the study by Grobler *et al.*, in 2005 was also used in the present study. These populations include pure blue wildebeest from the Klaserie, Musina and Vaalwater areas (Limpopo Province); and pure black wildebeest from S.A. Lombard Nature Reserve (North West Province).



**Figure 2.3: Localities of new reference populations. Pure blue wildebeest were sampled from the (1) Kruger National Park and (3) Kgalagadi Transfrontier National Park. Pure black wildebeest were sampled from (2) Benfontein game farm and (4) Grootte Schuur game farm (Image: [http://maps .google.com](http://maps.google.com))**

### 2.2.3 Sampling

One or more of blood, hair and muscle tissue samples were collected from a total of 607 animals. The populations to be screened for the presence of putative hybrid animals comprised of 503 black wildebeest (see Table 2.2). The new reference populations consisted of 57 black wildebeest and 47 blue wildebeest (see Table 2.3). Populations were sampled opportunistically during routine hunting and culling activities, i.e. no animals were killed or handled specifically for this study. All samples, with the exception of those from the Kruger and Kgalagadi National Parks, were collected and supplied by Mr. S. Vrahimis.

**Table 2.2: Black wildebeest hybrid populations sampled (excluding reference populations)**

<b>Location</b>	<b>N</b>
Caledon Nature Reserve	45
De Brug	19
Erfenisdam Nature Reserve	15
Florida Game Farm	2
Gariepdam Nature Reserve	23
Geluk Game Farm	47
Koppiesdam Nature Reserve	33
Maria Moroka Nature Reserve	80
Langkuil Game Farm	22
Reddersburg	6
Rustfonteindam Nature Reserve	7
Seekoeivlei Nature Reserve	12
Soetdoring Nature Reserve	27
Sterkfonteindam Nature Reserve	22
Tussen-die-Riviere Nature Reserve	41
Willem Pretorius Nature Reserve	102
<b>Total</b>	<b>503</b>
<b>Average no. of samples per population</b>	<b>31.44</b>

(Refer to Appendix A for a complete list of all the black wildebeest individual samples collected)

**Table 2.3: Number of reference animals sampled**

<b>Location</b>	<b>Species</b>	<b>N</b>
Benfontein Game Farm	<i>C. gnou</i>	18
Grootte Schuur Estate	<i>C. gnou</i>	25
Kgalagadi Transfrontier Park	<i>C. taurinus</i>	15
Klaserie, Vaalwater, Musina	<i>C. taurinus</i>	15
Kruger National Park	<i>C. taurinus</i>	17
S.A. Lombard Nature Reserve	<i>C. gnou</i>	14
<b>Total</b>		104
<b>Average no. of samples per population</b>		17.33

(Refer to Appendix B for a complete list of all the reference population samples collected)

The blood samples were collected in ethylenediamine tetra-acetic acid tubes (EDTA). The muscle tissue samples were collected and stored in tubes filled with 20% dimethyl sulfoxide (DMSO). Aliquots of the blood samples were made and then stored at -20°C whereas the tissue samples were stored at ambient temperature. Hair samples were stored in paper envelopes to avoid mould growth; these envelopes were also stored at ambient temperature.

## 2.3 Methods for molecular analysis

The methods used for DNA extraction, PCR optimization, data collection and statistical analysis are detailed in the following sections.

### 2.3.1 DNA extraction and quantification

Ultimately only muscle tissue samples were used in this study due to the poor quality of the blood and hair samples received. Genomic DNA was extracted using a commercial kit from Roche®<sup>1</sup>, the Applied Science High Pure PCR Preparation Kit, following the protocol for isolation of nucleic acid from mammalian tissue. Muscle samples were cut into small pieces of approximately 25 - 50 mg and placed in nuclease free microcentrifuge tubes. Tissue lysis buffer (200 µl) and Proteinase K (40 µl) were added to the tissue samples, mixed thoroughly and incubated overnight at 55°C. After incubation, 200 µl of Binding buffer was added and another incubation step of 10 min at 70°C followed. The final step of purification included the addition of 100 µl isopropanol; the samples were then mixed well and transferred to a

<sup>1</sup> Roche is a registered trademark of Roche Diagnostics GmbH, Mannheim Germany

High Filter Tube inserted into a collection tube. The samples were centrifuged for 1 min at 8,000 *g*. After this step, washing and elution steps followed.

For the washing procedure, the High filter tube was inserted into a new collection tube and the flow through liquid was discarded. To the upper reservoir of the filter tube, 500  $\mu$ l inhibitor removal buffer was added and the samples were centrifuged for 1 min at 8,000 *g*. Again the filter tube was inserted into a new collection tube and the flowthrough liquid was discarded. For washing, 500  $\mu$ l of washing buffer was added and samples were centrifuged for 1 min at 8,000 *g*. This washing step was repeated twice, with the filter tube inserted into a new collection tube and the flowthrough liquid discarded each time. After the final washing step, the filter tube was inserted into a new collection tube and centrifuged at 10,000 *g* for 10 sec to remove any residual wash buffer. The elution of the DNA followed the washing procedure. The filter tube was placed into a sterile microcentrifuge tube and 200  $\mu$ l of pre-warmed elution buffer were to the upper reservoir of the filter tube and the combination was centrifuged for 1 min at 8,000 *g*. The DNA was then stored at -20°C.

The quality and quantity of the DNA were determined with the use of the Nanodrop®<sup>2</sup> ND-1000 spectrophotometer. The calculated absorbance is correlated with the concentration with the use of the Beer-Lambert equation (Equation 2.1).

#### Equation 2.1: Beer-Lambert equation

$$A = E \cdot b \cdot c$$

**where**

**A** = absorbance in absorbance units (AU)

**E** = wavelength-dependent extinction coefficient with units of liter/mol-cm

**b** = path length in cm

**c** = analyte concentration in moles/liter or molarity (M)

The software (V3.3) of the Nanodrop® ND-1000 makes use of a modified version of this Beer-Lambert equation (Equation 2.2) for nucleic acid quantification.

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<sup>2</sup> Nanodrop is a registered trademark of Nanodrop Technologies, Inc., Wilmington, Delaware

**Equation 2.2: Modified Beer-Lambert equation**

$$C = (A \cdot e) / b$$

**where**

**C** = nucleic acid concentration in ng/μl

**A** = absorbance in AU

**e** = wavelength-dependent extinction coefficient in ng-cm/ μl

**b** = path length in cm

The quality of the DNA is measured at 260/230 and 260/280 ratios of absorbance and the quantity, in ng/μl, is based on absorbance at 260 nm. A value of 1.8 - 2.2 for the 260/230 ratio is an indication of pure nucleic acid. Similarly, a value of 1.8 for the 260/280 ratio indicate a pure nucleic acid. The concentrations of extracted DNA samples were standardized at 25 ng/μl before continuing with the polymerase chain reaction (PCR).

**2.3.2 Amplification of microsatellite loci**

The nucleotide sequences of the primers used for this study are listed in Table 2.4. Each of the primer pairs consisted of a fluorescently labelled forward primer and an unlabelled reverse primer. The primers were labelled with HEX<sup>TM3</sup> (green) and 6-FAM<sup>TM</sup> (blue) respectively. The signal produced by these fluorescent dyes is resolved using a fluorophore colour separation algorithm known as a matrix.

**Table 2.4: The nucleotide sequences of the primers used, GenBank accession numbers and references**

Primer	GenBank Reference	Sequence	Reference
BM1824 (HEX)	G18394	F: 5'-GAGCAAGGTGTTTTTCCAATC-3' R: 5'-CATTCTCCAAGTCTTCCTTG-3'	Bishop <i>et al.</i> , 1994
ETH10 (6'-FAM)	Z22739	F: 5'-GTTGAGGACTGGCCCTGCTAACA-3' R: 5'-CCTCCAGCCCACTTTCTCTTCTC-3'	Solinas-Toldo <i>et al.</i> , 1993

<sup>3</sup> HEX, 6-FAM & NED are registered trademarks of Applied Biosystems, Foster City California, USA

Optimization of both the annealing temperature and the  $MgCl_2$  concentration were necessary since these primers were not developed specifically for wildebeest. The  $Mg^{2+}$  concentration has an influence on the specificity and yield of the amplification reaction.  $Mg^{2+}$  forms soluble complexes with the dNTPs and this makes these molecules available and recognisable as substrate for the enzyme. Optimal concentrations of  $Mg^{2+}$  in PCR reactions vary between 0.5 - 2.5 mM, with the most commonly used concentration being 1.5 mM. The optimal concentration of  $Mg^{2+}$  should be determined for each PCR assay (Viljoen *et al.*, 2005). Three different concentrations (1.5 mM, 2 mM & 2.5 mM) were tested for both primers including a buffer already containing 1.0 mM  $MgCl_2$ . No amplification was obtained for the locus BM1824 using the buffer already containing 1.0 mM  $MgCl_2$ . However, after the concentration of  $MgCl_2$  was adjusted to 2.0 mM, amplification was successful. The locus ETH10 amplified best with the buffer already containing 1.0 mM  $MgCl_2$ .

The optimal annealing temperature ( $T_a$ ) also needed to be determined for both primers. Typically the  $T_a$  is 5°C below the true melting temperature ( $T_m$ ), but optimal annealing temperatures are often higher (5 - 10°C) than the  $T_m$  of the primer. It is best to select the highest possible annealing temperatures permitted by a specific primer set since increased annealing temperatures enhances discrimination and reduces mis-extension. These temperatures are usually in the range of 55 - 72°C (Viljoen *et al.*, 2005). The annealing temperatures ( $T_a$ ) for the selected primers were set at 5°C below the melting temperature, and then gradually adjusted until optimum specificity was obtained.

PCR amplification was performed in reactions with a total volume of 20  $\mu$ l. Each PCR reaction contained 2 mM deoxynucleotide triphosphate (dNTP's), 1  $\mu$ M forward primer as well as 1  $\mu$ M reverse primer, 1 unit (U) Super-Therm Gold DNA polymerase (Sepsci), Super-Therm Gold buffer (additional  $MgCl_2$  were added for the primer BM1824, with a final concentration of 2.0 mM) and 50 - 150 nanograms (ng) DNA. Double distilled water (ddH<sub>2</sub>O) was added to reach the final volume of 20  $\mu$ l.

PCR amplification was performed using the Applied Biosystems 2720 Thermal Cycler. The amplification cycles consisted of a denaturing step of 12 min at 94°C; followed by 35 cycles each of 40 sec at 94°C, 40 sec at 57°C, and 1 min at 72°C; and a final extension step of 72°C for 60 min.

The primary methodology for separating and detecting short tandem repeat (STR) alleles is capillary electrophoresis. Fragment analysis was done on the ABI 3130 Genetic Analyzer.

The internal size standard used was GeneScan™ 350 Rox™<sup>4</sup> (Applied Biosystems). An internal standard contains DNA fragments of known size and enables automated data analysis and accurate DNA fragment size comparisons between electrophoresis runs. The matrix used in this study was the Multi-Capillary D30 (Dye set D) Matrix Standard kits; this matrix is capable of analyzing DNA fragments labelled with the HEX™ and 6-FAM™ labels used in the current study, as well as the labels NED™ and ROX™.

The fragment analysis results were visualized and analyzed (scored) using GeneMarker®<sup>5</sup> software, version 1.6.

### **2.3.3 Populations sampled**

The 22 wildebeest populations were sampled and genotyped. The following abbreviations were used to designate the nature reserve populations: Caledon (CAL), Tussen-die-Riviere (TDR), Maria Moroka (MM), Willem Pretorius (WP), Gariëpdam (GD), Koppiesdam (KD), Erfenisdam (ERFD), Sterkfonteindam (SFD), De Brug (DB), Soetdoring (SOE), Rustfonteindam (RFD), Reddersburg (RED), and Seekoeivlei (SKV). The private game farm populations were designated as follows: Florida game farm (FPG) (this population was excluded from most of the statistical analysis since it consisted of only two individuals), Lankgkuil game farm (LAN) and the Geluk game farm (GEL). The black wildebeest reference populations were abbreviated as: S.A. Lombard (SAL), Benfontein game farm (BEN) and Grootte Schuur estate (GS). The following abbreviations were used for blue wildebeest reference populations: Kruger National Park (KNP), Kgalagadi Transfrontier National Park (KGAL) and the animals sampled from the Klaserie, Vaalwater and Musina area (KVM).

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<sup>4</sup> GeneScan and ROX are registered trademarks of Applied Biosystems Corporation, Foster City California, USA

<sup>5</sup> GeneMarker is a registered trademark of SoftGenetics LLC, State College Pennsylvania, USA

## 2.4 Statistical Analysis

Statistical analysis of the genotyping results involved the use of various statistical approaches and the relevant software. The different programmes as well as the various genetic measures used are discussed below.

### 2.4.1 Data organization

Genotypic data for all the populations were entered into a Microsoft Office Excel spreadsheet and further analyzed using Microsatellite Toolkit (Park, 2001), an add-in to Excel. Microsatellite Toolkit can be used to calculate parameters such as allele frequencies and various measures of the genetic diversity of populations. The software can also be used to prepare input files for various other software programmes. In this study it was used to prepare input files for ARLEQUIN version 3.1 (Excoffier *et al.*, 2005) and FSTAT (Goudet, 2001) software.

### 2.4.2 Genetic Diversity

For populations to evolve and adapt to environmental change it is important to conserve genetic diversity. Large, naturally outbreeding species have extensive genetic diversity, but this is usually reduced in small populations and species of conservation concern. Loss of genetic diversity is usually associated with inbreeding and reduction in reproduction and survival in species. Genetic diversity can be measured in terms of polymorphism, average heterozygosity and allelic diversity (Frankham *et al.*, 2010). Allelic frequency (the relative frequency of a particular allele in a population), average number of alleles per locus and heterozygosity (expressed as observed and expected heterozygosity) were calculated using Microsatellite Toolkit.

An additional (and valuable) measure of genetic diversity used is allelic richness. This measure is important in conservation genetics as it can be a good indicator of past demographic changes (Toro *et al.*, 2009). Furthermore, allelic richness compensates for unequal sample sizes among populations sampled. The FSTAT software program was used to calculate the overall allelic richness.

### 2.4.3 Hardy-Weinberg equilibrium (HWE)

Calculation of conformation to Hardy-Weinberg equilibrium is crucial to studies of conservation and evolutionary genetics since it is a very effective measure to detect deviations from random mating, selection and the effects of inbreeding. If none of these processes occur in a large random-mating population, the allele and genotype frequencies are expected to stay constant from generation to generation and the population is then said to be in HWE (Frankham *et al.*, 2010). To test for deviations from HWE, ARLEQUIN software was used.

### 2.4.4 Degree of genetic differentiation ( $F_{ST}$ ) between populations

The measure  $F_{ST}$  can be used to evaluate the degree of genetic differentiation or drift among populations, i.e. the genetic variation due to allele frequency differentiation amongst these specific populations (Wright, 1965).  $F_{ST}$  values were calculated for pair-wise combinations of the 22 populations, to detect patterns of gene flow and determine the significance of differentiation (if any) between populations. ARLEQUIN software was also used for these analyses.

The gene flow ( $Nm$ ) values among pair-wise combinations were calculated (Equation 2.3) using the  $F_{ST}$  values obtained from ARLEQUIN.

#### Equation 2.3: Equation for calculating gene flow

$$Nm = [0.25 (1 - F_{ST})] / F_{ST}$$

where

$Nm$  = gene flow

$F_{ST}$  = degree of genetic differentiation

### 2.4.5 Analysis of Molecular Variance (AMOVA)

Analysis of molecular variance is a method that uses available molecular data to estimate genetic diversity at different hierarchical levels. These hierarchical levels include components of diversity such as “within populations”, “among populations/within groups”,

“among groups” and “among individual” diversity. AMOVA analyses were applied in three different ways during the current study. For the first AMOVA analysis, only the reference populations were included to determine the genetic diversity between blue and black wildebeest. Group 1 consisted of only black wildebeest reference populations (S.A. Lombard nature reserve, Benfontein game farm and Grootte Schuur estate) and Group 2 consisted of the blue wildebeest reference populations (Kruger National Park, Kgalagadi Transfrontier Park and the combined Klaserie, Vaalwater and Musina population).

For the second AMOVA analysis the populations were divided into three groups where: Group 1 consisted of the black wildebeest reference populations (S.A. Lombard nature reserve, Benfontein game farm and Grootte Schuur estate); Group 2 consisted of the blue wildebeest reference populations (Kruger National Park, Kgalagadi Transfrontier Park and the combined Klaserie, Vaalwater and Musina population) and the populations grouped into Group 3 included all the remaining black wildebeest populations of unknown status (Florida game farm, Caledon, Tussen-die-Riviere, Maria Moroka, Willem Pretorius, Odendaalsrus, Gariepdam, Koppiesdam, Erfenisdam, Sterkfonteindam, De Brug, Soetdoring, Rustfonteindam, Reddersburg and Seekoeivlei nature reserves, as well as the Geluk game farm).

The final AMOVA analysis compared only black wildebeest populations. Group 1 consisted of the black reference populations and Group 2 consisted of the black wildebeest populations of unknown status, pooled to form one large population. ARLEQUIN software was used for these analyses.

#### **2.4.6 Assignment tests**

Conventional methods used in population genetics, such as  $F_{ST}$  and genetic distance, are based on the allele frequencies of entire populations. With highly variable markers such as microsatellites, a differentiation of individuals becomes feasible. Pritchard *et al.*, (2000) developed a method that infers population structure using genotypic data. A number of populations ( $K$ ) are chosen which are characterized by allele frequencies at each locus. Individuals can then be probabilistically assigned to each population or to joint parental populations in the case of admixed ancestry. The software developed (STRUCTURE v. 2.3.1), presents an estimate of the number of genetic clusters or true genetic populations in a

dataset. For the current study the program was used to determine the true number of genetic populations, and for the assignment of individuals to identified populations.

Individual genotypes were entered into an input file along with the assumed population of origin for each individual. The reference populations for black and blue wildebeest were pooled to represent one black and one blue reference population. The analysis of the data in STRUCTURE involved two separate calculations, for the first analysis data from both loci (ETH10 and BM1824) were used. For the second part only results obtained for the locus BM1824 were analyzed for all the populations. This was done to determine the power of resolution of the two loci combined as well as the power of the most promising locus. While the approaches implemented in STRUCTURE software is not necessarily aimed at such low numbers of loci, it was felt that a test using the available loci would be a strong test of the power of these loci.

For the STRUCTURE runs, the true number of population was determined by setting possible  $K$  values of between one and ten. Five independent runs for each assumed  $K$  was used, with a burn-in period of 100,000 steps followed by 200,000 MCMC (Markov Chain Monte Carlo) iterations. The output of these analyses was entered into STRUCTURE HARVESTER (Earl and von Holdt, 2012); this software package further analyses the information obtained by STRUCTURE to provide the true value of  $K$ , based on the measure deltaK (Evanno *et al.*, 2005).

After determination of the true number of populations, STRUCTURE was again run to determine the most likely assignment of each individual to each identified cluster. This second analysis was done with a burn-in period of 100,000 steps followed by 1,000,000 MCMC iterations. These analyses were performed for the dataset that consisted of results for both loci (ETH10 and BM1824) and the dataset which contained only results for the locus BM1824.

An older assignment method was also used to for the assignment of individuals to the most likely population of origin. GENECLASS version 2.0 software (Cornuet *et al.*, 1999) was used to assign or exclude individuals to reference populations. This program also computes the probabilities that each individual belong to each reference population. The test populations were compared to the two black and blue wildebeest reference populations and

the probabilities were calculated. The Monte Carlo re-sampling algorithm of Paetkau *et al.* (1995) was implemented for this analysis.

## 2.5 Results and Discussion

### 2.5.1 Genotyping

The amount of DNA extracted from the muscle samples was sufficient to yield reliable genotyping results. The two cross-species microsatellite markers, ETH10 and BM1824, were successful in profiling a total of 607 samples and both of these markers were polymorphic for blue and black wildebeest. The allele size range for each locus as well as the number of alleles observed for the reference populations of blue and black wildebeest are listed in Table 2.5. Similar values for the black wildebeest populations of the Free State Province and private game farms are listed in Table 2.6.

**Table 2.5: Allele size range and the number of alleles observed for the blue and black reference populations**

Locus	No. of alleles observed		Allele size range	
	Black Wildebeest	Blue Wildebeest	Black Wildebeest	Blue Wildebeest
ETH 10	3	3	203-209	205-211
BM 1824	6	12	192-200	178-218

**Table 2.6: Allele size range and the number of alleles observed for the black wildebeest test populations**

Locus	No. of alleles observed	Allele size range
ETH 10	2	203-205
BM 1824	8	192-214

The complete genotyping results all black wildebeest tested are provided in Appendix C; with the genetic profiles of the reference populations for both blue and black wildebeest given in Appendix D.

### 2.5.2 Statistical Analysis

The results obtained from the various statistical calculations, using the specified software was compared and interpreted. Results of the analysis for the various coefficients are given below.

#### 2.5.2.1 Genetic diversity

The highest unbiased heterozygosity for reference populations (Table 2.7) were observed for the blue wildebeest populations. The Kgalagadi Transfrontier National Park population showed the highest level of heterozygosity (0.693) as well as the highest number of alleles (5.5) per locus among all the populations. The lowest value among blue wildebeest populations was  $H = 0.558$  observed in the Kruger National Park. The Klaserie, Vaalwater and Musina pooled samples showed an intermediate heterozygosity of  $H = 0.562$ . This value is however perhaps artificially higher compared to the Kruger National Park, since heterozygosity would almost certainly be raised by pooling of three distinct populations.

Among black wildebeest reference populations a heterozygosity value of 0.412 was found for the Benfontein game farm population, with  $H = 0.310$  in the Grootte Schuur Estate population. This compares to  $H = 0.494$  previously reported for the pure S.A. Lombard black wildebeest population.

The trends observed from heterozygosity values were supported by values from the average number of alleles per locus, and the allelic richness. Allelic richness observed for the blue wildebeest populations ranged from 3.038 - 4.267, with an average of 3.5 - 5.5 alleles per locus before adjustment for unequal sample size (A). Lower levels were observed for the black wildebeest control populations, which had allelic richness levels of 2.484 - 2.901, and an average of three alleles per locus in all populations.

Overall the genetic diversity of black wildebeest in South Africa is expected to be lower than that of blue wildebeest due to the extreme decline in black wildebeest numbers experienced during early 1940's (Bigalke, 1947). This expectation was supported by the low levels of heterozygosity as well as allelic richness observed. Furthermore, blue wildebeest occur in much larger numbers than the black wildebeest (with a current population size of 150,000

compared to 18,000 for black wildebeest – IUCN, 2008), and never experienced the historic bottleneck encountered by the black wildebeest.

Among the black wildebeest test populations the unbiased heterozygosity values ranged from 0.132 - 0.628 with the highest value observed for the Maria Moroka population. The lowest value among the black wildebeest test populations was  $H = 0.132$  observed in the Rustfontein dam population, this population also had the overall lowest number of alleles per locus (1.5).

The average number of alleles per locus and allelic richness supported the unbiased heterozygosity results. Allelic richness observed for the black wildebeest test populations ranged from 1.495 - 3.470, with an average of 1.5 - 5 alleles per locus. The Maria Moroka population had the highest allelic richness (3.470) among the test populations, with the highest average alleles per locus (5) observed for the Willem Pretorius population.

The populations that contained introgressed blue wildebeest genetic material, did exhibit higher values of allelic richness and unbiased heterozygosity, compared to the pure black wildebeest populations. There were other black wildebeest test populations that also exhibited high allelic richness and levels of heterozygosity, but no introgressed blue wildebeest alleles were detected. The possibility still exists that these population could potentially also contain hybrid animals but due to small sample sizes and only two microsatellite markers used, these animals were not identified. This could be the case for the Tussen-die-Riviere, Sterkfontein dam, De Brug and Reddersburg populations.

The intermediate levels of heterozygosity observed for the putative hybrid populations, could also be an indication that the introgression of blue wildebeest into this populations is minor and not recent. As stated by Grobler *et al.* (2005), the heterozygosity observed in hybrid populations would be expected to exceed the value observed for blue wildebeest, under the assumption of a recent substantial introgression, as unique alleles from the two species would have been combined.

**Table 2.7: Unbiased ( $H_z$ ) and observed heterozygosity ( $H_o$ ), the standard deviations (SD), number of alleles per locus and the average allelic richness for all the populations. Highlighted in black is the three black wildebeest reference populations and in blue is the three blue wildebeest reference populations. The number of individuals per population is indicated in brackets next to each population name**

Population	Unbiased $H_z$	Unbiased $H_z$ SD	Observed $H_z$	Observed $H_z$ SD	No Alleles	No Alleles SD	Average Allelic Richness	Average Allelic Richness SD
<b>CAL (45)</b>	0.401	0.077	0.198	0.043	3.5	2.120	2.501	0.788
<b>TDR (41)</b>	0.475	0.115	0.171	0.042	4	2.830	2.885	1.296
<b>MM (80)</b>	0.628	0.152	0.333	0.038	4.5	3.540	3.470	2.082
<b>WP (102)</b>	0.458	0.230	0.276	0.032	5	4.240	2.855	1.462
<b>LAN (22)</b>	0.520	0.204	0.200	0.063	3.5	2.120	2.873	1.305
<b>GD (23)</b>	0.277	0.192	0.088	0.045	3	1.410	2.184	1.026
<b>KD (33)</b>	0.345	0.345	0.267	0.056	3	2.830	2.413	1.998
<b>ERFD (15)</b>	0.330	0.330	0.467	0.098	2.5	2.120	2.224	1.730
<b>SFD (22)</b>	0.581	0.125	0.214	0.063	4	2.830	3.268	1.797
<b>DB (19)</b>	0.511	0.068	0.237	0.069	3	1.410	2.656	0.933
<b>SOE (27)</b>	0.430	0.068	0.077	0.037	2.5	0.710	2.279	0.395
<b>RFD (7)</b>	0.132	0.132	0.143	0.094	1.5	0.710	1.495	0.699
<b>RED (6)</b>	0.394	0.394	0.417	0.142	3	2.830	3.000	2.828
<b>SKV (12)</b>	0.258	0.258	0.227	0.087	2.5	2.120	2.161	1.642
<b>GEL (47)</b>	0.488	0.245	0.217	0.044	4.5	3.540	3.059	1.701
<b>SAL (14)</b>	0.495	0.241	0.192	0.076	3	1.410	2.901	1.399
<b>BEN (18)</b>	0.412	0.191	0.125	0.058	3	0.0	2.520	0.486
<b>GS (25)</b>	0.310	0.310	0.239	0.065	3	2.830	2.484	2.099
<b>KNP (17)</b>	0.558	0.148	0.459	0.094	3.5	2.120	3.038	1.474
<b>KGAL (15)</b>	0.693	0.151	0.670	0.091	5.5	3.540	4.267	2.191
<b>KVM (15)</b>	0.562	0.192	0.567	0.091	4	2.830	3.258	1.801
<b>Average</b>	0.441	0.198	0.275	0.068	3.430	2.290	2.752	1.482

### 2.5.2.2 Hardy-Weinberg Equilibrium (HWE)

A high proportion of deviations from HWE were found for among the black wildebeest populations (Table 2.8). However, the De Brug, Rustfontein, Reddersburg and the Seekoeivlei populations were in HWE for the locus BM1824. Two of the blue wildebeest populations studied were in HWE for both of the loci, the Klaserie, Vaalwater and Musina blue combination, as well as the Kruger National Park blue wildebeest population. Observed numbers of genotypes in the third blue wildebeest reference population, Kgalagadi Transfrontier National Park, did not deviate from HWE at the locus ETH10, however for BM1824 a deviation from expected HW equilibrium of genotypes was observed.

**Table 2.8: Expected and observed heterozygosity values and the corresponding p-values per population. P-values of all the populations that are in Hardy-Weinberg equilibrium are indicated in green (N/A = not applicable – the locus was monomorphic for the population)**

Population	ETH10			BM1824		
	Obs. Het.	Exp. Het.	P-value	Obs. Het.	Exp. Het.	P-value
CAL	0.000	0.324	0.000	0.395	0.478	0.000
TDR	0.000	0.360	0.000	0.341	0.590	0.000
MM	0.000	0.475	0.000	0.667	0.780	0.000
WP	0.000	0.227	0.000	0.551	0.688	0.001
LAN	0.000	0.316	0.000	0.400	0.724	0.017
GD	0.000	0.085	0.022	0.176	0.469	0.001
KD	N/A	N/A	N/A	0.533	0.690	0.000
ERFD	N/A	N/A	N/A	0.933	0.660	0.007
SFD	0.000	0.455	0.000	0.429	0.706	0.000
DB	0.000	0.444	0.000	0.474	0.579	0.059
SOE	0.000	0.498	0.000	0.154	0.363	0.001
RFD	N/A	N/A	N/A	0.286	0.264	1.000
RED	N/A	N/A	N/A	0.833	0.788	1.000
SKV	N/A	N/A	N/A	0.455	0.515	0.057
GEL	0.000	0.243	0.000	0.435	0.733	0.000
SAL	0.000	0.254	0.004	0.385	0.735	0.000
BEN	0.000	0.221	0.001	0.250	0.603	0.000
GS	N/A	N/A	N/A	0.478	0.620	0.014
KNP	0.385	0.409	1.000	0.533	0.706	0.060
KGAL	0.769	0.542	0.227	0.571	0.844	0.000
KVM	0.467	0.370	0.528	0.667	0.754	0.091

The observed patterns of conformation / deviation from HWE are largely in agreement with known population parameters. The blue wildebeest populations from the Kruger National Park and Kgalagadi are large, free-ranging populations of a species with high levels of genetic diversity. By contrast, all extant black wildebeest belong to fragmented populations, in a species with already reduced levels of genetic diversity. These factors can combine to lead to a large number of deviations from HWE, as was indeed observed.

### **2.5.2.3 Species specific alleles**

The first objective of this study was to investigate the effectiveness of the microsatellite markers ETH10 and BM1824, previously used in a study by Grobler and colleagues (2005), for the identification of alleles specific to blue and black wildebeest respectively in a much larger sample.

One key point for the present study was to include more reference populations, as the reference populations used in the study by Grobler *et al.* (2005) were relatively small, and the possibility existed that alleles identified as specific to a species might in fact be present in both species.

The allele frequencies of all the populations tested in the Free State province, private farms as well as the reference populations are given in Table 2.9.

**Table 2.9: Number of alleles and allelic frequencies for the two loci, per population. The populations highlighted in black and blue represents the black wildebeest and blue wildebeest reference populations, respectively**

Locus	Alleles	Populations																						
		FPG	CAL	TDR	MM	WP	LAN	GD	KD	ERFD	SFD	DB	SOE	RFD	RED	SKV	GEL	SAL	BEN	GS	KNP	KGAL	KVM	
ETH10	203		0.200	0.231	0.382	0.130	0.190	0.043			0.333	0.316	0.423					0.860	0.143	0.059				
	205	1.000	0.800	0.769	0.618	0.870	0.810	0.957	1.000	1.000	0.667	0.684	0.577	1.000	1.000	1.000	0.140	0.857	0.882	1.000	0.269	0.615		
	209																			0.059			0.077	0.767
	211																					0.731	0.308	0.233
BM 1824	178																				0.467	0.143		
	180																						0.400	
	192		0.070	0.098	0.080	0.005	0.250		0.467	0.100	0.143	0.105	0.096		0.167	0.045	0.174		0.344	0.587			0.179	
	194		0.698	0.610	0.360	0.347	0.350	0.706	0.267	0.400	0.500	0.605	0.788	0.857	0.417	0.682	0.033	0.231	0.531	0.152			0.036	
	196		0.186	0.171	0.213	0.418		0.206	0.150	0.433	0.143	0.237	0.115	0.143	0.083	0.182	0.022	0.192						
	198		0.023	0.073	0.040	0.051	0.325	0.059	0.100		0.071						0.380	0.423	0.125	0.130			0.036	
	200		0.023	0.037	0.027	0.128					0.067	0.119	0.053			0.250	0.091	0.304	0.154		0.065	0.100	0.250	
	202																					0.133		0.167
	204																					0.267	0.036	
	206																					0.033	0.250	0.267
	210			0.012	0.173	0.036	0.050		0.017		0.024					0.083		0.076			0.065		0.071	0.033
	212				0.107	0.005	0.025	0.029										0.011						0.100
	214					0.010																		
	218																							0.033

The addition of new reference material revealed that some of the alleles previously assumed to be unique to a specific species were in fact shared between the two species. Overall the locus BM1824 is potentially more informative than ETH10 as can be seen by the number of alleles observed for all the populations at these loci. For ETH10 only four alleles were identified among all the populations, whereas for BM1824 a total of 14 alleles were identified in all the populations combined.

Analysis of the locus ETH10 revealed that allele 209, previously thought to be unique to blue wildebeest is in fact shared between these two species, although the frequency of this allele in the black wildebeest population from Benfontein game farm is very low. Allele 205, previously identified as unique to black wildebeest was also found to be shared between the two species. This re-classification of alleles from species-specific to shared strongly reinforces the need to use reference populations of adequate size and representatives of a full distribution area, when screening for hybrids. The results for locus BM1824 revealed that alleles 178 and 180, previously identified as diagnostic, remained unique for blue wildebeest. However, alleles 198 and 200, previously identified as specific to only black wildebeest, were found to be shared between the two species after inclusion of more reference populations. Alleles 202 - 206 as well as 212 and 218 remained unique for blue wildebeest. In the black wildebeest population from Willem Pretorius nature reserve an allele 214 was found that did not occur in either of the reference populations.

Allele frequencies for the black wildebeest test populations (reserves in the Free State Province as well as private game farms) for the locus ETH10 did not reveal any blue wildebeest specific alleles for any of these populations at this locus. Several of the black wildebeest test populations did however possess alleles that are nominally specific to only blue wildebeest populations at the locus BM1824. Allele 212 was identified in several test populations but at very low frequencies, these populations (with frequencies in brackets) were: Maria Moroka (0.107), Willem Pretorius (0.005), Langkuil (0.025) Gariëpdam (0.029) and Geluk (0.011).

The allele frequency results obtained for the black wildebeest populations throughout the Free State province as well as the private game farms provided valuable data on the status of the black wildebeest on these reserves and farms. For the locus ETH10, none of the black wildebeest test populations screened possessed any blue wildebeest specific alleles. Several

black wildebeest test populations did however possess alleles classified as typical of blue wildebeest at the locus BM1824. Five populations were identified that possessed the allele 212 for the locus BM1824. These populations were, Maria Moroka, Willem Pretorius, Langkuil and Gariepdam Nature Reserves as well as the Geluk game farm. The presence of these putative blue wildebeest alleles in black wildebeest populations can be viewed as an indication that these populations contain hybrid individuals. There had been previous controversy regarding the status of the black wildebeest on the Maria Moroka Nature Reserve, with these animals possibly being hybrid (Kotze, unpublished results). In fact, translocations from this locality were stopped based on the likely presence of hybrids. The current study provided additional proof that introgression of blue wildebeest genetic material into this black wildebeest population may have occurred.

The presence of nominal blue wildebeest alleles for BM1824 in the Willem Pretorius group is problematic. Superficially, this can be seen as an indication of introgression from blue wildebeest. However, the management history of the reserve suggests that historical hybridization of this locality is unlikely. There are two plausible explanations for this question. First, it may be that hybrids are indeed present on Willem Pretorius, based on unrecorded events. Alternatively, this proves that the search of reference populations (and thus species-specific alleles) should again be widened, to also include Willem Pretorius. In this regard, it is notable that an allele at BM1824, not identified in any of the reference populations was found in this black wildebeest population. The possibility does exist that this previously unidentified allele were mis-sampled, which would indicate that the definition reference populations should be widened.

#### **2.5.2.4 Degree of genetic differentiation ( $F_{ST}$ ) and gene flow ( $Nm$ ) between the populations**

The  $F_{ST}$  values among all pair wise combinations of population were calculated and are listed in Table 2.10.

Differentiation among populations is dependent on the levels of gene flow between these populations. Therefore gene flow was inferred from the  $F_{ST}$  values using Equation 2.3. Gene flow among the various populations is also given in Table 2.10 below the  $F_{ST}$  values.

The level of genetic differentiation and gene flow among the three blue wildebeest populations ranged from 0.157 - 0.362 and 0.441 - 1.342, respectively. The highest  $F_{ST}$  value and were observed between the Kruger National Park and the Klaserie, Vaalwater and Musina pooled population. The average  $F_{ST}$  and gene flow values among the three blue wildebeest populations were 0.274 and 0.786, respectively.

Overall the genetic differentiation among the pure black wildebeest populations remained low, with a high level of gene flow observed between the pair-wise combinations.  $F_{ST}$  values for the three pure black wildebeest populations ranged from 0.100 - 0.217. The gene flow values among these populations ranged from 0.902 - 2.250. The highest level of genetic differentiation was observed between the Grootte Schuur and S.A. Lombard populations. Overall, the average value of genetic differentiation among the black wildebeest reference populations was 0.152 with an average gene flow among the populations of 1.563.

The genetic differentiation and gene flow among the black wildebeest test populations varied significantly. The genetic differentiation values ranged from 0 - 0.578, with gene flow ranging from 0 - 113.905. Overall the average  $F_{ST}$  and  $N_m$  values among all the black wildebeest test population were 0.144 and 5.238. The management history of these populations could have had an effect on these values. Numerous translocations took place among the Nature Reserves in the Free State Province leading to lower genetic differentiation and higher level of gene flow among these populations. However, the possibility of introgression of blue wildebeest genetic material could be the cause for the higher levels of genetic differentiation observed between the pair-wise combinations involving the Maria Moroka, Willem Pretorius, Lankuil, Gariepdam, Koppiesdam and Geluk populations.

Comparison of the average genetic differentiation among the pure black wildebeest and the black wildebeest test populations remained low with a value of 0.179. Average  $F_{ST}$  among the blue wildebeest and the black wildebeest test populations was similar to the average  $F_{ST}$  observed among the pure blue and pure black wildebeest, with 0.402 and 0.383, respectively.

Table 2.10:  $F_{ST}$  and  $N_m$  values among pairs of wildebeest populations

	CAL	TDR	MM	WP	LAN	GD	KD	ERFD	SFD	DB	SOE	RFD	RED	SKV	GEL	SAL	BEN	GS	KNP	KGAL	KVM
CAL	0																				
TDR	-0.007 Nm = 0*	0																			
MM	0.091 Nm = 2.503	0.057 Nm = 4.105	0																		
WP	0.094 Nm = 2.402	0.081 Nm = 2.847	0.091 Nm = 2.489	0																	
LAN	0.124 Nm = 1.769	0.075 Nm = 3.085	0.080 Nm = 2.868	0.136 Nm = 1.591	0																
GD	0.008 Nm = 33.083	0.005 Nm = 47.460	0.115 Nm = 1.926	0.040 Nm = 6.076	0.127 Nm = 1.713	0															
KD	0.216 Nm = 0.906	0.180 Nm = 1.142	0.190 Nm = 1.067	0.156 Nm = 1.348	0.105 Nm = 2.135	0.187 Nm = 1.087	0														
ERFD	0.051 Nm = 4.674	0.046 Nm = 5.201	0.071 Nm = 3.282	-0.026 Nm = 0*	0.130 Nm = 1.670	-0.003 Nm = 0*	0.140 Nm = 1.540	0													
SFD	0.036 Nm = 6.788	0.005 Nm = 51.403	0.017 Nm = 14.570	0.086 Nm = 2.657	0.053 Nm = 4.463	0.089 Nm = 2.572	0.187 Nm = 1.085	0.059 Nm = 3.997	0												
DB	0.002 Nm = 113.905	-0.009 Nm = 0*	0.036 Nm = 6.691	0.081 Nm = 2.847	0.102 Nm = 2.206	0.058 Nm = 4.073	0.221 Nm = 0.883	0.034 Nm = 7.136	-0.008 Nm = 0*	0											
SOE	0.057 Nm = 4.157	0.039 Nm = 6.123	0.089 Nm = 2.572	0.207 Nm = 0.958	0.183 Nm = 1.114	0.197 Nm = 1.022	0.350 Nm = 0.465	0.186 Nm = 1.094	0.040 Nm = 5.947	0.014 Nm = 17.972	0										
RFD	0.044 Nm = 5.492	0.072 Nm = 3.245	0.202 Nm = 0.988	0.169 Nm = 1.225	0.236 Nm = 0.810	0.031 Nm = 7.802	0.331 Nm = 0.505	0.190 Nm = 1.068	0.158 Nm = 1.330	0.110 Nm = 2.014	0.187 Nm = 1.090	0									
RED	0.088 Nm = 2.601	0.071 Nm = 3.273	0.119 Nm = 1.859	0.064 Nm = 3.640	0.082 Nm = 2.796	0.007 Nm = 34.472	0.082 Nm = 2.795	0.059 Nm = 4.008	0.073 Nm = 3.185	0.094 Nm = 2.398	0.229 Nm = 0.842	0.177 Nm = 1.162	0								
SKV	0.034 Nm = 7.118	0.046 Nm = 5.154	0.154 Nm = 1.368	0.082 Nm = 2.798	0.166 Nm = 1.257	0.207 Nm = 0*	0.064 Nm = 0.956	0.113 Nm = 3.648	0.085 Nm = 1.963	0.203 Nm = 2.677	0.019 Nm = 0.983	0.023 Nm = 12.992	0								
GEL	0.459 Nm = 0.295	0.395 Nm = 0.383	0.255 Nm = 0.732	0.436 Nm = 0.324	0.340 Nm = 0.484	0.532 Nm = 0.220	0.517 Nm = 0.233	0.457 Nm = 0.297	0.292 Nm = 0.606	0.364 Nm = 0.437	0.383 Nm = 0.403	0.578 Nm = 0.183	0.470 Nm = 0.282	0.540 Nm = 0.213	0						
SAL	0.173 Nm = 1.195	0.121 Nm = 1.816	0.111 Nm = 2.002	0.080 Nm = 2.875	0.044 Nm = 5.432	0.149 Nm = 1.428	0.185 Nm = 1.101	0.103 Nm = 2.177	0.098 Nm = 2.301	0.144 Nm = 1.486	0.266 Nm = 0.690	0.287 Nm = 0.621	0.097 Nm = 2.327	0.190 Nm = 1.066	0.360 Nm = 0.444	0					
BEN	0.078 Nm = 2.955	0.050 Nm = 4.750	0.117 Nm = 1.887	0.139 Nm = 1.549	0.030 Nm = 8.083	0.088 Nm = 2.591	0.070 Nm = 3.321	0.114 Nm = 1.943	0.066 Nm = 3.538	0.086 Nm = 2.657	0.175 Nm = 1.179	0.174 Nm = 1.187	0.036 Nm = 6.694	0.101 Nm = 2.225	0.449 Nm = 0.307	0.140 Nm = 1.536	0				
GS	0.280 Nm = 0.643	0.226 Nm = 0.856	0.188 Nm = 1.080	0.226 Nm = 0.856	0.095 Nm = 2.382	0.320 Nm = 0.531	0.026 Nm = 9.365	0.272 Nm = 0.669	0.194 Nm = 1.039	0.257 Nm = 0.723	0.372 Nm = 0.422	0.460 Nm = 0.293	0.154 Nm = 1.373	0.336 Nm = 0.494	0.457 Nm = 0.297	0.217 Nm = 0.902	0.100 Nm = 2.250	0			
KNP	0.466 Nm = 0.286	0.408 Nm = 0.363	0.311 Nm = 0.554	0.412 Nm = 0.357	0.390 Nm = 0.391	0.556 Nm = 0.200	0.517 Nm = 0.234	0.437 Nm = 0.322	0.352 Nm = 0.460	0.396 Nm = 0.381	0.462 Nm = 0.291	0.587 Nm = 0.176	0.446 Nm = 0.311	0.540 Nm = 0.213	0.432 Nm = 0.329	0.403 Nm = 0.370	0.455 Nm = 0.299	0.489 Nm = 0.261	0		
KGAL	0.290 Nm = 0.612	0.232 Nm = 0.828	0.163 Nm = 1.284	0.223 Nm = 0.871	0.163 Nm = 1.284	0.311 Nm = 0.554	0.252 Nm = 0.742	0.206 Nm = 0.964	0.163 Nm = 1.284	0.221 Nm = 0.881	0.320 Nm = 0.531	0.375 Nm = 0.417	0.154 Nm = 1.373	0.300 Nm = 0.583	0.344 Nm = 0.477	0.171 Nm = 1.212	0.204 Nm = 0.975	0.195 Nm = 1.032	0.157 Nm = 1.342	0	
KVM	0.545 Nm = 0.209	0.490 Nm = 0.260	0.403 Nm = 0.370	0.518 Nm = 0.233	0.466 Nm = 0.286	0.610 Nm = 0.160	0.590 Nm = 0.174	0.485 Nm = 0.265	0.430 Nm = 0.331	0.465 Nm = 0.288	0.515 Nm = 0.235	0.600 Nm = 0.167	0.500 Nm = 0.250	0.582 Nm = 0.180	0.478 Nm = 0.273	0.478 Nm = 0.245	0.505 Nm = 0.208	0.546 Nm = 0.208	0.362 Nm = 0.441	0.303 Nm = 0.575	0

### 2.5.2.5 Analysis of Molecular Variance (AMOVA)

Three different analyses of molecular variance were performed. For the first AMOVA analysis, only the pure blue and pure black populations were compared to determine the variation between the two species (Table 2.11). The results for this analysis revealed that 20.67% variation is found among pure blue and pure black wildebeest with the highest level of variation was found within the various reference populations (55.93%).

**Table 2.11: AMOVA results for pure blue and pure black wildebeest only**

Source of variation	Percentage of variation
Among species	20.67
Among populations within groups	23.40
Within populations	55.93

For the second AMOVA analysis the populations were divided into three groups where: Group 1 consisted of the black wildebeest reference populations; Group 2 consisted of the blue wildebeest reference populations and the populations grouped into Group 3 included all the remaining black wildebeest populations of unknown status. The results of this analysis are given in Table 2.12. The variation observed between the black wildebeest test populations was 16.50%. This supports the pair-wise  $F_{ST}$  results, which showed high levels of genetic differentiation among some of the black wildebeest test populations. The variation among species decreased compared to the first AMOVA analysis, after the inclusion of the black wildebeest test populations. This could be due to possible introgression of blue wildebeest genetic material into these test populations.

**Table 2.12: AMOVA results for the populations grouped according to test populations and the two separate black and blue wildebeest reference populations**

Source of variation	Percentage of variation
Among species	15.39
Among populations within groups	16.50
Within populations	68.11

For the final AMOVA analysis only black wildebeest populations were compared. Two groups were created, one consisted of pure black wildebeest and the other consisted of the pooled test populations (Table 2.13). Negligible variation was found between the pure black wildebeest and the black wildebeest test populations (-1.84%). The highest level of variation was still observed within the populations (82.7%). More variation can be found among the various black wildebeest test populations than among the test and pure black populations.

**Table 2.13: AMOVA results for the pure black wildebeest and pooled black wildebeest test population grouping**

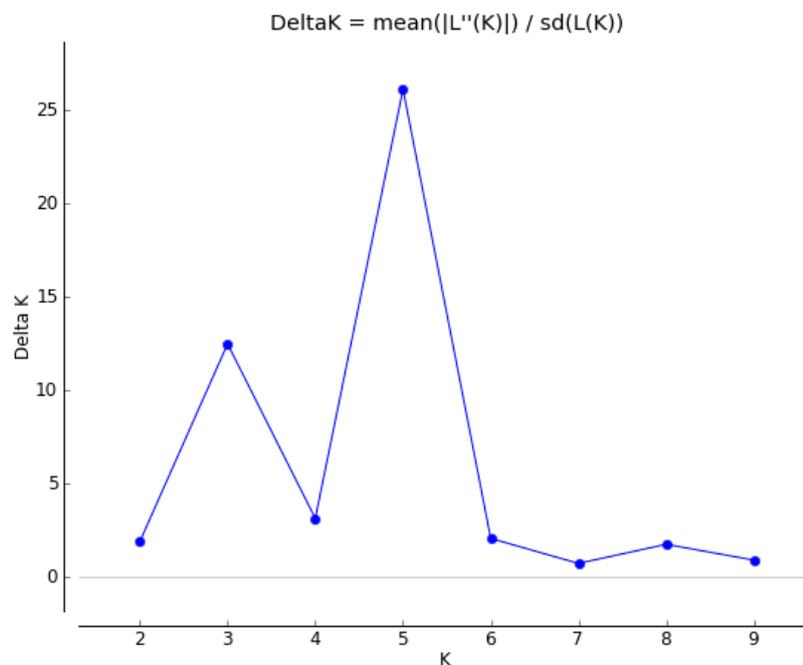
Source of variation	Percentage of variation
Between pure black and test black wildebeest populations	-1.84
Between black wildebeest test populations	19.14
Within populations	82.70

Overall the AMOVA results revealed that significant variation exists among the blue and black wildebeest reference populations and after inclusion of the black wildebeest test populations this value decreased slightly. This decrease could be an additional indication that nominally blue wildebeest alleles exist within some of the black wildebeest test populations.

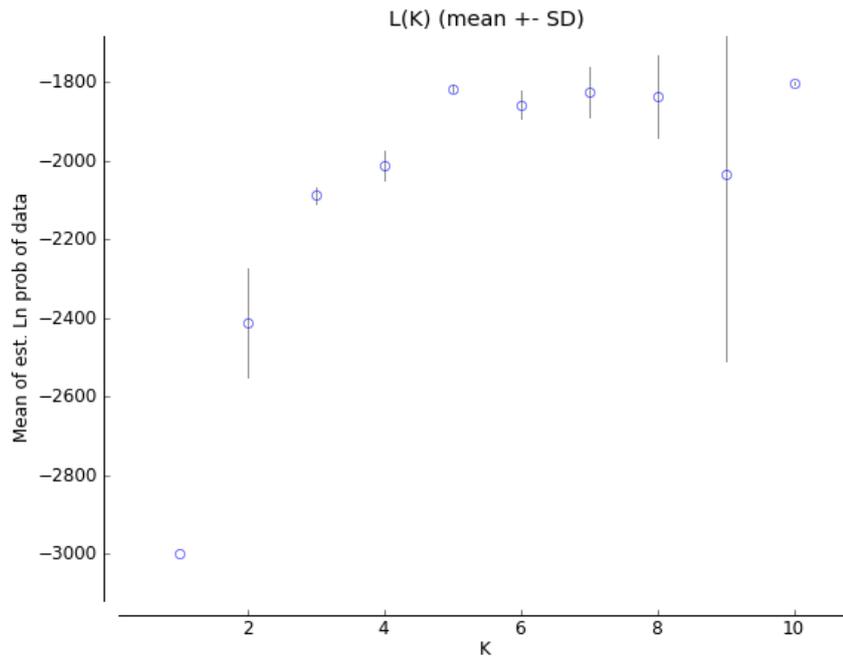
### 2.5.2.6 Individual assignment

Based on allele frequency results obtained for all populations, it was concluded that the locus ETH10 was not as informative as BM1824. Therefore the assignment tests were executed as two separate runs, with the first including results for both loci and the second run done using only data for BM1824. This was done to further assess the diagnostic power of the latter locus when used on its own. For all assignment tests, data obtained from the three black wildebeest and three blue wildebeest reference populations were combined to give just one black reference and one blue reference population (though STRUCTURE need not acknowledge such *prior* population boundaries).

Analysis of the genotyping results with both loci revealed that the most likely  $K$  value was  $K = 5$  as can be seen from the deltaK and mean  $-\ln Pr$  values over multiple runs, as presented in Figures 2.4 and 2.5.



**Figure 2.4: Plot for detecting the number of groups ( $K$ ) that best fit the data when both loci were considered. The peak observed at  $K = 5$  indicated that this is the true number of groups for this dataset (from STRUCTURE HARVESTER)**



**Figure 2.5: Plot of the mean likelihood  $L$  per  $K$  and variance per  $K$  value, obtained from STRUCTURE on the dataset containing 21 populations genotyped for two polymorphic loci. The graph includes standard deviation to display likelihood variance (from STRUCTURE HARVESTER)**

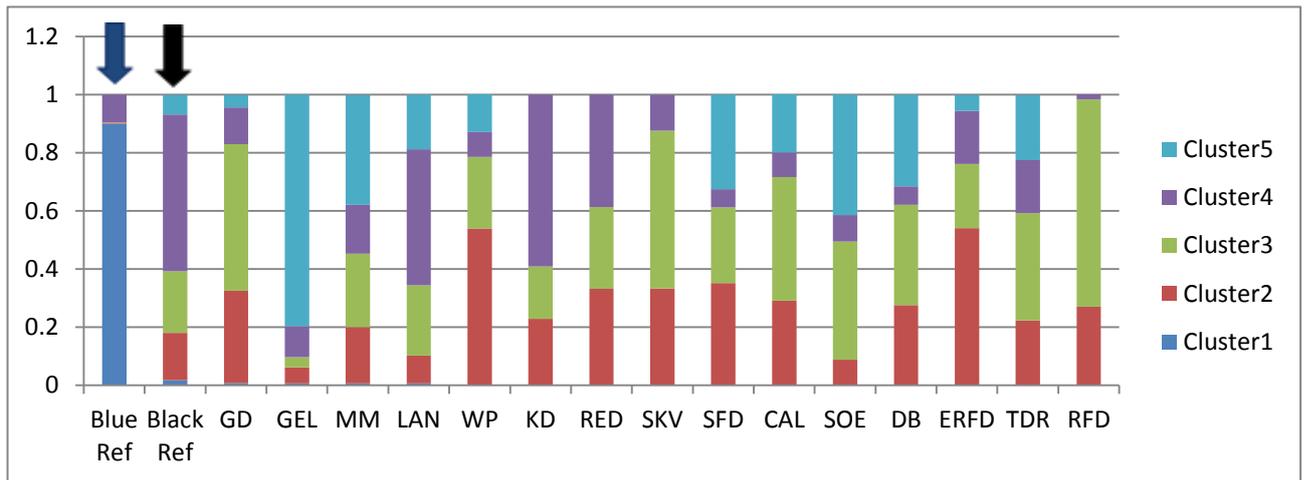
After the true number of populations were determined, a second run was executed, with a burn-in period of 100,000 steps followed by 1,000,000 MCMC iterations and with  $K = 5$ . Results obtained for  $K = 5$  are presented in Table 2.14. The blue wildebeest reference populations were grouped in a distinct cluster completely separate from all the black wildebeest populations, with a very high probability of 90%. The assignment for the black wildebeest reference population were however not as unambiguous. Individuals of this population shared similarities with all five of the identified clusters, but with the highest probability of belonging to cluster 4, with a value of 53.9% and only 1.8% assignment to the cluster dominated by blue wildebeest. Most of the black wildebeest populations from the various nature reserves and game farms were also assigned to all cluster, but again with insignificant assignment to the cluster dominated by blue wildebeest (0 - 0.8%). The Willem Pretorius, Erfenisdam and Sterkfontein Nature Reserve populations all grouped together with the highest probability of belonging to cluster 2. The Caledon, Tussen-die-Riviere, Gariepdam, De Brug, Rustfontein and Seekoeivlei Nature Reserve populations were assigned to cluster 3 with the strongest probabilities. The Langkuil, Koppiesdam and Reddersburg populations were assigned to the same cluster as the black wildebeest reference

population, with average probabilities ranging between 38.7% and 59.1%. The final cluster consisted of the Maria Moroka, Soetdoring and Geluk samples, with levels of assignment ranging from 37.8 - 79.6%, with the highest level observed for the Geluk population.

**Table 2.14: Proportion of membership of each pre-defined population to each of the five clusters, using data from both loci. The highest probability of belonging to a specific cluster, for each of the populations, is indicated in red**

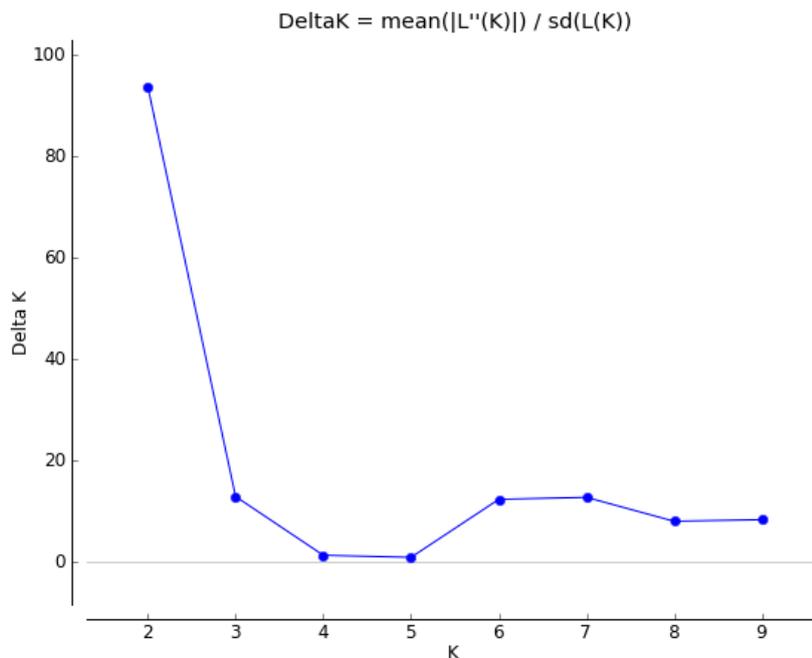
Populations	Inferred clusters				
	1	2	3	4	5
CAL	0.002	0.289	<b>0.425</b>	0.085	0.200
TDR	0	0.223	<b>0.369</b>	0.183	0.224
MM	0.007	0.193	0.253	0.168	<b>0.378</b>
WP	0.003	<b>0.536</b>	0.246	0.086	0.130
LAN	0.007	0.096	0.241	<b>0.468</b>	0.188
GD	0.008	0.317	<b>0.504</b>	0.127	0.043
KD	0.003	0.226	0.180	<b>0.591</b>	0
ERFD	0.001	<b>0.540</b>	0.220	0.183	0.055
SFD	0.002	<b>0.350</b>	0.260	0.063	0.325
DB	0.001	0.275	<b>0.345</b>	0.063	0.316
SOE	0.001	0.087	0.406	0.092	<b>0.414</b>
RFD	0	0.271	<b>0.712</b>	0.018	0
RED	0.003	0.329	0.281	<b>0.387</b>	0
SKV	0.003	0.330	<b>0.542</b>	0.124	0
GEL	0.007	0.055	0.035	0.107	<b>0.796</b>
<b>Black Ref</b>	0.018	0.163	0.211	<b>0.539</b>	0.068
<b>Blue Ref</b>	<b>0.900</b>	0.003	0.001	0.096	0

The graphical representation (Figure 2.6) of the assignment of the populations is inconclusive. The dark blue zone found in the blue wildebeest reference populations probably signify that some alleles are more predominant in blue wildebeest than in the pure black wildebeest reference material. The presence of these alleles in the black wildebeest populations is almost negligible. Similarly, the black reference (and test) populations contained several bands not observed in blue wildebeest. The black wildebeest populations are characterized by different patterns of assignment to different clusters that most likely reflect geographic and management-based differences. There was however no clear indication of introgression of blue material into black test populations.

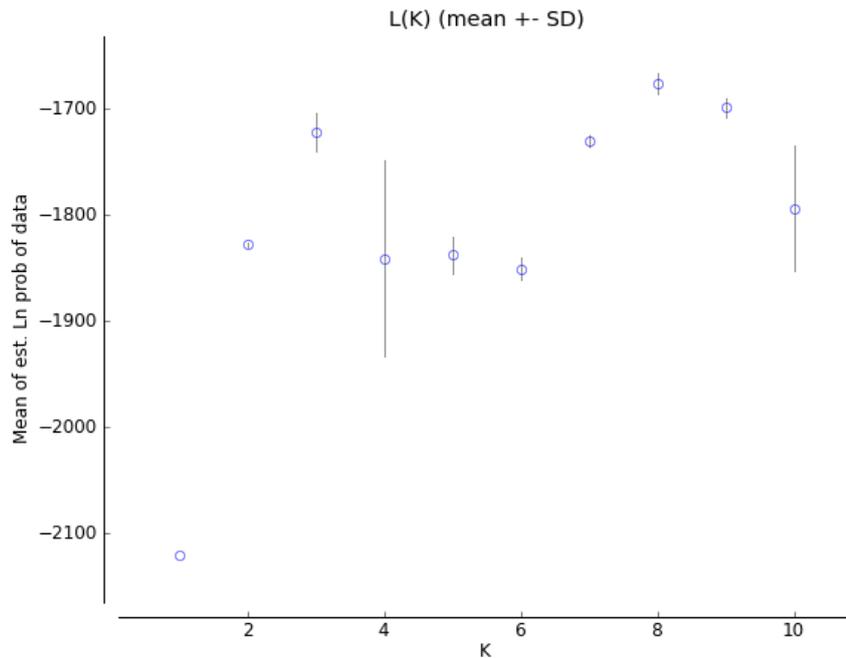


**Figure 2.6: A graphical representation of the assignment results for five clusters, with data from both loci**

The second analysis, based on the genotyping data for locus BM1824 only, suggested that the most likely  $K$  value was  $K = 2$  as illustrated in Figures 2.7 and 2.8.



**Figure 2.7: Plot for detecting the number of groups ( $K$ ) that best fit the data. Only data for the locus BM1824 was included (from STRUCTURE HARVESTER)**



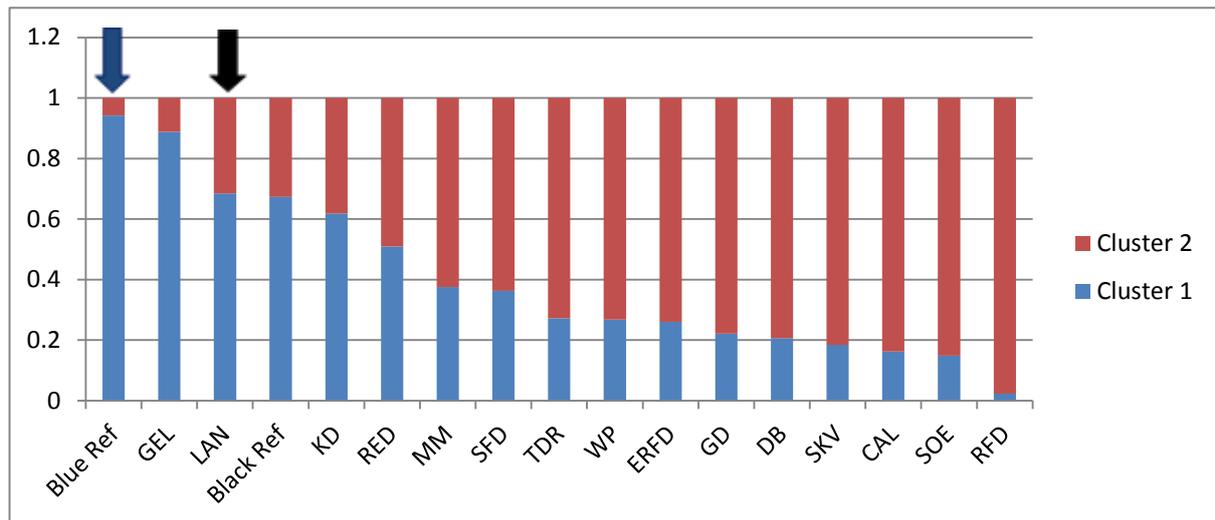
**Figure 2.8: Plot of the mean likelihood  $L(K)$  and variance per  $K$  value obtained from STRUCTURE on the dataset containing 21 populations genotyped for only the locus BM1824 (from STRUCTURE HARVESTER)**

Assignment of the populations (Table 2.15) to each of the two clusters revealed that the blue and black wildebeest reference populations were grouped in the same cluster, which limits the use of the data for further analysis. The black wildebeest reference population did however share 32.7% similarity with the second cluster (Cluster 2). The majority of the black wildebeest populations screened in the Free State Province and surrounding areas grouped together in Cluster 2. Four populations, Langkuil, Koppiesdam, Reddersburg and the Geluk, grouped with the two reference populations in the first cluster.

**Table 2.15: Proportion of membership for each pre-defined population in each of the two clusters, using data obtained from the locus BM1824. The highest probability of belonging to a specific cluster, for each of the populations, is indicated in red**

Populations	Inferred Clusters	
	1	2
<b>CAL</b>	0.162	<b>0.838</b>
<b>TDR</b>	0.272	<b>0.728</b>
<b>MM</b>	0.376	<b>0.624</b>
<b>WP</b>	0.268	<b>0.732</b>
<b>LAN</b>	<b>0.685</b>	0.315
<b>GD</b>	0.222	<b>0.778</b>
<b>KD</b>	<b>0.618</b>	0.382
<b>ERFD</b>	0.261	<b>0.739</b>
<b>SFD</b>	0.363	<b>0.637</b>
<b>DB</b>	0.207	<b>0.793</b>
<b>SOE</b>	0.149	<b>0.851</b>
<b>RFD</b>	0.023	<b>0.977</b>
<b>RED</b>	<b>0.510</b>	0.490
<b>SKV</b>	0.185	<b>0.815</b>
<b>GEL</b>	<b>0.888</b>	0.112
<b>Black Ref</b>	<b>0.673</b>	0.327
<b>Blue Ref</b>	<b>0.942</b>	0.058

From the patterns observed in Figure 2.9 it can be concluded that there is a significant amount of shared alleles between these two species.



**Figure 2.9: A graphical representation of the assignment of all the populations to two different clusters, using data from only BM1824**

The individual assignment of the animals was also done with the use of GeneClass software. Individuals (black wildebeest test populations) were compared to the two reference populations and assigned to the most probable group (see Appendix E for the complete set of results per individual animal). It was again found that in several populations, individuals had partial membership in more than one of the two assigned populations (black wildebeest and blue wildebeest). The majority of the test animals were however assigned to the black wildebeest cluster. Three animals in the Maria Moroka population were assigned to the blue wildebeest cluster with very low probabilities. Similar situations were encountered for the Willem Pretorius, Langkuil and Geluk populations, with two individual from Willem Pretorius, one from Langkuil and two from the Geluk population being assigned to the blue wildebeest cluster. Genotypic data did reveal the presence of nominally blue wildebeest, at low frequencies, in these black wildebeest test populations. The presence of these alleles even though at low frequencies attributed to the assignment of these individuals to the blue wildebeest cluster. Two possible explanations exist, either hybrids are present in these populations or these alleles identified as nominally blue wildebeest alleles, are indeed shared between the two species. The latter scenario would mean that the definition reference populations should be widened.

# CHAPTER THREE: APPLICATION OF MOLECULAR TECHNIQUES TO A KNOWN HYBRID POPULATION

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## 3.1 Application of molecular techniques

A confirmed hybrid population was used to determine the effectiveness with which the two microsatellite markers described in Chapter 2 can identify hybrid animals. Two hybrid male black wildebeest were identified on a privately owned game farm, Perdeberg, in the western Free State Province. These animals were identified based on external morphological characteristics (see Figure 3.1), with the most distinctive characteristic being the atypical shape of the horns. The horns of these two individuals turned downward for the first third of their length, similar to that of the black wildebeest, and then curled outwards, like that of the blue wildebeest. The ease of morphological identification suggests that these were  $F_1$  hybrids.



**Figure 3.1: Two  $F_1$  black wildebeest hybrids (Photo by Prof. J.P. Grobler)**

The two hybrid male individuals (labelled Male 1 and Male 2) along with other putative pure black wildebeest cows were culled and the samples sent to the Department of Genetics,

University of the Free State, for DNA analysis. An embryo was also retrieved from one of the female black wildebeest individuals during processing, and sampled. It was unfortunately not known to which of the female animals the embryo belonged.

### **3.2 Molecular analysis**

DNA was isolated from the samples using the High Pure PCR Preparation kit by Roche Applied Science<sup>1</sup> (see Chapter 2 for the complete protocol). In the case of the embryo, DNA was extracted from the embryo body as well as the umbilical cord. After extraction, fragment analysis was performed with the two microsatellite markers ETH10 and BM1824. The genotypes of all individuals sampled were then scored. All methods for fragment analysis were similar to methods described in Chapter 2. Possible introgression of blue wildebeest alleles into a black wildebeest population was studied using the classification established in Chapter 2, using alleles classified as “black”, “blue” or shared.

### **3.3 Results**

Fragment analysis of the Perdeberg samples revealed the presence of expected species-specific alleles in black wildebeest individuals, but also alleles specific to blue wildebeest (based on the results reported in Chapter 2) in this black wildebeest population (Table 3.1)

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<sup>1</sup> Roche is a registered trademark of Roche Diagnostics GmbH, Mannheim Germany

**Table 3.1: Alleles found in individuals from the Perdeberg black wildebeest population (alleles highlighted in blue and black were previously only identified in blue and black wildebeest respectively, with unshaded alleles considered shared alleles)**

Samples	Marker			
	ETH10		BM1824	
Male 1	203	209	198	206
Male 2	205	211	198	206
Female 1	203	203	194	194
Female 2	205	205	194	198
Female 3	203	203	194	198
Female 4	-	-	194	194
Embryo	203	209	198	206
Umbilical cord	203	209	198	206

The first known hybrid male individual (Male 1) possessed no blue wildebeest specific alleles at the locus ETH10. However for the locus BM1824, the allele 206 was found, which was only found in blue wildebeest populations before. The second hybrid male individual (Male 2) possessed allele 211 for the locus ETH10; this allele was previously only found in blue wildebeest populations. Similarly, allele 206 for the locus BM1824 that was previously only found in blue wildebeest populations was found in this individual. None of the female animals possessed any introgressed blue wildebeest alleles at either of the two loci. However, for the embryo, a blue wildebeest specific allele (206) was detected at the locus BM1824. Results for the locus ETH10 in the embryo did not indicate introgression of blue wildebeest genetic material. Results for the umbilical cord mirrored that of the embryo, which was expected since the umbilical cord of mammals is not of parental origin.

Scrutiny of alleles present in all individuals suggests that the embryo most likely inherited allele 203 at the locus ETH10 from Female 3 and allele 209 from Male 1. At the locus BM1824, allele 198 could have been inherited from the latter female and 206 from the same known hybrid male (Figure 3.2).

Male 1			Female 3		
Locus	Alleles		Locus	Alleles	
ETH 10	203	209	ETH 10	203	203
BM 1824	198	206	BM 1824	194	198

Embryo		
Locus	Alleles	
ETH 10	203	209
BM 1824	198	206

**Figure 3.2: Most plausible paternity of the embryo sampled (alleles highlighted in blue and black were previously only identified in blue and black wildebeest respectively, with unshaded alleles considered shared alleles)**

### 3.4 Discussion

Results of this small scale trial study show that the markers identified in Chapter 2 have significant potential to identify early generation hybrids ( $F_1$  and  $F_2$ ). The presence of an allele specific to blue wildebeest was found in this black wildebeest population for the locus ETH10. Similarly, an allele previously only found in blue wildebeest was also identified in this population at the locus BM1824. The detection of introgression of blue wildebeest genetic material in two known hybrid male animals, provide valuable insight into the ability of these two microsatellite markers to positively and accurately identify  $F_1$  hybrids.

No blue wildebeest specific alleles were detected in the female black wildebeest individuals, though with such a small number of markers, the latter individuals cannot be presumed to be pure. Nevertheless, the fact that nominally blue alleles were found in both the evidently hybrid males and the embryo is a strong indication that the embryo was a second generation hybrid. The ability to detect backcrossed hybrid individuals with molecular techniques is critical, since identification of hybrids beyond the first generation is no longer possible with the use of external morphological characteristics. The identification of backcrossed individuals and later generation hybrids can provide useful information for conservation

purposes, such as the recovery of parental individuals from hybrid swarms by removing the hybrid animals (Allendorf *et al.*, 2001).

The amount of markers recommended for the determination of the hybrid status of individuals vary, since fewer markers are needed when attempting to separate F<sub>1</sub> hybrids from parental taxa compared to situations where advanced backcrosses are involved. According to Boecklen and Howard (1997), four to five markers will be sufficient when coarsely classifying individuals into parental, F<sub>1</sub> and simple backcrosses. Although the classification of the animals in the Perdeberg populations was done with only two markers, the results revealed introgression of blue wildebeest genetic material in this instance. Not only did this case study provide proof of the ability of these microsatellite markers to positively identify F<sub>1</sub> hybrids, but also that the probability of identifying second generation hybrids with these loci is plausible. However, note that the results obtained reveal only one possible outcome of the independent assortment that accompanied meiosis in these specific animals. The embryo may equally have received only black alleles at the locus BM1824, resulting in the genotype 194/198 or 198/198. That would have resulted in an individual that is a known hybrid; yet this status would have escaped molecular screening at these loci. It is thus highly recommended that further analysis of hybrid animals should be done with additional microsatellite markers or alternative molecular techniques.

# CHAPTER FOUR: SIMULATION STUDY

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## 4.1 Introduction

Simulation studies can be used for a number of purposes in conservation genetics, in cases where individual based data are unavailable. For example, it can be applied to screen for hybridization, to test whether such hybridization was natural or of anthropogenic origin (Nielsen *et al.*, 2006).

When analysing data with simulation software, questions arise on to the amount of samples and loci necessary to obtain reliable results from these models. These questions can be addressed by simulating multilocus genotype data from specific allele frequencies and testing the inferences from programs such as STRUCTURE (Pritchard *et al.*, 2000) and NEWHYBRIDS (Anderson and Thompson, 2002). A simulation approach can be implemented for specific data sets for a case-by-case evaluation of the statistical power for correctly identifying the status of an individual as purebred or hybrid (Nielsen *et al.*, 2006).

Widely available software such as HYBRIDLAB (Nielsen *et al.*, 2006) can be used to create artificial parental and hybrid genotypes. This program will first estimate allele frequencies at each locus of the parental populations and then create the F<sub>1</sub> hybrids. The F<sub>1</sub> hybrid genotypes are created by randomly drawing one allele at a locus from each of the two hybridizing populations (as defined by the user). The options provided by this program are not limited to creating F<sub>1</sub> genotypes; further backcrosses can easily be generated. For example if a F<sub>2</sub> backcross is required, the output of the F<sub>1</sub> hybrids can be used in combination with one of the parental populations (Nielsen *et al.*, 2006). After the simulation of the various backcrosses, the persistence of introgression of hybrid alleles can be tested.

## 4.2 Case studies

This section highlights the potential application of simulation studies in conjunction with other statistical software for hybrid identification and conservation management.

*Individual admixture analysis of Atlantic cod (Gadus morhua)*

Nielsen *et al.*, (2003) used HYBRIDLAB to evaluate the parental or hybrid origin of individual Atlantic cod (*Gadus morhua*) in a transition area between the North Sea and Baltic Sea. Intermediate allele frequencies were found in this transition area and these authors estimated the most probable number of populations present and the admixture proportions.

Admixture proportions obtained from STRUCTURE identified a large number of hybrid genotypes. HYBRIDLAB was used to create two simulated samples of equal size compared to that found in the real transition area. One of the simulated samples consisted of a mixture of North Sea and Baltic Sea parental genotypes and the other consisted of a simulated hybrid swarm. The simulated data sets clearly showed that the hybrid swarm scenario matched the results obtained for the real populations. The authors were able to conclude that a hybrid zone was the most probable explanation of the cod population structure in that area (Nielsen *et al.*, 2003).

*Identification of individuals of hybrid origin in Australian bass (Macquaria novemaculeata)*

Breeding programmes have become a common practice in the management of declining species that are of economical importance. These breeding programmes need to ensure that the breeders do not include hybrid individuals, in order to maintain the genetic integrity of the species. Schwartz and Beheregaray (2008) researched the use of simulations and Bayesian analyses with molecular data to detect hybrids for exclusion from breeding programmes and to determine if introgression is taking place in hybridizing bass species. The two species involved in this study were Australian bass (*M. novemaculeata*) and the estuary perch (*M. colonorum*). These two species are related and there is a large overlap in their distributional ranges. Population numbers of the Australian bass declined drastically due to construction of dams and weirs on coastal streams. As a result, the stocking of waterways upstream of these dams and weirs with hatchery produced fish has been a common management response. Identification of hybrids between these two species is of great conservation concern. Morphologically these two species are extremely difficult to distinguish, therefore a very accurate and rapid method of hybrid identification is needed to avoid using individuals of hybrid origin as breeders in hatchery programmes. This study made use of microsatellite

markers specifically developed for Australian bass to identify hybrid individuals and more specifically pure individuals that can be used in breeding programmes. Statistical analysis of the data used a combination of methods, including a Bayesian clustering method to identify pure species from individuals with hybrid origin as well as a simulation approach to evaluate the effectiveness of the Bayesian method in identifying various classes of hybrids (Schwartz and Beheregaray, 2008).

A total of 119 individuals were included in the study - this consisted of 89 possible hybrids, 10 putative pure bass and 10 putative pure perch. An additional 10 purebred individuals from hybrid free areas were also included, six of the 10 were pure bass and four were pure perch. The ancestry of the individual fish was determined by using the model-based clustering method implemented in STRUCTURE. The genotyped fish were assigned to two species groups, bass or perch. The pure bass and pure perch individuals were then used as controls to evaluate the accuracy of the purebred assignment; therefore all 119 individuals were classified as “unknown” before analysis (Schwartz and Beheregaray, 2008).

Genotypes were simulated, using HYBRIDLAB, for 500 individuals in each parental population, using the fish that grouped with the known bass and perch in an analysis by STRUCTURE. The parental populations were then used to simulate backcrosses to each species. The simulated dataset was analysed using STRUCTURE, to determine the  $q$ -value ranges for each hybrid class (Schwartz and Beheregaray, 2008).

The  $q$ -values obtained for the simulated F1 hybrids were clearly distinct from the simulated parental ranges. These authors found that overall, 86% of the simulated genotypes with hybrid origin, in the last three generations, could clearly be distinguished from the perch- and bass-simulated parental populations. This study demonstrated that the use of microsatellite markers in conjunction with various statistical tests could discriminate between Australian bass and estuary perch, detect interspecific hybrids as well as assess the levels of introgression. It also highlighted the value of simulation of genotypes for predicting the probability to distinguish between backcrossed and purebreds (Schwartz and Beheregaray, 2008).

*Interbreeding between Mediterranean brown trout (Salmo trutta) and hatchery fish*

A similar study by Sanz *et al.*, (2009) tested the efficiency of different methods and markers (allozymes and microsatellite markers) to assess introgression. Interbreeding between Mediterranean and hatchery brown trout (*Salmo trutta*) provided valuable source material for their study. Data sets were simulated based on parental data, using HYBRIDLAB software. Parental populations for pure brown trout and pure hatchery fish were simulated. Simulated genotypes were also generated for F<sub>1</sub>, F<sub>2</sub> and backcrosses of F<sub>1</sub> with parental brown trout and parental hatchery fish. The simulated data was then used to carry out admixture analysis using STRUCTURE and NEWHYBRID. The efficiency of these methods was evaluated based on the proportion of hybrids correctly identified (Sanz *et al.*, 2009).

STRUCTURE correctly identified the majority of simulated hybrid individuals, with both marker types used. A very small proportion of parental hatchery individuals could not be distinguished. Similar results were obtained for NEWHYBRIDS; however, a proportion of F<sub>2</sub> hybrids could not be identified. The results obtained from the simulated data indicated that allozyme and microsatellite genotyping could identify hybrids and introgression at similar efficiencies with the different statistical methods used (Sanz *et al.*, 2009).

**4.3 Simulation study for wildebeest**

Two wildebeest datasets, with genotypic data for two and five markers respectively, were used to simulate hybrid individuals. The studies were designed to mimic as much as possible real life hybridization events that can take place on game farms and nature reserves where both species are kept. Various scenarios were created with the use of simulated data sets, as outlined below. The purpose of these simulation studies was to determine the long term outcome of hybridization events and the persistence of introgression of blue wildebeest genetic material into black wildebeest populations.

*Simulation study 1:*

A random sample of ten blue wildebeest and 50 black wildebeest individuals were selected from the original reference populations (see Chapter 2), to serve as parental populations in the simulation study. Random animals were selected using a function of POPTOOLS (Hood, 2010) that allows the user to select a random sample from a specific dataset. These specific numbers potentially mimic many hybridization events on game farms or reserves. HYBRIDLAB was used to simulate first generation hybrids between these two parental populations. The simulated F<sub>1</sub> hybrids were then combined with the blue and black wildebeest parental populations and backcrossed with the same combination (combination F<sub>1</sub> and parental populations), this was done to model a scenario where blue and black wildebeest are kept in close proximity and allowed to interbreed without intervention. The resulting second generation animals were allowed to interbreed and this process continued up to the tenth generation. In each of these generations, a total of 50 animals were generated.

*Simulation study 2*

The same parental populations created in the first simulation study were used and HYBRIDLAB was again used to simulate first generation hybrids between these two parental individuals. However, the simulated F<sub>1</sub> hybrids were then only combined with the black wildebeest parental population for further backcrossing. This simulates a hybridization event where managers become aware of the problem and intervenes by removing the original pure individuals. The same backcrosses as described in the first simulation study were then made, until tenth generation backcrosses were obtained.

*Simulation study 3*

The dataset used by Grobler *et al.* (2005) were analysed, to determine if an increased amount of loci would have a significant effect. These authors made use of five microsatellite markers, BM1824, BM2113, CSSM36, ETH10 and TGLA53. The reference populations for blue and black wildebeest used in the study by Grobler *et al.* (2005) were used to represent the parental populations in this simulation study. These authors made use of smaller reference population for their study, therefore less animals were selected for use in the simulation. A random sample of five blue wildebeest and 20 black wildebeest individuals were selected

from their reference populations using POPTOOLS. HYBRIDLAB was used to simulate first generation hybrids between these two parental populations. The simulated F<sub>1</sub> hybrids were then mixed with the blue and black wildebeest parental animals and backcrossed with the same combination (combination F<sub>1</sub> and parental populations), similar to the first simulation study. The resulting second generation animals were allowed to interbreed and this was continued up to the tenth generation. For each generation a total of 50 animals were generated.

#### *Simulation study 4*

The same scenario as described for simulation study 2 was repeated, using the multilocus dataset of Grobler *et al.* (2005).

Allele frequencies for the first, fifth and tenth generation hybrids were calculated using Microsatellite Toolkit (Park, 2001) and compared to the reference populations.

### **4.3.1 Results and discussion**

#### *Simulation study 1*

The allele frequencies calculated revealed that the nominally blue wildebeest alleles (identified in Chapter 2) were present at relatively high frequencies in the first generation hybrids, all of the blue wildebeest specific alleles were present in this generation (Table 4.1). After five generations of backcrosses these alleles could still be detected, however some of the nominally blue alleles were no longer present in this backcrossed generation. A slight decrease in the frequency of the blue wildebeest alleles were observed between the first and fifth generation hybrids. The frequency of the nominally blue wildebeest alleles in the fifth generation backcrosses were relatively low, except for allele 211 at the locus ETH10 and allele 206 for the locus BM1824 - these alleles occur in 16.7% and 26% of the fifth generation backcrossed individuals. The frequency of the blue alleles decreased slightly between the fifth and tenth generation, with some of the alleles still present at high frequencies, such as allele 206 at the BM1824 locus. It should be noted that for this simulation study the blue wildebeest individuals were kept in the simulated population and

allowed to interbreed again to obtain the second generation, this would have influenced the persistence and frequency of the blue wildebeest alleles in the tenth generation.

**Table 4.1: Allele frequencies for simulated first, fifth and tenth generation hybrid individuals obtained from simulation study 1. Included in this table are the allele frequencies for the blue and black reference populations (alleles highlighted in black and blue are specific to pure black and blue wildebeest, with unshaded alleles considered shared alleles)**

Markers	Alleles	Black reference	Blue reference	F1	F5	F10
<b>ETH10</b>	<b>203</b>	<b>0.059</b>	-	<b>0.020</b>	-	-
	<b>205</b>	0.922	0.281	0.490	0.708	0.802
	<b>209</b>	0.020	0.305	0.240	0.125	0.063
	<b>211</b>	-	0.415	0.250	0.167	0.135
<b>BM1824</b>	<b>178</b>	-	0.205	0.010	-	-
	<b>180</b>	-	0.136	0.094	0.050	0.100
	<b>192</b>	0.365	0.057	0.208	0.180	0.080
	<b>194</b>	0.289	0.011	0.177	0.220	0.260
	<b>196</b>	<b>0.048</b>	-	<b>0.021</b>	<b>0.090</b>	<b>0.100</b>
	<b>198</b>	0.202	0.011	0.042	0.080	0.060
	<b>200</b>	0.067	0.114	0.063	0.010	0.020
	<b>202</b>	-	0.102	0.115	0.060	0.010
	<b>204</b>	-	0.102	0.052	-	-
	<b>206</b>	-	0.182	0.146	0.260	0.310
	<b>210</b>	0.030	0.034	0.021	0.050	0.06
	<b>212</b>	-	0.034	0.052	-	-
	<b>218</b>	-	0.011	0.010	-	-

#### *Simulation study 2*

The allele frequencies calculated for this simulation study revealed a slight decrease in the overall frequency of nominally blue wildebeest alleles in the fifth generation compared to first generation hybrids (Table 4.2). After five generations of backcrossing blue alleles 212 and 218 at the locus BM1824, were no longer present in the hybrid populations. Even though the overall frequency of the blue wildebeest alleles in the fifth generation backcrossed individuals decreased slightly, some alleles were still present at high frequencies. Allele 211 at the locus ETH10 was found to persist at a frequency of 29%. For the locus BM1824 allele

206 was still found in 10% of the backcrossed individuals. Allele frequency results for the tenth generation revealed a similar trend, however a significant amount of blue alleles found in the first and fifth generation hybrids were no longer present after successive backcrosses. This is a good simulation of the persistence of introgressed alleles despite the removal of blue wildebeest.

**Table 4.2: Allele frequencies for simulated first, fifth and tenth generation hybrid individuals obtained from simulation study 2. Included in this table are the allele frequencies for the blue and black reference populations (alleles highlighted in black and blue are specific to pure black and blue wildebeest, with unshaded alleles considered shared alleles)**

Markers	Alleles	Black reference	Blue reference	F1	F5	F10
<b>ETH10</b>	<b>203</b>	<b>0.059</b>	-	<b>0.020</b>	-	-
	<b>205</b>	0.922	0.281	0.520	0.606	0.725
	<b>209</b>	0.020	0.305	0.214	0.106	0.092
	<b>211</b>	-	0.415	0.245	0.287	0.184
<b>BM1824</b>	<b>178</b>	-	0.205	0.010	0.010	-
	<b>180</b>	-	0.136	0.092	0.010	-
	<b>192</b>	0.365	0.057	0.204	0.260	0.210
	<b>194</b>	0.289	0.011	0.194	0.208	0.290
	<b>196</b>	<b>0.048</b>	-	<b>0.020</b>	<b>0.010</b>	-
	<b>198</b>	0.202	0.011	0.041	0.167	0.170
	<b>200</b>	0.067	0.114	0.051	0.073	0.060
	<b>202</b>	-	0.102	0.112	0.073	0.080
	<b>204</b>	-	0.102	0.051	0.042	0.050
	<b>206</b>	-	0.182	0.143	0.104	-
	<b>210</b>	0.030	0.034	0.020	0.042	0.140
	<b>212</b>	-	0.034	0.061	-	-
	<b>218</b>	-	0.011	0.010	-	-

*Simulation study 3*

The dataset used for this simulation study included more loci to provide additional information on the persistence of introgression over ten generations. Comparison of the allele frequencies obtained from the hybrid individuals and the pure populations showed that a considerable amount of nominally blue wildebeest alleles occurred in the hybrid populations, especially the first and fifth generation hybrids (Table 4.3). Allele frequency results for the tenth generation hybrids showed a decrease in the amount and frequency of the blue wildebeest alleles at most of the microsatellite markers compared to the first generation hybrids. However, between the fifth and tenth generation no significant difference in the amount and frequency of nominally blue alleles, were observed.

The presence of the blue alleles after the various backcrosses could be due to the fact that, similar to simulation study 1, the blue wildebeest were kept in the simulated population and allowed to interbreed again to obtain the second generation. This allowed for an additional stage of introgression of blue wildebeest alleles into the simulated hybrid population. Black wildebeest specific alleles and alleles shared between the two species were however still the predominant alleles in the simulated hybrid population.

**Table 4.3: Allele frequencies for simulated first, fifth and tenth generation hybrid individuals obtained from simulation study 3. Included in this table are the allele frequencies for the blue and black reference populations obtained from Grobler *et al.*, (2005) (alleles highlighted in black and blue are specific to pure black and blue wildebeest, with unshaded alleles considered shared alleles)**

Markers	Alleles	Black reference	Blue reference	F1	F5	F10
<b>BM2113</b>	<b>131</b>	1.000	0.667	0.670	0.720	0.640
	<b>133</b>	-	0.067	0.050	0.050	0.070
	<b>135</b>	-	0.167	0.210	0.210	0.250
	<b>141</b>	-	0.067	0.020	-	-
	<b>143</b>	-	0.033	0.050	0.020	0.040
<b>ETH10</b>	<b>203</b>	0.969	-	0.450	0.260	0.560
	<b>205</b>	0.031	-	0.050	0.100	0.050
	<b>209</b>	-	0.767	0.400	0.330	0.270
	<b>211</b>	-	0.233	0.100	0.310	0.120
<b>BM1824</b>	<b>180</b>	-	0.400	0.280	0.210	0.350
	<b>194</b>	0.047	-	0.030	0.040	0.050
	<b>196</b>	0.391	-	0.220	0.300	0.200
	<b>200</b>	0.563	-	0.250	0.330	0.340
	<b>202</b>	-	0.167	-	-	-
	<b>206</b>	-	0.267	0.220	0.120	0.060
	<b>210</b>	-	0.033	-	-	-
	<b>212</b>	-	0.100	-	-	-
	<b>218</b>	-	0.033	-	-	-
<b>TGLA53</b>	<b>150</b>	-	0.267	0.060	0.070	0.050
	<b>152</b>	0.219	-	0.100	0.120	0.160
	<b>154</b>	0.281	0.267	0.320	0.340	0.410
	<b>156</b>	0.297	0.100	0.100	0.160	0.150
	<b>158</b>	0.016	0.200	0.220	0.230	0.230
	<b>160</b>	0.188	-	0.090	-	-
	<b>162</b>	-	0.100	0.070	0.080	-
	<b>166</b>	-	0.033	0.040	-	-
	<b>168</b>	-	0.033	-	-	-
<b>CSSM36</b>	<b>156</b>	0.672	-	0.350	0.480	0.370
	<b>166</b>	0.078	-	0.09	0.100	0.290
	<b>172</b>	-	0.500	0.350	0.300	0.300
	<b>174</b>	-	0.100	0.030	-	-
	<b>176</b>	0.219	-	0.050	0.070	0.020
	<b>178</b>	0.031	-	0.010	0.010	-
	<b>180</b>	-	0.100	0.060	0.030	0.010
	<b>182</b>	-	0.267	0.060	0.010	0.010
	<b>186</b>	-	0.033	-	-	-

*Simulation study 4*

The results obtained for this simulation study was very similar to simulation study 3. Compared to the third simulation study the overall amount and frequency of blue wildebeest alleles in the fifth generation decreased slightly compared to the first generation hybrids. An even smaller decrease in allele frequencies was observed between the fifth and the tenth generation hybrids. However, this decrease was expected to be higher, since the blue wildebeest were not kept as possible parents for the simulation of the second generation hybrids, as was done in simulation study 3 (Table 4.4).

**Table 4.4: Allele frequencies for simulated first, fifth and tenth generation hybrid individuals obtained from simulation study 4. Included in this table are the allele frequencies for the blue and black reference populations obtained from Grobler *et al.*, (2005) (alleles highlighted in black and blue are specific to pure black and blue wildebeest, with unshaded alleles considered shared alleles)**

Markers	Alleles	Black reference	Blue reference	F1	F5	F10
<b>BM2113</b>	<b>131</b>	1.000	0.667	0.670	0.790	0.820
	<b>133</b>	-	0.067	0.050	0.080	0.110
	<b>135</b>	-	0.167	0.210	0.090	0.050
	<b>141</b>	-	0.067	0.020	0.040	0.020
	<b>143</b>	-	0.033	0.050	-	-
<b>ETH10</b>	<b>203</b>	0.969	-	0.450	0.550	0.540
	<b>205</b>	0.031	-	0.050	0.140	0.050
	<b>209</b>	-	0.767	0.400	0.280	0.360
	<b>211</b>	-	0.233	0.100	0.030	0.050
<b>BM1824</b>	<b>180</b>	-	0.400	0.280	0.160	0.120
	<b>194</b>	0.047	-	0.030	0.070	0.060
	<b>196</b>	0.391	-	0.220	0.320	0.220
	<b>200</b>	0.563	-	0.250	0.320	0.430
	<b>202</b>	-	0.167	-	-	-
	<b>206</b>	-	0.267	0.220	0.130	0.170
	<b>210</b>	-	0.033	-	-	-
	<b>212</b>	-	0.100	-	-	-
	<b>218</b>	-	0.033	-	-	-
	<b>TGLA53</b>	<b>150</b>	-	0.267	0.060	0.030
<b>152</b>		0.219	-	0.100	0.040	-
<b>154</b>		0.281	0.267	0.320	0.450	0.500
<b>156</b>		0.297	0.100	0.100	0.050	0.030
<b>158</b>		0.016	0.200	0.220	0.190	0.240
<b>160</b>		0.188	-	0.090	0.140	0.110
<b>162</b>		-	0.100	0.070	0.100	0.120
<b>166</b>		-	0.033	0.040	-	-
<b>168</b>		-	0.033	-	-	-
<b>CSSM36</b>	<b>156</b>	0.672	-	0.350	0.350	0.460
	<b>166</b>	0.078	-	0.090	0.010	-
	<b>172</b>	-	0.500	0.350	0.490	0.400
	<b>174</b>	-	0.100	0.030	-	-
	<b>176</b>	0.219	-	0.050	0.030	0.020
	<b>178</b>	0.031	-	0.010	-	-
	<b>180</b>	-	0.100	0.060	0.120	0.120
	<b>182</b>	-	0.267	0.060	-	-
	<b>186</b>	-	0.033	-	-	-

Overall, introgression of blue wildebeest alleles could still be detected after five generation in all the simulation studies. The decrease in the frequency of these alleles between the first generation and the fifth generation were almost negligible. However, between the first and tenth generations a more significant difference in the amount and frequency of nominally blue wildebeest alleles could be observed. No significant differences were found between the studies where blue wildebeest were allowed to interbreed for a second generation versus studies where these animals were removed after the initial hybridization event.

# CHAPTER FIVE: GROBLER *et al.* 2005

## REVISITED

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### 5.1 Introduction

In 2005, researchers at the University of Limpopo (J.P. Grobler) and the Agricultural Research Institute (A. Kotze and K. Ehlers) reported on a significant advance in wildebeest hybrid identification (Grobler *et al.*, 2005). These authors reported on the genetic status of an isolated black wildebeest population from the Abe Bailey Nature Reserve in the Gauteng Province. This population was founded by introducing ten animals translocated from the Pietersburg Nature Reserve in the Limpopo Province. The latter nature reserve hosted both blue and black wildebeest for many years before, in the early 1980s, concern over possible hybridization was raised. These animals were subsequently culled; however translocation of some of the animals already took place to six other localities, including the Abe Bailey Nature Reserve. This resulted in a high probability that the current population on Abe Bailey may contain introgressed blue wildebeest genetic material.

A set of five microsatellite markers were used by Grobler *et al.* (2005) to assess the level of introgression of blue wildebeest genetic material into the Abe Bailey population. Furthermore, these authors aimed to determine whether the genetic impact of this introgression was persistent after presumably pure black wildebeest were repeatedly added to the original Abe Bailey population. An assignment test was done with the genotyping results obtained using GENECLASS software (Cornuet *et al.*, 1999). All the black wildebeest animals from the Abe Bailey population were true to species. A visual inspection of the presence/absence of alleles did however suggest limited introgression of nominally blue alleles into black at one locus. Subsequent to the publishing of this work, more powerful methods for statistical analysis have been developed, notably in the form of STRUCTURE software (Pritchard *et al.*, 2000). The aim in this chapter was thus to re-analyze the results used by Grobler *et al.* (2005), taking advantage of newer developments in hybrid detection.

## 5.2 Reanalysis of microsatellite data

The dataset used by Grobler *et al.* in 2005 was obtained from the authors and reanalysed with more current software and statistical approaches. The dataset contained genotypes for 70 animals at five loci, BM1824, BM2113, CSSM36, ETH10 and TGLA53 (Table 5.1).

**Table 5.1: Allele frequency data generated by Grobler *et al.* (2005)**

Locus	Allele	Blue wildebeest (n = 22)	Abe Bailey (n = 12)	Black wildebeest (n = 36)
<b>BM1824</b>	180	0.43	-	-
	194	-	0.15	0.03
	196	-	0.45	0.35
	200	-	0.40	0.62
	202	0.18	-	-
	204	0.03	-	-
	206	0.18	-	-
	210	0.05	-	-
	212	0.13	-	-
	218	0.03	-	-
<b>BM2113</b>	129	0.02	-	-
	131	0.52	0.91	0.98
	133	0.26	0.09	0.02
	135	0.07	-	-
	139	0.05	-	-
	141	0.07	-	-
<b>CSSM36</b>	157	-	0.64	0.77
	167	-	0.29	0.07
	173	0.53	-	-
	175	0.08	-	-
	177	0.03	0.07	0.13
	179	0.03	-	0.04
	181	0.13	-	-
	183	0.20	-	-
	187	0.03	-	-
<b>ETH10</b>	203	-	0.64	0.97
	205	-	0.36	0.03
	209	0.81	-	-
	211	0.19	-	-
<b>TGLA53</b>	148	0.02	-	-
	150	0.20	0.15	-
	152	-	0.20	0.20
	154	0.25	0.20	0.34
	156	0.07	0.25	0.26
	158	0.25	0.20	0.01
	160	0.02	-	0.19
	162	0.09	-	-
	168	0.07	-	-
	170	0.02	-	-

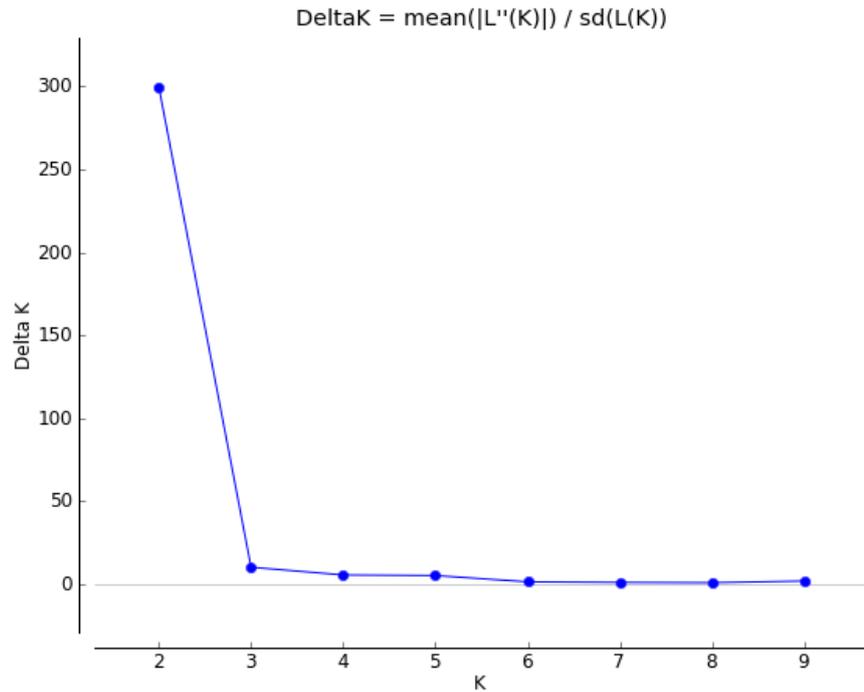
The animals analysed consisted of a reference blue set, a reference black set and the putative hybrid animals from Abe Bailey. An assignment test was done using STRUCTURE software. The true number of population was first determined by setting possible  $K$  values of between one and ten. Five independent runs for each assumed  $K$  was used, with a burn-in period of 100,000 steps followed by 200,000 MCMC iterations. The output of these analyses was entered into STRUCTURE HARVESTER (Earl and von Holdt, 2012). This software package further analyse all the information obtained by STRUCTURE and provides a graphical representation of the results.

After determination of the true number of populations, STRUCTURE was again run to determine the probability of assignment of each individual to each identified cluster. This analysis was done with a burn-in period of 100,000 steps followed by 1,000,000 MCMC iterations.

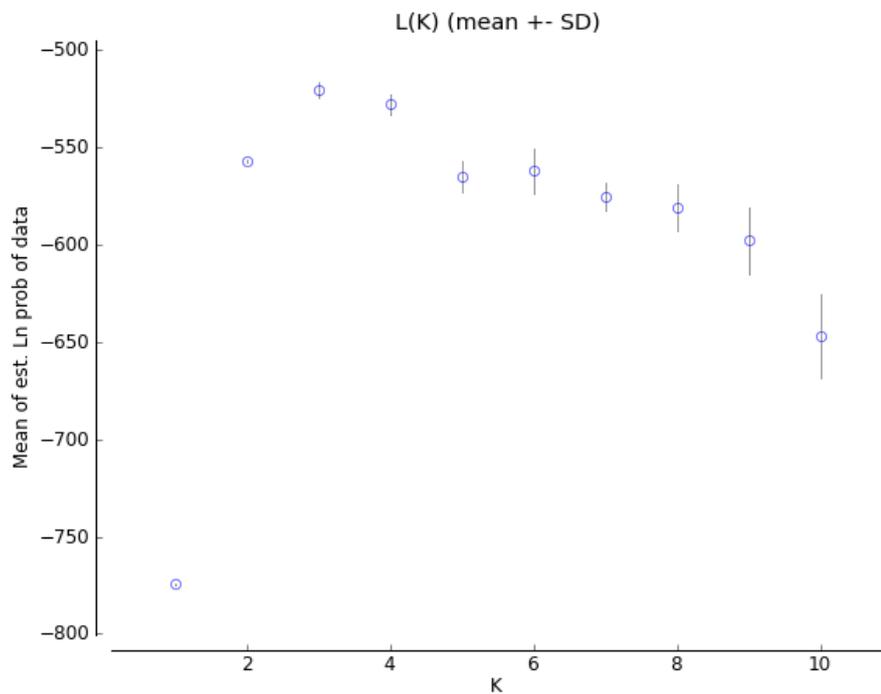
### **5.3 Results and Discussion**

For the analysis the samples were grouped into three groups, pure black wildebeest (consisting of animals from the SA Lombard and Suikerbosrand Nature Reserves), pure blue wildebeest (consisting of animals from the Klaserie, Vaalwater and Musina regions) and the animals from the Abe Bailey Nature Reserve.

Analysis of the STRUCTURE results revealed that the most likely  $K$  value was  $K = 2$ . The highest deltaK value was observed at  $K = 2$  (Figure 5.1) and the plot of the mean likelihood  $L(K)$  and variance per  $K$  value (Figure 5.2) also reached a plateau at  $K = 2$ .



**Figure 5.1: Plot for detecting the number of groups ( $K$ ) that best fit the data. The highest delta  $K$  value is reached at  $K = 2$  (from STRUCTURE HARVESTER)**



**Figure 5.2: Plot of the mean likelihood  $L(K)$  and variance per  $K$  value from STRUCTURE. A plateau is reached at  $K = 2$  (from STRUCTURE HARVESTER)**

After the true number of populations was determined, a second run was completed with  $K = 2$ . The results of the assignment of populations to each of the two clusters are given in Table 5.2.

**Table 5.2: Proportion of membership for each pre-defined population in each of the two clusters. The highest probability of belonging to a specific cluster, for each of the populations, is indicated in red**

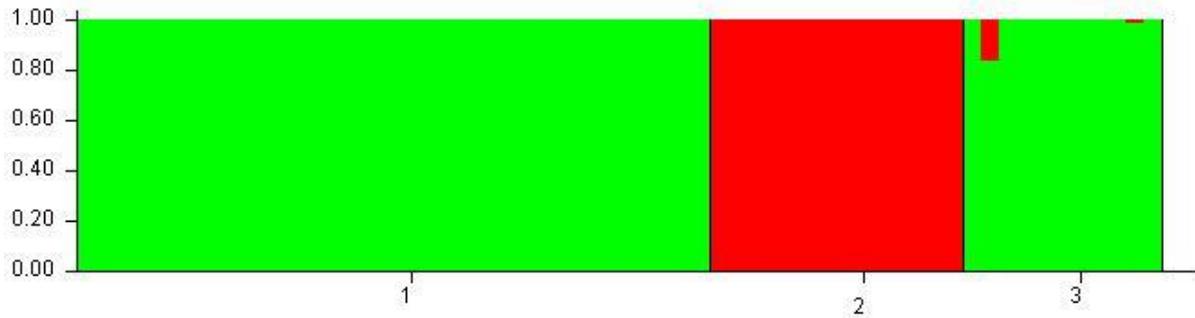
Populations	Inferred Clusters	
	1	2
<b>Black</b>	0.000	<b>1.000</b>
<b>Blue</b>	<b>1.000</b>	0.000
<b>Abe Bailey</b>	0.015	<b>0.985</b>

The assignment of these populations to their inferred clusters revealed a very clear distinction between the pure blue and black wildebeest. These two populations grouped into their separate clusters with a 100% probability. Assignment of the individuals from the Abe Bailey populations revealed a very small amount of similarity with the blue wildebeest individuals, with a 1.5% assignment to the cluster consisting of only blue wildebeest. Two individuals within the Abe Bailey population were identified that caused this occurrence. The results of the individual assignment for the Abe Bailey animals are given in Table 5.3.

**Table 5.3: Proportion of membership for each individual in the Abe Bailey population to two clusters containing pure black and blue wildebeest**

Populations	Inferred Clusters	
	Blue	Black
<b>AB1</b>	0.000	1.000
<b>AB2</b>	0.160	0.840
<b>AB3</b>	0.000	1.000
<b>AB4</b>	0.000	1.000
<b>AB5</b>	0.000	1.000
<b>AB6</b>	0.000	1.000
<b>AB7</b>	0.000	1.000
<b>AB8</b>	0.000	1.000
<b>AB9</b>	0.000	1.000
<b>AB10</b>	0.000	1.000
<b>AB11</b>	0.008	0.992
<b>AB12</b>	0.000	1.000

Only two individuals, AB2 and AB11, showed minor similarities to the blue wildebeest populations. This can also be seen from the bar plot depicting the individual proportion of membership of each of the animals to the two inferred clusters (Figure 5.3). There was a distinct difference between the pure black (green block) and pure blue wildebeest (red block) populations and members of each species grouped into their respective clusters with 100% certainty. However, two animals in the Abe Bailey population showed minor introgression from the blue wildebeest, as can be seen by the two small red bars observed in population three.



**Figure 5.3: Bar plot obtained from STRUCTURE, indicating the assignment of the individuals in the three populations, to the two inferred clusters represented by the green and red shading. The animals are grouped according to populations, 1 = pure black wildebeest, 2 = pure blue wildebeest and 3 = the Abe Bailey population**

The results obtained after this additional statistical analysis, confirmed the findings by Grobler *et al.* (2005) that minor historical introgression of blue wildebeest genetic material occurred in the Abe Bailey population. It cannot be determined whether the initial level of introgression persisted or decreased after the subsequent addition of pure black wildebeest to this population. It can however be concluded that the historical introgression of blue wildebeest material into black on Pietersburg and then Abe Bailey could be determined using five microsatellite markers and appropriate statistical analysis.

## CHAPTER SIX: DISCUSSION

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### 6.1 Genetic diversity in black wildebeest populations

Genetic diversity is a fundamental requirement for populations to adapt to changes in the environment. Large, naturally outbreeding species have extensive genetic diversity, but this is usually reduced in smaller populations (Frankham *et al.*, 2010). Typically black wildebeest populations are small and isolated, promoting inbreeding and genetic drift. These effects are further amplified when populations are founded from a limited number of individuals (Grobler *et al.*, 2005). This is the case for most black wildebeest populations in South Africa. Furthermore, the species has passed through two bottlenecks in the last 110 years, and the limited founders (approximately 300 individuals) that survived the last bottleneck may have already been highly related (Corbet and Robinson, 1991).

The statistical analysis of the genotypic data obtained for the 22 populations confirmed that the overall diversity of black wildebeest is lower than that of the blue wildebeest. These results complied with results obtained in earlier studies that made use of allozyme data and microsatellite data, respectively (Corbet and Robinson, 1991; Grobler and van der Bank, 1993; Grobler *et al.*, 2005). The lower level of genetic diversity in the black wildebeest can be expected due to the extreme decline in numbers experienced during 1930s, which brought the species to the brink of extinction (Kirkman, 1938). Management strategies implemented for black wildebeest should have the dual goal of conserving high levels of genetic diversity while still preserving the genetic integrity of black wildebeest populations (Grobler *et al.*, 2005).

### 6.2 Introgression of blue wildebeest alleles into black wildebeest populations

The addition of new reference material in the current study revealed that some of the alleles previously assumed to be unique to a specific species were in fact shared between the two species. This re-classification of alleles from species-specific to shared strongly reinforced

the need to use reference populations of adequate size and representative of a full distribution area, when screening for hybrids. In terms of identifying the hybrid status of populations the two microsatellite markers did identify nominally blue wildebeest alleles in some of these populations. The presence of these alleles could either be due to the fact that hybridization has occurred at these localities or alternatively, this proves that the search of reference populations (and thus species-specific alleles) should again be widened. Another alternative would be to screen these populations with additional microsatellite or alternative marker systems to confirm the results obtained with the current microsatellite markers.

The numbers of markers recommended for the determination of the hybrid status of individuals vary, since fewer markers are needed when attempting to separate  $F_1$  hybrids from parental taxa compared to situations where advanced backcrosses are involved. According to Boecklen and Howard (1997), four to five markers will be sufficient when coarsely classifying individuals into parental,  $F_1$  and simple backcrosses. Superficially, the Perdeberg case study provided proof that the two microsatellite markers used in this study could positively identify  $F_1$  hybrids. The identification of second generation hybrids with these microsatellite markers was also plausible in the Perdeberg scenario. The outcome of the Perdeberg study may however equally have been different (and un-informative) if independent assortment in these animals had resulted in other genotypic combinations. It is thus highly recommended that further analysis of hybrid animals should be done with additional microsatellite markers or alternative molecular techniques.

For management purposes, it is crucial to determine the scale and persistence of introgression in hybridization scenarios. Simulation studies enables researchers to model hybrid animals (and populations) to evaluate the power of methods used for identifying the status of individuals as purebred or hybrid, as well as the persistence of introgressed alleles after generations of backcrossing (Nielsen *et al.*, 2006). The simulations done in the current study were designed specifically to mimic possible real life hybridization events that can take place on game farms and nature reserves where both species are kept. Even though Senn and Pemberton (2009) stated that the possibility of still finding introgressed alleles after six generations of backcrossing is very slim, the simulations studies provided proof that introgressed alleles could still be detected after ten generations of backcrossing using five microsatellite markers. This is a significant outcome for the management of hybrid

populations. Evidently, reliable identification of individual hybrid animals will require much larger numbers of markers compared to populations.

### **6.3 Recommendations for the future management of black wildebeest populations**

Ideally, the most practical approach for dealing with hybrid animals would first be to accurately identify the populations that contain hybrid animals, and then cull or exclude these populations from future conservation efforts and reintroduction programs. This would prevent the problem of hybridization from being exacerbated by introducing hybrid individuals into more pure populations (Ward *et al.*, 1999). However, if hybridization in populations were rare the isolation of these animals would lead to populations that over time would only contain a small proportion of introgressed alleles. This is due to the backcrossing of the rare hybrids with parental individuals, thus diluting the effect of introgression (Senn and Pemberton, 2009).

Alternative recommendations, made by Hedrick (2009) for the reduction of cattle ancestry in bison herds could also be applied to the black wildebeest populations in South Africa. Similar to the recommendation by Ward *et al.* (1999), Hedrick (2009) stated that hybrid populations should be kept separate and no introductions of these animals should be made into pure populations. However, if surplus pure animals are available and problems such as inbreeding depression and low genetic variation arise in the isolated hybrid populations, pure animals can be introduced into these hybrid herds, which would lead to the genetic swamping of the introgressed alleles. This approach is however not viable for black wildebeest, due to the limited number of pure black wildebeest available in South Africa (Grobler *et al.*, 2005).

A more drastic approach would be to cull animals with hybrid ancestry; however, this would have a catastrophic effect on the already reduced genetic diversity of black wildebeest and would require a more accurate system for hybrid identification in black wildebeest than that currently used (Grobler *et al.*, 2011). Simberloff (1996) specific cautions against this extermination of hybrids as these nominally hybrid herds may be significant reservoirs of genetic diversity which will be lost if these animals are culled. Although black wildebeest are no longer threatened with extinction, the overall low level of genetic diversity remains a

problem and could potentially be addressed by hybrid animals and the possibility of unique alleles in putative hybrid populations.

Grobler *et al.* (2011) recommended a more realistic approach to ultimately prevent an additional genetic bottleneck from taking place in the black wildebeest. These authors recommended that the genetic purity of black wildebeest populations in South Africa should be tolerated and managed as it currently exists. Pure herds of black wildebeest should be kept on protected game ranches and government controlled protected areas. At the same time, black wildebeest with moderate introgression of blue wildebeest alleles should be allowed on game ranches used for local sport hunting. For this approach to be successful it is critical that accurate records be kept on all translocations between black wildebeest populations, in order to retain a distinction between pure and hybrid animals.

The preceding recommendations are aimed at the management of existing hybrid populations. It is however more important to prevent hybridization events before they occur. Grobler *et al.* (2011) formulated more specific recommendation for the prevention of hybridization in black wildebeest populations. These authors recommended that no game farm or nature reserve in South Africa should be permitted to keep both species on the same property. These authors also suggested that the restriction be extended to include neighbouring properties due to the high probability of animals not being contained by fences. Stricter regulations on the movement of individuals should be maintained and better record keeping of these movements is crucial to prevent hybrid animals from being sold and mixed with pure populations. Strict measures should also be implemented to protect the few known pure populations, especially regulations on the introduction of animals into these herds.

While management recommendations can already be implemented, the most important issue that needs to be dealt with is the development of additional molecular techniques for the identification of hybrid animals in black wildebeest populations. In this regard, various research projects are underway at the University of the Free State and the National Zoological Gardens (Pretoria) to supplement the current identification methods.

## CHAPTER SEVEN: SUMMARY

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Black wildebeest (*Connochaetes gnou*), a species endemic to South Africa, experienced two bottlenecks in the last century and the number of animals ultimately decreased to approximately 300. These bottlenecks led to a decrease in the genetic diversity of black wildebeest populations across South Africa. An additional threat to the genetic integrity of the black wildebeest was discovered between the 1960s and late 1980s, when researchers noted that hybridization between blue and black wildebeest occurs and that these hybrid animals are fertile. Identification of the hybrid individuals is crucial and various molecular techniques were researched, with microsatellite markers proving to be the most successful. The aim of the current study was to investigate the effectiveness of previously identified cross-species microsatellite markers and statistical approaches for the identification of hybrid herds and individuals on various Nature Reserves in the Free State Province as well as privately owned game farms in and around the Province. Two previously identified diagnostic microsatellite markers (BM1824 and ETH10) were used to screen the populations for putative hybrids. The genetic diversity of the black wildebeest populations studied supported earlier findings showing lower genetic diversity in black wildebeest compared to blue wildebeest. The addition of new reference material in the current study revealed that some of the alleles previously assumed to be unique to a specific species were in fact shared between the two species. This reinforced the need to use more reference populations of adequate size. Nominally blue wildebeest alleles were found in five populations on different game farms and Nature Reserves. The presence of these alleles could be an indication that hybrids are present at these localities or alternatively, support the finding that the number and distribution of reference populations should be increased. Assignment of populations to specific clusters using different software programmes revealed that, due to the large amount of genetic material shared between blue and black wildebeest, no clear assignment of individuals to a specific cluster could be obtained. Molecular analysis of two known hybrid animals did indicate that the two microsatellite markers chosen were able to identify first generation hybrids and possibly even second generation hybrids. The study also investigated the persistence of introgression of blue wildebeest genetic material into black wildebeest populations using simulation software. The simulation tests revealed that introgressed alleles could still be detected after ten generations of backcrossing. This has serious implications for

the management of hybrid populations. Various recommendations can be made in terms of the future management and conservation of black wildebeest on Nature Reserves and game farms. The most practical approach for dealing with hybrid animals would first be to develop additional molecular techniques for the accurate identification of populations that contain hybrid animals. Positively identified hybrid populations should be kept separate and no introductions of these animals should be made into pure populations. A more drastic approach would be to cull animals with hybrid ancestry. This would however have serious implications on the already reduced level of genetic diversity in the black wildebeest populations. The most pragmatic approach for dealing with hybrid populations would be to keep pure blue and black wildebeest in protected areas and allow black wildebeest with moderate introgression on game ranches exclusively used for sport hunting.

Key words: assignment tests, black wildebeest, blue wildebeest, cross-species microsatellite markers, genetic diversity, hybrid, hybrid identification, introgression, simulation

## CHAPTER EIGHT: OPSOMMING

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Die swartwildebees (*Connochaetes gnou*), 'n spesie endemies tot Suid-Afrika, het gedurende die laaste eeu twee bottelnekke ondergaan en die aantal diere het na die laaste bottelnek afgeneem tot ongeveer 300. Hierdie bottelnek het gelei tot 'n afname in die genetiese diversiteit van swartwildebees bevolkings regoor Suid-Afrika. Nog 'n bedreiging tot die genetiese integriteit van die swartwildebees is ontdek tussen 1960 en die laat 1980s. Navorsers het in die tydperk ontdek dat verbastering plaasvind tussen blou- en swartwildebeeste en dat die basters vrugbaar is. Dit is noodsaaklik om die baster individue te kan identifiseer en verskeie molekulêre tegnieke is reeds nagevors. Tot dusver het mikrosatelliet merkers die meeste sukses getoon. Die doel van die studie was om die effektiwiteit van voorheen geïdentifiseerde mikrosatelliet merkers te ondersoek, asook die betroubaarheid van statistiese berekenings, vir die identifikasie van basterpopulasies en individue op Natuurreservate en wildsplase in die Vrystaat. Twee voorheen geïdentifiseerde diagnostiese mikrosatelliet merkers (BM1824 and ETH10) is gebruik om bevolkings te ondersoek vir moontlik basters. Die genetiese diversiteit van die swart wildebees bevolkings wat bestudeer is, stem ooreen met vorige bevindings wat aangedui het dat die genetiese diversiteit laer is in swartwildebeeste in vergelyking met blou wildebees bevolkings. Die byvoeging van nuwe verwysingsmateriaal het getoon dat sommige allele wat voorheen aangedui is as uniek tot 'n spesifieke spesie eintlik gedeel word tussen die twee spesies. Dit bevestig dat meer verwysingsbevolkings van voldoende grootte en geskikte lokaliteite gebruik moet word. Blouwildebees allele is wel gevind in vyf bevolkings op verskillende wildsplase en natuurreservate. Die teenwoordigheid van hierdie allele kan 'n aanduiding wees dat basters teenwoordig is in hierdie bevolkings. 'n Alternatiewe verduideliking kan wees dat meer verwysingsbevolkings gebruik moet word vir die studie. Die aanwysing van bevolkings tot spesifieke groepe deur gebruik te maak van verskillende sagteware programme, het aangedui dat - as gevolg van die groot hoeveelheid genetiese materiaal wat gedeel word tussen swart- en blouwildebeeste - geen duidelike aanwysings gemaak is tot 'n spesifieke groep nie. Molekulêre analise van twee bevestigde basters het aangedui dat die twee mikrosatelliet merkers wat gekies is, daartoe instaat is om eerste generasie basters en moontlik ook tweede generasie basters te identifiseer. Die studie het ook die volharding van die introgressie van blouwildebees genetiese materiaal in 'n swartwildebees populasie getoets

deur gebruik te maak van simulasiestof. Hierdie simulasiestof het aangedui dat ingedringde allele steeds bespeur kon word na tien generasies se terug-kruisings. Dit het ernstige gevolge vir die bestuur van basterbevolkings. Verskeie aanbevelings kan gemaak word in terme van die toekomstige bestuur en bewaring van swartwildebeeste op natuurreservate en wildsplase. Die mees praktiese benadering sal wees om addisionele molekule teganne te ontwikkel vir die akkurate identifisering van bevolkings wat basters bevat. Indien 'n bevolking geïdentifiseer word wat wel basters bevat, moet hierdie bevolking apart gehou word en geen van hierdie diere moet verskuif word na ander suiwer bevolkings. 'n Meer drastiese benadering sal wees om al die basters uit te dun, maar dit kan moontlik lei tot verdere vermindering van die genetiese diversiteit van die swartwildebeeste bevolkings. 'n Gebalanseerde benadering sal wees om suiwer swart- en blouwildebeeste in beskermde areas te hou en om slegs toe te laat dat swartwildebeeste met matige hoeveelheid introgressie op wildsplase gehou word waar die diere slegs aangehou word vir jagdoeleindes.

Sleutelwoorde: aanwysingstoetse, baster, baster identifisering, blouwildebeeste, genetiese diversiteit, introgressie, oorkruis-spesie mikrosatelliet merkers, swartwildebeeste, simulasiestof

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## **APPENDIX A**

Sample list: Black wildebeest test populations

List of the black wildebeest test populations sampled from Nature Reserves in the Free State Province as well as private game farms across South Africa

<b>No.</b>	<b>Sample no.</b>	<b>Species</b>	<b>Sex</b>	<b>Age</b>	<b>Sample Type</b>	<b>Origin</b>	<b>Province</b>
1	FS 15	Black Wildebeest	Male	Adult	Tissue	Florida Private Game Farm	Free State
2	FS 16	Black Wildebeest	Female	Adult	Tissue	Florida Private Game Farm	Free State
3	FS 9	Black Wildebeest	Male	16 months	Tissue	Caledon Nature Reserve	Free State
4	FS 18	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
5	FS 19	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
6	FS 20	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
7	FS 21	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
8	FS 22	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
9	FS 27	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
10	FS 31	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
11	FS 32	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
12	FS 33	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
13	FS 35	Black Wildebeest	Male	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
14	FS 36	Black Wildebeest	Male	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
15	FS 130	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
16	FS 145	Black Wildebeest	Female	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
17	FS 147	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
18	FS 148	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
19	FS 149	Black Wildebeest	Male	Juvenile	Tissue	Caledon Nature Reserve	Free State
20	FS 150	Black Wildebeest	Male	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
21	FS 151	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
22	FS 152	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State

23	FS 153	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
24	FS 157	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
25	FS 158	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
26	FS 159	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
27	FS 160	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
28	FS 161	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
29	FS 162	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
30	FS 163	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
31	FS 164	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
32	FS 165	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
33	FS 166	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
34	FS 167	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
35	FS 169	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
36	FS 197	Black Wildebeest	Female	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
37	FS 200	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
38	FS 201	Black Wildebeest	Male	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
39	FS 202	Black Wildebeest	Male	Sub-Adult	Tissue	Caledon Nature Reserve	Free State
40	FS 211	Black Wildebeest	Male	Juvenile	Tissue	Caledon Nature Reserve	Free State
41	FS 212	Black Wildebeest	Male	Adult	Tissue	Caledon Nature Reserve	Free State
42	FS 215	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
43	FS 218	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
44	FS 219	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
45	FS 220	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
46	FS 423	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
47	FS 425	Black Wildebeest	Female	Adult	Tissue	Caledon Nature Reserve	Free State
48	FS 42	Black Wildebeest	Male	Calf	Tissue	Tussen die Riviere Nature Reserve	Free State
49	FS 44	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State

50	FS 49	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
51	FS 57	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
52	FS 59	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
53	FS 63	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
54	FS 65	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
55	FS 71	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
56	FS 72	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
57	FS 74	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
58	FS 75	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
59	FS 77	Black Wildebeest	Female	Sub-Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
60	FS 141	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
61	FS 178	Black Wildebeest	Male	Juvenile	Tissue	Tussen die Riviere Nature Reserve	Free State
62	FS 179	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
63	FS 180	Black Wildebeest	Unknown	Juvenile	Tissue	Tussen die Riviere Nature Reserve	Free State
64	FS 181	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
65	FS 182	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
66	FS 183	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
67	FS 184	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
68	FS 185	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
69	FS 186	Black Wildebeest	Unknown	Unknown	Tissue	Tussen die Riviere Nature Reserve	Free State
70	FS 187	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
71	FS 188	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
72	FS 189	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
73	FS 190	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
74	FS 191	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
75	FS 192	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
76	FS 193	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State

77	FS 194	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
78	FS 195	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
79	FS 196	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
80	FS 198	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
81	FS 199	Black Wildebeest	Female	Juvenile	Tissue	Tussen die Riviere Nature Reserve	Free State
82	FS 204	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
83	FS 205	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
84	FS 206	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
85	FS 207	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
86	FS 208	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
87	FS 209	Black Wildebeest	Male	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
88	FS 210	Black Wildebeest	Female	Adult	Tissue	Tussen die Riviere Nature Reserve	Free State
89	FS 29	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
90	FS 37	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
91	FS 38	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
92	FS 39	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
93	FS 40	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
94	FS 48	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
95	FS 51	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
96	FS 52	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
97	FS 55	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
98	FS 56	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
99	FS 58	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
100	FS 61	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
101	FS 64	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
102	FS 67	Black Wildebeest	Female	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
103	FS 68	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State

104	FS 69	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
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106	FS 73	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
107	FS 76	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
108	FS 79	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
109	FS 80	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
110	FS 81	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
111	FS 82	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
112	FS 83	Black Wildebeest	Male	Juvenile	Tissue	Maria Moroka Nature Reserve	Free State
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114	FS 85	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
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116	FS 87	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
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118	FS 89	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
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120	FS 91	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
121	FS 92	Black Wildebeest	Female	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
122	FS 93	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
123	FS 94	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
124	FS 95	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
125	FS 96	Black Wildebeest	Female	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
126	FS 97	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
127	FS 98	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
128	FS 99	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
129	FS 100	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
130	FS 101	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State

131	FS 102	Black Wildebeest	Female	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
132	FS 103	Black Wildebeest	Female	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
133	FS 104	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
134	FS 105	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
135	FS 106	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
136	FS 107	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
137	FS 108	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
138	FS 109	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
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141	FS 112	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
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143	FS 114	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
144	FS 115	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
145	FS 116	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
146	FS 118	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
147	FS 119	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
148	FS 120	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
149	FS 121	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
150	FS 122	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
151	FS 123	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
152	FS 124	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
153	FS 125	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
154	FS 126	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
155	FS 127	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
156	FS 128	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
157	FS 129	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State

158	FS 131	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
159	FS 135	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
160	FS 137	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
161	FS 139	Black Wildebeest	Female	Adult	Tissue	Maria Moroka Nature Reserve	Free State
162	FS 140	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
163	FS 142	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
164	FS 143	Black Wildebeest	Male	Adult	Tissue	Maria Moroka Nature Reserve	Free State
165	FS 144	Black Wildebeest	Male	Sub-Adult	Tissue	Maria Moroka Nature Reserve	Free State
166	FS 407	Black Wildebeest	Unknown	Unknown	Tissue	Maria Moroka Nature Reserve	Free State
167	FS 408	Black Wildebeest	Unknown	Unknown	Tissue	Maria Moroka Nature Reserve	Free State
168	FS 409	Black Wildebeest	Unknown	Unknown	Tissue	Maria Moroka Nature Reserve	Free State
169	FS 309	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
170	FS 310	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
171	FS 326	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
172	FS 327	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
173	FS 328	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
174	FS 329	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
175	FS 330	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
176	FS 331	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
177	FS 332	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
178	FS 333	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
179	FS 334	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
180	FS 336	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
181	FS 337	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
182	FS 338	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
183	FS 339	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
184	FS 340	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State

185	FS 341	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
186	FS 342	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
187	FS 343	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
188	FS 344	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
189	FS 345	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
190	FS 346	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
191	FS 347	Black Wildebeest	Male	Juvenile	Tissue	Willem Pretorius Nature Reserve	Free State
192	FS 348	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
193	FS 350	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
194	FS 351	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
195	FS 352	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
196	FS 353	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
197	FS 354	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
198	FS 355	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
199	FS 356	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
200	FS 357	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
201	FS 358	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
202	FS 359	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
203	FS 360	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
204	FS 361	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
205	FS 362	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
206	FS 363	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
207	FS 364	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
208	FS 365	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
209	FS 366	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
210	FS 367	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
211	FS 368	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State

212	FS 369	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
213	FS 370	Black Wildebeest	Female	Calf	Tissue	Willem Pretorius Nature Reserve	Free State
214	FS 371	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
215	FS 372	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
216	FS 373	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
217	FS 375	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
218	FS 376	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
219	FS 377	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
220	FS 378	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
221	FS 380	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
222	FS 381	Black Wildebeest	Male	Yearling	Tissue	Willem Pretorius Nature Reserve	Free State
223	FS 383	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
224	FS 386	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
225	FS 387	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
226	FS 390	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
227	FS 391	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
228	FS 394	Black Wildebeest	Male	Yearling	Tissue	Willem Pretorius Nature Reserve	Free State
229	FS 398	Black Wildebeest	Female	Yearling	Tissue	Willem Pretorius Nature Reserve	Free State
230	FS 449	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
231	FS 458	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
232	FS 469	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
233	FS 470	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
234	FS 471	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
235	FS 471 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
236	FS 475	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
237	FS 475 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
238	FS 476	Black Wildebeest	Male	Young Adult	Tissue	Willem Pretorius Nature Reserve	Free State

239	FS 477	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
240	FS 477 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
241	FS 478	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
242	FS 479	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
243	FS 479 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
244	FS 480	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
245	FS 480 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
246	FS 481	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
247	FS 484	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
248	FS 484 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
249	FS 485	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
250	FS 485 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
251	FS 486	Black Wildebeest	Male	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
252	FS 495	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
253	FS 495 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
254	FS 496	Black Wildebeest	Female	Sub-Adult	Tissue	Willem Pretorius Nature Reserve	Free State
255	FS 496 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
256	FS 497	Black Wildebeest	Male	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
257	FS 500	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
258	FS 500 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
259	FS 501	Black Wildebeest	Female	Adult	Tissue	Willem Pretorius Nature Reserve	Free State
260	FS 501 A	Black Wildebeest	Unknown	Foetus	Tissue	Willem Pretorius Nature Reserve	Free State
261	W 1	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
262	W 2	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
263	W 3	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
264	W 4	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
265	W 5	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State

266	W 6	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
267	W 7	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
268	W 8	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
269	W 9	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
270	W 10	Black Wildebeest	Unknown	Unknown	Tissue	Willem Pretorius Nature Reserve	Free State
271	L68	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
272	L69	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
273	L70	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
274	L71	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
275	L72	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
276	L73	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
277	L74	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
278	L75	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
279	L76	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
280	L77	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
281	L78	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
282	L79	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
283	L80	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
284	L82	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
285	L83	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
286	L85	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
287	L87	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
288	L88	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
289	L89	Black Wildebeest			Tissue	Odendaalsrus (Langkuil)	Free State
290	FS 451	Black Wildebeest	Male	Unknown	Tissue	Odendaalsrus (Langkuil)	Free State
291	FS 452	Black Wildebeest	Male	Unknown	Tissue	Odendaalsrus (Langkuil)	Free State
292	FS 453	Black Wildebeest	Male	Unknown	Tissue	Odendaalsrus (Langkuil)	Free State

293	G7948	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
294	G7952	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
295	G7954	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
296	G7964	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
297	G7969	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
298	G7973	Black Wildebeest			Tissue	Gariepdam Nature Reserve	Free State
299	FS 168	Black Wildebeest	Male	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
300	FS 170	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
301	FS 172	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
302	FS 228	Black Wildebeest	Female	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
303	FS 244	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
304	FS 245	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
305	FS 246	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
306	FS 253	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
307	FS 254	Black Wildebeest	Unknown	Adult	Tissue	Gariepdam Nature Reserve	Free State
308	FS 255	Black Wildebeest	Male	Adult	Tissue	Gariepdam Nature Reserve	Free State
309	FS 258	Black Wildebeest	Female	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
310	FS 259	Black Wildebeest	Female	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
311	FS 260	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
312	FS 261	Black Wildebeest	Female	Juvenile	Tissue	Gariepdam Nature Reserve	Free State
313	FS 262	Black Wildebeest	Female	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
314	FS 263	Black Wildebeest	Female	Adult	Tissue	Gariepdam Nature Reserve	Free State
315	FS 264	Black Wildebeest	Female	Sub-Adult	Tissue	Gariepdam Nature Reserve	Free State
316	FS 46	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
317	FS 66	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
318	FS 173	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
319	FS 237	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State

320	FS 241	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
321	FS 249	Black Wildebeest	Male	Sub-Adult	Tissue	Koppiesdam	Free State
322	FS 286	Black Wildebeest	Male	Sub-Adult	Tissue	Koppiesdam	Free State
323	FS 287	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
324	FS 289	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
325	FS 290	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
326	FS 291	Black Wildebeest	Female	Juvenile	Tissue	Koppiesdam	Free State
327	FS 292	Black Wildebeest	Male	Sub-Adult	Tissue	Koppiesdam	Free State
328	FS 293	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
329	FS 294	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
330	FS 295	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
331	FS 296	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
332	FS 298	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
333	FS 299	Black Wildebeest	Male	Calf	Tissue	Koppiesdam	Free State
334	FS 302	Black Wildebeest	Male	Juvenile	Tissue	Koppiesdam	Free State
335	FS 304	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
336	FS 305	Black Wildebeest	Female	Sub-Adult	Tissue	Koppiesdam	Free State
337	FS 306	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
338	FS 307	Black Wildebeest	Female	Sub-Adult	Tissue	Koppiesdam	Free State
339	FS 316	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
340	FS 317	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
341	FS 318	Black Wildebeest	Male	Adult	Tissue	Koppiesdam	Free State
342	FS 319	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
343	FS 320	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
344	FS 321	Black Wildebeest	Male	Sub-Adult	Tissue	Koppiesdam	Free State
345	FS 322	Black Wildebeest	Male	Sub-Adult	Tissue	Koppiesdam	Free State
346	FS 323	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State

347	FS 324	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
348	FS 325	Black Wildebeest	Female	Adult	Tissue	Koppiesdam	Free State
349	FS 1	Black Wildebeest	Female	Adult	Tissue	Erfenisdam	Free State
350	FS 2	Black Wildebeest	Male	Yearling	Tissue	Erfenisdam	Free State
351	FS 3	Black Wildebeest	Male	Yearling	Tissue	Erfenisdam	Free State
352	FS 4	Black Wildebeest	Male	Yearling	Tissue	Erfenisdam	Free State
353	FS 5	Black Wildebeest	Male	Yearling	Tissue	Erfenisdam	Free State
354	FS 6	Black Wildebeest	Male	Yearling	Tissue	Erfenisdam	Free State
355	FS 349	Black Wildebeest	Male	Sub-Adult	Tissue	Erfenisdam	Free State
356	FS 392	Black Wildebeest	Female	Sub-Adult	Tissue	Erfenisdam	Free State
357	FS 393	Black Wildebeest	Female	Sub-Adult	Tissue	Erfenisdam	Free State
358	FS 395	Black Wildebeest	Female	Sub-Adult	Tissue	Erfenisdam	Free State
359	FS 396	Black Wildebeest	Female	Adult	Tissue	Erfenisdam	Free State
360	FS 397	Black Wildebeest	Male	Sub-Adult	Tissue	Erfenisdam	Free State
361	FS 399	Black Wildebeest	Female	Sub-Adult	Tissue	Erfenisdam	Free State
362	FS 402	Black Wildebeest	Male	Sub-Adult	Tissue	Erfenisdam	Free State
363	FS 403	Black Wildebeest	Female	Sub-Adult	Tissue	Erfenisdam	Free State
364	FS 43	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
365	FS 174	Black Wildebeest	Female	Adult	Tissue	Sterkfonteindam	Free State
366	FS 175	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
367	FS 176	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
368	FS 177	Black Wildebeest	Male	Sub-Adult	Tissue	Sterkfonteindam	Free State
369	FS 238	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
370	FS 239	Black Wildebeest	Male	Juvenile	Tissue	Sterkfonteindam	Free State
371	FS 240	Black Wildebeest	Male	Juvenile	Tissue	Sterkfonteindam	Free State
372	FS 242	Black Wildebeest	Female	Adult	Tissue	Sterkfonteindam	Free State
373	FS 243	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State

374	FS 248	Black Wildebeest	Male	Juvenile	Tissue	Sterkfonteindam	Free State
375	FS 256	Black Wildebeest	Female	Juvenile	Tissue	Sterkfonteindam	Free State
376	FS 257	Black Wildebeest	Female	Adult	Tissue	Sterkfonteindam	Free State
377	FS 268	Black Wildebeest	Male	Juvenile	Tissue	Sterkfonteindam	Free State
378	FS 269	Black Wildebeest	Male	Sub-Adult	Tissue	Sterkfonteindam	Free State
379	FS 270	Black Wildebeest	Male	Juvenile	Tissue	Sterkfonteindam	Free State
380	FS 275	Black Wildebeest	Female	Adult	Tissue	Sterkfonteindam	Free State
381	FS 276	Black Wildebeest	Female	Adult	Tissue	Sterkfonteindam	Free State
382	FS 277	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
383	FS 278	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
384	FS 279	Black Wildebeest	Male	Sub-Adult	Tissue	Sterkfonteindam	Free State
385	FS 280	Black Wildebeest	Male	Adult	Tissue	Sterkfonteindam	Free State
386	DB 01	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
387	DB 03	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
388	DB 04	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
389	DB 05	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
390	DB 06	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
391	DB 07	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
392	DB 08	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
393	DB 09	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
394	DB 10	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
395	DB 11	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
396	DB 12	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
397	DB 13	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
398	DB 105	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
399	DB 107	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
400	DB 109	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State

401	DB 110	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
402	DB 111	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
403	DB 112	Black Wildebeest	Female	Unknown	Tissue	De Brug	Free State
404	DB 113	Black Wildebeest	Male	Unknown	Tissue	De Brug	Free State
405	FS 24	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
406	FS 26	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
407	FS 28	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
408	FS 41	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
409	FS 410	Black Wildebeest	Male	Sub-Adult	Tissue	Soetdoring	Free State
410	FS 411	Black Wildebeest	Male	Sub-Adult	Tissue	Soetdoring	Free State
411	FS 412	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
412	FS 413	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
413	FS 414	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
414	FS 415	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
415	FS 418	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
416	FS 419	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
417	FS 420	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
418	FS 421	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
419	FS 422	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
420	FS 426	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
421	FS 428	Black Wildebeest	Male	Sub-Adult	Tissue	Soetdoring	Free State
422	FS 429	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
423	FS 431	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
424	FS 437	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
425	FS 438	Black Wildebeest	Male	Calf	Tissue	Soetdoring	Free State
426	FS 439	Black Wildebeest	Male	Sub-Adult	Tissue	Soetdoring	Free State
427	FS 440	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State

428	FS 441	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
429	FS 446	Black Wildebeest	Male	Sub-Adult	Tissue	Soetdoring	Free State
430	FS 447	Black Wildebeest	Male	Adult	Tissue	Soetdoring	Free State
431	FS 450	Black Wildebeest	Female	Adult	Tissue	Soetdoring	Free State
432	FS 230	Black Wildebeest	Male	Sub-Adult	Tissue	Rustfontein	Free State
433	FS 231	Black Wildebeest	Female	Adult	Tissue	Rustfontein	Free State
434	FS 232	Black Wildebeest	Male	Sub-Adult	Tissue	Rustfontein	Free State
435	FS 233	Black Wildebeest	Male	Adult	Tissue	Rustfontein	Free State
436	FS 234	Black Wildebeest	Female	Adult	Tissue	Rustfontein	Free State
437	FS 235	Black Wildebeest	Male	Sub-Adult	Tissue	Rustfontein	Free State
438	FS 236	Black Wildebeest	Female	Sub-Adult	Tissue	Rustfontein	Free State
439	FS 271	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
440	FS 272	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
441	FS 273	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
442	FS 274	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
443	FS 283	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
444	FS 284	Black Wildebeest	Female	Adult	Tissue	Reddersburg	Free State
445	FS 424	Black Wildebeest	Female	Calf	Tissue	Seekoeivlei	Free State
446	FS 432	Black Wildebeest	Female	Adult	Tissue	Seekoeivlei	Free State
447	FS 433	Black Wildebeest	Female	Adult	Tissue	Seekoeivlei	Free State
448	FS 434	Black Wildebeest	Female	Sub-Adult	Tissue	Seekoeivlei	Free State
449	FS 435	Black Wildebeest	Male	Calf	Tissue	Seekoeivlei	Free State
450	FS 444	Black Wildebeest	Female	Calf	Tissue	Seekoeivlei	Free State
451	FS 445	Black Wildebeest	Male	Calf	Tissue	Seekoeivlei	Free State
452	FS 448	Black Wildebeest	Female	Adult	Tissue	Seekoeivlei	Free State
453	FS 454	Black Wildebeest	Male	Adult	Tissue	Seekoeivlei	Free State
454	FS 455	Black Wildebeest	Male	Adult	Tissue	Seekoeivlei	Free State

455	FS 456	Black Wildebeest	Female	Adult	Tissue	Seekoeivlei	Free State
456	FS 457	Black Wildebeest	Male	Adult	Tissue	Seekoeivlei	Free State
457	FS 712	Black Wildebeest	Female	Adult	Tissue	Geluk	
458	FS 713	Black Wildebeest	Male	Sub-Adult	Tissue	Geluk	
459	FS 721	Black Wildebeest	Female	Adult	Tissue	Geluk	
460	FS 722	Black Wildebeest	Female	Calf	Tissue	Geluk	
461	FS 723	Black Wildebeest	Female	Adult	Tissue	Geluk	
462	FS 724	Black Wildebeest	Male	Unknown	Tissue	Geluk	
463	FS 725	Black Wildebeest	Female	Sub-Adult	Tissue	Geluk	
464	FS 726	Black Wildebeest	Male	Sub-Adult	Tissue	Geluk	
465	FS 727	Black Wildebeest	Male	Unknown	Tissue	Geluk	
466	FS 728	Black Wildebeest	Female	Unknown	Tissue	Geluk	
467	FS 729	Black Wildebeest	Male	Unknown	Tissue	Geluk	
468	FS 730	Black Wildebeest	Male	Unknown	Tissue	Geluk	
469	FS 731	Black Wildebeest	Unknown	Unknown	Tissue	Geluk	
470	FS 732	Black Wildebeest	Female	Adult	Tissue	Geluk	
471	FS 733	Black Wildebeest	Female	Adult	Tissue	Geluk	
472	FS 734	Black Wildebeest	Female	Adult	Tissue	Geluk	
473	FS 735	Black Wildebeest	Female	Adult	Tissue	Geluk	
474	FS 736	Black Wildebeest	Unknown	Unknown	Tissue	Geluk	
475	FS 737	Black Wildebeest	Female	Adult	Tissue	Geluk	
476	FS 738	Black Wildebeest	Female	Adult	Tissue	Geluk	
477	FS 740	Black Wildebeest	Female	Adult	Tissue	Geluk	
478	FS 741	Black Wildebeest	Female	Adult	Tissue	Geluk	
479	FS 743	Black Wildebeest	Female	Adult	Tissue	Geluk	
480	FS 744	Black Wildebeest	Male	Calf	Tissue	Geluk	
481	FS 745	Black Wildebeest	Female	Adult	Tissue	Geluk	

482	FS 746	Black Wildebeest	Female	Calf	Tissue	Geluk	
483	FS 747	Black Wildebeest	Female	Calf	Tissue	Geluk	
484	FS 748	Black Wildebeest	Female	Adult	Tissue	Geluk	
485	FS 749	Black Wildebeest	Male	Calf	Tissue	Geluk	
486	FS 750	Black Wildebeest	Female	Calf	Tissue	Geluk	
487	FS 751	Black Wildebeest	Female	Adult	Tissue	Geluk	
488	FS 752	Black Wildebeest	Male	Sub-Adult	Tissue	Geluk	
489	FS 753	Black Wildebeest	Female	Calf	Tissue	Geluk	
490	FS 754	Black Wildebeest	Female	Calf	Tissue	Geluk	
491	FS 755	Black Wildebeest	Male	Calf	Tissue	Geluk	
492	FS 756	Black Wildebeest	Male	Calf	Tissue	Geluk	
493	FS 757	Black Wildebeest	Female	Calf	Tissue	Geluk	
494	FS 758	Black Wildebeest	Female	Adult	Tissue	Geluk	
495	FS 760	Black Wildebeest	Male	Calf	Tissue	Geluk	
496	FS 761	Black Wildebeest	Male	Calf	Tissue	Geluk	
497	FS 762	Black Wildebeest	Female	Adult	Tissue	Geluk	
498	FS 763	Black Wildebeest	Male	Calf	Tissue	Geluk	
499	FS 764	Black Wildebeest	Female	Adult	Tissue	Geluk	
500	FS 765	Black Wildebeest	Female	Sub-Adult	Tissue	Geluk	
501	FS 766	Black Wildebeest	Male	Sub-Adult	Tissue	Geluk	
502	FS 767	Black Wildebeest	Male	Sub-Adult	Tissue	Geluk	
503	FS 768	Black Wildebeest	Female	Adult	Tissue	Geluk	
504	FS 702	Black Wildebeest		Adult	Tissue	Perdeberg (Kimberly)	Northern Cape
505	FS 703	Black Wildebeest	Male	Adult	Tissue	Perdeberg (Kimberly - F1 Hybrid)	Northern Cape
506	FS 704	Black Wildebeest	Male	Adult	Tissue	Perdeberg (Kimberly - F1 Hybrid)	Northern Cape
507	FS 705	Black Wildebeest	Female	Adult	Tissue	Perdeberg (Kimberly)	Northern Cape
508	FS 710	Black Wildebeest	Female	Adult	Tissue	Perdeberg (Kimberly)	Northern Cape

509	FS 711	Black Wildebeest		Adult	Tissue	Perdeberg (Kimberly)	Northern Cape
510	Embryo 1	Black Wildebeest	Unknown	Embryo	Tissue	Perdeberg (Kimberly)	Northern Cape
511	Umbilical cord 1	Black Wildebeest	Unknown	Unknown	Tissue	Perdeberg (Kimberly)	Northern Cape

## **APPENDIX B**

Sample list: Reference populations – Blue and  
Black wildebeest

List of reference samples collected from pure blue and pure black wildebeest populations across South Africa

No.	Sample no.	Species	Sex	Age	Sample Type	Origin	Province
1	SA Lom 1	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
2	SA Lom 2	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
3	SA Lom 3	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
4	SA Lom 4	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
5	SA Lom 5	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
6	SA Lom 6	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
7	SA Lom 7	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
8	SA Lom 8	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
9	SA Lom 9	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
10	SA Lom 10	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
11	SA Lom 11	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
12	SA Lom 12	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
13	SA Lom 13	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
14	SA Lom 14	Black Wildebeest	Unknown	Unknown	Tissue	SA Lombard Nature Reserve	North West
15	07/420	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
16	07/421	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
17	07/422	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
18	07/423	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
19	07/424	Blue Wildebeest	Male	Juvenile	Tissue	Kruger National Park	Limpopo
20	07/425	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
21	07/426	Blue Wildebeest	Male	Adult	Tissue	Kruger National Park	Limpopo
22	07/427	Blue Wildebeest	Male	Sub-Adult	Tissue	Kruger National Park	Limpopo
23	07/428*	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
24	07/428	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo

25	07/429	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
26	07/430	Blue Wildebeest	Female	Sub-Adult	Tissue	Kruger National Park	Limpopo
27	07/432	Blue Wildebeest	Female	Sub-Adult	Tissue	Kruger National Park	Limpopo
28	07/433	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
29	07/434	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
30	07/435	Blue Wildebeest	Male	Adult	Tissue	Kruger National Park	Limpopo
31	07/436	Blue Wildebeest	Female	Adult	Tissue	Kruger National Park	Limpopo
32	Blue1	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
33	Blue2	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
34	Blue3	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
35	Blue4	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
36	Blue5	Black Wildebeest	Female	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
37	Blue6	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
38	Blue7	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
39	Blue8	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
40	Blue9	Black Wildebeest	Female	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
41	Blue10	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
42	Blue11	Black Wildebeest	Female	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
43	Blue12	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
44	Blue13	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
45	Blue14	Black Wildebeest	Female	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
46	Blue15	Black Wildebeest	Male	Adult	Hair	Kgalagadi Transfrontier Park	Northern Cape/Botswana
47	B529	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
48	B530	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
49	B531	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
50	B532	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape

51	B533	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
52	B534	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
53	B535	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
54	B536	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
55	B537	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
56	B538	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
57	B540	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
58	B541	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
59	B542	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
60	B544	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
61	B545	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
62	B546	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
63	B547	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
64	B548	Black Wildebeest	Unknown	Unknown	Hair	Benfontein	Northern Cape
65	BW 159	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
66	BW 160	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
67	BW 161	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
68	BW 162	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
69	BW 163	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
70	BW 164	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
71	BW 165	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
72	BW 166	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
73	BW 167	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
74	BW 168	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
75	BW 169	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
76	BW 170*	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
77	BW 170	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape

78	BW 171	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
79	BW 172	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
80	BW 173	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
81	BW 174	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
82	BW 175	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
83	BW 176	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
84	BW 178	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
85	BW 179	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
86	BW 182	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
87	BW 183	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
88	BW 185	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape
89	BW 186	Black Wildebeest	Unknown	Unknown	Tissue	Groote Schuur Estate	Western Cape

## **APPENDIX C**

Genetic profiles: Black wildebeest test  
populations

Genetic profiles of all the black wildebeest test populations sampled on Nature Reserves in the Free State Province and private game farms, for both loci BM1824 and ETH10.

<b>Sample no.</b>	<b>Origin</b>	<b>Eth 10</b>		<b>BM 1824</b>	
FS 15	Florida Private Game Farm	205	205	-	-
FS 16	Florida Private Game Farm	205	205	-	-
FS 9	Caledon NR	203	203	194	196
FS 20	Caledon NR	205	205	-	-
FS 32	Caledon NR	205	205	194	196
FS 33	Caledon NR	203	203	194	194
FS 35	Caledon NR	205	205	194	200
FS 18	Caledon NR	205	205	194	196
FS 19	Caledon NR	203	203	194	194
FS 21	Caledon NR	205	205	194	194
FS 22	Caledon NR	203	203	194	194
FS 27	Caledon NR	205	205	194	194
FS 31	Caledon NR	205	205	194	194
FS 36	Caledon NR	205	205	194	194
FS 130	Caledon NR	205	205	194	194
FS 145	Caledon NR	203	203	194	194
FS 147	Caledon NR	205	205	194	196
FS 148	Caledon NR	205	205	194	194
FS 149	Caledon NR	205	205	194	194
FS 150	Caledon NR	205	205	196	200
FS 157	Caledon NR	203	203	194	196
FS 158	Caledon NR	205	205	194	196
FS 159	Caledon NR	205	205	194	194
FS 160	Caledon NR	205	205	194	196
FS 165	Caledon NR	203	203	192	192
FS 151	Caledon NR	205	205	194	194
FS 152	Caledon NR	205	205	194	196
FS 153	Caledon NR	205	205	196	198
FS 161	Caledon NR	205	205	194	194
FS 162	Caledon NR	205	205	196	198
FS 163	Caledon NR	203	203	194	194
FS 164	Caledon NR	205	205	194	196
FS 166	Caledon NR	205	205	194	196
FS 167	Caledon NR	205	205	194	196
FS 169	Caledon NR	205	205	194	196
FS 197	Caledon NR	205	205	194	196
FS 200	Caledon NR	205	205	194	194
FS 201	Caledon NR	205	205	194	194
FS 202	Caledon NR	205	205	194	194

FS 211	Caledon NR	203	203	194	194
FS 212	Caledon NR	205	205	192	192
FS 215	Caledon NR	205	205	192	192
FS 218	Caledon NR	205	205	194	194
FS 219	Caledon NR	205	205	194	194
FS 220	Caledon NR	205	205	194	194
FS 423	Caledon NR	205	205	-	-
FS 425	Caledon NR	205	205	194	194
FS 42	Tussen die Riviere NR	-	-	192	192
FS 44	Tussen die Riviere NR	205	205	194	198
FS 49	Tussen die Riviere NR	205	205	192	194
FS 57	Tussen die Riviere NR	205	205	192	194
FS 59	Tussen die Riviere NR	205	205	194	198
FS 63	Tussen die Riviere NR	205	205	192	192
FS 65	Tussen die Riviere NR	205	205	194	194
FS 71	Tussen die Riviere NR	205	205	192	192
FS 72	Tussen die Riviere NR	203	203	194	194
FS 74	Tussen die Riviere NR	205	205	194	194
FS 75	Tussen die Riviere NR	205	205	194	196
FS 77	Tussen die Riviere NR	203	203	194	194
FS 141	Tussen die Riviere NR	205	205	194	194
FS 178	Tussen die Riviere NR	203	203	194	194
FS 179	Tussen die Riviere NR	205	205	196	196
FS 180	Tussen die Riviere NR	205	205	194	200
FS 181	Tussen die Riviere NR	205	205	194	210
FS 182	Tussen die Riviere NR	203	203	194	194
FS 183	Tussen die Riviere NR	205	205	194	194
FS 184	Tussen die Riviere NR	205	205	194	194
FS 185	Tussen die Riviere NR	205	205	196	196
FS 186	Tussen die Riviere NR	205	205	194	194
FS 187	Tussen die Riviere NR	205	205	194	194
FS 188	Tussen die Riviere NR	205	205	194	194
FS 189	Tussen die Riviere NR	203	203	196	200
FS 190	Tussen die Riviere NR	205	205	196	200
FS 191	Tussen die Riviere NR	205	205	194	194
FS 192	Tussen die Riviere NR	205	205	194	196
FS 193	Tussen die Riviere NR	205	205	194	196
FS 194	Tussen die Riviere NR	205	205	194	194
FS 195	Tussen die Riviere NR	205	205	196	196
FS 196	Tussen die Riviere NR	205	205	198	198
FS 198	Tussen die Riviere NR	205	205	194	194
FS 199	Tussen die Riviere NR	203	203	196	198

FS 204	Tussen die Riviere NR	203	203	196	198
FS 205	Tussen die Riviere NR	205	205	194	194
FS 206	Tussen die Riviere NR	205	205	194	194
FS 207	Tussen die Riviere NR	-	-	194	194
FS 208	Tussen die Riviere NR	205	205	194	194
FS 209	Tussen die Riviere NR	203	203	194	194
FS 210	Tussen die Riviere NR	203	203	194	196
FS 29	Maria Moroka NR	203	203	210	212
FS 37	Maria Moroka NR	205	205	196	210
FS 38	Maria Moroka NR	205	205	196	212
FS 39	Maria Moroka NR	203	203	194	194
FS 40	Maria Moroka NR	205	205	196	212
FS 48	Maria Moroka NR	205	205	194	210
FS 51	Maria Moroka NR	203	203	210	210
FS 52	Maria Moroka NR	205	205	196	196
FS 55	Maria Moroka NR	205	205	196	196
FS 56	Maria Moroka NR	-	-	194	210
FS 58	Maria Moroka NR	-	-	194	196
FS 61	Maria Moroka NR	203	203	196	196
FS 64	Maria Moroka NR	203	203	192	210
FS 67	Maria Moroka NR	203	203	196	212
FS 68	Maria Moroka NR	203	203	194	212
FS 69	Maria Moroka NR	205	205	194	210
FS 70	Maria Moroka NR	203	203	196	212
FS 73	Maria Moroka NR	203	203	194	196
FS 76	Maria Moroka NR	205	205	194	196
FS 79	Maria Moroka NR	205	205	194	210
FS 80	Maria Moroka NR	205	205	194	196
FS 81	Maria Moroka NR	205	205	196	196
FS 82	Maria Moroka NR	203	203	196	196
FS 83	Maria Moroka NR	203	203	194	210
FS 84	Maria Moroka NR	205	205	194	212
FS 85	Maria Moroka NR	203	203	210	210
FS 86	Maria Moroka NR	205	205	194	212
FS 87	Maria Moroka NR	205	205	196	200
FS 88	Maria Moroka NR	205	205	192	210
FS 89	Maria Moroka NR	205	205	210	212
FS 90	Maria Moroka NR	203	203	194	196
FS 91	Maria Moroka NR	205	205	194	196
FS 92	Maria Moroka NR	203	203	210	212
FS 93	Maria Moroka NR	205	205	212	212
FS 94	Maria Moroka NR	205	205	194	210

FS 95	Maria Moroka NR	205	205	-	-
FS 96	Maria Moroka NR	205	205	194	210
FS 97	Maria Moroka NR	203	203	194	210
FS 98	Maria Moroka NR	205	205	192	210
FS 99	Maria Moroka NR	203	203	194	194
FS 100	Maria Moroka NR	203	203	194	196
FS 101	Maria Moroka NR	205	205	192	196
FS 102	Maria Moroka NR	203	203	194	210
FS 103	Maria Moroka NR	203	203	194	212
FS 104	Maria Moroka NR	205	205	196	196
FS 105	Maria Moroka NR	205	205	194	194
FS 106	Maria Moroka NR	205	205	196	200
FS 107	Maria Moroka NR	203	203	192	198
FS 108	Maria Moroka NR	203	203	196	210
FS 109	Maria Moroka NR	205	205	194	196
FS 110	Maria Moroka NR	205	205	194	210
FS 111	Maria Moroka NR	205	205	196	200
FS 112	Maria Moroka NR	205	205	194	194
FS 113	Maria Moroka NR	205	205	210	212
FS 114	Maria Moroka NR	203	203	194	196
FS 115	Maria Moroka NR	205	205	194	196
FS 116	Maria Moroka NR	203	203	210	212
FS 118	Maria Moroka NR	205	205	192	210
FS 119	Maria Moroka NR	205	205	194	212
FS 120	Maria Moroka NR	203	203	194	194
FS 121	Maria Moroka NR	205	205	192	192
FS 122	Maria Moroka NR	205	205	194	198
FS 123	Maria Moroka NR	205	205	-	-
FS 124	Maria Moroka NR	205	205	194	194
FS 125	Maria Moroka NR	203	203	194	194
FS 126	Maria Moroka NR	205	205	-	-
FS 127	Maria Moroka NR	203	203	-	-
FS 128	Maria Moroka NR	205	205	194	194
FS 129	Maria Moroka NR	203	203	194	194
FS 131	Maria Moroka NR	203	203	-	-
FS 135	Maria Moroka NR	-	-	194	194
FS 137	Maria Moroka NR	-	-	194	198
FS 139	Maria Moroka NR	203	203	194	194
FS 140	Maria Moroka NR	205	205	198	198
FS 142	Maria Moroka NR	205	205	192	200
FS 143	Maria Moroka NR	205	205	194	210
FS 144	Maria Moroka NR	205	205	194	194

FS 407	Maria Moroka NR	205	205	192	192
FS 408	Maria Moroka NR	205	205	192	198
FS 409	Maria Moroka NR	205	205	194	194
W 1	Willem Pretorius NR	205	205	194	196
W 2	Willem Pretorius NR	205	205	196	196
W 3	Willem Pretorius NR	205	205	196	196
W 4	Willem Pretorius NR	205	205	196	196
W 5	Willem Pretorius NR	205	205	194	194
W 6	Willem Pretorius NR	205	205	194	194
W 7	Willem Pretorius NR	205	205	196	214
W 8	Willem Pretorius NR	205	205	194	196
W 9	Willem Pretorius NR	205	205	194	196
W 10	Willem Pretorius NR	205	205	196	196
FS 309	Willem Pretorius NR	205	205	-	-
FS 310	Willem Pretorius NR	203	203	196	200
FS 326	Willem Pretorius NR	205	205	200	200
FS 327	Willem Pretorius NR	205	205	196	200
FS 328	Willem Pretorius NR	205	205	194	194
FS 329	Willem Pretorius NR	205	205	194	196
FS 330	Willem Pretorius NR	205	205	196	200
FS 331	Willem Pretorius NR	205	205	196	200
FS 332	Willem Pretorius NR	205	205	196	196
FS 333	Willem Pretorius NR	205	205	196	196
FS 334	Willem Pretorius NR	205	205	196	196
FS 336	Willem Pretorius NR	205	205	196	200
FS 337	Willem Pretorius NR	-	-	196	196
FS 338	Willem Pretorius NR	205	205	194	200
FS 339	Willem Pretorius NR	205	205	198	198
FS 340	Willem Pretorius NR	205	205	194	198
FS 341	Willem Pretorius NR	205	205	194	200
FS 342	Willem Pretorius NR	205	205	200	200
FS 343	Willem Pretorius NR	205	205	196	200
FS 344	Willem Pretorius NR	205	205	196	196
FS 345	Willem Pretorius NR	205	205	194	196
FS 346	Willem Pretorius NR	205	205	196	196
FS 347	Willem Pretorius NR	205	205	196	196
FS 348	Willem Pretorius NR	205	205	196	200
FS 350	Willem Pretorius NR	203	203	196	200
FS 351	Willem Pretorius NR	205	205	196	196
FS 352	Willem Pretorius NR	205	205	194	200
FS 353	Willem Pretorius NR	205	205	194	196
FS 354	Willem Pretorius NR	205	205	196	200

FS 355	Willem Pretorius NR	205	205	194	196
FS 356	Willem Pretorius NR	205	205	196	200
FS 357	Willem Pretorius NR	205	205	196	196
FS 358	Willem Pretorius NR	205	205	196	200
FS 359	Willem Pretorius NR	205	205	194	196
FS 360	Willem Pretorius NR	205	205	194	196
FS 361	Willem Pretorius NR	205	205	194	196
FS 362	Willem Pretorius NR	205	205	196	200
FS 363	Willem Pretorius NR	205	205	196	196
FS 364	Willem Pretorius NR	205	205	196	196
FS 365	Willem Pretorius NR	205	205	194	196
FS 366	Willem Pretorius NR	205	205	200	214
FS 367	Willem Pretorius NR	203	203	196	196
FS 368	Willem Pretorius NR	205	205	196	200
FS 369	Willem Pretorius NR	203	203	194	194
FS 370	Willem Pretorius NR	205	205	194	196
FS 371	Willem Pretorius NR	205	205	194	196
FS 372	Willem Pretorius NR	205	205	196	196
FS 373	Willem Pretorius NR	-	-	194	194
FS 375	Willem Pretorius NR	205	205	194	196
FS 376	Willem Pretorius NR	203	203	196	200
FS 377	Willem Pretorius NR	205	205	194	196
FS 378	Willem Pretorius NR	203	203	194	196
FS 380	Willem Pretorius NR	203	203	194	196
FS 381	Willem Pretorius NR	205	205	194	194
FS 383	Willem Pretorius NR	205	205	-	-
FS 386	Willem Pretorius NR	205	205	196	212
FS 387	Willem Pretorius NR	205	205	194	196
FS 390	Willem Pretorius NR	205	205	194	194
FS 391	Willem Pretorius NR	205	205	194	196
FS 394	Willem Pretorius NR	205	205	194	194
FS 398	Willem Pretorius NR	205	205	194	196
FS 449	Willem Pretorius NR	203	203	198	198
FS 458	Willem Pretorius NR	203	203	194	194
FS 469	Willem Pretorius NR	203	203	-	-
FS 470	Willem Pretorius NR	205	205	194	200
FS 471	Willem Pretorius NR	205	205	196	198
FS 471 A	Willem Pretorius NR	205	205	196	198
FS 475	Willem Pretorius NR	205	205	196	196
FS 475 A	Willem Pretorius NR	205	205	196	196
FS 476	Willem Pretorius NR	205	205	194	194
FS 477	Willem Pretorius NR	205	205	194	194

FS 477 A	Willem Pretorius NR	205	205	194	210
FS 478	Willem Pretorius NR	205	205	194	194
FS 479	Willem Pretorius NR	203	203	194	194
FS 479 A	Willem Pretorius NR	203	203	194	194
FS 480	Willem Pretorius NR	205	205	194	210
FS 480 A	Willem Pretorius NR	205	205	194	210
FS 481	Willem Pretorius NR	203	203	196	196
FS 484	Willem Pretorius NR	205	205	196	210
FS 484 A	Willem Pretorius NR	205	205	210	210
FS 485	Willem Pretorius NR	205	205	192	198
FS 485 A	Willem Pretorius NR	205	205	194	194
FS 486	Willem Pretorius NR	205	205	194	194
FS 495	Willem Pretorius NR	205	205	196	196
FS 495 A	Willem Pretorius NR	205	205	194	198
FS 496	Willem Pretorius NR	205	205	194	194
FS 496 A	Willem Pretorius NR	205	205	-	-
FS 497	Willem Pretorius NR	205	205	194	210
FS 500	Willem Pretorius NR	205	205	194	200
FS 500 A	Willem Pretorius NR	205	205	196	200
FS 501	Willem Pretorius NR	205	205	194	198
FS 501 A	Willem Pretorius NR	205	205	194	194
L68	Odendaalsrus (Langkuil)	205	205	194	194
L69	Odendaalsrus (Langkuil)	205	205	192	192
L70	Odendaalsrus (Langkuil)	205	205	198	212
L71	Odendaalsrus (Langkuil)	205	205	194	194
L72	Odendaalsrus (Langkuil)	-	-	194	194
L73	Odendaalsrus (Langkuil)	205	205	198	198
L74	Odendaalsrus (Langkuil)	205	205	194	198
L75	Odendaalsrus (Langkuil)	205	205	198	198
L76	Odendaalsrus (Langkuil)	205	205	-	-
L77	Odendaalsrus (Langkuil)	205	205	192	192
L78	Odendaalsrus (Langkuil)	205	205	192	194
L79	Odendaalsrus (Langkuil)	205	205	198	210
L80	Odendaalsrus (Langkuil)	205	205	192	194
L82	Odendaalsrus (Langkuil)	205	205	192	198
L83	Odendaalsrus (Langkuil)	203	203	192	210
L85	Odendaalsrus (Langkuil)	205	205	198	198
L87	Odendaalsrus (Langkuil)	203	203	198	198
L88	Odendaalsrus (Langkuil)	203	203	194	198
L89	Odendaalsrus (Langkuil)	205	205	194	194
FS 451	Odendaalsrus (Langkuil)	205	205	-	-
FS 452	Odendaalsrus (Langkuil)	203	203	192	192

FS 453	Odendaalsrus (Langkuil)	205	205	194	194
G7948	Gariepdam NR	205	205		
G7952	Gariepdam NR	205	205		
G7954	Gariepdam NR	205	205		
G7964	Gariepdam NR	205	205		
G7969	Gariepdam NR	205	205		
G7973	Gariepdam NR	205	205		
FS 168	Gariepdam NR	205	205	196	196
FS 170	Gariepdam NR	205	205	196	196
FS 172	Gariepdam NR	205	205	196	198
FS 228	Gariepdam NR	205	205	194	194
FS 244	Gariepdam NR	205	205	194	194
FS 245	Gariepdam NR	205	205	194	194
FS 246	Gariepdam NR	205	205	194	194
FS 253	Gariepdam NR	205	205	194	194
FS 254	Gariepdam NR	205	205	196	196
FS 255	Gariepdam NR	205	205	194	194
FS 258	Gariepdam NR	205	205	194	194
FS 259	Gariepdam NR	205	205	194	212
FS 260	Gariepdam NR	203	203	194	194
FS 261	Gariepdam NR	205	205	194	194
FS 262	Gariepdam NR	205	205	194	194
FS 263	Gariepdam NR	205	205	194	198
FS 264	Gariepdam NR	205	205	194	194
FS 46	Koppiesdam	205	205	192	198
FS 66	Koppiesdam	205	205	192	192
FS 173	Koppiesdam	205	205	192	210
FS 237	Koppiesdam	205	205	194	196
FS 241	Koppiesdam	205	205	194	196
FS 249	Koppiesdam	205	205	194	196
FS 286	Koppiesdam	205	205	192	192
FS 287	Koppiesdam	205	205	192	192
FS 289	Koppiesdam	205	205	192	198
FS 290	Koppiesdam	205	205	198	198
FS 291	Koppiesdam	205	205	192	192
FS 292	Koppiesdam	205	205	192	192
FS 293	Koppiesdam	205	205	194	198
FS 294	Koppiesdam	205	205	192	192
FS 295	Koppiesdam	205	205	194	196
FS 296	Koppiesdam	205	205	194	196
FS 298	Koppiesdam	205	205	194	194
FS 299	Koppiesdam	205	205	192	192

FS 302	Koppiesdam	205	205	192	192
FS 304	Koppiesdam	205	205	-	-
FS 305	Koppiesdam	205	205	-	-
FS 306	Koppiesdam	205	205	194	196
FS 307	Koppiesdam	205	205	-	-
FS 316	Koppiesdam	205	205	192	198
FS 317	Koppiesdam	205	205	194	194
FS 318	Koppiesdam	205	205	192	192
FS 319	Koppiesdam	205	205	192	192
FS 320	Koppiesdam	205	205	194	196
FS 321	Koppiesdam	205	205	194	196
FS 322	Koppiesdam	205	205	192	192
FS 323	Koppiesdam	205	205	192	194
FS 324	Koppiesdam	205	205	192	194
FS 325	Koppiesdam	205	205	194	196
FS 1	Erfenisdam	-	-	194	196
FS 2	Erfenisdam	205	205	194	196
FS 3	Erfenisdam	-	-	196	196
FS 4	Erfenisdam	205	205	194	196
FS 5	Erfenisdam	205	205	194	196
FS 6	Erfenisdam	-	-	194	196
FS 349	Erfenisdam	205	205	194	196
FS 392	Erfenisdam	-	-	192	194
FS 393	Erfenisdam	205	205	192	200
FS 395	Erfenisdam	205	205	194	196
FS 396	Erfenisdam	205	205	194	196
FS 397	Erfenisdam	205	205	196	200
FS 399	Erfenisdam	205	205	192	194
FS 402	Erfenisdam	205	205	194	196
FS 403	Erfenisdam	205	205	194	196
FS 43	Sterkfonteindam	205	205	196	196
FS 174	Sterkfonteindam	205	205	194	200
FS 175	Sterkfonteindam	205	205	196	196
FS 176	Sterkfonteindam	205	205	194	200
FS 177	Sterkfonteindam	205	205	196	200
FS 238	Sterkfonteindam	205	205	194	194
FS 239	Sterkfonteindam	205	205	-	-
FS 240	Sterkfonteindam	205	205	194	194
FS 242	Sterkfonteindam	205	205	194	200
FS 243	Sterkfonteindam	205	205	194	194
FS 248	Sterkfonteindam	205	205	194	194
FS 256	Sterkfonteindam	205	205	196	200

FS 257	Sterkfonteindam	-	-	194	194
FS 268	Sterkfonteindam	205	205	194	194
FS 269	Sterkfonteindam	203	203	192	198
FS 270	Sterkfonteindam	205	205	194	198
FS 275	Sterkfonteindam	203	203	194	194
FS 276	Sterkfonteindam	203	203	192	192
FS 277	Sterkfonteindam	203	203	194	194
FS 278	Sterkfonteindam	203	203	194	198
FS 279	Sterkfonteindam	203	203	192	192
FS 280	Sterkfonteindam	203	203	192	210
DB 01	De Brug	205	205	194	196
DB 03	De Brug	205	205	194	194
DB 04	De Brug	203	203	194	196
DB 05	De Brug	203	203	194	194
DB 06	De Brug	205	205	196	200
DB 07	De Brug	205	205	194	194
DB 08	De Brug	205	205	194	194
DB 09	De Brug	205	205	194	194
DB 10	De Brug	205	205	194	196
DB 11	De Brug	203	203	194	194
DB 12	De Brug	205	205	194	194
DB 13	De Brug	205	205	194	194
DB 105	De Brug	205	205	194	196
DB 107	De Brug	203	203	192	192
DB 109	De Brug	205	205	194	196
DB 110	De Brug	203	203	194	196
DB 111	De Brug	205	205	192	200
DB 112	De Brug	205	205	196	196
DB 113	De Brug	203	203	192	194
FS 24	Soetdoring	205	205	194	194
FS 26	Soetdoring	203	203	194	194
FS 28	Soetdoring	205	205	192	196
FS 41	Soetdoring	203	203	192	192
FS 410	Soetdoring	205	205	192	192
FS 411	Soetdoring	203	203	194	194
FS 412	Soetdoring	203	203	194	194
FS 413	Soetdoring	205	205	194	194
FS 414	Soetdoring	205	205	194	194
FS 415	Soetdoring	205	205	194	194
FS 418	Soetdoring	205	205	194	194
FS 419	Soetdoring	205	205	194	194
FS 420	Soetdoring	205	205	194	194

FS 421	Soetdoring	203	203	194	194
FS 422	Soetdoring	203	203	196	196
FS 426	Soetdoring	205	205	194	194
FS 428	Soetdoring	205	205	194	194
FS 429	Soetdoring	205	205	194	194
FS 431	Soetdoring	203	203	194	196
FS 437	Soetdoring	203	203	194	196
FS 438	Soetdoring	203	203	194	194
FS 439	Soetdoring	205	205	194	194
FS 440	Soetdoring	205	205	-	-
FS 441	Soetdoring	205	205	194	194
FS 446	Soetdoring	-	-	194	196
FS 447	Soetdoring	203	203	194	194
FS 450	Soetdoring	203	203	194	194
FS 230	Rustfonteindam	205	205	194	194
FS 231	Rustfonteindam	205	205	194	194
FS 232	Rustfonteindam	205	205	194	194
FS 233	Rustfonteindam	205	205	194	196
FS 234	Rustfonteindam	205	205	194	196
FS 235	Rustfonteindam	205	205	194	194
FS 236	Rustfonteindam	205	205	194	194
FS 271	Reddersburg	205	205	194	200
FS 272	Reddersburg	205	205	194	194
FS 273	Reddersburg	205	205	194	210
FS 274	Reddersburg	205	205	192	200
FS 283	Reddersburg	205	205	196	200
FS 284	Reddersburg	205	205	192	194
FS 424	Seekoeivlei	205	205	-	-
FS 432	Seekoeivlei	205	205	194	194
FS 433	Seekoeivlei	205	205	194	194
FS 434	Seekoeivlei	205	205	196	200
FS 435	Seekoeivlei	205	205	194	194
FS 444	Seekoeivlei	205	205	194	194
FS 445	Seekoeivlei	205	205	194	194
FS 448	Seekoeivlei	205	205	194	194
FS 454	Seekoeivlei	205	205	194	196
FS 455	Seekoeivlei	205	205	194	196
FS 456	Seekoeivlei	205	205	192	200
FS 457	Seekoeivlei	205	205	194	196
FS 712	Geluk	203	203	200	200
FS 713	Geluk	203	203	198	198
FS 721	Geluk	203	203	198	198

FS 722	Geluk	203	203	192	198
FS 723	Geluk	-	-	200	200
FS 724	Geluk	203	203	200	200
FS 725	Geluk	203	203	200	200
FS 726	Geluk	203	203	192	198
FS 727	Geluk	203	203	192	200
FS 728	Geluk	203	203	200	200
FS 729	Geluk	203	203	198	198
FS 730	Geluk	203	203	192	198
FS 731	Geluk	203	203	198	198
FS 732	Geluk	203	203	198	210
FS 733	Geluk	205	205	194	194
FS 734	Geluk	205	205	194	212
FS 735	Geluk	205	205	200	210
FS 736	Geluk	203	203	200	200
FS 737	Geluk	-	-	192	192
FS 738	Geluk	-	-	192	192
FS 740	Geluk	-	-	192	192
FS 741	Geluk	203	203	200	200
FS 743	Geluk	203	203	192	198
FS 744	Geluk	203	203	198	198
FS 745	Geluk	203	203	198	198
FS 746	Geluk	203	203	192	198
FS 747	Geluk	203	203	192	198
FS 748	Geluk	205	205	196	198
FS 749	Geluk	203	203	198	210
FS 750	Geluk	203	203	192	198
FS 751	Geluk	205	205	198	210
FS 752	Geluk	205	205	196	200
FS 753	Geluk	203	203	198	198
FS 754	Geluk	203	203	200	210
FS 755	Geluk	203	203	198	210
FS 756	Geluk	203	203	198	210
FS 757	Geluk	203	203	200	200
FS 758	Geluk	203	203	198	198
FS 760	Geluk	203	203	198	198
FS 761	Geluk	203	203	200	200
FS 762	Geluk	203	203	200	200
FS 763	Geluk	203	203	192	198
FS 764	Geluk	203	203	198	198
FS 765	Geluk	203	203	200	200
FS 766	Geluk	203	203	192	198

FS 767	Geluk	203	203	-	-
FS 768	Geluk	203	203	200	200
FS 702	Perdeberg (Kimberly)	203	203	194	194
FS 703	Perdeberg (Kimberly - F1 Hybrid)	203	209	198	206
FS 704	Perdeberg (Kimberly - F1 Hybrid)	205	211	198	206
FS 705	Perdeberg (Kimberly)	205	205	194	198
FS 710	Perdeberg (Kimberly)	203	203	194	198
FS 711	Perdeberg (Kimberly)	-	-	194	194
Embryo 1	Perdeberg (Kimberly)	203	209	198	206
Umbilical cord 1	Perdeberg (Kimberly)	203	209	198	206

## **APPENDIX D**

Genetic profiles: Reference populations – Blue  
and Black wildebeest

Genetic profiles obtained for the blue and black wildebeest reference populations using the loci BM1824 and ETH10.

Sample no.	Origin	Eth 10		BM 1824	
SA Lom 2	SA Lombard NR	205	205	194	194
SA Lom 3	SA Lombard NR	203	203	-	-
SA Lom 4	SA Lombard NR	205	205	196	198
SA Lom 5	SA Lombard NR	205	205	194	194
SA Lom 6	SA Lombard NR	205	205	196	198
SA Lom 7	SA Lombard NR	205	205	198	198
SA Lom 8	SA Lombard NR	205	205	194	194
SA Lom 9	SA Lombard NR	205	205	196	198
SA Lom 10	SA Lombard NR	203	203	196	198
SA Lom 11	SA Lombard NR	205	205	198	198
SA Lom 12	SA Lombard NR	205	205	200	200
SA Lom 13	SA Lombard NR	205	205	196	198
SA Lom 14	SA Lombard NR	205	205	198	198
07/420	Kruger National Park	205	211	-	-
07/421	Kruger National Park	211	211	178	202
07/422	Kruger National Park	205	211	178	178
07/423	Kruger National Park	211	211	-	-
07/424	Kruger National Park	211	211	202	204
07/425	Kruger National Park	205	211	178	200
07/426	Kruger National Park	205	211	178	200
07/427	Kruger National Park	211	211	178	202
07/428*	Kruger National Park	205	211	178	200
07/428	Kruger National Park	211	211	178	178
07/429	Kruger National Park	211	211	202	206
07/430	Kruger National Park	211	211	204	204
07/432	Kruger National Park	205	205	178	204
07/433	Kruger National Park			178	178
07/434	Kruger National Park			178	178
07/435	Kruger National Park			204	204
07/436	Kruger National Park			204	204
Blue1	Kgalagadi	205	205	178	204
Blue2	Kgalagadi	205	211	206	206
Blue3	Kgalagadi	205	211	-	-
Blue4	Kgalagadi	205	209	210	210
Blue5	Kgalagadi	205	211	178	206
Blue6	Kgalagadi	205	211	200	200
Blue7	Kgalagadi	-	-	178	206

Blue8	Kgalagadi	205	211	200	200
Blue9	Kgalagadi	205	205	206	206
Blue10	Kgalagadi	205	209	192	192
Blue11	Kgalagadi	205	211	192	200
Blue12	Kgalagadi	205	211	192	200
Blue13	Kgalagadi	205	205	192	200
Blue14	Kgalagadi	205	211	194	198
Blue15	Kgalagadi	-	-	178	206
B529	Benfontein	205	205	194	194
B530	Benfontein	205	205	192	192
B531	Benfontein	205	205	192	192
B532	Benfontein	205	205	194	198
B533	Benfontein	209	209	194	198
B534	Benfontein	205	205	194	198
B535	Benfontein	203	203	-	-
B536	Benfontein	205	205	-	-
B537	Benfontein	-	-	192	192
B538	Benfontein	205	205	194	194
B540	Benfontein	205	205	194	194
B541	Benfontein	205	205	192	192
B542	Benfontein	205	205	194	194
B544	Benfontein	205	205	192	198
B545	Benfontein	205	205	192	192
B546	Benfontein	205	205	194	194
B547	Benfontein	205	205	194	194
B548	Benfontein	205	205	194	194
BW 159	Groote Schuur Estate	205	205	-	-
BW 160	Groote Schuur Estate	205	205	194	194
BW 161	Groote Schuur Estate	205	205	198	210
BW 162	Groote Schuur Estate	205	205	192	192
BW 163	Groote Schuur Estate	205	205	192	198
BW 164	Groote Schuur Estate	205	205	192	198
BW 165	Groote Schuur Estate	205	205	194	200
BW 166	Groote Schuur Estate	205	205	192	192
BW 167	Groote Schuur Estate	-	-	192	192
BW 168	Groote Schuur Estate	205	205	192	198
BW 169	Groote Schuur Estate	205	205	192	200
BW 170*	Groote Schuur Estate	205	205	192	192
BW 170	Groote Schuur Estate	205	205	192	192
BW 171	Groote Schuur Estate	205	205	192	192
BW 172	Groote Schuur Estate	205	205	194	198
BW 173	Groote Schuur Estate	205	205	192	192

BW 174	Groote Schuur Estate	205	205	192	210
BW 175	Groote Schuur Estate	-	-	194	200
BW 176	Groote Schuur Estate	-	-	192	192
BW 178	Groote Schuur Estate	-	-	194	194
BW 179	Groote Schuur Estate	-	-	192	198
BW 182	Groote Schuur Estate	205	205	-	-
BW 183	Groote Schuur Estate	205	205	192	192
BW 185	Groote Schuur Estate	205	205	192	192
BW 186	Groote Schuur Estate	205	205	192	210

## **APPENDIX E**

GeneClass Assignment test results

Individual assignment of the black wildebeest test populations (per individual) to one of the two reference populations (pure blue or pure black wildebeest). The highlighted individuals were assigned to the blue wildebeest cluster.

Individual	Populations	Assignment	
		Black	Blue
FPG1	Florida Private Game Farm	1.000	0.338
FPG2	Florida Private Game Farm	1.000	0.316
CAL1	Caledon	0.077	0.000
CAL2	Caledon	1.000	0.365
CAL3	Caledon	0.558	0.002
CAL4	Caledon	0.110	0.000
CAL5	Caledon	0.593	0.221
CAL6	Caledon	0.558	0.002
CAL7	Caledon	0.110	0.000
CAL8	Caledon	0.733	0.040
CAL9	Caledon	0.110	0.000
CAL10	Caledon	0.733	0.040
CAL11	Caledon	0.733	0.040
CAL12	Caledon	0.733	0.040
CAL13	Caledon	0.733	0.040
CAL14	Caledon	0.110	0.000
CAL15	Caledon	0.558	0.002
CAL16	Caledon	0.733	0.040
CAL17	Caledon	0.733	0.040
CAL18	Caledon	0.418	0.029
CAL19	Caledon	0.077	0.000
CAL20	Caledon	0.558	0.002
CAL21	Caledon	0.733	0.040
CAL22	Caledon	0.558	0.002
CAL23	Caledon	0.146	0.000
CAL24	Caledon	0.733	0.040
CAL25	Caledon	0.558	0.002
CAL26	Caledon	0.516	0.002
CAL27	Caledon	0.733	0.040
CAL28	Caledon	0.516	0.002
CAL29	Caledon	0.110	0.000
CAL30	Caledon	0.558	0.002
CAL31	Caledon	0.558	0.002
CAL32	Caledon	0.558	0.002
CAL33	Caledon	0.558	0.002

CAL34	Caledon	0.558	0.002
CAL35	Caledon	0.733	0.040
CAL36	Caledon	0.733	0.040
CAL37	Caledon	0.733	0.040
CAL38	Caledon	0.110	0.000
CAL39	Caledon	0.928	0.298
CAL40	Caledon	0.928	0.298
CAL41	Caledon	0.733	0.040
CAL42	Caledon	0.733	0.040
CAL43	Caledon	0.733	0.040
CAL44	Caledon	1.000	0.309
CAL45	Caledon	0.733	0.040
TDR1	Tussen-die-Riviere	0.722	0.204
TDR2	Tussen-die-Riviere	0.867	0.041
TDR3	Tussen-die-Riviere	1.000	0.124
TDR4	Tussen-die-Riviere	1.000	0.124
TDR5	Tussen-die-Riviere	0.867	0.041
TDR6	Tussen-die-Riviere	0.928	0.298
TDR7	Tussen-die-Riviere	0.733	0.040
TDR8	Tussen-die-Riviere	0.928	0.298
TDR9	Tussen-die-Riviere	0.110	0.000
TDR10	Tussen-die-Riviere	0.733	0.040
TDR11	Tussen-die-Riviere	0.558	0.002
TDR12	Tussen-die-Riviere	0.110	0.000
TDR13	Tussen-die-Riviere	0.733	0.040
TDR14	Tussen-die-Riviere	0.110	0.000
TDR15	Tussen-die-Riviere	0.296	0.001
TDR16	Tussen-die-Riviere	0.593	0.221
TDR17	Tussen-die-Riviere	0.500	0.099
TDR18	Tussen-die-Riviere	0.110	0.000
TDR19	Tussen-die-Riviere	0.733	0.040
TDR20	Tussen-die-Riviere	0.733	0.040
TDR21	Tussen-die-Riviere	0.296	0.001
TDR22	Tussen-die-Riviere	0.733	0.040
TDR23	Tussen-die-Riviere	0.733	0.040
TDR24	Tussen-die-Riviere	0.733	0.040
TDR25	Tussen-die-Riviere	0.032	0.000
TDR26	Tussen-die-Riviere	0.418	0.029
TDR27	Tussen-die-Riviere	0.733	0.040
TDR28	Tussen-die-Riviere	0.558	0.002
TDR29	Tussen-die-Riviere	0.558	0.002
TDR30	Tussen-die-Riviere	0.733	0.040

TDR31	Tussen-die-Riviere	0.296	0.001
TDR32	Tussen-die-Riviere	0.605	0.040
TDR33	Tussen-die-Riviere	0.733	0.040
TDR34	Tussen-die-Riviere	0.055	0.000
TDR35	Tussen-die-Riviere	0.055	0.000
TDR36	Tussen-die-Riviere	0.733	0.040
TDR37	Tussen-die-Riviere	0.733	0.040
TDR38	Tussen-die-Riviere	0.521	0.027
TDR39	Tussen-die-Riviere	0.733	0.040
TDR40	Tussen-die-Riviere	0.110	0.000
TDR41	Tussen-die-Riviere	0.077	0.000
MM1	Maria Moroka	0.000	0.000
MM2	Maria Moroka	0.301	0.009
MM3	Maria Moroka	0.046	0.009
MM4	Maria Moroka	0.110	0.000
MM5	Maria Moroka	0.046	0.009
MM6	Maria Moroka	0.500	0.099
MM7	Maria Moroka	0.009	0.000
MM8	Maria Moroka	0.296	0.001
MM9	Maria Moroka	0.296	0.001
MM10	Maria Moroka	0.228	0.044
MM11	Maria Moroka	0.323	0.005
MM12	Maria Moroka	0.019	0.000
MM13	Maria Moroka	0.060	0.000
MM14	Maria Moroka	0.000	0.000
MM15	Maria Moroka	0.000	0.000
MM16	Maria Moroka	0.500	0.099
MM17	Maria Moroka	0.000	0.000
MM18	Maria Moroka	0.077	0.000
MM19	Maria Moroka	0.558	0.002
MM20	Maria Moroka	0.500	0.099
MM21	Maria Moroka	0.558	0.002
MM22	Maria Moroka	0.296	0.001
MM23	Maria Moroka	0.019	0.000
MM24	Maria Moroka	0.049	0.000
MM25	Maria Moroka	0.100	0.099
MM26	Maria Moroka	0.009	0.000
MM27	Maria Moroka	0.100	0.099
MM28	Maria Moroka	0.418	0.029
MM29	Maria Moroka	0.520	0.303
MM30	Maria Moroka	0.036	0.192
MM31	Maria Moroka	0.077	0.000

MM32	Maria Moroka	0.558	0.002
MM33	Maria Moroka	0.000	0.000
<b>MM34</b>	Maria Moroka	<b>0.010</b>	<b>0.138</b>
MM35	Maria Moroka	0.500	0.099
MM36	Maria Moroka	1.000	0.356
MM37	Maria Moroka	0.500	0.099
MM38	Maria Moroka	0.049	0.000
MM39	Maria Moroka	0.520	0.303
MM40	Maria Moroka	0.110	0.000
MM41	Maria Moroka	0.077	0.000
MM42	Maria Moroka	0.588	0.013
MM43	Maria Moroka	0.049	0.000
MM44	Maria Moroka	0.000	0.000
MM45	Maria Moroka	0.296	0.001
MM46	Maria Moroka	0.733	0.040
MM47	Maria Moroka	0.418	0.029
MM48	Maria Moroka	0.154	0.000
MM49	Maria Moroka	0.019	0.000
MM50	Maria Moroka	0.558	0.002
MM51	Maria Moroka	0.500	0.099
MM52	Maria Moroka	0.418	0.029
MM53	Maria Moroka	0.733	0.040
<b>MM54</b>	Maria Moroka	<b>0.036</b>	<b>0.192</b>
MM55	Maria Moroka	0.077	0.000
MM56	Maria Moroka	0.558	0.002
MM57	Maria Moroka	0.000	0.000
MM58	Maria Moroka	0.520	0.303
MM59	Maria Moroka	0.100	0.099
MM60	Maria Moroka	0.110	0.000
MM61	Maria Moroka	0.928	0.298
MM62	Maria Moroka	0.867	0.041
MM63	Maria Moroka	1.000	0.327
MM64	Maria Moroka	0.733	0.040
MM65	Maria Moroka	0.110	0.000
MM66	Maria Moroka	1.000	0.357
MM67	Maria Moroka	0.037	0.000
MM68	Maria Moroka	0.733	0.040
MM69	Maria Moroka	0.110	0.000
MM70	Maria Moroka	0.033	0.000
MM71	Maria Moroka	0.524	0.027
MM72	Maria Moroka	0.691	0.036
MM73	Maria Moroka	0.110	0.000

MM74	Maria Moroka	0.605	0.040
MM75	Maria Moroka	0.620	0.498
MM76	Maria Moroka	0.500	0.099
MM77	Maria Moroka	0.733	0.040
MM78	Maria Moroka	0.928	0.298
MM79	Maria Moroka	0.941	0.124
MM80	Maria Moroka	0.733	0.040
WP1	Willem Pretorius	0.558	0.002
WP2	Willem Pretorius	0.296	0.001
WP3	Willem Pretorius	0.296	0.001
WP4	Willem Pretorius	0.296	0.001
WP5	Willem Pretorius	0.733	0.040
WP6	Willem Pretorius	0.733	0.040
WP7	Willem Pretorius	0.046	0.000
WP8	Willem Pretorius	0.558	0.002
WP9	Willem Pretorius	0.558	0.002
WP10	Willem Pretorius	0.296	0.001
WP11	Willem Pretorius	1.000	0.323
WP12	Willem Pretorius	0.032	0.000
<b>WP13</b>	Willem Pretorius	<b>0.396</b>	<b>0.502</b>
WP14	Willem Pretorius	0.418	0.029
WP15	Willem Pretorius	0.733	0.040
WP16	Willem Pretorius	0.558	0.002
WP17	Willem Pretorius	0.418	0.029
WP18	Willem Pretorius	0.418	0.029
WP19	Willem Pretorius	0.296	0.001
WP20	Willem Pretorius	0.296	0.001
WP21	Willem Pretorius	0.296	0.001
WP22	Willem Pretorius	0.418	0.029
WP23	Willem Pretorius	0.048	0.000
WP24	Willem Pretorius	0.593	0.221
WP25	Willem Pretorius	0.605	0.040
WP26	Willem Pretorius	0.867	0.041
WP27	Willem Pretorius	0.593	0.221
<b>WP28</b>	Willem Pretorius	<b>0.396</b>	<b>0.502</b>
WP29	Willem Pretorius	0.418	0.029
WP30	Willem Pretorius	0.296	0.001
WP31	Willem Pretorius	0.558	0.002
WP32	Willem Pretorius	0.296	0.001
WP33	Willem Pretorius	0.296	0.001
WP34	Willem Pretorius	0.418	0.029
WP35	Willem Pretorius	0.032	0.000

WP36	Willem Pretorius	0.296	0.001
WP37	Willem Pretorius	0.593	0.221
WP38	Willem Pretorius	0.558	0.002
WP39	Willem Pretorius	0.418	0.029
WP40	Willem Pretorius	0.558	0.002
WP41	Willem Pretorius	0.418	0.029
WP42	Willem Pretorius	0.296	0.001
WP43	Willem Pretorius	0.418	0.029
WP44	Willem Pretorius	0.558	0.002
WP45	Willem Pretorius	0.558	0.002
WP46	Willem Pretorius	0.558	0.002
WP47	Willem Pretorius	0.418	0.029
WP48	Willem Pretorius	0.296	0.001
WP49	Willem Pretorius	0.296	0.001
WP50	Willem Pretorius	0.558	0.002
WP51	Willem Pretorius	0.052	0.029
WP52	Willem Pretorius	0.019	0.000
WP53	Willem Pretorius	0.418	0.029
WP54	Willem Pretorius	0.110	0.000
WP55	Willem Pretorius	0.558	0.002
WP56	Willem Pretorius	0.558	0.002
WP57	Willem Pretorius	0.296	0.001
WP58	Willem Pretorius	0.523	0.031
WP59	Willem Pretorius	0.558	0.002
WP60	Willem Pretorius	0.032	0.000
WP61	Willem Pretorius	0.558	0.002
WP62	Willem Pretorius	0.077	0.000
WP63	Willem Pretorius	0.077	0.000
WP64	Willem Pretorius	0.733	0.040
WP65	Willem Pretorius	1.000	0.343
WP66	Willem Pretorius	0.046	0.009
WP67	Willem Pretorius	0.558	0.002
WP68	Willem Pretorius	0.733	0.040
WP69	Willem Pretorius	0.558	0.002
WP70	Willem Pretorius	0.733	0.040
WP71	Willem Pretorius	0.558	0.002
WP72	Willem Pretorius	0.083	0.000
WP73	Willem Pretorius	0.110	0.000
WP74	Willem Pretorius	0.030	0.000
WP75	Willem Pretorius	0.593	0.221
WP76	Willem Pretorius	0.516	0.002
WP77	Willem Pretorius	0.516	0.002

WP78	Willem Pretorius	0.296	0.001
WP79	Willem Pretorius	0.296	0.001
WP80	Willem Pretorius	0.733	0.040
WP81	Willem Pretorius	0.733	0.040
WP82	Willem Pretorius	0.500	0.099
WP83	Willem Pretorius	0.733	0.040
WP84	Willem Pretorius	0.110	0.000
WP85	Willem Pretorius	0.110	0.000
WP86	Willem Pretorius	0.500	0.099
WP87	Willem Pretorius	0.500	0.099
WP88	Willem Pretorius	0.019	0.000
WP89	Willem Pretorius	0.301	0.009
WP90	Willem Pretorius	0.176	0.138
WP91	Willem Pretorius	0.941	0.124
WP92	Willem Pretorius	0.733	0.040
WP93	Willem Pretorius	0.733	0.040
WP94	Willem Pretorius	0.296	0.001
WP95	Willem Pretorius	0.867	0.041
WP96	Willem Pretorius	0.733	0.040
WP97	Willem Pretorius	1.000	0.333
WP98	Willem Pretorius	0.500	0.099
WP99	Willem Pretorius	0.593	0.221
WP100	Willem Pretorius	0.418	0.029
WP101	Willem Pretorius	0.867	0.041
WP102	Willem Pretorius	0.733	0.040
ODE1	Odendaalsrus	0.733	0.040
ODE2	Odendaalsrus	0.928	0.298
ODE3	Odendaalsrus	0.083	0.099
ODE4	Odendaalsrus	0.733	0.040
ODE5	Odendaalsrus	0.553	0.027
ODE6	Odendaalsrus	0.605	0.040
ODE7	Odendaalsrus	0.867	0.041
ODE8	Odendaalsrus	0.605	0.040
ODE9	Odendaalsrus	1.000	0.307
ODE10	Odendaalsrus	0.928	0.298
ODE11	Odendaalsrus	1.000	0.124
ODE12	Odendaalsrus	0.454	0.099
ODE13	Odendaalsrus	1.000	0.124
ODE14	Odendaalsrus	0.941	0.124
ODE15	Odendaalsrus	0.060	0.000
ODE16	Odendaalsrus	0.605	0.040
ODE17	Odendaalsrus	0.083	0.000

ODE18	Odendaalsrus	0.141	0.000
ODE19	Odendaalsrus	0.733	0.040
ODE20	Odendaalsrus	1.000	0.343
ODE21	Odendaalsrus	0.146	0.000
ODE22	Odendaalsrus	0.733	0.040
GD1	Gariepdam	1.000	0.322
GD2	Gariepdam	1.000	0.334
GD3	Gariepdam	1.000	0.339
GD4	Gariepdam	1.000	0.346
GD5	Gariepdam	1.000	0.335
GD6	Gariepdam	1.000	0.329
GD7	Gariepdam	0.296	0.001
GD8	Gariepdam	0.296	0.001
GD9	Gariepdam	0.516	0.002
GD10	Gariepdam	0.733	0.040
GD11	Gariepdam	0.733	0.040
GD12	Gariepdam	0.733	0.040
GD13	Gariepdam	0.733	0.040
GD14	Gariepdam	0.733	0.040
GD15	Gariepdam	0.296	0.001
GD16	Gariepdam	0.733	0.040
GD17	Gariepdam	0.733	0.040
GD18	Gariepdam	0.100	0.099
GD19	Gariepdam	0.110	0.000
GD20	Gariepdam	0.733	0.040
GD21	Gariepdam	0.733	0.040
GD22	Gariepdam	0.867	0.041
GD23	Gariepdam	0.733	0.040
KD1	Koppiesdam	0.941	0.124
KD2	Koppiesdam	0.928	0.298
KD3	Koppiesdam	0.520	0.303
KD4	Koppiesdam	0.558	0.002
KD5	Koppiesdam	0.558	0.002
KD6	Koppiesdam	0.558	0.002
KD7	Koppiesdam	0.928	0.298
KD8	Koppiesdam	0.928	0.298
KD9	Koppiesdam	0.941	0.124
KD10	Koppiesdam	0.605	0.040
KD11	Koppiesdam	0.928	0.298
KD12	Koppiesdam	0.928	0.298
KD13	Koppiesdam	0.867	0.041
KD14	Koppiesdam	0.928	0.298

KD15	Koppiesdam	0.558	0.002
KD16	Koppiesdam	0.558	0.002
KD17	Koppiesdam	0.733	0.040
KD18	Koppiesdam	0.928	0.298
KD19	Koppiesdam	0.928	0.298
KD20	Koppiesdam	1.000	0.339
KD21	Koppiesdam	1.000	0.323
KD22	Koppiesdam	0.558	0.002
KD23	Koppiesdam	1.000	0.339
KD24	Koppiesdam	0.941	0.124
KD25	Koppiesdam	0.733	0.040
KD26	Koppiesdam	0.928	0.298
KD27	Koppiesdam	0.928	0.298
KD28	Koppiesdam	0.558	0.002
KD29	Koppiesdam	0.558	0.002
KD30	Koppiesdam	0.928	0.298
KD31	Koppiesdam	1.000	0.124
KD32	Koppiesdam	1.000	0.124
KD33	Koppiesdam	0.558	0.002
ERFD1	Erfenisdam	0.322	0.003
ERFD2	Erfenisdam	0.558	0.002
ERFD3	Erfenisdam	0.052	0.001
ERFD4	Erfenisdam	0.558	0.002
ERFD5	Erfenisdam	0.558	0.002
ERFD6	Erfenisdam	0.339	0.005
ERFD7	Erfenisdam	0.558	0.002
ERFD8	Erfenisdam	0.985	0.059
ERFD9	Erfenisdam	0.620	0.498
ERFD10	Erfenisdam	0.558	0.002
ERFD11	Erfenisdam	0.558	0.002
ERFD12	Erfenisdam	0.418	0.029
ERFD13	Erfenisdam	1.000	0.124
ERFD14	Erfenisdam	0.558	0.002
ERFD15	Erfenisdam	0.558	0.002
SFD1	Sterkfonteindam	0.296	0.001
SFD2	Sterkfonteindam	0.593	0.221
SFD3	Sterkfonteindam	0.296	0.001
SFD4	Sterkfonteindam	0.593	0.221
SFD5	Sterkfonteindam	0.418	0.029
SFD6	Sterkfonteindam	0.733	0.040
SFD7	Sterkfonteindam	1.000	0.367
SFD8	Sterkfonteindam	0.733	0.040

SFD9	Sterkfonteindam	0.593	0.221
SFD10	Sterkfonteindam	0.733	0.040
SFD11	Sterkfonteindam	0.733	0.040
SFD12	Sterkfonteindam	0.418	0.029
SFD13	Sterkfonteindam	0.519	0.036
SFD14	Sterkfonteindam	0.733	0.040
SFD15	Sterkfonteindam	0.154	0.000
SFD16	Sterkfonteindam	0.867	0.041
SFD17	Sterkfonteindam	0.110	0.000
SFD18	Sterkfonteindam	0.146	0.000
SFD19	Sterkfonteindam	0.110	0.000
SFD20	Sterkfonteindam	0.141	0.000
SFD21	Sterkfonteindam	0.146	0.000
SFD22	Sterkfonteindam	0.060	0.000
DB01	De Brug	0.558	0.002
DB03	De Brug	0.733	0.040
DB04	De Brug	0.077	0.000
DB05	De Brug	0.110	0.000
DB06	De Brug	0.418	0.029
DB07	De Brug	0.733	0.040
DB08	De Brug	0.733	0.040
DB09	De Brug	0.733	0.040
DB10	De Brug	0.558	0.002
DB11	De Brug	0.110	0.000
DB12	De Brug	0.733	0.040
DB13	De Brug	0.733	0.040
DB14	De Brug	0.558	0.002
DB15	De Brug	0.146	0.000
DB16	De Brug	0.558	0.002
DB17	De Brug	0.077	0.000
DB18	De Brug	0.620	0.498
DB19	De Brug	0.296	0.001
DB20	De Brug	0.172	0.000
SOE1	Soetdoring	0.733	0.040
SOE2	Soetdoring	0.110	0.000
SOE3	Soetdoring	0.588	0.013
SOE4	Soetdoring	0.146	0.000
SOE5	Soetdoring	0.928	0.298
SOE6	Soetdoring	0.110	0.000
SOE7	Soetdoring	0.110	0.000
SOE8	Soetdoring	0.733	0.040
SOE9	Soetdoring	0.733	0.040

SOE10	Soetdoring	0.733	0.040
SOE11	Soetdoring	0.733	0.040
SOE12	Soetdoring	0.733	0.040
SOE13	Soetdoring	0.733	0.040
SOE14	Soetdoring	0.110	0.000
SOE15	Soetdoring	0.019	0.000
SOE16	Soetdoring	0.733	0.040
SOE17	Soetdoring	0.733	0.040
SOE18	Soetdoring	0.733	0.040
SOE19	Soetdoring	0.077	0.000
SOE20	Soetdoring	0.077	0.000
SOE21	Soetdoring	0.110	0.000
SOE22	Soetdoring	0.733	0.040
SOE23	Soetdoring	1.000	0.325
SOE24	Soetdoring	0.733	0.040
SOE25	Soetdoring	0.325	0.003
SOE26	Soetdoring	0.110	0.000
SOE27	Soetdoring	0.110	0.000
RFD1	Rustfonteindam	0.733	0.040
RFD2	Rustfonteindam	0.733	0.040
RFD3	Rustfonteindam	0.733	0.040
RFD4	Rustfonteindam	0.558	0.002
RFD5	Rustfonteindam	0.558	0.002
RFD6	Rustfonteindam	0.733	0.040
RFD7	Rustfonteindam	0.733	0.040
RED1	Reddersburg	0.593	0.221
RED2	Reddersburg	0.733	0.040
RED3	Reddersburg	0.500	0.099
RED4	Reddersburg	0.620	0.498
RED5	Reddersburg	0.418	0.029
RED6	Reddersburg	1.000	0.124
SKV1	Seekoeivleidam	1.000	0.335
SKV2	Seekoeivleidam	0.733	0.040
SKV3	Seekoeivleidam	0.733	0.040
SKV4	Seekoeivleidam	0.418	0.029
SKV5	Seekoeivleidam	0.733	0.040
SKV6	Seekoeivleidam	0.733	0.040
SKV7	Seekoeivleidam	0.733	0.040
SKV8	Seekoeivleidam	0.733	0.040
SKV9	Seekoeivleidam	0.558	0.002
SKV10	Seekoeivleidam	0.558	0.002
SKV11	Seekoeivleidam	0.620	0.498

SKV12	Seekoeivleidam	0.558	0.002
GEL1	Geluk	0.024	0.001
GEL2	Geluk	0.083	0.000
GEL3	Geluk	0.083	0.000
GEL4	Geluk	0.154	0.000
<b>GEL5</b>	Geluk	<b>0.110</b>	<b>0.384</b>
GEL6	Geluk	0.024	0.001
GEL7	Geluk	0.024	0.001
GEL8	Geluk	0.154	0.000
GEL9	Geluk	0.083	0.001
GEL10	Geluk	0.024	0.001
GEL11	Geluk	0.083	0.000
GEL12	Geluk	0.154	0.000
GEL13	Geluk	0.083	0.000
GEL14	Geluk	0.044	0.000
GEL15	Geluk	0.733	0.040
GEL16	Geluk	0.100	0.099
<b>GEL17</b>	Geluk	<b>0.356</b>	<b>0.431</b>
GEL18	Geluk	0.024	0.001
GEL19	Geluk	0.743	0.163
GEL20	Geluk	0.761	0.181
GEL21	Geluk	0.731	0.180
GEL22	Geluk	0.024	0.001
GEL23	Geluk	0.154	0.000
GEL24	Geluk	0.083	0.000
GEL25	Geluk	0.083	0.000
GEL26	Geluk	0.154	0.000
GEL27	Geluk	0.154	0.000
GEL28	Geluk	0.516	0.002
GEL29	Geluk	0.044	0.000
GEL30	Geluk	0.154	0.000
GEL31	Geluk	0.454	0.099
GEL32	Geluk	0.418	0.029
GEL33	Geluk	0.083	0.000
GEL34	Geluk	0.021	0.000
GEL35	Geluk	0.044	0.000
GEL36	Geluk	0.044	0.000
GEL37	Geluk	0.024	0.001
GEL38	Geluk	0.083	0.000
GEL39	Geluk	0.083	0.000
GEL40	Geluk	0.024	0.001
GEL41	Geluk	0.024	0.001

GEL42	Geluk	0.154	0.000
GEL43	Geluk	0.083	0.000
GEL44	Geluk	0.024	0.001
GEL45	Geluk	0.154	0.000
GEL46	Geluk	0.039	0.000
GEL47	Geluk	0.024	0.001