Development and implementation of the Rhinoceros DNA Index System (RhODIS®) for the forensic analysis and biological management of African rhinoceros

by

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Supervisor: Prof. Peter N. Thompson

Co-supervisor: Dr Stephen J. O'Brien

Dedication

This thesis is dedicated to all the rhinoceros that have died at the hands of poachers, the orphans and the survivors. In the hope that this work has made a small contribution to realize an end to the slaughter of these, most charismatic and noble of creatures, that have suffered to satisfy the vilest of human qualities, greed.

Declaration

I, Cindy Kim Harper, declare that the thesis/dissertation, which I hereby submit for the degree Philosophiae Doctor at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: ……

Date: 15 October 2018

Ethics statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval from the Animal Ethics Committee of the University of Pretoria (v054-16).

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the Policy guidelines for responsible research.

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Summary

There has been a steady increase in the numbers of rhinoceros poached since 2008 in South Africa, when 83 rhinos were poached, increasing to over 1000 animals poached each year in 2014, 2015, 2016 and 2017. This increase has occurred in other rhinoceros range states as well as in Asia, with all rhinoceros populations under severe threat due to illegal hunting for their horns. Rhinoceros horn has been used for centuries in Traditional Chinese Medicine (TCM) as cure for fever and other ailments. Recently the market for rhinoceros horn has evolved to include items such as jewellery. Forensic tools have been used extensively to support the investigation of crime and DNA is one of these tools. DNA technology has also been applied to parentage testing in domestic animals and population studies of wildlife. DNA profiling for individual identification is commonly performed using Short Tandem Repeat (STR) markers or loci, also called microsatellite markers. This study describes the development of a technique to individually identify rhinoceros horn linking it to a specific animal and providing information to support the prosecution of a poacher or trafficker found in possession of a horn. Part of the study includes the implementation of a system for DNA sampling from the rhinoceros animal or horn following chain of custody requirements and developing a database which includes representative samples of white and black African rhinoceros of the various species and subspecies. This data supports the calculation of a match probability statistic that provides a weight to the match between the horn and animal by indicating the rarity of the genotype in the source population. The size and structure of the database and characteristics of the genotyping test influence the calculation and accuracy

of the match probability. The genetic data is also applied to assist the biological management of extant rhinoceros populations further aiding the survival of the species through supporting genetic health and diversity of captive and free ranging populations. This study presents the data collected, technical and analytical methods used, match statistics and case information that support forensic investigation of rhinoceros crime and the application of the data to biological management of selected populations and, therefore, the overall utility of the RhODIS® system as an important tool in protecting and sustaining rhinoceros populations.

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I owe my two amazing daughters, Charna and Jayden-Belle, and my Mom, my deepest gratitude for their love and support throughout this project. You have, and continue to, inspire and motivate me.

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A special thank you to my colleagues in the Veterinary Genetics Laboratory, who tirelessly extracted DNA, did thousands of PCR tests, trained people and entered mountains of data. Those that have walked this RhODIS® path with me, know how difficult it has been at times and to those that have persevered, you are awesome and continue to motivate me.

RhODIS® has relied on many donors throughout the project and I am deeply grateful to these people and organizations, public and private, large and small. Their support has meant that RhODIS® could survive and grow and provide the information needed to put some of those responsible for so much suffering behind bars. South African National Parks, World Wide Fund for Nature, Global Environment Facility of the United Nations Environment Programme, Mark Boucher and the many private donors.

And finally a special thank you to everyone that collected samples and that I had the opportunity to teach and that taught me about forensics and rhinoceros.

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Abbreviations

AfRSG	African Rhino Specialist Group
CITES	The Convention on International Trade in Endangered Species of Wild Fauna and Flora
CoC	Chain of Custody
CODIS	Combined DNA Index System
DEA	Department of Environmental Affairs
DNA	Deoxyribonucleic acid
HWE	Hardy-Weinberg Equilibrum
ISFG	International Society of Forensic Genetics
IUCN SSC	International Union for the Conservation of Nature Species Specialist Group
KNP	Kruger National Park
KZN	KwaZulu Natal
LD	Linkage Disequilibrium
LOD	Logarithm of the odds
MAF	Minimum allele frequency
MP	Match Probability
NEMBA	National Environmental Management Biodiversity Act
NRC	National Research Council
PCR	Polymerase Chain Reaction
RhODIS®	Rhinoceros DNA Index System
SANParks	South African National Parks
SNP	Single-nucleotide polymorphism
STR	Short Tandem Repeat
SWFS	Society of Wildlife Forensic Science
TCM	Traditional Chinese Medicine
TOPS	Threatened or protected species regulations

CHAPTER 1

1.1 General Introduction

There has been a steady increase in the numbers of rhinoceros poached since 2008 in South Africa, when 83 rhinos were poached to over 1000 animals poached each year from 2013 to 2017 (https://www.environment.gov.za/mediarelease/molewa_progressonintegrated_strategicmanagement_ofrhinoceros) (Figure 1). The poaching of rhinoceros has become a widespread threat to the African species across the continent including other rhino range states with more than 8000 rhinoceros poached across the African continent between 2006 and 2017 (Emslie et al., 2016). Forensic tools have been used extensively to support the investigation of crime, particularly human crime. In animal related crimes, the animal could be the silent witness, the perpetrator or the victim of the crime and the same forensic techniques can be adapted and utilised in the investigation of these crimes. DNA is one of these tools and the technology has been applied widely to parentage testing in domestic animals and population studies of wildlife (Andreassen et al., 2012, Caniglia et al., 2010, Miller et al., 2014). The use of DNA forensic evidence in the investigation of animal related crime is becoming increasingly sophisticated (Johnson et al., 2014). This work is supported by specialised organizations, such as the International Society of Forensic Genetics (ISFG) (http://www.isfg.org/) and the Society of Wildlife Forensic Science (SWFS) (http://www.wildlifeforensicscience.org/) that assist by providing guidelines and through encouraging research.

The National Research Council second report on the Evaluation of forensic DNA evidence (Weir, 1996) states that one cannot claim a DNA profile match in the absence of data to give an indication of the rarity of the matching characteristics, which implies the use of a representative population DNA database of the species in question, including sub-populations of that species. The same report also states that there is no other forensic evidence that can, with the same level of consistency and degree of certainly, show that evidence items match their source as DNA testing.

The rhinoceros is a charismatic megafauna that has always been under extreme pressure to survive due to uncontrolled hunting by mainly colonialist hunters in the past (Walker and Walker, 2012) and poachers financed through organized criminal syndicates (Milliken and Shaw, 2012). Rhinoceros are killed for their horns which are removed from the carcass of the rhinoceros and are trafficked through the various levels of the crime syndicate to their final destination, mainly the Asian consumer countries. Rhinoceros horns are purported to be of use as Traditional Chinese Medicine (TCM). They are carved into high value items or sold as status symbols to indicate wealth and stature of the buyer (Milliken and Shaw, 2012, Moneron et al., 2017). It has been extremely difficult to link individuals in the trafficking syndicates to specific crime

scenes unless the trafficked item can be traced to its origin. DNA is a powerful tool to provide the individual identity of both animal and its horn thereby linking the confiscated items and those who possess them to crime scenes.

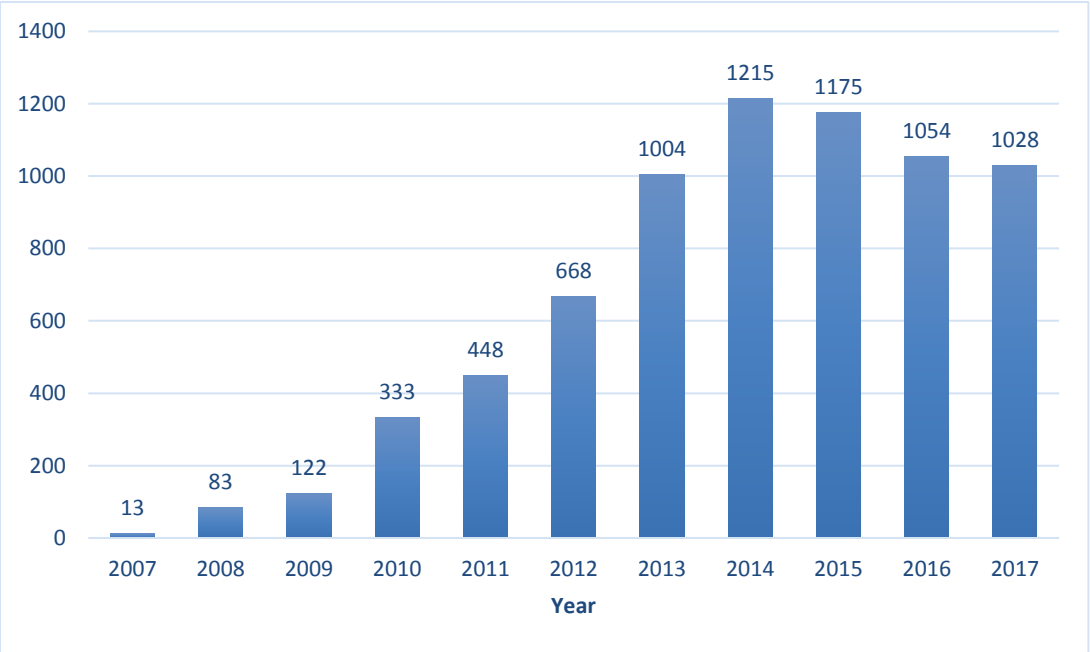

Figure 1: Number of rhinoceros poached annually since 2007 in South Africa

The development of an extraction method to obtain DNA from rhinoceros horn is a first step in producing the DNA evidence that links the horn to a specific crime scene. Underpinning the application of this information in the prosecution of rhinoceros crime, is the need for: 1) an extensive and representative rhinoceros DNA profile database; 2) a sampling methodology that follows chain of custody principles and 3) statistical support of DNA matches in cases.

The same DNA data can be used to support management of rhinoceros populations by evaluating the genetic viability and selection of individuals to ensure that the diversity of the remaining, increasingly isolated and fragmented populations, is maintained. The genetic information captured within a comprehensive database, therefore, supports conservation of the species at both the enforcement side and by enhancing reproductive efficiency to offset the losses due to poaching.

The purpose of this project was to provide, primarily, a DNA based forensic tool to support rhinoceros crime investigations with additional benefits in terms of genetic population management. The specific aims of the project being:

1) The development and validation of a method to extract DNA from rhinoceros horn.

2) The development of a standardised sampling methodology for sampling of live rhinoceros the carcass at a crime scene and rhinoceros horns from stockpiled horns.

3) The development of a representative DNA profile database of African rhinoceros species and subspecies.

4) The characterisation of a set of rhinoceros Short Tandem Repeat (STR) markers to provide statistical support for the use of these data in rhinoceros crime cases.

5) The application of these data to support the genetic management of rhinoceros populations.

1.2 Outline of the thesis

In this work, I present a method to obtain high quality nuclear DNA from rhinoceros horn and utilising this information to link horns removed from rhinoceros animals to the individual animal in order to establish the origin of the horn after separation from the animal. The application of this technique is mainly to serve as evidence in the forensic investigation of rhinoceros crime following the significant escalation in rhinoceros poaching in the African and Asian rhinoceros range states. The DNA profiling method has been established using previously isolated and published microsatellite markers in a multiplex PCR test that identifies the sex and species of the rhinoceros as well as its individual identity. This information is stored on a database identified as RhODIS® (The Rhinoceros DNA Index System) that serves as a repository for rhinoceros DNA information from all African rhinoceros range states. Samples collected for this dataset are collected in sampling kits developed and distributed as part of the RhODIS® programme and follow chain of custody requirements and biodata accompanying each sample is collected using an android based app identified as eRhODIS™ also designed as part of the RhODIS® programme. The RhODIS® genotyping data has a further application as a genetic management tool for individual rhinoceros populations and also as a meta-population management tool. The thesis is presented as a general introduction, a literature review followed by three chapters that cover the specific objectives of the study and a conclusion.

Chapter 1 is a general introduction that provides the context and the specific aims of the study.

Chapter 2 provides a literature review that covers the description of the problem of rhinoceros poaching, the reasons for this trend and the history of the rhinoceros and extant populations. This chapter also details the methodology for producing the DNA profile from rhinoceros tissue, the calculation of locus and population parameters and the use of these data in the forensic investigation of rhinoceros crimes. The literature review also includes standardization and validation procedures for DNA profiling for forensic purposes and the use of the data to calculate genotype match probabilities.

Chapter 3 describes the method to obtain and extract nuclear DNA from rhinoceros horn and matching the genotype to a specific animal. The study included material supplied by SANParks as part of a project to demonstrate the DNA profile matches between rhinoceros horn samples and tissue collected from rhinoceros animals. A small background dataset is used to characterise the markers and to provide initial match probability values. Chapter 3 is published in Forensic Science International: Genetics (2013).

Chapter 4 expands on the data presented in Chapter 3 by providing a large background dataset using the RhODIS® data collected since 2010 and provides strong support to the match probability calculations derived from this dataset. Further characterisation of the microsatellite marker panel used to obtain the RhODIS® genotypes is provided including an assignment of the markers to chromosomes based on the horse as a reference genome. A Structure analysis of the background dataset supports the subdivision of the rhinoceros population into a panmictic White rhinoceros population and a Black rhinoceros population consistent with the current IUCN subspecies subdivisions into *D.b. bicornis, D.b. michaeli* and *D.b. minor* subgroups with an additional partition between the *D.b. minor* subspecies originating in Kwazulu-Natal, Zimbabwe, and a third group that are an admixture of these two groups in the Kruger National Park. The use of the RhODIS® data in investigations that were used in rhinoceros cases and resulted in successful convictions and sentencing is described and specific match probability calculations provided for each case. Chapter 4 is published in Current Biology (2018) and included extensive collaboration with researchers in other countries and rhinoceros managers in the rhinoceros range states.

Chapter 5 presents a detailed study of an individual extralimital rhinoceros population in South Africa that includes signatures of historic admixture between the *D.b. minor* and *D.b. michaeli* Black rhinoceros subpopulations. The information presented includes the verification of the population pedigree using the RhODIS® DNA profiling data and comparing the individual admixture results from the pedigree to the inferred ancestry of individual animals using a Structure analysis. The study also highlights the value of using genotyping data to evaluate and manage extant populations and the potential for using this data as a meta-population management tool. The presentation of the reproductive success of this population also highlights the value of subspecies admixture in improving fitness in the Black rhinoceros where individual subspecies and populations have become genetically depauperate due a population crash caused by over harvesting. A manuscript will be prepared from this chapter for publication.

Chapter 6 integrates the information presented in this thesis and a discussion on the contribution of these findings to the specific field of science with a critical look at the limitations and shortcomings and where further research could improve on this work in the future.

CHAPTER 2

2. Literature Review

2.1 Rhinoceros species and subspecies

Rhinoceros-like animals thrived during the Tertiary period of the Earth's history about 60 million years ago (Joubert, 1996). During the Pliocene rhinoceros disappeared from North America. They disappeared from Europe and most of Asia during the Late Pleistocene (Cerdeño, 1998). By the end of the Pleistocene almost all the representatives of the rhinoceros ancestral lines had disappeared with only five species remaining, thought to be due to climate and habitat changes (Joubert, 1996). Present day African rhinoceros lineages were established in the Late Middle Miocene and Late Miocene period (Cerdeño, 1998). The families Rhinocerotidae and Equidae are found within the same order of Perissodactyls due to various anatomical similarities (Joubert, 1996). Fossil remains of the ancestors of today's rhinos are abundant and indicate that the common ancestor of the black and white rhinoceros was distributed across Africa.

2(a)

2(b)

Figure 2: (a) Map of Africa with the 13 rhinoceros range states coloured and (b) Detailed map of South Africa showing the major game reserves in the region (Hluhluwe-Imfolozi, Kruger National Park and Addo Elephant National Parks are circled) (http://www.safari.co.za/African_Safari_Guide-travel/south-africa-safari-map.html).

African black rhinoceros were split into 7 (1967) and later 8 (2011) subspecies previously, based on skeletal differences and distribution, of which the majority are now considered to be extinct (Groves and Grubb, 2011). These subspecies included; (http://www.rhinoresourcecenter.com/species/black-rhino/)

- *Diceros bicornis bicornis*; Western South Africa, Southern Namibia. Extinct since about 1800. This name was given to the black rhinoceros that occurred from the Cape to Southern Namibia and which is now extinct. The name has subsequently been transferred to the Namibian black rhinoceros and is now commonly used for this subspecies of black rhinoceros.
- *Diceros bicornis chobiensis*; Angola, Botswana. Individuals classified in this subspecies were found in the Okovango region and Southern Angola, Northern Botswana and genetically not well differentiated from *D.b. minor*.
- *Diceros bicornis minor*; Northern Namibia, Eastern South Africa, Botswana, Zimbabwe, Mozambique, Zambia, Malawi, Southern Uganda, Tanzania.
- *Diceros bicornis occidentalis*; Northern Namibia. Kaokoveld and Cunene region. May be distinguishable from the *D.b. bicornis* if re-examined using genetic data.
- *Diceros bicornis michaeli*; Eastern and Northern Kenya, Northern Tanzania.
- *Diceros bicornis brucii;* Somalia Ethiopia, Eastern Sudan. If distinct from *D.b.michaeli* then probably extinct.
- *Diceros bicornis ladoensis;* Southern Sudan, Uganda, Western Kenya.
- *Diceros bicornis longipes;* West African countries. Chad, Central African Republic and North-Eastern Nigeria. Declared extinct in 2011.

The African Rhino Specialist Group (AfRSG) of the IUCN currently only recognizes four subspecies of black rhinoceros of which *Diceros bicornis longipes* was declared extinct in 2011 (Emslie and Brooks, 1999). These are; (numbers remaining in 2015 given in brackets) (Emslie et al., 2016);

- *Diceros bicornis bicornis*; South-western subspecies occurring in Namibia, Northern Cape and Eastern Cape provinces of South Africa (2200).
- *Diceros bicornis minor*; South-central subspecies occurring in Zimbabwe, Botswana, KwaZulu-Natal province, Kruger National Park and remaining provinces of South Africa, Malawi, Southern Tanzania, Swaziland and Zambia (2164).
- *Diceros bicornis michaeli*; Eastern subspecies occurring in Kenya, Northern Tanzania and an extra-limital population in South Africa (886).
- *Diceros bicornis longipes;* Western subspecies occurred in central and western Africa, Cameroon, Chad, the Central African Republic, Sudan (Extinct).

The numbers of rhinoceros of all species and subspecies are low due to habitat encroachment and unlimited exploitation by, especially, European colonial hunters (Walker and Walker, 2012). The African species of rhinoceros include the White and Black rhinoceros that show clear morphological distinction. The White rhinoceros (*Ceratotherium simum simum*) is a grazer and its numbers dropped to below 50 in the early 1900's with only a few animals remaining in the Hluhluwe-Imfolozi game reserve where these animals were protected from being hunted to extinction due mainly to the presence of the tsetse fly in the area. Extensive efforts to increase the numbers of the species in the 1960's by the Natal Parks Board, were extremely successful with numbers of White rhinoceros at around 20 000 in 2012 (Player, 2013). The Northern White rhinoceros (*C.s. cottoni*) numbers have dwindled to only 2 remaining in a small population under intense protection in the Ol Pejeta Conservancy in Kenya (Howard, 2015).

The Black rhinoceros species can be divided into subspecies based on geographic origin. By the late 1960's about 70 000 remained on the African continent. By 1981 only 10 000 to 15 000 remained and by 1995 this number had decreased to 2410, the population having been decreased by over 90% in 30 years as a result of hunting. In 1933 only two populations of black rhinoceros, with a total of 110 animals, remained in South Africa (Milliken and Shaw, 2012). The population has subsequently showed a steady increase to 5055 in 2013, with 98% of black rhinoceros present in just four countries, Namibia, Kenya, Zimbabwe and South Africa (http://wwf.panda.org/knowledge_hub/endangered_species/rhinoceros/african_rhinos/black_rhinoceros/#threats). South Africa is the only country that has all three remaining black rhinoceros subspecies, *D.b. minor*, *D.b. bicornis* and *D.b. michaeli*. All three have shown an increase in numbers since 1992. The recognised subspecies of Black rhinoceros include the Southern-central black rhinoceros (*D.b. minor*) whose range included central Tanzania, Zambia, Zimbabwe, North-eastern South Africa and Mozambique. Small numbers have been reintroduced to Zambia, Malawi, Swaziland and Botswana from South Africa. The South-western black rhinoceros (*Diceros bicornis bicornis*) that occurred in Namibia, Angola, Western Botswana and the Northern Cape province of South Africa. Namibia is now the stronghold of this subspecies and it no longer occurs in Angola. The East African black rhinoceros (*Diceros bicornis michaeli*) whose range included South Sudan, Somalia, Ethiopia, Kenya and North-central Tanzania, but has been reduced to Kenya and small numbers in Tanzania (http://wwf.panda.org/knowledge_hub/endangered_species/rhinoceros/african_rhinos/black_rhinoceros/#threats). Recently black rhinoceros have been translocated from South Africa to Rwanda (18 *D.b. michaeli* animals) (https://www.iol.co.za/the-star/return-of-the-rhino-to-rwanda-welcomed-8966860) and Chad (6 *D.b. minor* animals) (https://www.news24.com/Africa/News/chad-gets-6-rhinos-nearly-50-years-after-losing-the-species-20180503) increasing the number of African rhinoceros range states from 11 (Botswana, Kenya, Malawi, Mozambique, Namibia, South Africa, Swaziland, Uganda, Tanzania, Zambia and Zimbabwe) to 13.

The three Asian rhinoceros species include the *Rhinoceros unicornis* (Greater One-horned rhinoceros) that occurs in India and Nepal (~3000) and is classified as vulnerable, the *Rhinoceros sondaicus* (Javan rhinoceros) that is found in a single population in the Ujung Kulon National Park in Java, Indonesia and is critically endangered (~60) and the *Dicerorhinus sumatrensis* (Sumatran rhinoceros) that occurs on the islands of Borneo and Sumatra and is critically endangered (~100) (https://www.worldwildlife.org/pages/rhino-facts-and-species).

2.2 Rhinoceros poaching

Poaching in Zimbabwe increased following independence in 1980. Following concerted efforts to consolidate rhinos into Intensive Protection Zones and widespread dehorning, poaching decreased and remained relatively low in Zimbabwe and South Africa between 1994 and 2002. Between 1990 and 2005 an average of 14 rhinoceros were poached in South Africa per year. Poaching in Zimbabwe started to increase again in 2003, driven by illegal trade dynamics in South Africa that included legal internal trade of horns, pseudo-hunting and illegal hunting in Zimbabwe due to political destabilizing factors that undermined the security of wildlife in the area. At this time Dawie Groenewald set up safari hunting operations in Zimbabwe which were linked to rhino poaching. These factors drove up horn prices until the poaching spilled over into neighbouring South Africa and eventually Namibia as well (Raoul du Toit, personal communication, 2017). In February 2009 a moratorium prohibiting the legal sale of rhinoceros horn was implemented in South Africa and this, in addition to, the factors already mentioned, appeared to trigger the dramatic increase in poaching levels in South Africa after 2008 (Milliken and Shaw, 2012).

In 2008, 83 rhinos were poached in South Africa increasing steadily to 1215 by the end of 2014, 1175 in 2015 and 1054 in 2016 (Milliken and Shaw, 2012). The most extensive losses have occurred in the Kruger National Park with the greatest threat coming from its eastern border with Mozambique (Milliken and Shaw, 2012). An increase in poaching across the African continent with more than 7000 animals killed in the last 10 years in African rhinoceros range states, has caused concern for the survival of the species in spite of significant security measures to protect the animals (Moneron et al., 2017). A recent decrease in the number of rhinoceros poached has occurred, in not only South Africa, in which official poaching statistics for 2017 indicated a decrease of 26 rhinoceros poached in 2017 compared to 2016, but also in other African countries. Kenya had 9 rhinoceros poached in 2017 compared to 14 in 2016 and Namibia showed a declining poaching trend from 95 in 2016, compared to 60 in 2016 and 32 in 2017 (*https://www.savetherhino.org/latest_news/blog/1791_kenya_poaching_stats_out*).

Rhinoceros poaching has become an increasingly sophisticated activity involving organized crime syndicates. Rhinoceros horn trade syndicates are divided into 5 levels of operation with the poacher being

at level 1. Level 2 represents the local buyer, courier or organizer who supplies the horn to Level 3, the national buyer, courier or exporter. The local exporter connects with Level 4, the international courier or buyer who sells to the international buyer or end consumer at Level 5. The national buyer, importer and international buyer are mainly of Asian nationality (Milliken and Shaw, 2012). The poaching operations have included using local sex workers in pseudo-hunting operations and recruiting foreign hunters to obtain horns for the illegal market (Milliken and Shaw, 2012). The Department of Environmental Affairs (South Africa) implemented a ban on internal trade in rhinoceros horn though a moratorium in 2009. This moratorium was lifted by a decision made after lengthy litigation by private rhinoceros owners, by the Constitutional Court, in April 2017 and internal legal trade was initiated shortly after (Reuters, 2017).

2.3 Rhinoceros horn use and structure

In traditional Chinese Medicine (TCM), rhino horn (Xi Jiao / Cornu Rhinoceri) is described as a cold drug, used to cool the blood and thus reduce fevers, stop bleeding and counteract toxins (https://www.sacredlotus.com/go/chinese-herbs/substance/xi-jiao-rhinoceros-horn). Rhinoceros horn has been valued in Chinese tradition as an ingredient to increase longevity as well as for its aesthetic value as drinking cups and other ornamental items since the 7th century AD. The use of rhinoceros horn in TCM was banned by the Chinese government in 1993 and rhinoceros horn was removed from the Chinese Pharmacopeia. A more recent and non-traditional claim for the use of rhinoceros horn has been that it can cure cancer and this rumour, which was started by an unnamed Vietnamese politician, has been said to have been one of the contributing factors in the spike in rhinoceros poaching. Rhinoceros horn was never used as an aphrodisiac in TCM and this belief appears to have originated in the west and may have its origins in the influence of Indian culture on the myths surrounding the rhinoceros and its horn (Patton, 2011).

Yemen used to be a significant importer of rhinoceros horn where the material was used for the handles of daggers known as jambiyas. These daggers were given to Yemeni boys at the age of 12 as a sign of manhood. The imports of rhinoceros horn were banned in Yemen in 1982 and the economic decline in this country also reduced its influence on the decline of the rhinoceros population significantly (http://www.pbs.org/wnet/nature/rhinoceros-rhino-horn-use-fact-vs-fiction/1178/).

In the early 1990's rhinoceros horns cost about $250 to $500 per kilogram but this has increased to over $60 000 per kilogram making it the most valuable illegally traded commodity in the world (Caulderwood, 2014). Vietnam is considered to be the most important consumer country for rhinoceros horn and rhinoceros horn products and the increase in wealth of this region has contributed significantly to this. Vietnam is Asia's fastest growing economy after China. Vietnam's increasing wealth and insatiable

consumerism for western as well as high end rare and valuable products has seen a rapid increase in retail sales and 150% increase in the number of multimillionaires in a 5 year period (Haworth, 2013). The cancer rate is also increasing by 20 to 30% each year in Vietnam, due to increasingly unhealthy lifestyles and pollution, but also due to increasing diagnosis of the condition. The mortality rate from cancer in Vietnam is over 73%, one of the highest in the world, however, the availability of western cancer treatments is very limited. This is rumoured to be a reason for the increasing use of traditional and non-western medicine in the country (https://qz.com/82302/theres-a-country-that-will-pay-300000-per-rhino-horn-to-cure-cancer-and-hangovers-and-its-wiping-out-rhinos/).

Some consider TCM to be a less important reason for the demise of the rhinoceros than the increasing use of rhinoceros horn as a lifestyle enhancing drug by young newly rich Vietnamese that include the powdered horn in alcoholic drinks and serve it to special guests. It is believed to cure and prevent hangovers. It is also powdered in special ceramic bowls and served by adding it to water (Karl Ammann, personal communication, 2014). Rhinoceros horn is also considered a status symbol and a rare item in a country where luxury goods are sought after and provide gifts to secure political favour (Guilford, 2013). The carving of horn into various items including cups, combs, beads and ornaments has increased and the value of these items has increased with the powder, which is a by-product to the manufacture of these goods, becoming the less valuable and sold for medicinal use. The carving of the horns has moved to supply countries such as South Africa with horn smuggled as ornaments and jewellery that makes monitoring and enforcement extremely difficult (Moneron et al., 2017). The rarity value of the horn is increasing with decreasing numbers of rhinoceros.

Hieronymus et al. (2006) used computed tomography (CT scanning), gross observation of sectioned horn and light microscopy to examine rhinoceros horn structure. Rhinoceros horns lack a bony core and are anchored to the dermis through keratinized cells that form tubular cellular structures and grow from a generative layer of epidermis covering dermal papillae. All horn growth takes place from the base of the horn and damage to the growth plate causes abnormalities in the structure of the horn. The structure of the horn has been compared to ungulate hoof walls, baleen plates and cockatoo bills. Rhinoceros horn continues to grow at a rate of approximately 5 to 6 cm per year and can be harvested without harm to the animal as long as the cut is made above the conically shaped growth plate at the base. The horn matrix consists of calcium salts and melanin. Keratins are damaged by prolonged exposure to UV light and the melanin's role appears to be to protect the horn from such damage. The centre of the horn has an area of increased melanisation and calcification resulting in this part being most resistant to damage and wear and contributing to the conical shape of the horn (Hieronymus et al., 2006).

A study by Boy et al. (2015), shows the horn originating from elongated dermal papillae and consisting of tightly packed corneocytes from the base to the tip of the horn. The corneocytes show vertical and horizontal orientation and no hollow spaces or intercellular matrix present. This study also shows the epidermal base membrane dipping into the underlying connective tissue at the edges of the horn base resulting in an inward slant to the outer dermal papillae which contributes to the conical shape of the horn (Boy et al., 2015). The presence of corneocytes throughout the horn structure supports the extraction of DNA and ability to obtain a nuclear DNA profile from any part of the horn.

2.4 DNA profiling for individual identification

DNA profiling for individual identification is commonly performed using STR markers or loci in a multiplex PCR (Johnson et al., 2014). PCR primers, designed to co-amplify a set of di-nucleotide repeat markers that are rhinoceros species specific have been previously published in population studies (Brown and Houlden, 1999, Cunningham et al., 1999, Florescu et al., 2003, Nielsen et al., 2008, Scott et al., 2004, Scott, 2008). Capillary electrophoresis using a 3500 Genetic Analyser (ThermoFisher Scientific) is used as a standard instrument in many forensic DNA laboratories. GeneMapper® software (ThermoFisher Scientific) is a genotyping analysis software package commonly used to analyse STR data including for forensic purposes particularly in the human environment. GeneMarker® software (Softgenetics®) is another commonly used genotyping analysis software package that can analyse a wide variety of data from various sequencing platforms. STRand Analysis Software (Toonen and Hughes, Dec 2001) was developed by the University of California, Davis, Veterinary Genetics Laboratory and is used to analyse and compare multiplexed STR data across various platforms. STRand can be downloaded and used at no cost (http://www.vgl.ucdavis.edu/ informatics/download_strand.php).

The DNA profile of an animal is found in each nucleated cell in the body and can therefore also be used when only traces of tissue such as rhinoceros horn are present or when the horn has been powdered for medicinal use. STR or microsatellite markers have been used extensively in population genetic studies of humans and animals and are also used in human identification and criminal investigation (Butler, 2015). STR marker data are, however, not readily transferrable between laboratories and requires inter-laboratory and between platform standardization using known control genotype samples. In order for laboratories to share STR based genotyping data they must use the same loci in their profiling test and they must ensure that allele calls are standardised. Human DNA test kits include known genotype standards and allelic ladders such as the GlobalFiler™ (ThermoFisher Scientific) human DNA profiling kit that includes and allelic ladder with 343 alleles. The Qiagen human DNA profiling kit, the Investigator 24 Plex QS kit also includes a novel quality sensor - a set of primers and artificial DNA that amplifies a known

short and long fragment indicating successful PCR, absence of DNA, DNA degradation and presence of inhibitors.

2.5 The application of DNA profiling in Veterinary Forensic Science

Forensics is commonly defined as the application of scientific methods to the investigation of crime (https://en.oxforddictionaries.com/definition/forensic). When animals are involved in crime, they may be the victim, examples include wildlife poaching and stock theft, the perpetrator, including cases of murder and hunting or the witness when the animal DNA becomes evidence (Menotti-Raymond et al., 1997) or when the animal identifies a criminal including cases where tracking dogs are used to identify poachers (https://lowvelder.co.za/295966/knp-tracking-dogs-conduct-noted-in-court/).

Forensic DNA analysis contributes to the overall evidence in a case and is rarely directly associated with the charging of a suspect. DNA matching is also often used to elicit a guilty plea or used to direct an investigation. In such cases the DNA evidence may be undervalued. Some of the limitations of the use of DNA as evidence include that it is probabilistic, i.e. only a small section of the overall DNA of an individual human or animal is considered and therefore more than one human or animal could potentially match to the DNA profile observed. This has implications on the presentation of DNA evidence in reports and in the court. The DNA analysist must determine and present the relative statistical power of the DNA evidence. This relative value is called the match probability.

Snowball the cat represents the first criminal case in which animal DNA was successfully used for conviction in a human murder trial (Menotti-Raymond et al., 1997). It, therefore, set many precedents, especially regarding the requirements for setting up the profiling test and developing a background DNA database of the animal species involved (O'Brien, 2003). In 1994 a young woman was killed by her ex-boyfriend on Prince Edward Island, Canada. The most compelling evidence to link the suspect to the crime in this case was hair found in the lining of a leather jacket found in the woods that had the blood of the victim on it. The white hair on the jacket was not human but feline and the investigating officer suspected that it was the hair of a cat named Snowball that belonged to the parents of the suspect with whom he had been living at the time of the murder. At that stage animal DNA profile matching had not been utilised in a human criminal case. Dr O'Brien's laboratory at the National Cancer Institute in the USA was approached to test the hair samples found on the jacket and compare the DNA profile obtained to the DNA profile from blood collected from Snowball. In order to do this the laboratory had to select an appropriate and informative set of microsatellite loci that amplified in cats and had to set up a background database of cats living in on Prince Edward Island to calculate the match probability or likelihood that a cat, other than Snowball could randomly have a DNA profile match to that obtained from

the hair sample. The evidence, meticulous test procedures and testimony by Dr O'Brien and his team resulted in a successful prosecution and a guilty verdict in this case. The case also emphasized the need for having a representative database of a species in order to calculate match statistics to present to court that indicate the relative rarity of that DNA profile in the population of that species (O'Brien, 2003).

2.6 DNA databases underpin forensic DNA testing

The Combined DNA Index System (CODIS) database is the human DNA database used by the FBI in the USA to link human DNA profiles to specific individuals involved in human crime (https://www.fbi.gov/services/laboratory/biometric-analysis/codis). The USA introduced the DNA Identification Act of 1994 giving the FBI authority to establish a National DNA database (NDIS) to support law enforcement using DNA profiling technology. CODIS comprises genotypes of people from various sectors. The program is constantly expanding and requires updated systems to support kinship analysis, Y STR data, mitochondrial data, mini STR panels, additional STR markers as well as Next Generation Sequencing data and SNP technology. Following the introduction of the DNA Identification Act of 1994 in the USA, the DNA Advisory Board was established that was responsible for the development and implementation of the Quality Assurance Standards for forensic DNA testing and DNA databasing laboratories that participate in the NDIS program. Participating laboratories must comply with these standards and are audited accordingly. These standards overlap with and correspond to the standards described in the American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD/LAB) standards. The standards used are regularly updated and publically available (https://www.fbi.gov/services/laboratory/biometric-analysis/codis). Systems including national and international DNA databases utilising similar and sharable data have been developed in several other countries and regions and most of these DNA profiling systems use the commercial typing systems and can thus be considered standardised. European Network of Forensic Science Institutes (ENFSI) was established in 1995 and has a similar mandate to CODIS but in the European context and including other forensic technologies and not only DNA, which is supported by the ENFSI DNA working group.

The International Forensic Strategic Alliance (IFSA) represents a collaborative partnership between regional forensic networks and operational forensic laboratories worldwide. The Southern Africa Regional Forensic Network (SARFN) that represents 12 southern African countries formed under guidance of the United Nations Office of Drugs and Crime (UNODC) is a member of the IFSA. Although these organizations are mainly involved in human forensic applications the same forensic technologies are used to investigate crimes involving animals particularly organized criminal activities such as wildlife crime. This organization also focusses on standards and guidelines for forensic laboratories within its member organizations (http://www.ifsa-forensics.org/).

2.7 Standardizing STR based DNA profiling

Inter-laboratory and between platform standardization has been very limited in terms of animal, and particularly wild animal, DNA profiling and even when specific species have been extensively DNA profiled, these databases may not represent all isolated populations (Johnson et al., 2014). Examples of animal DNA profiling systems that have been standardised and validated include dogs (Wictum et al., 2013b), horses (Van De Goor et al., 2010), cats (Menotti-Raymond et al., 2005), hen harriers (van Hoppe et al., 2016), pigs (Lin et al., 2014) and cattle (Van De Goor et al., 2009).

Steps in the development of an STR (or other) marker system for forensic purposes:

1. Identify appropriate loci by isolation, using previously published markers or isolating from the genome.

2. Select most informative and robust loci for the final panels by sensitivity, species specificity, stability, repeatability testing.

3. Re-design the primers and dye labels in efficient multiplex panels.

4. Quantify DNA in the extracts to determine if it is possible to obtain a DNA profile and provide a reason for sample failure if there is no amplification.

5. Collect samples for a background database and genotype them using the selected markers in a standard method and bin the alleles correctly.

6. Collect samples representing various populations across as wide a range of the species as possible and characterise the markers.

Developmental validation studies for DNA profiling tests must include the following:

1) The selection of and multiplexing of polymorphic and informative markers from previous studies, isolation projects or genome information.

2) The design of appropriate primers to amplify the selected markers in a multiplex PCR.

3) The selection of an appropriate sample set for the validation testing and background databasing.

4) DNA quantification methods.

5) Construction of an allelic ladder utilizing homozygote genotypes for all markers tested, single amplification and creation of a working stock of that amplicon, combining all working stocks into a multi-allelic working solution that is balanced and diluted for future use.

6) Sequencing of each identified allele to determine the repeat sequence and internal sequence variation.

7) Match probability calculations per locus using a background database and formulae that account for population structure (Balding and Nichols, 1995) and inbreeding within subpopulations (Ayres and Overall, 1999).

8) Species specificity testing includes using the test panel to screen for species that could most commonly be encountered in an environment or as a contaminant or alternative to the species in question to establish whether cross amplification occurs.

9) Sensitivity testing of a test panel involves the assessment of peak height and peak imbalance in serial dilutions of DNA extracts to determine the lower limit of the DNA concentration that supports amplification of all alleles.

10) Stability is evaluated by degrading extracted DNA using DNase at set time intervals to determine the ability of the test panel to amplify loci even on highly degraded and fragmented DNA.

11) Reproducibility of the test is determined by repeat testing of control samples, testing of the same sample sets by different personnel and the testing of the panels by different laboratories to ensure that the results are consistent under different conditions.

12) Variable case-type samples must be analysed.

13) Population study data must be collected to provide a background dataset that can be used to characterise the markers, determine sub-population structure in the dataset and provide match probability statistics.

14) Mixture studies must be done to determine the effect of DNA mixtures in a sample by using 2 well-characterised DNA profiles and mixing the extracts and variable ratios.

15) Precision is evaluated by measuring the results of multiple runs of a specific test and determining the closeness of the measurements to each other.

16) Accuracy is evaluated by measuring the results of a test to the actual results of a control or known sample.

17) PCR reaction condition assessment including differential and preferential amplification is determined by observing the electropherogram results and evaluating whether markers co-amplify in the multiplex

efficiently without interfering with each other. PCR Mastermixes have fixed concentrations of reagents and individual evaluation of these is therefore not done.

Newer technologies, including the use of next generation sequencing methods (Jäger et al., 2017) are evolving and becoming more cost effective and useful across a broader spectrum of users but STR based technology will remain a highly effective, widely used technology for the purpose of forensic testing for the foreseeable future with large databases of human and animal STR profiles available to various research and investigative authorities (Butler, 2015, Johnson et al., 2014).

The use of dinucleotide repeats in forensic testing is not recommended due to increased stutter in these loci and heterozygote imbalance that can make allele calling difficult. An exception is made in cases where these markers have been used widely and large databases already exist. Further recommendations include the preferred use of tetranucleotide markers and the sequencing of individual alleles in order to provide a nomenclature based on repeat numbers (Linacre et al., 2011). The accurate calling of alleles should be based on the use of an allelic ladder. A control DNA sample with known allele calls should be used with each run to confirm that allele calls are as expected and to identify any electrophoretic variation between runs (Linacre et al., 2011).

2.8 Population analysis to support Match Probabilities (MP) in rhinoceros

Probability is defined as the likelihood or measure of certainty that an event will occur with the measure given as a value between 0 and 1. Bayesian probability uses prior knowledge to predict future events and is a conditional probability. Bayes Theorem provides a method to quantify the most likely outcome of an event by factoring in previous information – quantifying the likelihood that a specific hypothesis is true compared to the likelihood that it is not true.

The match probability (MP) can be defined as the probability of observing the same DNA profile given that the DNA profile has already been observed in a population. The match probability of a DNA profile depends on the allele frequencies at the various loci within the population and subpopulations (Gittelson et al., 2017). The genotype frequency is calculated by first calculating the genotype frequency at each locus, where homozygotes are estimated as p^2 or q^2 with heterozygotes as $2pq$, where p and q represent individual allele frequencies in a panmictic populations where the allele frequencies conform to HWE. The individual locus genotype match probabilities are then multiplied across all the loci in a calculation which is known as the product rule.

DNA evidence can be reported as a match probability or likelihood ratio. Population structure is one of the factors that must be taken into account when calculating the match probability of DNA evidence. In

1994 Balding and Nichols proposed incorporating a measure of the population substructure in the calculation of match probability by a formula which included the correlation coefficient F_{ST} or θ value (Balding and Nichols, 1994). Theta (θ) provides an estimate of the co-ancestry between sub-populations and therefore, reflects the population sub-structure. Initially match probability calculations assumed that the genotype at a locus within a DNA profile was independently inherited compared to a similar genotype in another individual's DNA profile and MP is simply the product of allele frequencies at a locus, p^2 or $2pq$ for homozygotes and heterozygotes, respectively, as described above.

Balding and Nichols suggested that similar genotypes at a locus from individuals in a subpopulation are not independently inherited but have a common ancestry and thus, a factor should be incorporated into the formula to determine the match probability that accounts for the population sub-structuring and relatively higher probability of individuals with similar genotypes in a subpopulation. This factor is the subpopulation correlation coefficient F_{ST} that indicates the relative genetic similarity between individuals within the subpopulation relative to the larger population. When (F_{ST}) $\theta > 0$, it indicates that there are large differences between allele frequencies within the subpopulations relative to the total population (Butler, 2015). To account for this greater level of relatedness in a sub-population relative to the total population, Balding and Nichols proposed a revised formula to calculate the MP at one locus by including the θ value (Balding and Nichols, 1994).

In the case of a homozygote (allele P_i), MP = $$\frac{(2\theta + (1-\theta)p_i)(3\theta + (1-\theta)p_i)}{(1+\theta)(1+2\theta)}$$

In the case of a heterozygote (allele P_i and P_j), MP = $$\frac{2(\theta + (1-\theta)p_i)(\theta + (1-\theta)p_j)}{(1+\theta)(1+2\theta)}$$

And If $\theta = 0$ in this equation then there is no differentiation between the sub-populations due to inbreeding within them and; $MP = 2P_iP_j$ or $MP = P_i^2$ and P_j^2

However, their formulae did not account specifically for the inbreeding within a subpopulation. Ayres and Overall (Ayres and Overall, 1999) expanded on the Balding and Nichols calculation by including the within-population inbreeding coefficient Fis or *f* (the correlation of genes within individuals relative to the subpopulation or an increase in homozygosity within a subpopulation, over and above the between subpopulation differentiation due to ancestral sharing of genotypes), to account for inbreeding within the subpopulation from which the individual's DNA profile is obtained.

In the case of a homozygote (allele P_i), MP =

$$\frac{(\theta+(1-\theta)p_i)}{f+(1-f)(\theta+(1-\theta)p_i)}\left[f^2+2f(1-f)\frac{(2\theta+(1-\theta)p_i)}{(1+\theta)}+(1-f)^2\frac{(2\theta+(1-\theta)p_i)(3\theta+(1-\theta)p_i)}{(1+\theta)(1+2\theta)}\right]$$

In the case of a heterozygote (allele P_i and P_j), MP =

$$2(1-f)\frac{(\theta+(1-\theta)p_i)(\theta+(1-\theta)p_j)}{(1+\theta)(1+2\theta)}$$

Including the correlation coefficient (F_{ST} / θ) within the calculation of MP increases the MP and thus the likelihood of a match to the genotype in question. This provides a more conservative approach to the calculation of MP.

When the formula containing the inbreeding coefficient F_{IS} / *f* is used; as F_{IS} / *f* increases, the MP for homozygotes will increase and the MP for heterozygotes will decrease, since the sub-population is inbred it will include fewer heterozygous genotypes and thus a lower likelihood of getting a heterozygous match. For a very heterozygous individual the probability of a match in an inbred population will decrease.

Estimating values of *f* and θ

The correlation coefficient, $F_{ST,}$ which is a measure of genetic diversity between populations varies between 0 (which means that no differences are observed between 2 populations and they are simply part of a single larger population) and 1 (which means that 2 populations have distinct alleles and allele frequencies and are completely different). When gene flow between sub-populations is low, the populations will diverge and become inbred and F_{ST} values will be high. Such high values that support complete separation of populations are seen for example between the African forest and savanna elephant (F_{ST} = 0.243) (Kenine E. Comstock et al., 2002). Differences in genetic variation between populations (F_{ST}) can be calculated using various packages including the AMOVA (Analysis of Molecular Variance) test in GenAlEx (Peakall and Smouse, 2012). In human populations a conservative θ is 0.01 with 0.03 used in some isolated populations (NRC recommendation 4.1) (Weir, 1996). If an F_{ST} (θ) value is selected from the upper end of a range of values for a set of sub-populations, it can be considered appropriately conservative for the calculation of MP and therefore fair (Ayres and Overall, 1999).

The size and representativeness of the dataset and characterisation of the markers

The size of the reference dataset and its representativeness of the population, therefore, influence the allele frequencies observed and thus the strength of the match and the selection of the method of reporting the match. The number of markers and their individual characteristics as well as strict

conformance to chain of custody in sample handling further influence the strength of the result. The presentation of such evidence in court is important. One should carefully consider how lay people grasp the match evidence in terms of the frequency of a profile occurring in a population compared to the probability of finding a matching genotype in the population (Butler, 2015). All these aspects must be considered in the systems developed to support the investigations of wildlife crime and specifically rhinoceros crime, to ensure strength of evidence and its use across international borders. Guidelines for the publication of genetic population data were provided in 2013 by **Forensic Science International: Genetics** which recommended a minimum of 500 samples when reporting autosomal and X-chromosome data (Anonymous, 2013). This may be impossible especially in small remaining populations of endangered species.

The use of samples that provide only small amounts of DNA or degraded samples must also be considered in forensic case work when samples are of poor quality. Degraded or small amounts of DNA may cause artefacts in the profile including allelic drop-out in which one or more alleles fail to amplify. Repeated amplification of the same sample could assist in removing this error since the missing allele may amplify in one of the repeats (Wasser et al., 2004). However, case work samples may not provide enough material for several replicates.

The number of alleles for each marker and allele frequencies must be determined for all markers in all species and subspecies separately and can be done using various software packages including GenAlEx software (Peakall and Smouse, 2012). The number of effective alleles for each sub-group that is an estimate of the number of equally frequent alleles at that marker can also be calculated with GenAlEx software (Peakall and Smouse, 2012). Alleles that occur at a low frequency or rare alleles will decrease the MP. The rarity of the allele may be a reflection of the number of samples of a population group which has the specific allele in higher frequency than other populations and, in order not to overestimate the rarity of an allele in MP calculations the minimum allele frequency (MAF) of alleles in a given population are calculated using the formula 5/(2N), where N is the number of individuals in the sampled population (Butler, 2015). The NRC II report (Weir, 1996) recommended that an allele should be observed at least 5 times in a population to be included in the MP calculations. However, the application of MAF will not be applied in calculating MP in the rhinoceros due to the low genetic variability of the remaining population and rapid continued loss of genetic material as a result of poaching and the possibility that the horns of rare and extinct rhinoceros could be included in case material. The rarity of alleles in this material should be used as indicative of how unique the DNA profile of the specific item is and how unlikely the chances of randomly finding a match to it given that it does not occur in the extant population.

Observed heterozygosity of each marker must be determined for each population (species and subspecies) as well as the mean observed heterozygosity for a population. Expected heterozygosity is a measure of the diversity of a marker in a population and thus also its informativeness and mean expected heterozygosity of a population is a measure of the diversity of the population. Expected heterozygosity is calculated assuming Hardy-Weinberg Equilibrium of the population and the unbiased value is usually reported which includes sample size in the calculation. These values can be calculated using GenAlEx software (Peakall and Smouse, 2012) and several other software packages.

In large, random mating a.k.a.panmictic populations allele and genotype frequencies are in equilibrium and will remain so unless factors act on the population that affect this equilibrium, including, inbreeding, assortive or selective mating, genetic isolation with genetic drift, mutations and population bottle-necks with a rapid loss of genetic diversity. The Hardy-Weinberg Equilibrium (HWE) provides a measure of whether a marker in a given population is behaving as expected if the population were to be mating randomly in which case the expected and observed heterozygosities would be very similar. A test for conformity with HWE is calculated for each marker and if this deviates significantly from expectations, it may indicate that the marker information must be interpreted with caution. The deviation may be as a result of null alleles (non-amplification of alleles due to binding problems in primers in specific populations), mutations in specific populations, homoplasy (alleles of similar length but differing sequence and thus not similar by descent), genotyping errors or X-linkage of markers. The deviation from HWE may, however, also be due to sub-structuring within the population where some allele frequencies are skewed and this can be corrected by analysing the sub-populations separately. HWE can also be calculated using GenAlEx software (Peakall and Smouse, 2012) or other software packages.

The program STRUCTURE assigns individuals to populations based on allele frequency differences and linkage equilibria. The number of populations (K) is given for a dataset and the model tested for the given number of populations (Pritchard et al., 2000). The most appropriate number of K can be determined using the method implemented in Evanno in the program STRUCTURE Harvester that calculates the delta K and plots the median value of K (Earl and von Holdt, 2012, Evanno et al., 2005).

Published and own reference sequence data of the markers selected for DNA profiling in the RhODIS® panels were aligned to the genome scaffolds of the Southern white rhinoceros (*C.s. simum*) (http://www.ncbi.nlm.nih.gov/genome/24631) that are available on GenBank. Using a reference assisted chromosome alignment tool, Chromosomer (Tamazian et al., 2016), the rhinoceros scaffolds were aligned to the domestic horse (*Equus ferus caballus*) genome as reference (Wade et al., 2009). The horse was selected as the reference species, since it is the closest relative of the rhinoceros with a complete and annotated genome. The two X-linked markers (IR12 and SR74) were consistently hemizygous in males

and either homo- or heterozygous in females in both black and white rhinoceros genotyped, supporting the referenced alignment of these 2 markers to the X-chromosome using this technique.

2.9 Match probability, probable match and likelihood ratio

The role of the forensic scientist as an expert in a criminal proceedings is to present evidence from an unbiased and balanced perspective and provide an opinion related to the evidence within their scope of expertise including the strength of calculated match values. The presentation of the DNA match evidence should be carefully worded and should indicate the relative rarity, or not, of a profile in a population. An example of such a phrase is “a conservative estimate of the occurrence of the profile in the population is 1 in X”.

2.10 Parentage, admixture and genetic population management

2.10.1 History and recent status of black and white rhinoceros populations

Rhinoceros are threatened species and remained in small pockets of isolated animals following their widespread decimation throughout their previous range on the African continent. The rapid decline in numbers of both the white and black rhinoceros resulted in a significant loss of genetic diversity (Moodley et al., 2017). The survival of the black rhinoceros in South Africa, similar to the white rhinoceros, is due essentially to the work of the Natal Parks Board. The last black rhinoceros in the Cape Province was shot near Addo in 1853, the last in the Free State in 1842 and the last living black rhinoceros was seen in the area of the now Kruger Park in 1936. Less than 100 black rhinoceros survived in the area of the Hluhluwe, Umfolozi and Mkuzi Game Reserves in 1930. In 1971 20 black rhinoceros (10 males and 10 females) were moved from Hluhluwe to the Kruger National Park, followed by 12 animals in 1972 from the Zambesi Valley in Zimbabwe (then Rhodesia) in order to re-introduce these animals into the area. These animals were all classified as the *D.b. minor* subspecies. Black rhinoceros were returned to the Addo Elephant National Park in 1961 and 1962 from the Kiboko region in Kenya. These animals were of the Eastern black rhinoceros (*D.b. michaeli*) subspecies and this re-introduction, therefore, represented an extralimital movement of animals into a region in which the specific subspecies had not previously occurred. In 1979 rhinoceros were introduced from KwaZulu-Natal (Zululand) into the Great Fish River reserve in the Eastern Cape, and area that fell between the former range of the Cape rhino (*D.b. bicornis*) to the west and the Zululand rhino (*D.b. minor*) to the east and could thus have formerly been an area in which intergradation of the subspecies was expected (Hall-Martin, 2009).

The black rhinoceros in Namibia was under similar threat by 1966 with only about 90 remaining in the Northwest corner of the country. In 1970 to 1972 an effort was made to protect these animals and most

were moved to the Etosha National Park, with about 20 remaining in the Kaokoveld region and 30 in Damaraland (Hall-Martin, 2009).

2.10.2 Species, subspecies and admixture

Admixed individuals belong to more than one genetic cluster. Variable stringency can be applied to identify admixed or inter-crossed individuals and the level of stringency where an animal is considered pure can be selected. A stringent threshold above which an animal is classified as pure is generally considered more than 0.95 (van Wyk et al., 2013). Taking into account the critically endangered status of the species and subspecies, the IUCN African Rhino Specialist Group recommended selecting animals with 0.85 and higher *D.b.michaeli* ancestry to provide a selection of founders for a newly translocated population with sufficient variability (Richard Emslie, personal communication, 2018).

How a species or subspecies is defined can have a major impact on how that species is managed and even protected. Subspecies admixture has been studied in the bontebok (*Damaliscus pygargus pygargus*) and blesbok (*Damaliscus pygargus phillipsi*). Classification of an individual as pure Bontebok or pure Blesbok was based on a coefficient of membership of 0.90 using the Bayesian clustering program STRUCTURE (van Wyk et al., 2013). Similarly the hybridization between the blue wildebeest (*Connochaetes taurinus*) and the black wildebeest (*C. gnou*) has been shown. The authors in this case again suggest the selection of animals based on a threshold Q value in STRUCTURE (Grobler et al., 2018). However, Boecklen and Howard (1997), with their model, cautioned that one only needs a few markers to identify recent hybridization and F1 hybrids but that the number of markers required to identify advanced back-crosses and more specifically the level of purity of these back-crossed animals, increases significantly and may be in excess of 70 independent markers. Linkage between the markers will also affect the result (Boecklen and Howard, 1997).

Concern expressed by van Wyk et al. (2013) that admixture of the blesbok and bontebok may cause a reduction in reproductive fitness and alteration in gene complexes leading to reduced adaptability seemed counterintuitive considering that the diversity of the bontebok populations was low (Ho = 0.21) and the population in the Bontebok National Park in South Africa with 17 founders, had shown long-term reduced reproductive fitness and signs of reduced immunity and adaptability. These characteristics are not discussed in terms of the hybrid or admixed bontebok / blesbok populations. Keller and Waller (2002) note that populations kept small and isolated for many generations suffer the deleterious effects of inbreeding that include reduced survival rate, reduced reproduction, reduced resistance to disease, reduced adaptability to environmental stressors and increased predation. The question, therefore, follows, whether anthropogenic admixture in small vulnerable populations of threatened species is more

detrimental to species survival than anthropogenic species separation leading to inbreeding, genetically depauperate animals and eventually local extinction.

The problem with keeping small vulnerable populations isolated for long periods without the addition of genes that would likely have occurred in natural circumstances though migrants, is a loss of diversity and evolutionary potential over time. The concept of genetic rescue of such populations is discussed by Whiteley et al. (2015). The aim of improving genetic diversity through admixture, is to avoid outbreeding depression, diluting a locally adapted population causing a loss of adaptation and population decline. This is done by ensuring that the restoration of genetic diversity is from populations that have similar environmental and behavioural backgrounds and where genetic divergence values are not significantly large. The long term benefits of an outcrossing event that introduced novel genes to a population are not always considered, for example, populations of inbred *Drosophila* showed improved population fitness benefits 5 to 10 generations after the introduction of genes from other inbred populations (Bijlsma et al., 2010). Significant improvements in animal numbers, heterozygosity, survival rate and other fitness parameters were observed following the introduction of eight female Texas puma (*Puma concolor stanleyana*) to an inbred population of approximately 22 Florida panthers (*P.c. coryi*) in Southern Floria, USA (Johnson et al., 2010). A successful rescue of a small population of Bighorn sheep (*Ovis Canadensis)* through 2 separate introductions is described by Miller et al. (2012). A feature of the Miller study is that all the animals were individually identified and a comprehensive pedigree was constructed using behavioural and genetic data.

With next generation sequencing techniques becoming simpler and more cost-effective, genome-wide analysis following genetic rescue or introduction events would add to the understanding of the effect on selection of different genes from founders and introduced animals and the correlation with fitness traits. The grey wolf was re-introduced to the Scandinavian Peninsula with a founding population of a single pair of animals (Åkesson et al., 2016). These animals bred and offspring closely inbred to produce a small population of animals whose numbers remained low and showed signs of inbreeding depression. The introduction of 1 and later 2 immigrant males caused immediate improvements in fitness and rapid increase in numbers of offspring. This population also showed the increased breeding success of offspring from immigrant animals compared to native animal offspring (Åkesson et al., 2016). The inadvertent genetic rescue of the black rhinoceros in the Addo Elephant National Park (AENP) is another example of the beneficial effects of hybridization and more uniquely in a large, slow reproducing mammal. It would appear that the criteria for selection into a new population should not be based on the relative purity of an individual but on the physical, behavioural and genetic characteristics of an individual which may instead positively correlated with its level of hybridization or admixture. The implementation of genetic

rescue can be better managed in future by using novel genomic tools available to monitor introgression and select specific individuals for translocation.

2.10.3 Population analysis methods

2.10.3.1 Parentage analysis

Cervus software

Cervus software is commonly used to assign parents to offspring in various species using co-dominant genetic markers including microsatellites and SNP's (Kalinowski et al., 2007). Cervus is not suited to using X-linked markers and may not provide accurate results if markers with null alleles or linked markers are used. Therefore, markers must be evaluated critically by reviewing the Hardy-Weinberg and linkage data before they are used in parentage assignment tests.

Cervus uses a likelihood statistical method based on allele frequency and simulation data to assign a parentage within a specified confidence interval. Likelihood is used when exclusions cannot exclude one or more candidate parents. The likelihood ratio evaluates one hypothesis relative to another and in parentage assignment this is evaluating the probability of the DNA profile if a candidate parent is the true parent divided by the probability of the DNA profile if the candidate parent is not the true parent. A large likelihood ratio, therefore, supports the assignment of a candidate parent to an offspring. When only the father is assigned the likelihood ratio is also called the Paternity Index. The likelihood ratio is calculated at each locus and the overall likelihood ratio is calculated by multiplying the likelihood ratios at each locus in the genotype and is expressed as the LOD score. If more than one candidate parent has a positive LOD score than Delta can be used as a criterion for parent assignment. Delta is the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent. Thus, if two candidate parents have LOD scores that are almost the same then Delta would be close to 0 and either parent could be assigned with almost equal certainty, making parentage assignment in such a case impossible.

During the simulation test performed in Cervus prior to doing parentage assignment testing using a real dataset, Cervus uses the allele frequency information from a given dataset to generate a pair of true "parent" genotypes, a set of offspring genotypes by sampling these "parent" genotypes and a set of random unrelated individual genotypes. The software then uses this simulated data to calculate the likelihood of assigning a true parent to an offspring out of the available parent genotypes by using a LOD or Delta score. By comparing the distribution of the LOD or Delta score for offspring where the most likely parent assigned is the true parent to the distribution of LOD or Delta for offspring where the most likely

parent is not the true parent at a given confidence level a critical LOD or Delta score is identified that is large enough to distinguish true and not true parents at a given confidence level (95%). When the LOD or Delta score in the real assignment exceeds the critical LOD or Delta score in the simulated assignment, then that animal can be assigned as a parent to an offspring at a given confidence level.

The simulation data that Cervus calculates also supports the feasibility of using a specific set of markers to assign parentages and accounts for un-sampled parents in a dataset and genotyping errors. Single parents or both parents can be assigned from a list of candidate parents. The accuracy of the parentage assignment will be reduced when genotyping errors occur, data is missing at multiple loci, too few markers are used, potential parents are highly related and a small proportion of the potential candidate parents are sampled. In cases where parents are related, such as in inbred stock, the likelihood that a relative of the true parent can be assigned is increased. The only way to overcome this problem is to increase the number of markers in the test. When parentage assignment is done in a forensic environment, the likelihood statistic must always be calculated in order to provide an evaluation of the strength of the evidence. It is during the process of exclusion that genotyping errors, null alleles and X-linked markers can cause errors due to false exclusions of candidate parents.

Cervus also identifies duplicate DNA profiles in a database. The duplicate profiles can be identified even when the profiles do not match completely by using a fuzzy match feature in the program. Matching of genotypes in RhODIS® are done using this feature in Cervus.

Colony software

Colony, similar to Cervus, uses likelihood to assign parentage from co-dominant marker data (Wang, 2004, Wang and Santure, 2009). Colony infers parental genotypes if parents cannot be found amongst the candidate parents and also offspring genotypes if data is missing or genotyping errors are found. The program then produces sibling data for the offspring that includes full-sib and half-sib information in text and graphic form and also provides a visual pedigree output using free pedigree drawing software such as Pedigree Viewer. Parental assignment includes probability values.

2.10.3.2 Population analysis

STRUCTURE software

A review article on STRUCTURE (Falush et al., 2003) analysis by Porras-Hurtado et al. (2013) provides a detailed description of the assumptions upon which the STRUCTURE analysis is based. It also highlights common issues which compromise these assumptions and can result in increased variability within

individual ancestry assignments. STRUCTURE uses a Bayesian iterative algorithm to split a set of individuals into Hardy-Weinberg and Linkage Equilibrium populations of individuals with distinct allele frequencies. The allele frequency data of the baseline database utilised for the analysis is therefore of fundamental importance, since individuals with similar allele frequencies are placed into the same groups. STRUCTURE uses a Markov Chain Monte Carlo estimation by splitting the population into groups and then using allele frequency variation to re-assign individuals. This is done a number of times – 100 000 times for example, that is defined as the burn-in period, after which the analysis is repeated several times (1 000 000 times for example) to support the assignment of the individuals to a pre-set number of populations (the K value). Typically various K values are used, since it is seldom known into how many populations a group of animals or humans can be divided. These set of K values from 1 to several are run multiple times and the most appropriate K value for a population dataset is calculated using the function in STRUCTURE Harvester. STRUCTURE Harvester collates results from STRUCTURE and assists in identifying the most likely population subdivision using ΔK (Evanno et al., 2005). The most appropriate K value for the extant Black rhinoceros population included in this analysis is 3, which follows the generally accepted split of *Diceros bicornis* into *D.b. minor*, *D.b. bicornis* and *D.b. michaeli* subspecies. Recent admixture can be determined in STRUCTURE if the ancestral populations diverged much earlier.

Porras-Hurtado et al. (2013) caution that Bayesian methods of population assignment are limited by small sample sizes and a small number of genetic markers used and that linkage between the markers must be minimal, particularly if microsatellite markers are used and that the minimum number of samples in the base sample set must exceed 500 in order to obtain reliable estimates of individual membership especially when admixed individuals are included. Evanno et al. (2005) also caution against small sample sets and a low number of markers when evaluating STRUCTURE results. Markers should also not be sex linked and thus the removal of the 2 sex linked markers from the analysis of the Black rhinoceros had a marked effect on individual assignments, with significant variation in results seen in admixed female animals when sex-linked markers are included or excluded from the analysis. Markers with the maximum allele frequency differences between populations (also called Ancestry Informative Markers – AIMs) are the preferred marker type to use in a STRUCTURE analysis.

If a DNA profile lacks an informative marker in a specific run, this will affect the ancestry coefficient of that individual. It is for this reason that one should exclude samples without complete profiles from the analysis as far as possible. Duplicates and the inclusion of closely related animals can also affect the allele frequencies and therefore affect the ancestry coefficients of individuals, particularly in admixed animals. In admixed animals the degree of variability between runs is the most pronounced and it is with this in mind that caution must be used when this data is applied to a commercial value of an animal (Sable

antelope for example) or conservation value of animals (Blesbok and Bontebok) and a threshold must be selected that is not too conservative which will lead to the exclusion of animals, especially in critically endangered species.

The most efficient way of running the STRUCTURE analysis for this latter purpose is to use a single reference database without duplicates and randomly selected individuals and test each unknown animal individually against this reference database in order to avoid significant deviations in the allele frequencies of the populations. Large autosomal SNP datasets that represent the diversity of a species with enough samples per population group will provide the most accurate ancestry information. This is, however, not available for many wild species yet. STRUCTURE is the most commonly used population analysis program and can utilise a number of different marker types. It has application in conservation management of populations, case control association studies in disease mapping and forensics if utilised with sufficient understanding to interpret results effectively. The forensic application is used to assign an individual to a specific population group, usually in humans but we have used this in assigning unknown Black rhinoceros horns to subspecies. This does not provide definitive evidence of origin but assists in directing investigations.

2.11 DNA extraction from rhinoceros horn

Species identification using mitochondrial DNA has traditionally been used when rhinoceros horn is recovered to provide evidence that it is from a rhinoceros and to identify the species of rhinoceros (Hsing-Mei et al., 2003). However, since rhinoceros horn was traditionally believed to be a clump of hair, no attempt had been made to obtain a nuclear based, individual DNA profile from this material in order to link the horn to the animal that it originated from. A commercially available DNA extraction kit that is used extensively in human forensic DNA applications with some modifications was used to provide a reliable and repeatable method to extract DNA from rhinoceros horn. A set of rhinoceros STR markers which have been published previously and used in various population studies in different rhinoceros species and have shown to amplify in both White and Black rhinoceros species were applied to rhinoceros samples to provide a unique DNA profile for each animal (Brown and Houlden, 1999, Cunningham et al., 1999, Florescu et al., 2003, Nielsen et al., 2008, Scott et al., 2004, Scott, 2008). The Zinc Finger locus was included in the multiplex PCR. The Zinc Finger locus occurs on the X and Y chromosomes and is used, based on fragment length differences between sexes, to identify the sex of the animal from which the sample is derived (Peppin et al., 2010).

The samples used in this study included hair, tissue, bone, toenail, horn and blood. Samples were obtained from live rhinoceros of both species from various provinces in South Africa, from private and

state owned reserves to obtain basic marker characteristics in each species. Blood and horn samples from live rhinoceros in the Kruger National Park were used to show that the DNA profiles obtained from the horns match the DNA profiles from the blood samples from the same animal (SANParks approved protocol and UP AUCC approval V034-08: 6 June 2008). The results are reported in Chapter 3.

The optimal sampling position in the horn was determined by sampling two horns of animals of approximately the same age and collecting drilling samples from various depths and positions across the length of the horn. The amount of horn required to provide a complete DNA profile was determined by using various amounts of powdered horn in the extraction assay from 0.1 to 35 mg and evaluating the DNA profile for amplification success and allelic drop out.

The effect of horn age on DNA was evaluated by testing horns of various ages received during routine trophy sampling. One item, a walking stick dated 1888, that appeared to be made completely from one rhinoceros horn was tested by drilling and provided a complete DNA profile. Another horn in a trophy stockpile was tested and provided a complete DNA profile. The horn was a hunt trophy from an animal shot in the Sudan in 1938 and was from a Northern white rhinoceros. This indicates the potential value of horn stockpiles in providing a genetic reconstruction of rhinoceros populations that are now extinct.

The repeatability of the assay was evaluated by repeating the test several times using the same known sample at a fixed DNA concentration and confirming that the DNA profile obtained was consistent. Each plate of 96 rhinoceros samples run in the Veterinary Genetics Laboratory includes four positive controls, a known White rhinoceros, Black rhinoceros, female and male rhinoceros, an extraction negative and two run or PCR negatives. Amplification success is determined by the number of markers that have successfully amplified, absence of allelic drop out and a peak height of more than 200 relative fluorescence units (RFU) and a balanced profile.

Standard spectrophotometry measures were done, using a Nanodrop (Thermo Scientific) instrument to determine the amount of DNA (ng/µl) and quality of the DNA (A260/A280 ratio) in the extract.

Primers used for STR amplification were tested against other commonly occurring and genetically related species and any cross amplification identified to ensure specificity of the test. A species in which several primers cross-amplify with the target species in a test could be incorrectly identified as the target species or, alternatively, a sample from the target species may be contaminated with DNA from the species in which cross amplification occurs, invalidating the results.

2.12 Rhinoceros horn trade and legislation and the application of molecular tools to support wildlife trade investigation

Trade in rhinoceros horn is enabled through a network of sophisticated organized crime syndicates that operate across international borders. Vietnam and China are the dominant end-user markets of rhinoceros horn (Crosta et al., 2017, Emslie et al., 2016, Moneron et al., 2017). The insatiable demand is driven by increasing wealth and disposable income in the consumer countries and a demand for rarities, with carved items, including cups, bangles beads, ornaments increasing and the powder generated by the production of these items sold for lower prices as a by-product for medicinal and social use (Crosta et al., 2017, Emslie et al., 2016, Moneron et al., 2017). Traceability of the carved products and powder is more complicated, since the horns are smuggled in pieces which cannot be identified physically by the unique shape of the whole horn and microchips are lost in the process of cutting and shaping the horn products. DNA profiling remains the only method to trace the origin of these items to a specific animal or animals providing evidence of source and trade routes. Emslie et al. (2016) state that the origin of horn seizures would improve knowledge of source and trade routes, if these could be sampled and identified more extensively and this information was made publicly available.

Illegal trade in rhinoceros horn has been implicated in funding, amongst others, North Korea's nuclear arms program and North Korean embassy officials have used diplomatic immunity laws to smuggle rhinoceros horn out of South Africa (Carey, 2017). Smuggling of horn by syndicates has been shown to be highly organized, adaptive and utilises various methods of travel and routes, including hand luggage, shipping containers, courier freight and postal services. The cross-border movement of the product is simplified by high levels of corruption and lack of cooperation between enforcement and border control agencies and delayed response times in transit countries (Moneron et al., 2017).

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an agreement between 183 countries known as Parties. Signatories to the CITES agreement are bound by the conventions adopted by the Parties at meetings held every four years. These conventions do not stipulate to countries what laws to implement but rather provides the framework for each country to adapt and adopt its own legislation to underpin the agreements of the convention. CITES was formerly established in 1975 as a means of regulating international trade in wild fauna and flora in order to ensure the conservation of species (https://www.cites.org/eng). Species listed by the convention are divided into three appendices; Appendix I includes species threatened with extinction, Appendix II includes those species not immediately threatened with extinction but may become threatened if trade is not controlled and Appendix III includes species where the range state has requested international support in monitoring trade. All Rhinocerotidae are listed as Appendix I (threatened by extinction) except

Ceratotherium simum simum in South Africa and Swaziland that are listed on Appendix II (controlled trade allowed). The CITES authority in each country is responsible for issuing permits required to trade in CITES listed species and parts of these species.

The National Environmental Management: Biodiversity Act (NEMBA) (No 10 of 2004) legislation governs the protection and utilisation of South Africa's biological resources. Under this act, the minister may issue norms and standards that must be followed in order to meet specific objectives of the act. A list of species is included under this act that require specific protection and are classified as critically endangered, endangered, vulnerable and protected and rules that govern the activities that may or may not be allowed in terms of these species (Anonymous, 2004). The Threatened or Protected Species Regulations (TOPS) stipulate the specific permitting and restricted activities in terms of listed threatened and endangered species. Laboratories that receive, test and store TOPS listed species must be registered in terms of TOPS with the South African Department of Environmental Affairs and have a TOPS permit allowing these activities. Samples from rhinoceros that are imported into and exported out of South Africa must have the appropriate CITES import and export permit from the country of origin and the receiving country. Additionally a Veterinary Import Permit issued by the Department of Agriculture must accompany the samples imported into South Africa and they must be packaged as specified.

2.13 Requirements to collect, analyse and report forensic molecular evidence in wildlife crime cases

The ISFG (International Society for Forensic Genetics) at its 23rd congress formed a commission to provide recommendations on the use of non-human DNA in forensic investigation (Linacre et al., 2011). The term non-human DNA includes plant, microbial and animal DNA. Animal DNA in forensic investigation generally includes the identification of a species from an item of unknown origin and the individual identification of the item in order to assign it to a specific animal. In some case the forensic investigation may also include kinship analysis in order to determine ownership of the animal. Species identification is performed using the mitochondrial genes, most commonly the cytochrome *b* (cyt *b*) gene and the cytochrome oxidase gene (COI). The cyt *b* locus has been used extensively in species identification. A short piece of either gene is sequenced and compared to a reference database or voucher specimen to identify the species origin. Reference sequence databases include GenBank (https://www.ncbi.nlm.nih.gov/genbank/). GenBank is the database of the National Institutes of Health (USA) and provides public access to sequence data deposited in it by researchers from all over the world with relevant data and references to associated publications. GenBank® forms part of a collaboration with other public sequence databases that include the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL) database, and GenBank at NCBI that exchange data on a daily basis. A The COI locus was proposed as a reference standard to identify species in 2003 to underpin the Barcode

of Life project (iBOL) using a 648 base pair section of the COI gene to provide a reference barcode for every species on earth (http://www.barcodinglife.org/).

Individual identification and assignment has utilised mainly STR (Short Tandem Repeat) loci and more recently SNP (Single Nucleotide Polymorphism) loci in forensic investigations. STR reference databases have been developed for various animal species mainly in domesticated animals such as cats (Menotti-Raymond et al., 2005), dogs (Kun et al., 2013, Wictum et al., 2013a), cattle (Van De Goor et al., 2009), horses (Chen et al., 2010) and pigs (Lin et al., 2014), but is limited in wild species. RhODIS® provides such a reference database for African rhinoceros and is distinctive in that it represents multiple populations across almost all the range of African rhinoceros. The use of a single reference database of STR loci requires extensive sharing of data, control samples to provide reference genotypes for standardization and regular inter-laboratory proficiency testing. The International Society of Animal Genetics (ISAG) provides a mechanism for this for domestic animal STR data sharing and biennial comparison testing (http://www.isag.us/).

The ISFG recommendations published in 2011 represented a starting point for international standardization and guidelines for non-human forensic DNA testing (Linacre et al., 2011). The recommendations included:

1) Procedures must be established to ensure the integrity and traceability of samples collected for analysis and voucher reference specimens similar to those used in any forensic investigation.

2) Validation studies must be done using voucher specimens.

3) The loci used in species identification must be justified based on the ability to identify and distinguish closely related species.

4) The sequence and genetic map showing the location of primers used in species identification must be available and referenced.

5) Inter and intra-species validation studies must be available for novel primers used in species identification.

6) Primers used to amplify loci in STR profiling must be available in the public domain and must be tested for reproducibility and specificity. The various alleles identified must be sequenced. The use of tetranucleotide repeats is recommended due to the increased stutter observed in dinucleotide STR profiling and the amount of stutter recorded as well as the selection of allele calling in cases where single base additions occur.

7) The use of an allelic ladder is recommended in cases where tetranucleotide repeat STR's are used to designate alleles. In order to generate an allelic ladder a suitably large proportion of the population must be sampled to provide the scope of the allelic ladder and a control genotype must be used to show that the alleles have separated as expected.

8) The number of repeats is recommended as a basis for naming alleles rather than using only the size based on the number of base pairs.

9) Particularly if the STR genotypes are to be used in determining parentage, the probability of mutations in that STR locus must be determined.

10) The frequency of alleles in various populations of the species must be determined as well as other population genetic parameters including Hardy-Weinberg Equilibrium by ensuring that an appropriate number of individuals of the species are sampled.

11) The probability that two individuals share a common allele as a result of their having a single ancestor in common is known as a kinship factor and is related to the level of inbreeding within a population and also the degree of relatedness between sub-populations due to historic common ancestors. This factor is called F_{ST} or Theta (θ). The factor used in human populations most often is 0.01 or 0.03, but is usually higher in inbred animal populations. An accurate kinship factor must be calculated and applied to the relevant population analysis.

12) A comprehensive case file must be maintained.

13) DNA laboratories performing non-human forensic testing must aim to be accredited in terms of ISO17025 standard.

The DNA profile

DNA profiles in forensic analysis may be defined as a full profile (contains all alleles in the genotype), a partial profile (a genotype with at least 1 allele missing) and a mixed profile (DNA profiles of more than 1 donor are present) (Puch-Solis et al., 2012).

2.14 The development of a rhinoceros DNA database and supporting tools

A SANParks approved project (UP AUCC approval V034-08: 6 June 2008) completed at the end of 2009 included the DNA extraction and genotyping of rhinoceros horn and tissue samples to evaluate the genotyping success and comparison between the samples. The VGL was approached in early 2010 by SANParks Crime Investigation to utilise the method to link recovered horns to specific rhinoceros

carcasses of rhinoceros poached in the park. In 2010 SAPS submitted the first case of horns seized from OR Tambo International airport to the laboratory to identify a possible link to private rhinoceros illegally killed on a private reserve in South Africa. A total of 7 horn samples were submitted and 2 of these horns linked to 2 rhinoceros from a farm in the Vaalwater district. The DNA evidence supported the conviction and sentencing of the suspect to 10 years imprisonment (https://southafricanews.wordpress.com/2010/06/30/vietnamese-man-gets-hefty-sentence-for-rhino-horn-smuggling/). Following this the number of samples from rhinoceros crime cases submitted to the laboratory increased and with the support of SANParks and the provincial wildlife authorities a decision was made to support the development of a single rhinoceros database. This database was named RhODIS®, an acronym for the Rhinoceros DNA Index System, based on the example of the CODIS program of the FBI in the USA (https://www.fbi.gov/services/laboratory/biometric-analysis/codis). Legal services of the University of Pretoria suggested that the name be protected through a trademark and the RhODIS® trademark was registered on 25 July 2011. A decision was also made to increase the size of the database as rapidly as possible by sampling all routine live rhinoceros, rhinoceros horns and rhinoceros carcasses from both legal hunts and illegal killings in order to ensure that the database could provide a traceability system for wildlife and enforcement authorities as numbers of poached rhinoceros were increasing. The profiling data would also provide statistical support to matches found.

In 2012 the South African Department of Environmental Affairs published a new set of norms and standards for the marking of rhinoceros and rhinoceros horns under the Nemba Act. These included that samples from rhinoceros translocated, dehorned, notched, hunted and those that died from natural mortalities and illegal hunting must be sampled and submitted to the VGL for profiling and adding to the RhODIS® database and that these samples had to be collected in sampling kits supplied by the VGL. The number of samples submitted to the VGL increased significantly reaching 50 000 by August 2018.

Sampling kits were developed in collaboration with the Environmental Crime Investigation Unit (ECI) of South African National Parks (SANParks) and the South African Police Services (SAPS) and the kits were launched in 2011 when 1000 were officially handed to SANParks (https://www.timeslive.co.za/news/south-africa/2011-06-23-tukkies-vets-launch-plan-to-save-rhinos/). The kits were developed in order to ensure that chain of custody (CoC) requirements would be fulfilled during both the routine collection of samples from live animals and the collection of samples during the investigation of a crime scene from a rhinoceros carcass. CoC is the documented movement and location of physical evidence from the time and place it is obtained until it is presented in court (https://legal-dictionary.thefreedictionary.com/chain+of+custody). This must form an unbroken chain from collection to laboratory through processing and final reporting with sealing and identification of each evidence item

and identification of each person handling the evidence item underpinning the process. Factors that can affect the CoC include the collection method of samples at the crime scene or from the carcass, mixing of samples, contamination of samples and mislabelling. Correct CoC procedures rule out tampering with the samples and substitution of samples. Substantial alteration of the sample as a result of incorrect handling and storage also constitutes breaks in the CoC since the samples are changed from the original sample collected. All persons handling the evidence items must be accounted for. By October 2017 more than 60 000 kits had been distributed throughout Southern Africa and other African rhinoceros range states. A number of training courses on the use of the sampling kits and the incorporation of the RhODIS® DNA evidence collection in terms of rhinoceros crime scene investigation followed and included countries Namibia, Swaziland, Kenya, India and South Africa. Three kits were developed, a horn kit to sample rhinoceros horns, a routine kit to sample live and hunted rhinoceros and a forensic kit to sample from rhinoceros that had been illegally killed. An instructional video showing the use of the kits and DNA sampling as part of the crime scene investigation was developed and launched. The kits were reduced to 2 types in 2017, the horn sampling kit and an animal sampling kit that could be used for routine and forensic sampling and had the option of the use of a sealed plastic bucket depending on the preference of the authority that used the kit and these have been specifically adapted and packed for individual clients. (Appendix 1: Horn sampling kit form, Appendix 2: Animal sampling kit form, Appendix 3: Animal sampling kit instructions).

The sampling kits contain all the sample collection material and containers required for collecting a full set of DNA samples from an animal. Needles, syringes, EDTA vacutainer blood collection tubes, gloves and disposable scalpels are commercially obtained and pre-sterilized. The sample bottles, drill bits, plastic containers and bleeding shoulders are decontaminated and all packing is done in a clean and separate area. Each kit includes a form in a sealable plastic pouch that must be completed so that the data can be entered into the RhODIS® database if not collected using the eRhODIS™ app. The form also includes the instructions for use of the kit and shipment information to the laboratory. The kits are sent in a sealed forensic evidence bag with a unique identification number which is also be applied as a barcoded label to all containers in the kit to link them to a specific kit. Each kit contains a second unsealed forensic evidence container with related number that must be used to return the samples and sampling items and must be sealed at the side of the animal or at the crime scene. All steps in sampling at the crime scene and receipt of the kit in the laboratory are photographed. A drill bit is included in the kits to collect horn material from the rhinoceros when providing a hole for the insertion of a microchip into the horn.

In order to improve the accuracy and efficiency of the system a data collection app was developed and named eRhODIS™ for android devices and donor support assisted in the distribution of initial tablets with

the app to specific authorities and officials to assist in collecting the biodata. By August 2018 more than 22 000 eRhODIS™ submissions had been received (https://erhodis.org/index.php).

CHAPTER 3

Extraction of nuclear DNA from rhinoceros horn and characterization of DNA profiling systems for white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros.

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Abstract:

Rhinoceros horn is now worth more, per unit weight, than gold, diamonds, or cocaine. Rhinoceros horn has been used in traditional Asian medicine as a presumed cure for a wide range of ailments. Rhinoceros poaching in South Africa has, on average, more than doubled each year over the past 5 years with the rapid economic growth in east and southeast Asia being assumed to be the primary factor driving the increased demand for horn. Here we report on the characterization of methods for genomic DNA extraction from rhinoceros horn and on DNA profiling systems for white (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros. The DNA profiling system described includes 22 short tandem repeat (STR), or microsatellite, markers and a gender marker (ZF1), which have been used previously in various studies on rhinoceros. Using a u value of 0.1, a conservative estimate of random match probability in 5 white rhinoceros ranged from 1:7.3 x 10^6 to 1:3.0 x 10^8. Given that the total population of white rhinoceros is approximately 20,000 such random match probabilities indicate that the genotyping system described provides data which can be used for evidentiary purposes. Furthermore, the methods are appropriate for use in investigations involving trace amounts of rhinoceros horn and the matching of profiles obtained from seized rhinoceros horn with material collected from live animals or poached carcasses.

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3.1 Introduction

The analytical techniques capable of providing DNA evidence to assist in conservation law enforcement have developed in parallel to human forensic genetics. Short tandem repeat (STR) markers are commonly used to establish a link between an evidence sample and an individual through a unique DNA profile consisting of a subset of these markers. Such systems are used in human forensics and are being applied increasingly to criminal investigations involving domestic and wild animals (Lin et al., 2014, Van De Goor et al., 2009, Van De Goor et al., 2010, van Hoppe et al., 2016). Illegal trade in rhinoceros horn poses a serious and increasing threat to the long-term survival of the rhinoceros (Biggs et al., 2013). Rhinoceros horn is used in traditional Asian medicine (TAM) in South-East Asia and as dagger handles in mainly Yemen. The demand for horn has escalated as a result of the economic boom in South-East Asia and endemic poverty in the habitat of the rhinoceros (Milliken and Shaw, 2012). The structure of rhinoceros horn has been described as an epidermal derivative, consisting of keratinized tubules of cells connected with a matrix of melanin and calcium. It continues to grow at a rate of 5–6 cm/year and can be harvested from the live animal (Hieronymus et al., 2006). Techniques have been described for the extraction of mitochondrial DNA from rhinoceros horn which then allows for the subsequent confirmation of the species of origin (Hsing-Mei et al., 2003). To date, methods to extract genomic DNA from rhinoceros horn and marker systems for the individual identification of rhinoceros from their horns have not been described. The objective of this study was to develop and characterize a method to extract nuclear DNA from rhinoceros horn of sufficient quality and quantity to allow the amplification of STRs producing a DNA profile capable of uniquely identifying an individual rhinoceros. This, in turn, could provide a mechanism for the matching of a DNA profile obtained from seized rhinoceros horn with that obtained from other samples collected from the same animal when it was alive or when samples were collected following poaching.

3.2 Materials and methods

3.2.1 Sample materials

Matching blood and horn samples were obtained from 6 white rhinoceros during routine capture operations in the Kruger National Park. Blood was collected into vacutainer tubes with EDTA (Ethylenediaminetetraacetic acid) (BD Vacutainer1) using 20 gauge needles from the ear vein. Horn samples were collected from the same animals from the tip, middle or base of the horn by excision of a piece of horn approximately 2 cm^3 using a saw. These pieces of horn weighed between 2.1 g and 4.8 g. In addition, 5 horn and hair samples collected from animals during routine identification and translocation procedures in Mpumalanga Province, South Africa and were submitted to the Veterinary Genetics

Laboratory for routine genotyping and their DNA profiles were compared. Two horns, one from a black rhinoceros (*Diceros bicornis minor*) and one from a southern white rhinoceros (*Ceratotherium simum simum*), that were donated by the Ezemvelo KZN Wildlife from two rhinoceros of approximately the same age and size were used to investigate the variation in DNA extracts from different parts of the horn. Samples submitted to the Veterinary Genetics Laboratory for routine genotyping were used for the further characterization of DNA profiling systems for white and black rhinoceros.

3.2.2 DNA extraction

Approximately 200 mg of rhinoceros horn was obtained by drilling into the horn with either a new drill bit or a drill bit decontaminated by washing with soap followed by soaking in an undiluted solution of commercial household bleach (Jik/Sodium Hypochlorite) and rinsed with deionised water and allowed to dry before using on a new sample and the drill shavings transferred to a labelled plastic tube (4 ml screw cap tube, J-Plast). The horn shavings were homogenized to a fine powder using a tissue homogenizer (Omni International TH). Approximately 20 mg of the powder was transferred to a labelled eppendorf tube. A total of 500 ml of Prepfiler™ lysis buffer (Life Technologies) and 5 ml of DTT (Dithiothreitol, Sigma) was added to each tube. The tubes were placed on a heated shaker (Vortemp 56, Labnet) for 1 hour at 70°C. Tubes were centrifuged (M-240 Boeco Germany) at 10,000 rpm for 2 minutes. A total of 300 ml of supernatant was transferred to individual wells in a Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) deepwell plate and 15 ml of Prepfiler™ Magnetic Beads (Life Technologies) were added to each well. The plate was vortexed at 1000 rpm for 10 seconds on a shaker and 180 ml of Isopropanol (Sigma) was added to each well and vortexed again at 1000 rpm for 10 seconds. The DNA extraction was completed on a Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) according to the Prepfiler™ V2 protocol (supplied by Applied Biosystems). Briefly, DNA binding was performed for 10 minutes followed by 3 washes using 300 ml Prepfiler$_{TM}$ Wash Solution per wash, 5 min drying at room temperature and elution into 75 ml of elution buffer performed at 70°C. Blood was extracted using 50 ml of whole blood as described in the Prepfiler™ protocol (Life Technologies). Further processing was performed on the Kingfisher 96 Magnetic Particle Processor (Thermo Scientific) as described above. The hair was extracted using NaOH (sodium hydroxide) and heat as described previously (Rudbeck and Dissing, 1998). The DNA concentration and quality of extracts were measured spectrophotometrically in triplicate using a Nanodrop™ 1000 spectrophotometer (Thermo Scientific).

3.2.3 DNA extraction from different positions in rhinoceros horn

Each horn was mounted in a drill press so that the median plane of the horn was horizontal and the drill press was set to stop at the median plane. Drilling was done from the side of the horn to the medial plane.

Drillings were performed using a 7 mm drill bit at distances of approximately 10%, 25%, 50%, 75% and 90% from the base to the tip of the horn. Up to 3 separate samples representing drillings at different depths were collected into separate sample tubes and extracted individually. The depth of each drilling was recorded in millimetres from the scale on the drill press.

3.2.4 Comparison of DNA profiles obtained from horn and other samples of the same animal

The DNA profiles were obtained from the blood and horn samples collected from 6 white rhinoceros during routine capture operations in the Kruger National Park and the 5 horn and hair samples collected from animals during routine identification and translocation procedures in Mpumalanga Province and compared.

3.2.5 Sensitivity of DNA extraction method

The sensitivity of the DNA extraction method was tested using variable amounts of horn powder in the extraction protocol. A single piece of rhinoceros horn was used that was obtained from the tip of a horn that was part of a horn stockpile. The piece of horn was drilled using a new 4 mm drill bit. Shavings from this piece of horn were collected into a plastic tube and homogenized to a fine powder using a tissue homogenizer. The powder was weighed and an amount of 0.1 mg, 1 mg, 2 mg, 5 mg, 10 mg, 15 mg, 20 mg, 25 mg and 35 mg was placed into separate labelled Eppendorf tubes and processed as described above.

3.2.6 Marker selection and PCR amplification Analysis

PCR amplification was performed using 22 dinucleotide STR markers published previously (Brown and Houlden, 1999, Cunningham et al., 1999, Florescu et al., 2003, Nielsen et al., 2008, Scott et al., 2004, Scott, 2008). Details of the markers and multiplexes are provided in Table 1. The zinc finger (ZF) locus (Peppin et al., 2010) was used to determine the gender of the animal from which the sample originated. STR analysis was performed using 4 multiplex reactions with between 5 and 8 markers included in each multiplex (see Table 1). Extracted DNA (1 µl diluted to approximately 30 ng/µl or undiluted at less than 30 ng/µl) was added to a PCR mastermix consisting of 5 µl of KAPA2G Fast Multiplex PCR Kit (Roche) and 4 µl of primer mix in a 10 µl reaction volume. PCR was performed using a thermal cycler (GeneAmp1PCR System 9700, Life Technologies) with cycling conditions standardized as follows: 3 min at 95°C, 30 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s followed by an extension step at 72°C for 10 min.

Table 1: Summary of the forward and reverse primers, repeat motifs, GenBank accession numbers, reference, dye label, size range and multiplex in which the loci used for genotyping of white and black rhinoceros.

Locus	Forward primer (5′-3′)	Reverse primer (5′-3′)	Repeat motif	Accession	Reference	Dye	Size (Bp)	Multiplex
BlRh1B	GATCAGTAACACCAAAGTCC	AGTGAAGACAGAAGGATCAC	$(GT)_{13}GCA(TG)_3$	AY606078	(Nielsen et al., 2008)	NED	230–250	3
BlRh1C	AGATTCTTGGAAAGGTCACT	AACATTGGGTTTCACCTC	$(AC)_{17}G(CA)_4$	AY606079	(Nielsen et al., 2008)	NED	120–160	2
BlRh37D	ACATGTGTAAACTTGGGAAC	TGGTTCATTGATCTCTTCTC	$(TG)_6(AG)_{11}GA(AG)_5$	AY606083	(Nielsen et al., 2008)	NED	200–250	1
BR6	TCATTTCTTTGTTCCCCATAGCAC	AGCAATATCCACGATATGTGAAGG	$(CA)_{15}$		(Cunningham et al., 1999)	PET	150–165	3
DB1	TAAGTCACAGGGACTAATCTG	GAGGGTTTATTGTGAATGAG	$(CA)_{14}$	AF129724	(Brown and Houlden, 1999, Nielsen et al., 2008)	VIC	230–250	3
DB23	ATCTTCCTCAGCAATAAGG	ATCATCAGAGTTTCCAGTTC	$(CA)_{12}$	AF129734	(Brown and Houlden, 1999, Nielsen et al., 2008)	FAM	180–214	4
DB44	AGGGTGGAATGTCAAGTAG	CTTCTAGAGGGAGACTAGGAG	$(TG)_4C\ (GT)_{16}$	AF129730	(Brown and Houlden, 1999, Nielsen et al., 2008)	VIC	200–230	3
DB52	CATGTGAAATGGACCGTCAGG	ATTTCTGGGAAGGGGCAGG	$(CA)_{21}$	AF129732	(Brown and Houlden, 1999, Nielsen et al., 2008)	PET	110–140	1
DB66	CCAGGTGAAGGGTCTTATTATTAGC	GGATTGGCATGGATGTTACC	$(CA)_7TA(CA)_{16}$	AF129733	(Brown and Houlden, 1999)	PET	210–230	3
IR10	CAGTGAGGAAGATTGGTTGC	CCTGACTCACACATCACCAG	$(CA)_{22}$		(Scott, 2008)	NED	120–140	4
IR12	GAATGCTGATCATTTAGTGAC	GGGTCCAGTTGAGATATCAC	$(CA)_{18}$		(Scott, 2008)	PET	170–200	4
IR22	ATGGTGGAAGAAGTGCAGCC	ACTTCTGTGTCTCTAGCGCC	$(CA)_{22}$		(Scott, 2008)	VIC	200–230	2

Locus	Forward primer (5'-3')	Reverse primer (5'-3')	Repeat motif	Accession	Reference	Dye	Size (Bp)	Multiplex
SR63	CTTGAGCAGAGTAGAATTTGG	CTCTGTATCCACCTCATTCC	$(AC)_{19}$	AY427965	(Scott et al., 2004)	FAM	180–210	4
SR74	CAGCACAATGTTTGGCACTTG	TTGGAGTCTTATGTCACCACC	$(CA)_{19}$	AY427967	(Scott et al., 2004)	NED	160–180	2
SR262	CTGCCTTAACAACTGAACTGC	TGGAGGTTATCTCATGCCAC	$(TG)_{28}$	AY606077	(Scott, 2008)	FAM	80–110	3
SR268	GTTTATACTATGCCCTGCAC	GGATGCTACCGAATAGATTG	$(CA)_{25}$	AY427972	(Scott, 2008)	VIC	170–200	4
SR281	AGGTGATTAGGGAATTGCTGG	TTCTTCTGTCCTGGCATTGC	$(GT)_{23}$	AY427974	(Scott et al., 2004)	FAM	220–250	2
7B	AACCAACTTGTAATGAGAGG	AATGAACAGGAAGGAAGAC	$(TG)_{16}A(GT)_{5}$	AY138544	(Florescu et al., 2003, Nielsen et al., 2008)	PET	220–230	3
7 C	GTCAGTTCAAGTTTTTGCTC	CTCATCCATGCTTCTTCTAC	$(CT)_{14}(AT)_{11}$	AY138543	(Florescu et al., 2003, Nielsen et al., 2008)	FAM	130–170	3
12F	ACAGCTAGAATCACCAAAAC	TCCTGCTGCATAAATCTC	$(TA)_{8}(AA)_{4}$	AY138545	(Florescu et al., 2003, Nielsen et al., 2008)	VIC	220–240	1
32A	CTAGCAAAATCTCAAAGAGG	TTACTAAGGGAATCACCAAG	$(AC)_{6}...(AC)_{15}$	AY138541	(Florescu et al., 2003, Nielsen et al., 2008)	FAM	190–210	1
32F	GGCAAAACTAAGAGAACTTG	GATACCAAACTGGAAATGG	$(AC)_{18}$	AY138542	(Florescu et al., 2003, Nielsen et al., 2008)	VIC	170–240	1
ZF1	GATTTGGAASCTAGGCATTTCC	GCCATGATACTCATGAATGACA			(Peppin et al., 2010)	FAM	95–105	4

3.2.7 Capillary electrophoresis and genotyping

PCR product (0.5 µl) was loaded with 10 µl Hi-Di™ formamide and 0.25 µl GeneScan™ 500 LIZ1 size standard (Life Technologies) and run on an 3130xl Genetic Analyzer (Applied Biosystems) and data transferred to a personal computer and analyzed using STRand software (University of California, Davis) (Toonen and Hughes, 2001). A set of bins for each locus within the four different panels were set up in STRand using fixed bin sizes to determine and standardize the allele calls between samples. Known control samples for both black and white rhinoceros were included with each sample set that was run to ensure the accuracy of allele calls between runs.

3.2.8 Population genetic analysis

The genotypes from a total of 367 samples from southern white rhinoceros (*C.s. simum*) and 33 samples from black rhinoceros of 3 subspecies (*Diceros bicornis bicornis* (n = 5), *Diceros bicornis minor* (n = 25) and *Diceros bicornis michaeli* (n = 3)) submitted to the Veterinary Genetics Laboratory for routine genotyping were used and genotyped using the procedures described above. Allele frequencies, observed (HObs) and expected (HExp) heterozygosities were calculated using Cervus V3.03 (Kalinowski et al., 2007). F statistics were calculated using FSTAT (Goudet, 1995) and GENEPOP (Raymond and Rousset, 1995) for the white and black rhinoceros populations without population subdivision to calculate a Fis value for each population. Probability of identity (PI) for each locus, and over all loci, for the white and black rhinoceros populations was calculated using GenAlEx (Peakall and Smouse, 2012).

3.2.9 Match probability

Five DNA profiles from white rhinoceros were selected and the random match probabilities calculated using the formula of Balding and Nichols (Balding and Nichols, 1995) at different values of theta for each locus and the multilocus match probability was calculated as the product of the locus specific match probabilities.

3.3 Results

Figure 3 shows the positions of the holes drilled in the horns from the black and white rhinoceros. Table 2 summarizes the DNA concentrations and number (and percentage) of alleles that amplified in extracts from powdered horn obtained from different locations from the base to the tip of the horns and at different depths from the median of the horn. The DNA concentration in extracts from powdered horn obtained from incurred horn samples that were compared with the DNA profiles of blood (n = 6) and plucked hairs (n = 5) of the same animal ranged from 14.8 to 149.5 ng/µl and all horn, blood and plucked

hair samples provided full DNA profiles that matched in the same animal. An example of the DNA profile obtained from a horn and matching blood sample is provided in Figure 4. The DNA concentration in extracts from between 0.1 and 35 mg of horn powder varied from 0.5 to 20.8 ng/µl and all extracts gave full DNA profiles except that from 0.1 mg of horn powder which gave a profile with 21 of the possible 23 loci amplifying successfully with a single locus, DB1 showing non-amplification of the second allele of a heterozygous pair. The allele frequencies for each locus are summarized using a standardized nomenclature system (Olaisen et al., 1998) in Table 3 for the white (n = 367) and black rhinoceros (n = 33). The number of alleles (Na), observed (H_{Obs}) and expected (H_{Exp}) heterozygosities, polymorphic information content (PIC), inter-individual inbreeding coefficient (F_{is}) and probability of identity for individuals (PI) and siblings (PI_{Sibs}) for each locus and the population means are provided in Table 3. The calculated random match probabilities for 5 individual white rhinoceros varied between 1.6 X 10^{8} and 2.1 X 10^{11} when θ was set at 0, between 7.3 X 10^{6} and 3.0 X 10^{8} when θ was set at 0.1 and between 1.7 X 10^{5} and 6.0 X 10^{6} when θ was set at 0.3.

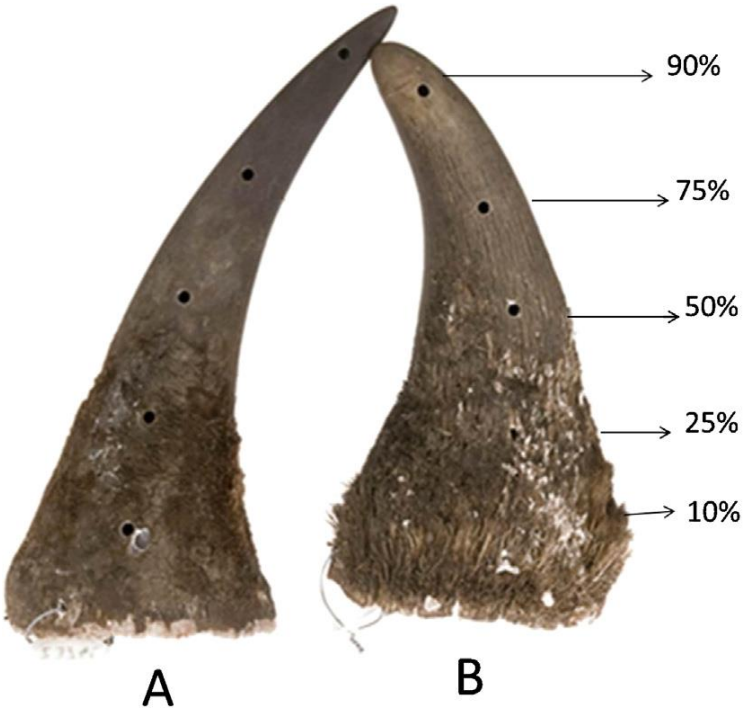

Figure 3: Two horns, one from a black rhinoceros (A) and one from a white rhinoceros (B) indicating the position of drillings taken for DNA analysis along the length of the horn.

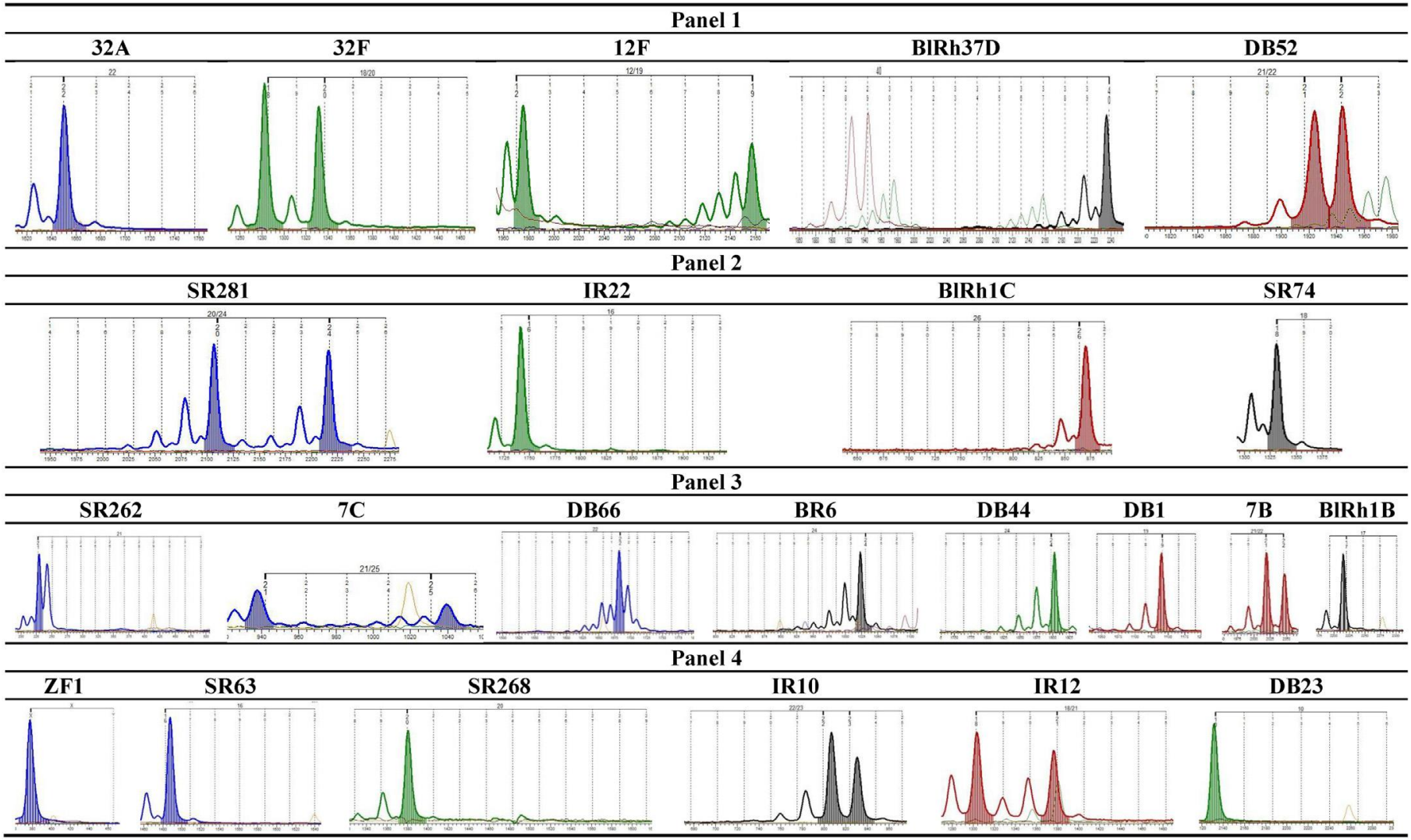

Figure 4: DNA profile of a white rhinoceros determined from an extract from the horn. The profile comprises 4 separate panels consisting of 22 STR markers and a gender marker.

Table 2: Summary of the drill depths from the median (mm) at five different levels from the base (10%) to the tip (90%) of the horn with the DNA concentration (in ng/µl), the number of alleles that amplified and the percentage of alleles that amplified for the horns from the white rhinoceros and the black rhinoceros.

Position	White rhinoceros				Black rhinoceros			
	From median	[DNA] ng/µl	Alleles	% amplified	From median	[DNA] ng/µl	Alleles	% amplified
90	0–8	89.9	46	100	0–7	170.9	46	100
	8–13	60.2	46	100	7–12	217.4	46	100
75	0–8	139.8	46	100	0–5	302.1	46	100
	8–13	31.2	46	100	5–12	179.8	46	100
	13–18	21.9	32	70	12–17	43.5	40	87
50	0–8	226.9	46	100	0–8	68.3	46	100
	8–16	20.5	36	78	8–15	22.4	32	70
	16–21	17.8	11	24	15–20	20.3	32	70
25	0–14	247.7	46	100	0–12	135.1	46	100
	14–25	99.7	46	100	12–19	40.8	46	100
	25–30	48.3	46	100	19–24	13.7	12	26
10	0–20	217.5	46	100	0–18	261.2	46	100
	20–45	104.5	46	100	18–30	241.9	46	100
	45–50	30.0	46	100	30–35	31.9	40	87

Table 3: Allele frequencies for 22 microsatellite markers investigated in white (n=367) and black rhinoceros (n=33) using a standardized nomenclature system.

Locus	Species	Alleles																								
		10	12	14	15	16	17	18	19	20	21	21.1	22	22.1	23	24	25	26	27	28	29	30	31	32	35	40
BlRh1B	White						0.759	0.241																		
	Black					0.030		0.818		0.152																
BlRh1C	White																	0.727	0.273							
	Black						0.197				0.500		0.167		0.076	0.045	0.015									
BlRh37D	White																									1.000
	Black								0.636	0.333			0.030													
BR6	White			0.618	0.118			0.042								0.223										
	Black				0.561			0.273		0.015								0.091	0.061							
DB1	White								0.847	0.153																
	Black				0.015		0.030	0.500	0.030	0.394	0.030															
DB23	White	1.000																								
	Black		0.015		0.545	0.439																				
DB44	White									0.255	0.018					0.727										
	Black							0.045		0.742	0.136		0.015			0.061										
DB52	White									0.324	0.354		0.322													
	Black						0.030		0.091	0.258	0.561		0.030		0.030											
DB66	White											0.020	0.293	0.130	0.557											
	Black				0.015				0.030	0.030	0.242		0.045		0.470		0.030	0.136								
IR10	White												0.298		0.702											
	Black						0.015			0.015	0.015		0.061			0.879	0.015									
IR12	White							0.373			0.627															
	Black							0.015		0.076			0.152		0.621	0.061	0.076									
IR22	White					1.000																				
	Black										0.045		0.530		0.424											
SR63	White					0.566	0.434																			
	Black							0.045	0.485	0.348	0.015		0.106													
SR74	White							1.000																		
	Black							0.197	0.561	0.242																
SR262	White										0.732						0.268									
	Black																		0.106	0.333	0.045	0.212	0.288	0.015		
SR268	White							0.161	0.038	0.801																
	Black																0.348	0.091	0.348	0.152	0.061					
SR281	White			0.346						0.382						0.272										
	Black						0.242		0.045				0.606		0.091			0.015								
7B	White								0.090		0.655		0.255													
	Black								0.015	0.091	0.894															
7C	White										0.083					0.258	0.660									
	Black																				0.076	0.212	0.455	0.167	0.091	
12F	White		0.513						0.487																	
	Black			0.091	0.076	0.742	0.076	0.015																		
32A	White										0.506		0.447		0.048											
	Black										0.076		0.015		0.864	0.015		0.030								
32F	White							0.416		0.123						0.003	0.458									
	Black								1.000																	

3.4 Discussion

Extraction of DNA from powdered horn of white and black rhinoceros using the Prepfiler™ kit on a Kingfisher Magnetic Particle Processor produced DNA extracts with DNA concentrations often in excess of 200 ng/µl. Samples collected from the centre of the horn anywhere from the base to the tip of the horn consistently produced DNA extracts with the highest concentration. Extractions further from the centre of the horn were less efficient and extractions closest to the outside surface of the horn sometimes resulted in incomplete DNA profiles. When collecting a sample from a detached horn it is recommended that one collects the sample by drilling into the dark area (increased melanisation) in the centre of the horn base to a depth of approximately 50 mm. When collecting horn samples from live rhinoceros by drilling into horn from the outside only the drillings from deeper in the horn should be collected. These are easy to identify as they have are a darker brown to black colour when compared to the white material from the periphery of the horn. DNA extracts from horn samples collected in the field and powdered in the laboratory resulted in extracts with concentrations between approximately 15 and 150 ng/µl. The DNA profiles obtained from these samples matched the profiles obtained from the blood and hair samples collected simultaneously from the same animal on all 23 loci in all cases. Whilst previous studies have documented the extraction of mitochondrial DNA form rhinoceros horn which was subsequently used to identify the species of origin of the horn (Hsing-Mei et al., 2003), and we have shown previously that nuclear DNA extracted from rhinoceros horn can be used to identify the gender of the animal of origin (Peppin et al., 2010).

This paper provides the first description of a technique which can extract nuclear DNA from rhinoceros horn which is of adequate quantity and quality to allow STR analysis to be applied to generate profiles to individually identify the animal of origin. The Prepfiler™ kit recommends that one should extract DNA from sample material weighing approximately 20 mg. However, pieces of material resembling rhinoceros horn may be substantially smaller than this and in an attempt to investigate the smallest sample size from which a DNA profile can be obtained we used the kit to extract DNA from 0.1 to 35 mg of powdered horn. Full DNA profiles were obtained from extracts of samples ranging from 1 to 35 mg and a partial profile which included 41 of the possible 46 alleles was obtained from a sample of 0.1 mg. These results show that one can generate complete DNA profiles from extremely small amounts of rhinoceros horn which may be of great value in matching a horn, or part of a horn, back to the animal from which it originated.

The STR markers investigated in this study included loci originally identified in white, black, Indian (*Rhinoceros unicornis*) and Sumatran (*Dicerorhinus sumatrensis*) rhinoceros (Brown and Houlden, 1999, Cunningham et al., 1999, Florescu et al., 2003, Scott et al., 2004, Scott, 2008). Eighteen of the 22 markers investigated were polymorphic STR markers with between 2 and 4 alleles observed in the white

rhinoceros. The remaining 4 markers (BlRh37D, DB23, IR22 and SR74) were monomorphic in the white rhinoceros but were polymorphic in the black rhinoceros. These four markers were all originally isolated from the black (BlRh37D, DB23), Indian (IR22) and Sumatran (SR74) rhinoceros. The marker 32F originally isolated from the white rhinoceros was polymorphic with 4 alleles in the white rhinoceros but was monomorphic in the black rhinoceros. In the case of SR74 the monomorphic allele in the white rhinoceros was of similar size to one of the 3 alleles observed in the black rhinoceros whereas for all other monomorphic loci the size of the monomorphic allele was unique in the species in which it was monomorphic providing a mechanism for confirming the species of origin for the sample investigated. The marker DB66 was highly polymorphic in the black rhinoceros with 8 different alleles observed in this study and a PIC value of 0.658 indicating that this is a highly informative marker in the black rhinoceros. In the white rhinoceros this marker provided 4 alleles but two of these differed from the other two alleles by a single base pair. The mechanism for this observed difference warrants further investigation. The marker 7B originally isolated from the white rhinoceros was polymorphic with 3 alleles in the white and black rhinoceros. However 29 of the 33 black rhinoceros included in this study were homozygous for the 21 allele. The 20 allele only occurred in the 3 *D.b. michaeli* and all were homozygous for this allele. This locus may have specific alleles fixed within the black rhinoceros subspecies, but a larger number of individuals from each subspecies will need to be investigated to confirm this. A single *D.b. minor* from the Kruger National Park had the 19 allele.

The southern white rhinoceros population was reduced to between 20 and 40 animals in the early 1900s with all these animals being confined to the Hluhluwe/iMfoloza area within the KwaZulu-Natal Province of South Africa (Walker and Walker, 2012). The current southern white rhinoceros population in Africa is between 18,000 and 20,000 (Emslie et al., 2016) and all are descended from this single founder population. The low genetic diversity observed in our study (mean Na = 2.722 and mean PIC = 0.329) is similar to that reported previously (Scott et al., 2004) and is a direct result of this bottleneck. In contrast, the genetic diversity was higher in the black rhinoceros (mean Na = 4.857 and mean PIC = 0.456). Due to this bottleneck, the discriminatory power of the marker set used in this study was considerably higher when applied to black rhinoceros.

The random match probability calculations were only performed for the white rhinoceros and were calculated using 17 polymorphic markers (the 4 monomorphic markers and the marker DB66 were excluded from the calculations). Using these data, the random match probability calculated for five white rhinoceros ranged from 1:1.56 X 10^8 to 1:2.1 X 10^{11} without any correction for inbreeding and from 1:1.7 X 10^5 to 6.0 X 10^6 using a u value of 0.3 to correct for significant inbreeding (Linacre et al., 2011). With θ set at 0.1 in the five animals investigated the estimated random match probability ranged from 1:7.3 X

10^6 to 1:3.0 X 10^8. Given that the total population of white rhinoceros in the world is between 18,000 and 20,000 (Emslie et al., 2016) such random match probabilities indicate that the genotyping system described provides data which can be used for evidentiary purposes. Until such time that reliable estimates of Fst are obtained for the white and black rhinoceros, taking into account sub-structuring within the black rhinoceros population, we recommend that random match probabilities are calculated with u set at 0.1 and 0.3 for the white and black rhinoceros, respectively.

The observed heterozygosity was lower than the expected heterozygosity in the black rhinoceros indicating an excess of homozygote loci. The inter-individual inbreeding coefficient was higher in the black (0.2879) than in the white (0.0760) rhinoceros population. The data from the 33 black rhinoceros included animals from all 3 sub-species of black rhinoceros and may indicate that there is significant sub-structuring within the black rhinoceros which could not be investigated in this study but warrants further study. The DNA extraction and genotyping system described produces highly repeatable results even with small amounts of sample material. These data show that these methods are appropriate for use in investigations involving trace amounts of rhinoceros horn and the matching of profiles obtained from seized rhinoceros horn with material collected from live animals or poached carcasses.

CHAPTER 4

Robust forensic matching of confiscated horns to individual poached African rhinoceros

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Abstract:

Black and white rhinoceros (*Diceros bicornis* and *Ceratotherium simum*) are iconic African species that are classified by the International Union for the Conservation of Nature (IUCN) as Critically Endangered and Near Threatened (http://www.iucnredlist.org/), respectively. At the end of the 19th century, Southern white rhinoceros (*Ceratotherium simum simum*) numbers had declined to fewer than 50 animals in the Hluhluwe-iMfolozi region of the KwaZulu-Natal (KZN) province of South Africa, mainly due to uncontrolled hunting. Efforts by the Natal Parks Board facilitated an increase in population to over 20,000 in 2015 through aggressive conservation management. Black rhinoceros (*Diceros bicornis*)

populations declined from several hundred thousand in the early 19th century to ~65,000 in 1970 and to ~2,400 by 1995 with subsequent genetic reduction, also due to hunting, land clearances and later poaching. In South Africa, rhinoceros poaching incidents have increased from 13 in 2007 to 1,215 in 2014. This has occurred despite strict trade bans on rhinoceros products and strict enforcement in recent years. Harper *et.al.* use DNA to match confiscated rhinoceros tissue to specific poaching crime scenes.

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4.1 Introduction

The genotypes and population analyses for 3,085 White rhinoceros (*Ceratotherium simum*) and 883 Black rhinoceros (*Diceros bicornis*) sampled since 2010 were selected from the RhODIS® database and used to provide a background dataset from which the random match probability of two matching genotypes was calculated to support the presentation of evidence in rhinoceros poaching cases. Four methods were applied to forensic matching of confiscated tissue evidence to specific crime scenes: 1) sequence characterization and optimization of STR panels informative for White and Black rhinoceros; 2) development and application of the RhODIS® (Rhinoceros DNA Index System) database containing genotypes and demographic information of some 50,000 rhinoceros acquisitions, including over 100 000 specimens connected to rhinoceros crimes and samples from live rhinoceros and rhinoceros horns; 3) analysis of the population genetic structure of White and Black Rhinoceros species, subspecies and structured populations, and 4) computation of match probability statistics for rhinoceros populations useful for assessing the chance of a random match within the studied population. The judicial prosecution, conviction and sentencing of suspects in South Africa and in other countries, where confiscated rhinoceros horns matched a specific crime scene carcass with a robust "match probability", affirm the utility of the RhODIS® approach in criminal prosecutions of the perpetrators of illegal rhinoceros trade.

Application of DNA profiling as a forensic tool depends upon the statistical power of composite genotypes to identify and differentiate individuals in the population. Rhinoceros population structure, historic contractions or expansions, migration, translocation and population fragmentation caused by poaching and habitat reduction have been reported but not on a comprehensive scale (Anderson-Lederer et al., 2012, Guerier et al., 2012, Karsten et al., 2011, Kotzé et al., 2014, Moodley et al., 2017, Van Coeverden de Groot et al., 2011). The partitioning of population genetic variation within and between free-ranging White and Black rhinoceros populations will influence the forensic match likelihood of DNA profiles and therefore the effectiveness of the DNA evidence in rhinoceros criminal prosecutions. This study applied a panel of 23 STR loci to genotype 3,968 individual rhinoceros DNA specimens from distinct populations of White and Black rhinoceros. The population genetic structure and divergence of these populations was assessed with the primary objective to evaluate the use of the RhODIS® database to support investigations of rhinoceros crimes and to provide robust match probability statistics which can be used to support prosecutions of these crimes.

4.2 Materials and methods

4.2.1 Samples

A total of 883 Black rhinoceros (*Diceros bicornis*) and 3,085 White rhinoceros (*Ceratotherium simum*) samples were included in this study. Tissue specimens were submitted to the Veterinary Genetics Laboratory (VGL), University of Pretoria as part of the RhODIS® (Rhinoceros DNA Index System) database from various African rhinoceros range states. Recognized living subspecies of Black rhinoceros were included: 1) 51 samples of the Eastern subspecies (*D.b. michaeli*); 2) 357 samples of the Southwestern subspecies (*D.b. bicornis*); and 3) 475 samples of the South-Central subspecies (*D.b. minor*) (Figure 5). The White rhinoceros has two extant subspecies, the Southern White rhinoceros (*C.s. simum*) and the Northern White rhinoceros (*C.s. cottoni*) (Emslie and Knight, 2014). Because only 2 animals remain of the Northern White subspecies they were not included in this study. Figure 5 lists the number of each subspecies and sub-population present and sampled in each range state in 2015 (Emslie et al., 2016).

The majority of samples submitted to RhODIS® were collected in RhODIS® sampling kits during forensic investigation of poaching scenes (~47%), during routine translocation, notching, dehorning for identification or hunting (~49%) or from rhinoceros horn stockpile identification operations (~4%) according to the RhODIS® guidelines. Samples received in the VGL were assigned individual barcode sample numbers. Sample quality varied from highly degraded, particularly in the case of samples from old carcasses, to highest quality blood samples from live animals.

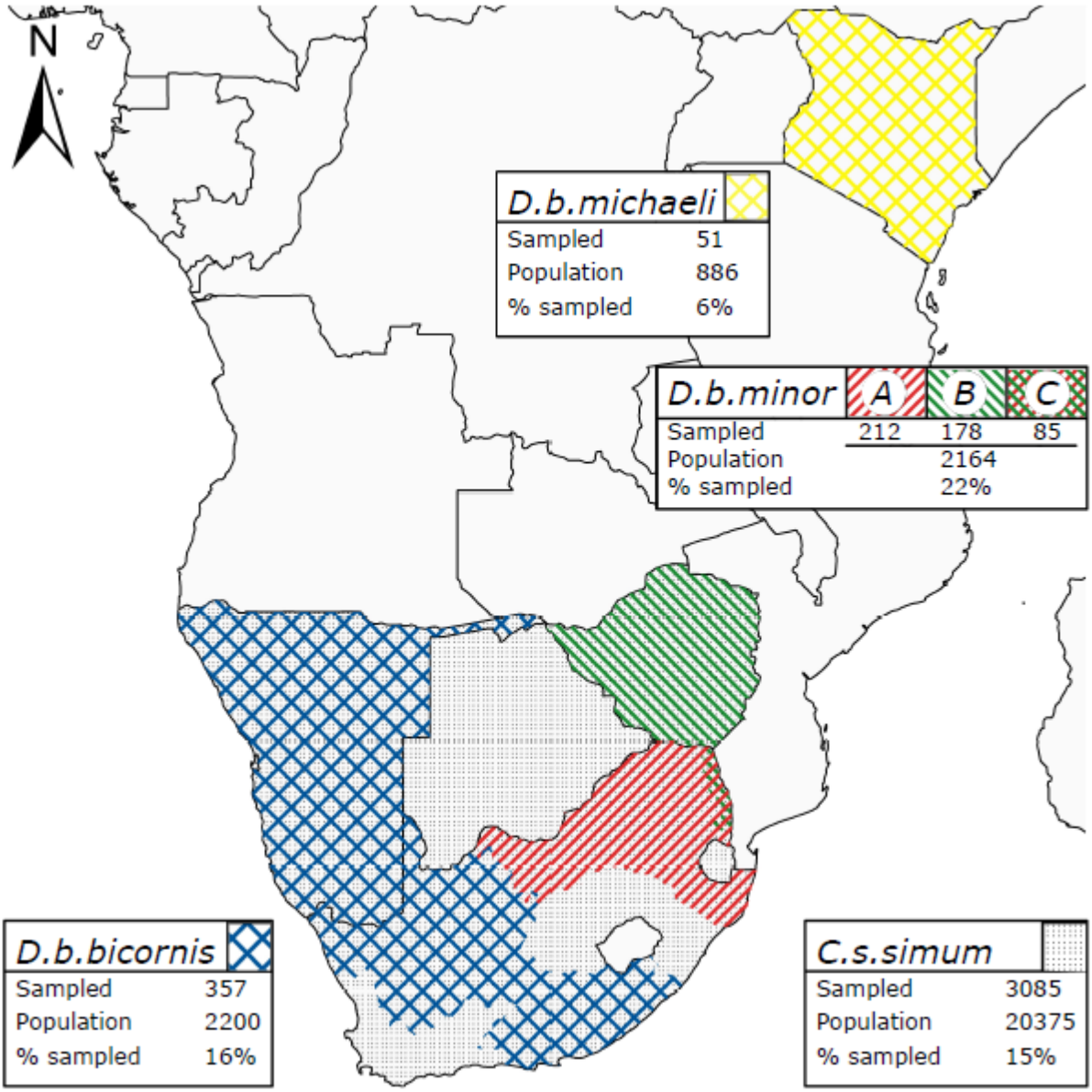


Figure 5: A total of 883 Black rhinoceros (*Diceros bicornis*), including the three recognized subspecies, and 3085 Southern White rhinoceros (*Ceratotherium simum simum*) samples are included in this study. The maps indicate the rhinoceros range states included in the sampling for this study and the number of rhinoceros of the specific subspecies in these range states in 2015.

4.2.2 DNA extraction and STR genotyping

DNA was extracted from blood and tissue samples using the Prepfiler®kit (ThermoFisher Scientific) according to the manufacturer's instructions (Harper et al., 2013). Hair samples were extracted using a modified alkaline extraction method (Rudbeck and Dissing, 1998), where 1 to 3 hair roots were cut into a 1.5 ml tube and 100 µl of 0.2M NaOH was added and heated at 97°C for 15 minutes, following which 100µl of 0.2M Tris-HCl at pH 8.5 was added. PCR was performed in four multiplex reactions, using the 22 loci previously described (Harper et al., 2013), with the addition of the Rh12 locus in multiplex 2 (Forward Primer, CTGGTGCATTCATCAGGGCT, Reverse Primer, AGAAGAGGTAGGAGAGGAAGTCA) (https://www.ncbi.nlm.nih.gov/nuccore/37496513) and the zinc finger (ZF) locus which was used to determine the gender of the animal from which the sample originated (Peppin et al., 2010).

4.2.3 Chromosome assignment imputation

The rhinoceros chromosome position of each locus was imputed based upon identifying the primer and flanking sequence in the whole genome sequence of the Southern white rhinoceros (*C.s. simum*) (http://www.ncbi.nlm.nih.gov/genome/24631) and then using a reference assisted chromosome assembly of the white rhinoceros scaffolds aligned against the domestic horse (*Equus ferus caballus*) genome (Wade et al., 2009) using Chromosomer (Tamazian et al., 2016). The chromosome assignment, albeit indirect involving two distantly related Perissodactyla species, allowed for an indication of likely chromosome linkage in detecting linkage disequilibria between STR loci.

4.2.4 Measures of genetic diversity

Allele frequencies were obtained using Cervus Version 3.03 (Kalinowski et al., 2007) and number of alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He) per locus (using the unbiased formula of Nei (Nei, 1987)), the fixation index (F) and Hardy-Weinberg Equilibrium (HWE) using the Bonferroni correction were calculated in Cervus for all loci (Kalinowski et al., 2007). Linkage disequilibrium (LD) of the loci was tested using Genepop Version 4.2 (Raymond and Rousset, 1995). Population structure was examined using three approaches: 1) an individual-based tree was constructed using NEIGHBOR of PHYLIP package (Felsenstein, 2005) based on allele-sharing, DPS (Proportion of shared alleles) (Bowcock et al., 1994) distance-matrix generated in MSA 4.05 software (Dieringer and Schlötterer, 2003) with 1-DPS correction and visualized in FigTree software (Rambaut, 2017), 2) Principal Component Analysis was performed in PAST 3 software (Hammer et al., 2001) using a variance-covariance matrix; before the analysis each allele for every loci was labelled as 0, 0.5, or 1 for allele absence, heterozygote or

homozygote in a given individual and 3) we detected population partitions using the STRUCTURE algorithm which clusters individuals with minimal deviation from genetic and linkage equilibrium (Falush et al., 2003). For Figure 6a and b, sex-linked markers and three loci with missing data were excluded leaving 18 loci (32A, DB44, 7B, 7C, BlRh1B, DB52, BR6, DB1, BlRh1C, 12F, BlRh37D, 32F, DB23, SR63, IR10, IR22, SR262, SR268). For STRUCTURE, K-values were evaluated for K=2 to K=8, with a burn-in of 50,000 iterations and 500,000 iterations at each value of K. Each K was run 10 times. The division of the Black subspecies is supported by the Delta K value calculated in STRUCTURE Harvester using the Evanno method (Earl and von Holdt, 2012). Between population differentiation (F_{ST}), which is a measure of the difference in heterozygosity due to population subdivision (Michalakis and Excoffier, 1996, Weir and Cockerham, 1984) was determined using GenAlEx 6.5 (Peakall and Smouse, 2012). Differences between the pairwise F_{ST} were tested for significance using GENODIVE (Meirmans and Van Tienderen, 2004) with 1000 permutations.

Figure 6a

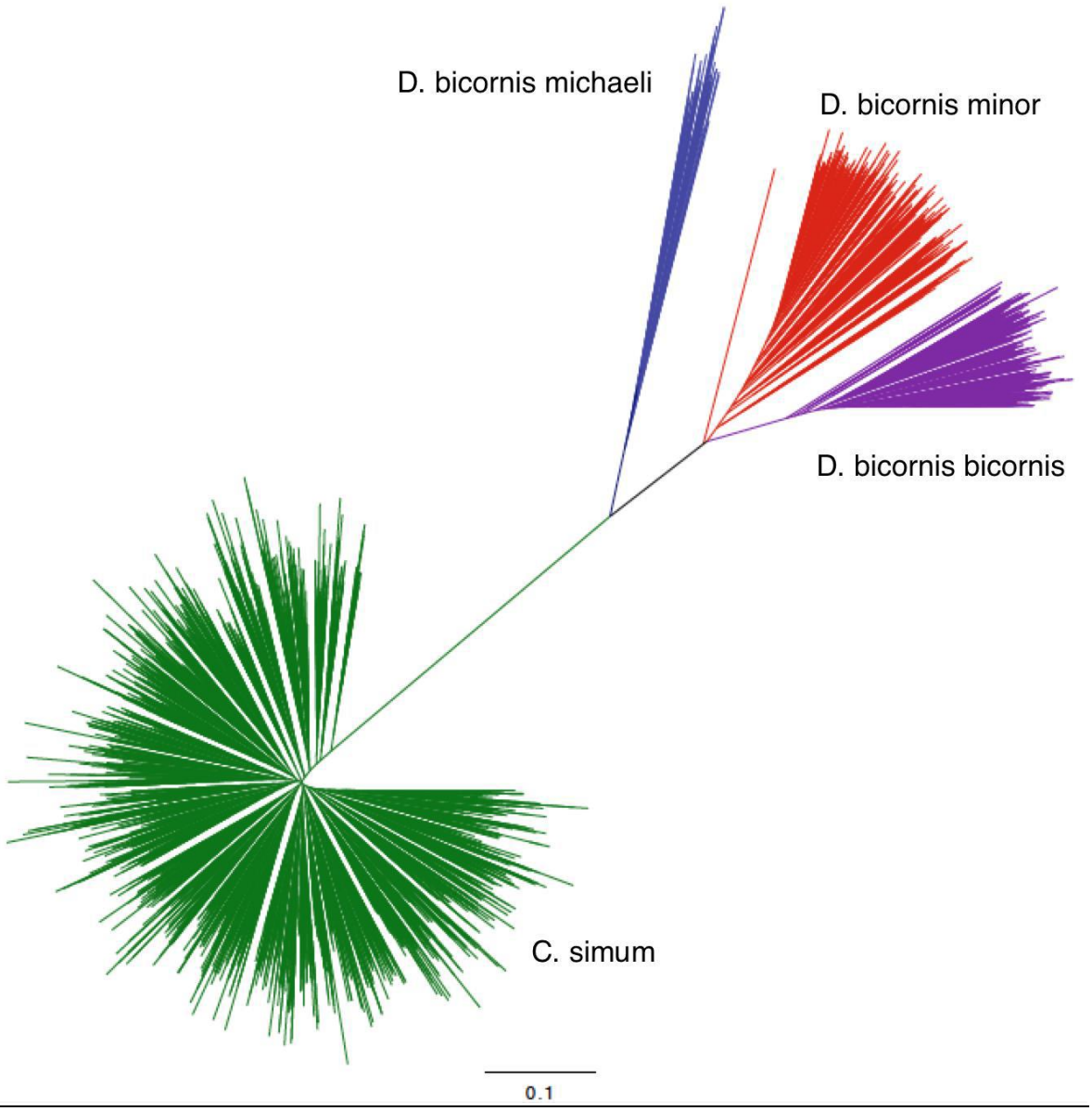


Figure 6b

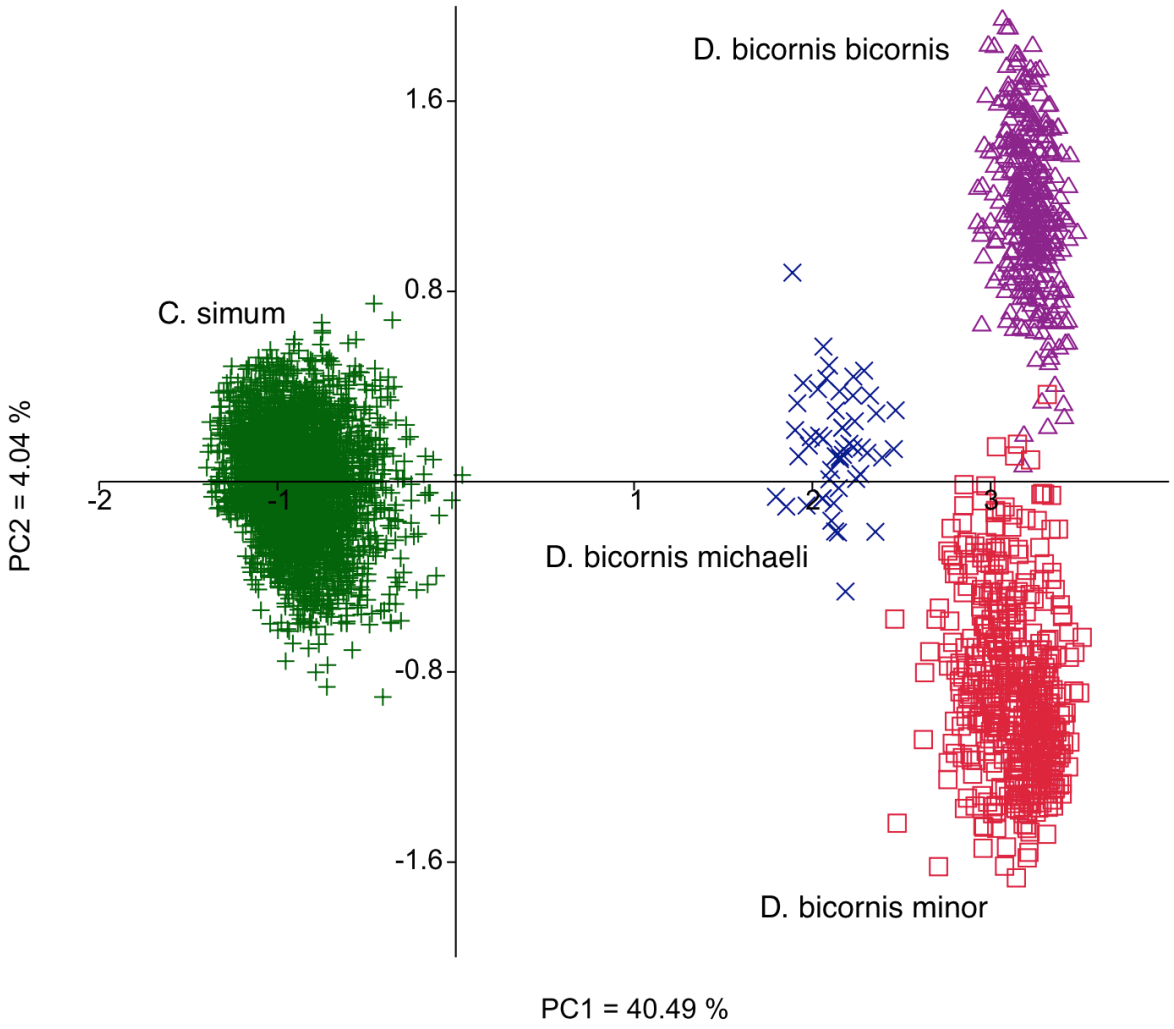
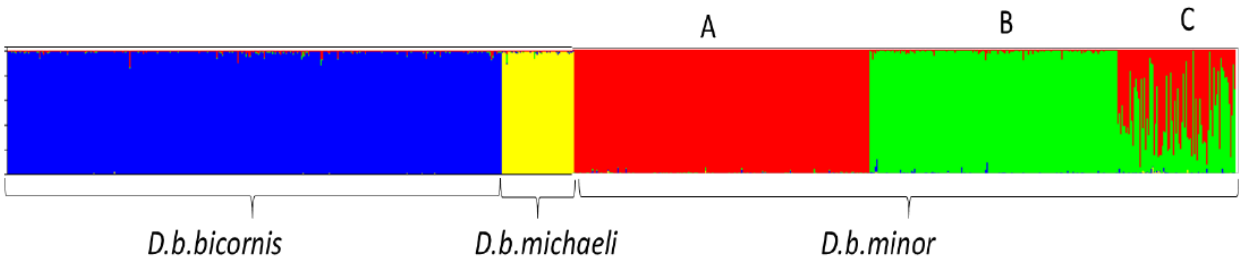


Figure 6c



A KwaZulu Natal

B Zimbabwe

C Kruger origin admixed group

Figure 6: Southern white rhinoceros (*C. simum simum*) comprise a single panmictic subspecies with the Black rhinoceros subdivided into three subspecies *D.b. bicornis*, *D.b. michaeli* and *D.b. minor*. This data is supported by individual based tree and principle component analysis in Figures 6a and 6b. The Structure diagram in Figure 6c supports the Black rhinoceros subdivision with an additional partition between the *D.b. minor* subspecies originating in Kwazulu-Natal (South Africa) (A), Zimbabwe (B), and a third group that are an admixture of these two groups (C).

4.2.5 Forensic match application

Matching of specific DNA profiles provide evidentiary support that two samples are derived from the same individual if underlying data are available to permit an estimate of the rarity of the profile (Weir, 1996). Single locus match probability was calculated using the formulae of Balding and Nichols (Balding and Nichols, 1995):

$$\Pr(A_u A_u | A_u A_u)_i = \frac{[2\theta_i + (1-\theta_i)P_u][3\theta_i + (1-\theta_i)P_u]}{(1+\theta_i)(1+2\theta_i)}$$

$$\Pr(A_u A_v | A_u A_v)_i = \frac{2[\theta_i + (1-\theta_i)P_u][\theta_i + (1-\theta_i)P_v]}{(1+\theta_i)(1+2\theta_i)}, u \neq v$$

The cumulative match probability across several STR loci was then obtained by the product rule. The between population allelic variation was quantified by F_{ST} and based on these data a Theta (θ) value of 0.1 was selected for use in calculation of match probability of African rhinoceros species in this study. The use of this sufficiently conservative θ also compensates departures from Hardy-Weinberg equilibrium at specific loci due to allelic variation in populations sampled (Buckleton et al., 2016).

4.3 Results

A total of 3,085 White rhinoceros and 883 Black rhinoceros from three recognized subspecies were genotyped using 23 STR loci (Table 1) and the genotypes were deposited into the RhODIS® database. The number of alleles and allele frequency of the Black and White rhinoceros and Black rhinoceros subspecies using this data are provided in Table 4.

Table 4: Allele frequencies for all STR loci tested in each species, subspecies and population including allele frequencies of the Black rhinoceros subspecies and the *D.b. minor* populations (A, B and C). Private alleles in each Black rhinoceros population are highlighted and bolded.

Locus	White (3085)				D.b.bicornis (357)				D.b.michaeli (51)				D.b.minor A (212)				D.b.minor B (178)				D.b.minor C (85)				D.b.minor All (475)				Black (883)			
32A																																
Allele	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq	Count	Hets	Homs	Freq
196	2932	1462	735	0.4953	1	1	0	0.0014	20	14	3	0.1961					68	62	3	0.2012	21	17	2	0.1265	89	79	5	0.0978	110	94	8	0.0637
198	2720	1470	625	0.4595					**26**	**22**	**2**	**0.2549**																	26	22	2	0.0151
200	268	248	10	0.0453	710	4	353	0.9944	15	13	1	0.1471	404	2	201	0.9951	213	75	69	0.6302	129	27	51	0.7771	746	104	321	0.8198	1471	121	675	0.8523
202									18	14	2	0.1765					3	3	0	0.0089	1	1	0	0.006	4	4	0	0.0044	22	18	2	0.0127
204					3	3	0	0.0042	11	11	0	0.1078					23	17	3	0.068	5	5	0	0.0301	28	22	3	0.0308	42	36	3	0.0243
206									12	10	1	0.1176	2	2	0	0.0049	31	25	3	0.0917	10	8	1	0.0602	43	35	4	0.0473	55	45	5	0.0319
DB44																																
206					16	12	2	0.0224	16	14	1	0.1569					28	28	0	0.0787	9	7	1	0.0529	37	35	1	0.0389	69	61	4	0.0391
210	1284	986	149	0.2108	93	85	4	0.1303					423	1	211	0.9976	212	88	62	0.5955	143	23	60	0.8412	778	112	333	0.8189	871	197	337	0.4932
212	160	152	4	0.0263	525	145	190	0.7353	3	3	0	0.0294	1	1	0	0.0024	86	52	17	0.2416	18	18	0	0.1059	105	71	17	0.1105	633	219	207	0.3584
214					80	70	5	0.112	12	10	1	0.1176																	92	80	6	0.0521
216									**4**	**4**	**0**	**0.0392**																	4	4	0	0.0023
218	4648	1072	1788	0.763					42	24	9	0.4118					9	9	0	0.0253					9	9	0	0.0095	51	33	9	0.0289
220									**21**	**19**	**1**	**0.2059**																	21	19	1	0.0119
222																	**21**	**15**	**3**	**0.059**					21	15	3	0.0221	21	15	3	0.0119
226									**4**	**4**	**0**	**0.0392**																	4	4	0	0.0023
7B																																
223	543	483	30	0.0907																												
225					94	84	5	0.1328	89	13	38	0.8725																	183	97	43	0.1049
227	3870	1372	1249	0.6461	614	84	265	0.8672	13	13	0	0.1275	410	0	205	1	356	0	178	1	168	0	84	1	934	0	467	1	1561	97	732	0.8951
229	1577	1137	220	0.2633																												
7C																																
132					1	1	0	0.0014	13	5	4	0.1275					115	73	21	0.323	45	29	8	0.2848	160	102	29	0.1782	174	108	33	0.1024
142	433	383	25	0.0759																												
148	1496	1098	199	0.2621																												
150	3779	1271	1254	0.6621																												
154					5	5	0	0.0071	3	3	0	0.0294					14	14	0	0.0393	1	1	0	0.0063	15	15	0	0.0167	23	23	0	0.0135
156									**27**	**21**	**3**	**0.2647**																	27	21	3	0.0159
158					458	168	145	0.6543	10	8	1	0.098					150	94	28	0.4213	21	17	2	0.1329	171	111	30	0.1904	639	287	176	0.3759
160					4	4	0	0.0057	21	17	2	0.2059	229	87	71	0.5964	29	27	1	0.0815	64	34	15	0.4051	322	148	87	0.3586	347	169	89	0.2041
161									11	7	2	0.1078	1	1	0	0.0026									1	1	0	0.0011	12	8	2	0.0071
162					2	2	0	0.0029	7	5	1	0.0686	154	86	34	0.401	42	28	7	0.118	27	19	4	0.1709	223	133	45	0.2483	232	140	46	0.1365
164									6	6	0	0.0588					6	6	0	0.0169					6	6	0	0.0067	12	12	0	0.0071
166					23	23	0	0.0329	3	3	0	0.0294																	26	26	0	0.0153
168					207	153	27	0.2957	1	1	0	0.0098																	208	154	27	0.1224
BlRh1B																																
234									**22**	**16**	**3**	**0.22**																	22	16	3	0.0126
238									**10**	**6**	**2**	**0.1**																	10	6	2	0.0057
240	4848	918	1965	0.7906					**2**	**2**	**0**	**0.02**																	2	2	0	0.0011
242	1284	918	183	0.2094	688	24	332	0.9636	29	21	4	0.29	335	75	130	0.7901	243	69	87	0.7232	125	27	49	0.744	703	171	266	0.7575	1420	216	602	0.8152
244									**10**	**8**	**1**	**0.1**																	10	8	1	0.0057
246					26	24	1	0.0364	23	13	5	0.23	89	75	7	0.2099	93	69	12	0.2768	43	27	8	0.256	225	171	27	0.2425	274	208	33	0.1573
248									**4**	**2**	**1**	**0.04**																	4	2	1	0.0023
DB66																																
182									**11**	**9**	**1**	**0.1078**																	11	9	1	0.0062
188					**124**	**92**	**16**	**0.1737**																					124	92	16	0.0704
190																	45	37	4	0.1264	43	35	4	0.2529	88	72	8	0.093	88	72	8	0.0499
192									**26**	**14**	**6**	**0.2549**																	26	14	6	0.0148
196									13	9	2	0.1275					41	35	3	0.1152	13	13	0	0.0765	54	48	3	0.0571	67	57	5	0.038
198					132	120	6	0.1849									19	19	0	0.0534					19	19	0	0.0201	151	139	6	0.0857

201	117	109	4	0.0207																											
202	1373	323	525	0.2432					5	5	0	0.049					11	9	1	0.0309	2	2	0	0.0118	13	11	1	0.0137	18	16	1	0.0102
203	1030	66	482	0.1824																											
204	3126	368	1379	0.5537	126	96	15	0.1765	4	4	0	0.0392	161	89	36	0.3833	59	51	4	0.1657	23	23	0	0.1353	243	163	40	0.2569	373	263	55	0.2117
206									**14**	**12**	**1**	**0.1373**																	14	12	1	0.0079
208					332	170	81	0.465	18	16	1	0.1765																	350	186	82	0.1986
210													99	73	13	0.2357	17	15	1	0.0478	24	24	0	0.1412	140	112	14	0.148	140	112	14	0.0795
DB52																															
207									**1**	**1**	**0**	**0.0098**																	1	1	0	0.0006
211									**29**	**19**	**5**	**0.2843**																	29	19	5	0.0165
213									20	16	2	0.1961					167	87	40	0.4691	6	6	0	0.0353	173	93	40	0.1821	193	109	42	0.1097
215					213	147	33	0.3008	14	10	2	0.1373					15	15	0	0.0421	3	3	0	0.0176	18	18	0	0.0189	245	175	35	0.1392
217	1915	1259	328	0.3114	1	1	0	0.0014	11	11	0	0.1078	161	87	37	0.3797	52	44	4	0.1461	57	33	12	0.3353	270	164	53	0.2842	282	176	53	0.1602
219	2177	1391	393	0.354	264	158	53	0.3729	20	12	4	0.1961	262	88	87	0.6179	120	72	24	0.3371	103	41	31	0.6059	485	201	142	0.5105	769	371	199	0.4369
221	2058	1350	354	0.3346	230	146	42	0.3249	4	4	0	0.0392	1	1	0	0.0024	2	2	0	0.0056	1	1	0	0.0059	4	4	0	0.0042	238	154	42	0.1352
225									**3**	**3**	**0**	**0.0294**																	3	3	0	0.0017
BR6																															
132	3957	1325	1316	0.6438																											
134	621	521	50	0.101	564	120	222	0.7899					251	109	71	0.592	108	68	20	0.3034	84	34	25	0.4941	443	211	116	0.4663	1007	331	338	0.5702
136					53	49	2	0.0742									31	29	1	0.0871	6	6	0	0.0353	37	35	1	0.0389	90	84	3	0.051
140	296	280	8	0.0482					7	5	1	0.0686	109	75	17	0.2571	48	46	1	0.1348	38	30	4	0.2235	195	151	22	0.2053	202	156	23	0.1144
142									**45**	**29**	**8**	**0.4412**																	45	29	8	0.0255
144									42	32	5	0.4118					59	41	9	0.1657	5	5	0	0.0294	64	46	9	0.0674	106	78	14	0.06
146									8	8	0	0.0784					64	52	6	0.1798	24	18	3	0.1412	88	70	9	0.0926	96	78	9	0.0544
150	1272	996	138	0.207													18	16	1	0.0506	4	4	0	0.0235	22	20	1	0.0232	22	20	1	0.0125
152													6	6	0	0.0142	17	17	0	0.0478	2	2	0	0.0118	25	25	0	0.0263	25	25	0	0.0142
154													50	48	1	0.1179	11	11	0	0.0309	7	7	0	0.0412	68	66	1	0.0716	68	66	1	0.0385
156					97	81	8	0.1359					8	8	0	0.0189									8	8	0	0.0084	105	89	8	0.0595
DB1																															
152					**209**	**163**	**23**	**0.2927**																					209	163	23	0.1183
156									**20**	**16**	**2**	**0.1961**																	20	16	2	0.0113
158					460	182	139	0.6443	26	16	5	0.2549	260	106	77	0.6132	110	72	19	0.309	82	42	20	0.4824	452	220	116	0.4758	938	418	260	0.5311
160	5302	712	2295	0.8604					**15**	**15**	**0**	**0.1471**																	15	15	0	0.0085
162	860	712	74	0.1396	45	43	1	0.063	6	6	0	0.0588	164	106	29	0.3868	246	72	87	0.691	88	42	23	0.5176	498	220	139	0.5242	549	269	140	0.3109
164									**35**	**23**	**6**	**0.3431**																	35	23	6	0.0198
BlRh1C																															
118					173	131	21	0.2423					117	99	9	0.2773	13	13	0	0.0365	27	21	3	0.1588	157	133	12	0.1656	330	264	33	0.1871
124									**6**	**6**	**0**	**0.0588**																	6	6	0	0.0034
126					261	151	55	0.3655	15	9	3	0.1471	235	119	58	0.5569	224	72	76	0.6292	90	34	28	0.5294	549	225	162	0.5791	825	385	220	0.4677
128					212	144	34	0.2969	34	18	8	0.3333	36	32	2	0.0853	2	2	0	0.0056	18	16	1	0.1059	56	50	3	0.0591	302	212	45	0.1712
130					68	62	3	0.0952	9	9	0	0.0882	16	16	0	0.0379	107	69	19	0.3006	32	26	3	0.1882	155	111	22	0.1635	232	182	25	0.1315
132									16	14	1	0.1569	18	18	0	0.0427					3	3	0	0.0176	21	21	0	0.0222	37	35	1	0.021
134																	**1**	**1**	**0**	**0.0028**					1	1	0	0.0011	1	1	0	0.0006
136	4433	1221	1606	0.7199					4	4	0	0.0392					9	9	0	0.0253					9	9	0	0.0095	13	13	0	0.0074
138	1725	1221	252	0.2801					**17**	**11**	**3**	**0.1667**																	17	11	3	0.0096
140									**1**	**1**	**0**	**0.0098**																	1	1	0	0.0006
12F																															
217									**1**	**1**	**0**	**0.01**																	1	1	0	0.0006
221									**9**	**9**	**0**	**0.09**																	9	9	0	0.0052

223	2888	1328	780	0.5297																											
227					61	57	2	0.0859	37	19	9	0.37	3	3	0	0.0072	37	37	0	0.1039	17	15	1	0.1024	57	55	1	0.0609	155	131	12	0.0888
229					322	160	81	0.4535	10	4	3	0.1	7	1	3	0.0169	17	15	1	0.0478	1	1	0	0.006	25	17	4	0.0267	357	181	88	0.2045
231					207	127	40	0.2915	24	18	3	0.24	341	55	143	0.8237	196	104	46	0.5506	108	42	33	0.6506	645	201	222	0.6891	876	346	265	0.5017
233					10	8	1	0.0141					63	53	5	0.1522	106	70	18	0.2978	40	30	5	0.241	209	153	28	0.2233	219	161	29	0.1254
235					110	92	9	0.1549	19	13	3	0.19																	129	105	12	0.0739
237	2564	1328	618	0.4703																											
BIRh37D																															
201					292	178	57	0.4113	61	17	22	0.6354	321	73	124	0.7829	249	71	89	0.6994	118	40	39	0.6941	688	184	252	0.735	1041	379	331	0.5976
203					418	178	120	0.5887	17	9	4	0.1771	89	73	8	0.2171	107	71	18	0.3006	52	40	6	0.3059	248	184	32	0.265	683	371	156	0.3921
207									**18**	**8**	**5**	**0.1875**																	18	8	5	0.0103
243	6149	1	3074	0.9998																											
245	1	1	0	0.0002																											
32F																															
171	2763	1503	630	0.4519																											
173					714	0	357	1	102	0	51	1	424	0	212	1	356	0	178	1	170	0	85	1	950	0	475	1	1766	0	883	1
175	775	671	52	0.1268																											
185	2576	1514	531	0.4213																											
DB23																															
237	6104	0	3052	1																											
241									**14**	**12**	**1**	**0.14**																	14	12	1	0.0082
247					618	78	270	0.8854	57	23	17	0.57	196	94	51	0.5	265	71	97	0.7571	92	46	23	0.5476	553	211	171	0.6077	1228	312	458	0.719
249					80	78	1	0.1146	26	14	6	0.26	196	94	51	0.5	85	71	7	0.2429	76	46	15	0.4524	357	211	73	0.3923	463	303	80	0.2711
253									**3**	**3**	**0**	**0.03**																	3	3	0	0.0018
SR63																															
188	3543	1457	1043	0.5767																											
190	2601	1457	572	0.4233																											
192									43	19	12	0.4388	1	1	0	0.0024	10	10	0	0.0281	4	4	0	0.0235	15	15	0	0.0158	58	34	12	0.0329
194					330	174	78	0.4622					224	104	60	0.5283	90	62	14	0.2528	66	32	17	0.3882	380	198	91	0.4	710	372	169	0.403
196					19	19	0	0.0266	10	8	1	0.102	198	104	47	0.467	207	93	57	0.5815	85	41	22	0.5	490	238	126	0.5158	519	265	127	0.2946
198					53	51	1	0.0742	2	2	0	0.0204									1	1	0	0.0059	1	1	0	0.0011	56	54	1	0.0318
200					312	188	62	0.437	40	18	11	0.4082	1	1	0	0.0024	49	45	2	0.1376	14	14	0	0.0824	64	60	2	0.0674	416	266	75	0.2361
202									**3**	**3**	**0**	**0.0306**																	3	3	0	0.0017
IR10																															
122					172	144	14	0.2409									5	5	0	0.014					5	5	0	0.0053	177	149	14	0.1007
128									**26**	**18**	**4**	**0.2766**																	26	18	4	0.0148
129									**12**	**8**	**2**	**0.1277**																	12	8	2	0.0068
130									**19**	**13**	**3**	**0.2021**																	19	13	3	0.0108
132	1770	1246	262	0.2997					37	25	6	0.3936									2	2	0	0.0118	2	2	0	0.0021	39	27	6	0.0222
134	4134	1248	1443	0.7													35	31	2	0.0983	15	15	0	0.0882	50	46	2	0.0526	50	46	2	0.0284
136	2	2	0	0.0003	522	146	188	0.7311					424	0	212	1	211	93	59	0.5927	132	38	47	0.7765	767	131	318	0.8074	1289	277	506	0.7332
138					20	12	4	0.028									103	69	17	0.2893	21	21	0	0.1235	124	90	17	0.1305	144	102	21	0.0819
140																	**2**	**2**	**0**	**0.0056**					2	2	0	0.0021	2	2	0	0.0011
IR12																															
156									**5**	**1**	**2**	**0.049**																	5	1	2	0.0028
170									**3**	**1**	**1**	**0.0294**																	3	1	1	0.0017
174	2301	665	818	0.375	56	30	13	0.0784	38	16	11	0.3725																	94	46	24	0.0533
176									**10**	**6**	**2**	**0.098**																	10	6	2	0.0057
178													8	6	1	0.019	111	37	37	0.3118	27	11	8	0.1607	146	54	46	0.1543	146	54	46	0.0829
180	3835	665	1585	0.625																											
182					341	85	128	0.4776	8	4	2	0.0784					28	12	8	0.0787	9	7	1	0.0536	37	19	9	0.0391	386	108	139	0.2191

184					39	21	9	0.0546	3	1	1	0.0294	322	34	144	0.763	44	20	12	0.1236	93	23	35	0.5536	459	77	191	0.4852	501	99	201	0.2843
186					51	27	12	0.0714	17	5	6	0.1667	63	29	17	0.1493	10	4	3	0.0281	11	5	3	0.0655	84	38	23	0.0888	152	70	41	0.0863
188					227	77	75	0.3179	18	10	4	0.1765	29	13	8	0.0687	48	20	14	0.1348	21	9	6	0.125	98	42	28	0.1036	343	129	107	0.1947
190																	83	37	23	0.2331	7	3	2	0.0417	90	40	25	0.0951	90	40	25	0.0511
192																	**21**	**13**	**4**	**0.059**					21	13	4	0.0222	21	13	4	0.0119
200																	**11**	**7**	**2**	**0.0309**					11	7	2	0.0116	11	7	2	0.0062
IR22																																
207	6116	0	3058	1																												
209									**5**	**3**	**1**	**0.049**																	5	3	1	0.0029
217					319	167	76	0.4557	5	3	1	0.049					1	1	0	0.0028					1	1	0	0.0011	325	171	77	0.1881
219					104	86	9	0.1486	29	21	4	0.2843	176	96	40	0.4335	236	70	83	0.6629	87	45	21	0.5305	499	211	144	0.5389	632	318	157	0.3657
221					277	173	52	0.3957	63	25	19	0.6176	230	96	67	0.5665	118	70	24	0.3315	77	45	16	0.4695	425	211	107	0.459	765	409	178	0.4427
223																	**1**	**1**	**0**	**0.0028**					1	1	0	0.0011	1	1	0	0.0006
SR74																																
158									1	1	0	0.0102					11	3	4	0.0309	1	1	0	0.0059	12	4	4	0.0126	13	5	4	0.0074
164									**7**	**7**	**0**	**0.0714**																	7	7	0	0.004
170													3	3	0	0.0071	91	35	28	0.2556	23	15	4	0.1353	117	53	32	0.1232	117	53	32	0.0664
172																	**41**	**19**	**11**	**0.1152**					41	19	11	0.0432	41	19	11	0.0233
174	5698	0	2849	1					37	13	12	0.3776	27	13	7	0.0637	58	28	15	0.1629	38	14	12	0.2235	123	55	34	0.1295	160	68	46	0.0908
176					257	71	93	0.3599	4	2	1	0.0408	313	43	135	0.7382	89	37	26	0.25	87	27	30	0.5118	489	107	191	0.5147	750	180	285	0.4257
178					457	71	193	0.6401	31	11	10	0.3163	81	31	25	0.191	33	7	13	0.0927	21	13	4	0.1235	135	51	42	0.1421	623	133	245	0.3536
180									12	4	4	0.1224					13	3	5	0.0365					13	3	5	0.0137	25	7	9	0.0142
182									6	2	2	0.0612					20	10	5	0.0562					20	10	5	0.0211	26	12	7	0.0148
SR262																																
86	4515	1127	1694	0.748																												
92	1521	1127	197	0.252																												
96									7	7	0	0.0686	4	2	1	0.0095					3	1	1	0.0185	7	3	2	0.0074	14	10	2	0.008
98					308	174	67	0.4375	2	2	0	0.0196	175	113	31	0.4147	240	88	76	0.6742	69	41	14	0.4259	484	242	121	0.5149	794	418	188	0.4548
100					156	116	20	0.2216	1	1	0	0.0098					27	23	2	0.0758	2	2	0	0.0123	29	25	2	0.0309	186	142	22	0.1065
102					12	8	2	0.017	24	22	1	0.2353	6	2	2	0.0142	2	2	0	0.0056	2	2	0	0.0123	10	6	2	0.0106	46	36	5	0.0263
104					228	146	41	0.3239	63	25	19	0.6176	237	113	62	0.5616	86	74	6	0.2416	86	42	22	0.5309	409	229	90	0.4351	700	400	150	0.4009
106									**5**	**5**	**0**	**0.049**																	5	5	0	0.0029
SR268																																
176	1043	837	103	0.1762					**10**	**8**	**1**	**0.1163**																	10	8	1	0.006
178	201	183	9	0.034																												
180	4674	970	1852	0.7898																												
186									**7**	**7**	**0**	**0.0814**																	7	7	0	0.0042
188					6	6	0	0.0088									72	42	15	0.2105	7	5	1	0.0449	79	47	16	0.0886	85	53	16	0.0511
190									5	3	1	0.0581	160	90	35	0.4061	65	39	13	0.1901	85	39	23	0.5449	310	168	71	0.3475	315	171	72	0.1895
192					250	162	44	0.3655	35	13	11	0.407	9	9	0	0.0228	70	40	15	0.2047	3	3	0	0.0192	82	52	15	0.0919	367	227	70	0.2208
194					87	69	9	0.1272	3	3	0	0.0349	185	89	48	0.4695	75	43	16	0.2193	45	31	7	0.2885	305	163	71	0.3419	395	235	80	0.2377
196					341	163	89	0.4985	9	5	2	0.1047	22	22	0	0.0558	60	40	10	0.1754	15	15	0	0.0962	97	77	10	0.1087	447	245	101	0.269
198									17	11	3	0.1977	18	18	0	0.0457					1	1	0	0.0064	19	19	0	0.0213	36	30	3	0.0217
SR281																																
222	2091	1343	374	0.3453																												
228													131	89	21	0.3104					20	20	0	0.1176	151	109	21	0.1593	151	109	21	0.0858
232					57	53	2	0.0803	38	24	7	0.3725					31	23	4	0.0871					31	23	4	0.0327	126	100	13	0.0716
234	2204	1400	402	0.3639					**4**	**4**	**0**	**0.0392**																	4	4	0	0.0023
238					622	80	271	0.8761	37	19	9	0.3627	291	89	101	0.6896	306	38	134	0.8596	147	23	62	0.8647	744	150	297	0.7848	1403	249	577	0.7972
240					29	29	0	0.0408	14	10	2	0.1373																	43	39	2	0.0244
242	1761	1215	273	0.2908																												

246									7	7	0	0.0686									1	1	0	0.0059	1	1	0	0.0011	8	8	0	0.0045
256									2	2	0	0.0196					19	19	0	0.0534	2	2	0	0.0118	21	21	0	0.0222	23	23	0	0.0131
RH12																															
108	1612	314	649	0.7941																											
112	141	133	4	0.0695																											
114	277	237	20	0.1365																											
116									**4**	**0**	**2**	**1**																	4	0	2	0.0044
122													81	55	13	0.27					15	13	1	0.1271	96	68	14	0.1273	96	68	14	0.1048
124					122	18	52	0.7722					116	70	23	0.3867	58	50	4	0.1726	29	25	2	0.2458	203	145	29	0.2692	325	163	81	0.3548
126					36	18	9	0.2278					99	63	18	0.33	240	66	87	0.7143	59	35	12	0.5	398	164	117	0.5279	434	182	126	0.4738
128																	35	31	2	0.1042	13	13	0	0.1102	48	44	2	0.0637	48	44	2	0.0524
132													4	4	0	0.0133	3	3	0	0.0089	2	2	0	0.0169	9	9	0	0.0119	9	9	0	0.0098

Table 5: Placement of the 23 rhinoceros microsatellite loci on chromosomes of the horse reference horse genome, the repeat sequence and scaffold position on the White rhinoceros reference genome with the species and nature of repeat sequence.

White rhinoceros reference genome				Original STR Description	
Chromosome	Locus	Repeat Sequence	Scaffold position	Species	Repeat
Chr 1	DB52	$(CA)_{12}$	JH767728:49,254,161-49,254,310	WR	S
Chr 2	7C	$(CT)_{14}(AT)_{11}$	JH767727:10,444,144-10,444,335	WR	C
Chr 3	BlRh37D	$(TG)_6CA(TG)_{17}(AG)_6$	JH767760:5,803,569-5,803,831	BR	C
Chr 4	BlRh1B	$(GT)_{13}$	JH767724:8,299,703-8,299,802	BR	C
Chr 5	BlRh1C	$(TG)_6(CG)_2(TG)_{11}$	JH767759:3,308,328-3,308,518	BR	C
Chr 6	32A	$(AC)_6$.....$(AC)_{16}$	JH767776:13,563,190-13,563,319	WR	C
Chr 7	7B	$(TG)_{15}$	JH767853:1,478,532-1,478,698	WR	C
Chr 9	SR268	$(AC)_{13}$	JH767757:15,973,899-15,974,168	SR	S
Chr 11	DB44	$(TG)_{19}$	JH767765:2,229,186-2,229,302	BR	C
Chr 13	BR6	$(TG)_{15}$	JH767793:958,941-959,116	BR	S
Chr 13	SR281	$(AC)_{17}$	JH767802:6,346,286-6,346,528	SR	S
Chr 14	DB1	$(GT)_{16}$	JH767726:55,212,707-55,212,874	BR	S
Chr 14	SR63	$(TG)_8$.....$(AG)_6$	JH767726:49,561,075-49,561,304	SR	S
Chr 15	12F	$(GT)_{10}A(TG)_6$.....$(TA)_9$	JH767782:5,765,753-5,766,031	WR	C
Chr 15	DB23	$(CA)_6$	JH767779:4,018,091-4,018,257	BR	S
Chr 17	32F	$(AC)_{11}$	JH767732:27,885,389-27,885,530	WR	S
Chr 20	RH12	$(TC)_8TT(TC)_8$	JH767735:34,492,757-34,492,857	IR	C
Chr X	IR12	$(AC)_{12}$	JH767774:6,674,158-6,674,305	IR	S
Chr X	SR74	$(AC)_{19}$	JH767831:1,676,337-1,676,424	SR	S
Not Found	DB66	$(CA)_{10}T(AC)_{12}$	JH767780:644,242-644,385	BR	C
Not Found	IR22	$(CA)_{13}$	JH767747:9,369,432-9,369,535	IR	S
Not Found	SR262	$(AC)_{11}$	JH767723:55,939,772-55,939,942	SR	S
Unknown	IR10	$(AC)_{15}$	Unplaced scaffold00031	IR	S

Abbreviations: WR - White rhinoceros, BR - Black rhinoceros, IR - Indian rhinoceros, SR - Sumatran rhinoceros, S - simple repeat, C - complex repeat

A brief characterization of the 23 diagnostic STRs is listed in Table 5. The repeat motif in the whole genome sequenced reference White rhinoceros (http://www.ncbi.nlm.nih.gov/genome/11839), scaffold number and imputed chromosome position are presented in this table. Loci DB66, IR22, and SR262, could not be placed on chromosomes and locus IR10 could not be placed on a scaffold. Table 5 also shows the species origin (White, Black, Indian or Sumatran rhinoceros), citations for each STR marker isolation (Brown and Houlden, 1999, Cunningham et al., 1999, Florescu et al., 2003, Harper et al., 2013, Nielsen et al., 2008, Scott et al., 2004, Scott, 2008), the sequence motif of each locus in the White rhinoceros reference genome sequence, and the repeat motif structure (complex or simple) in the rhinoceros species of origin. The two X-linked markers (IR12 and SR74) were consistently hemizygous in males and either homo- or heterozygous in females. Monomorphic STR loci were identified in White (BlRh37D, DB23, SR74 and IR22) and Black (32F) rhinoceros.

Southern White rhinoceros are traditionally considered panmictic and comprising a single subspecies *Ceratotherium simum simum*, as a result of the severe founder effect in the late 19th century [2]. Black rhinoceros are generally subdivided into three modern subspecies *D.b. bicornis, D.b. michaeli and D.b. minor* (Moodley et al., 2017). Population structure of White and Black rhinoceros based upon three different analyses (Composite STR phylogeny, Principal Component Analysis, and STRUCTURE) affirmed the partition of White versus Black rhinoceros species and further the separation of the three Black rhinoceros subspecies (Figure 6). In addition, the STRUCTURE algorithm revealed a fine grain distinctiveness between Black rhinoceros *D.b. minor* populations from Zimbabwe (green in Figure 6c) and Kwazulu-Natal (KZN), South Africa (red in Figure 6c). STRUCTURE analysis further indicated that Black rhinoceros in the Kruger National Park (KNP) are comprised of a mix of KZN and Zimbabwe rhinoceros as expected since KNP Black rhinoceros founders originated from these two locales (Hall-Martin, 1988). *D.b. bicornis* (blue in Figure 6c) occur in Namibia and in South Africa in the Northern Cape and Eastern Cape provinces. All *D.b. bicornis* in South Africa were introduced from Namibia (Hall-Martin, 1988). Table 6 shows the population differentiation (F_{ST}) between White and Black rhinoceros subspecies. The results in Table 6 and Figure 6 support the recognition of one White rhinoceros subspecies (*Ceratotherium simum simum*), and three Black rhinoceros subspecies, *D.b. bicornis*, *D.b. michaeli* and *D.b. minor*, with significant partitioning of the Zimbabwe versus Kwazulu-Natal (KZN) *D.b. minor* populations in the present African rhinoceros populations. Similar partitions were observed previously with fewer (8-11) STR loci (Moodley et al., 2017).

Table 6: Population differentiation (F_{ST}) between White and Black rhinoceros species, subspecies and populations. All F_{ST} values were significantly differentiated at a P-Value < 0.001.

	C.s. simum	***D.b. bicornis***	***D.b. michaeli***	***D.b. minor* A**	***D.b. minor* B**	***D.b. minor* C**
C.s. simum	0.000					
D.b. bicornis	0.401	0.000				
D.b. michaeli	0.302	0.197	0.000			
***D.b. minor* A**	0.418	0.156	0.224	0.000		
***D.b. minor* B**	0.351	0.120	0.171	0.096	0.000	
***D.b. minor* C**	0.362	0.126	0.179	0.031	0.036	0.000

For White rhinoceros, Linkage Disequilibrium (LD) was revealed between loci DB1 and SR63 on chromosome 14 ($p<0.05$) but not between BR6 and SR281 on chromosome 13 or DB23 and 12F on chromosome 15 (Table 5). Match probability calculations for the White rhinoceros species are calculated excluding monomorphic loci (BlRh37D, DB23, SR74 and IR22), X-linked loci (IR12 and SR74), locus DB66 that shows significant deviation from Hardy-Weinberg Equilibrium and locus SR63 due to LD with locus DB1.

Most STR loci within the three Black rhinoceros subspecies diverged from HWE when the three subspecies were pooled due to the population subdivision. However, when the Black rhino subspecies and populations identified as panmictic were assessed separately, most polymorphic STR loci passed the requirement of conformance to Hardy-Weinberg and Linkage equilibria. Three STR loci (32F, 7B and IR10) are monomorphic in the *D.b. minor* in KZN, South Africa. The single fixed allele at each of these loci in the KZN population also occurs in the Zimbabwe and admixed Kruger National Park (KNP) population at the highest frequency. Private alleles are found in the *D.b. bicornis* population (N=2), the *D.b. michaeli* population (N=42) and the Zimbabwe *D.b. minor* population (N=7), but not in the other *D.b. minor* populations. The private alleles are highlighted in Table 4. Deviations from HWE in the three *D.b. minor* populations are probably related to inbreeding and genetic drift within the respective populations.

Because the genotyping data were developed principally for forensic match applications, we computed single locus match probabilities for all polymorphic loci for White rhinoceros (3,085 genotypes) and Black rhinoceros (883 genotypes) using theta values from 0 to 0.3 (Table 7). These estimates, as well as the number of alleles (Na), the number of effective alleles (Ne), the observed heterozygosity (Ho) and expected heterozygosity (He) per locus and the fixation index (F) are presented in Table 7 for each respective rhinoceros species and study population. In Table 8 we present the Composite Match Probability (π), considering all informative STR loci that are not sex linked. The π value estimate in Table 8 reflects the product of the mean observed match likelihood per locus across all informative STR loci for each population/subspecies, allowing an *a priori* discernment of the power and utility of the STR locus set for forensic match identification for each study population.

Table 7: STR locus statistics for each species or population including Hardy-Weinberg equilibrium (HWE) statistics and match probabilities at various Theta values for each locus. The loci listed are informative for each species or population, conform to HWE and are not monomorphic or X-linked. Na: number of alleles, Ne: number of effective alleles, Ho: observed heterozygosity, He: expected heterozygosity, F: fixation index, DF: degrees of freedom, P: Probability of deviation, Sign: Significance of deviation; ns (not significant), * (significant at the 5% level).

		Locus statistics						HWE statistics				MP at various Theta (θ) values						
Species/ population	Locus	Sample size	Na	Ne	Ho	He	F	DF	ChiSq	P	Sign	Observed	0	0.01	0.05	0.1	0.2	0.3
White (*C.s. simum*)	DB52	3075	3.000	2.992	0.650	0.666	0.023	3	6.355	0.0956	ns	0.182674	0.185795	0.190330	0.209854	0.237091	0.299523	0.370535
	SR281	3028	3.000	2.974	0.654	0.664	0.015	3	3.031	0.3869	ns	0.185082	0.187186	0.191754	0.211384	0.238704	0.301184	0.372137
	32F	3057	3.000	2.514	0.603	0.602	-0.002	3	2.349	0.5031	ns	0.244013	0.243028	0.247796	0.267911	0.295211	0.355937	0.423342
	7B	2995	3.000	2.020	0.499	0.505	0.011	3	4.661	0.1984	ns	0.309660	0.310834	0.317714	0.344564	0.377133	0.441074	0.505360
	32A	2960	3.000	2.181	0.537	0.542	0.008	3	4.094	0.2515	ns	0.315088	0.315607	0.319455	0.336249	0.359967	0.414465	0.475970
	7C	2854	3.000	1.950	0.482	0.487	0.010	3	5.434	0.1426	ns	0.329660	0.328973	0.335872	0.362701	0.395066	0.458125	0.521063
	12F	2726	2.000	1.993	0.487	0.498	0.022	1	1.347	0.2459	ns	0.370362	0.375888	0.378565	0.391231	0.410732	0.458658	0.514783
	IR10	2953	3.000	1.725	0.423	0.420	-0.006	3	0.947	0.8142	ns	0.424498	0.424146	0.429284	0.449847	0.475703	0.528372	0.582624
	BlRh1C	3079	2.000	1.676	0.397	0.403	0.017	1	0.863	0.3530	ns	0.435837	0.437370	0.442876	0.464588	0.491287	0.544295	0.597758
	DB44	3046	3.000	1.594	0.363	0.373	0.027	3	4.043	0.2569	ns	0.446312	0.446005	0.453311	0.480906	0.512604	0.570382	0.624408
	SR262	3018	2.000	1.605	0.373	0.377	0.009	1	0.268	0.6045	ns	0.458585	0.459211	0.465224	0.488489	0.516274	0.569507	0.621562
	RH12	1015	3.000	1.529	0.337	0.346	0.026	3	5.322	0.1497	ns	0.462580	0.457489	0.466123	0.498027	0.533313	0.594271	0.648257
	DB1	3081	2.000	1.316	0.231	0.240	0.038	1	4.403	0.0359	*	0.608712	0.606178	0.612875	0.637531	0.664600	0.710713	0.750770
D.b. bicornis	BlRh1C	357	4.000	3.453	0.683	0.710	0.038	6	3.914	0.6883	ns	0.132188	0.138537	0.143664	0.165252	0.194594	0.260506	0.334835
	DB66	357	4.000	3.208	0.669	0.688	0.027	6	16.392	0.0118	*	0.144368	0.144510	0.150674	0.175701	0.208055	0.277051	0.351992
	DB52	354	4.000	2.984	0.638	0.665	0.040	6	3.750	0.7105	ns	0.178310	0.185902	0.190489	0.210185	0.237573	0.300161	0.371209
	IR22	350	3.000	2.588	0.609	0.614	0.008	3	2.234	0.5253	ns	0.229685	0.230379	0.235260	0.255794	0.283572	0.345212	0.413580
	SR268	342	4.000	2.510	0.585	0.602	0.028	6	4.825	0.5664	ns	0.238377	0.237539	0.242756	0.264379	0.293051	0.355401	0.423556
	SR63	357	4.000	2.434	0.605	0.589	-0.027	6	12.614	0.0496	*	0.262471	0.255358	0.260169	0.280383	0.307653	0.367924	0.434482
	DB1	357	3.000	1.981	0.543	0.495	-0.097	3	4.803	0.1868	ns	0.341485	0.329853	0.336085	0.360709	0.391136	0.452146	0.514501
	7C	350	7.000	1.935	0.509	0.483	-0.052	21	12.549	0.9236	ns	0.347655	0.343070	0.349252	0.373650	0.403739	0.463901	0.525209
	BlRh37D	355	2.000	1.939	0.501	0.484	-0.035	1	0.446	0.5045	ns	0.389735	0.383245	0.386439	0.400775	0.421659	0.470754	0.526724
	BR6	357	3.000	1.543	0.350	0.352	0.005	3	1.092	0.7791	ns	0.446385	0.449930	0.458618	0.490741	0.526312	0.587878	0.642526
	7B	354	2.000	1.299	0.237	0.230	-0.030	1	0.328	0.5670	ns	0.615803	0.618981	0.625600	0.649923	0.676526	0.721586	0.760471
	DB23	349	2.000	1.255	0.223	0.203	-0.101	1	3.575	0.0586	ns	0.647466	0.655877	0.662195	0.685292	0.710319	0.752090	0.787525
	BlRh1B	357	2.000	1.075	0.067	0.070	0.042	1	0.631	0.4270	ns	0.869008	0.867033	0.870030	0.880805	0.892115	0.910019	0.924226

D.b.michaeli	DB66	51	8.000	6.313	0.765	0.842	0.091	28	30.356	0.3464	ns	0.036078	0.044093	0.048451	0.067476	0.094741	0.160013	0.237846
	7C	51	10.000	6.252	0.745	0.840	0.113	45	59.525	0.0721	ns	0.043137	0.043927	0.048407	0.067834	0.095456	0.161110	0.239057
	DB52	51	8.000	5.244	0.745	0.809	0.079	28	19.007	0.8979	ns	0.045490	0.062746	0.067516	0.087951	0.116497	0.182995	0.260634
	32A	51	6.000	5.505	0.824	0.818	-0.006	15	12.902	0.6099	ns	0.051765	0.058537	0.063080	0.082753	0.110624	0.176446	0.254029
	BlRh1C	51	8.000	5.050	0.706	0.802	0.120	28	32.419	0.2578	ns	0.053333	0.064143	0.069263	0.090864	0.120451	0.188068	0.266006
	DB1	51	5.000	4.061	0.745	0.754	0.011	10	7.492	0.6784	ns	0.085490	0.101237	0.106424	0.128257	0.157978	0.225127	0.301492
	DB44	51	7.000	3.932	0.765	0.746	-0.026	21	20.128	0.5132	ns	0.087843	0.098016	0.104015	0.128550	0.160676	0.230472	0.307747
	SR268	43	7.000	4.160	0.581	0.760	0.235	21	36.702	0.0182	*	0.090808	0.086265	0.092299	0.116960	0.149232	0.219415	0.297292
	12F	50	6.000	4.019	0.640	0.751	0.148	15	25.200	0.0473	*	0.093878	0.100275	0.105716	0.128393	0.158864	0.226837	0.303481
	IR10	47	4.000	3.465	0.681	0.711	0.043	6	7.658	0.2643	ns	0.133210	0.134778	0.140131	0.162478	0.192512	0.259260	0.334011
	SR281	51	6.000	3.380	0.647	0.704	0.081	15	14.981	0.4528	ns	0.134118	0.138094	0.143726	0.166983	0.197788	0.265239	0.339991
	SR63	49	5.000	2.696	0.510	0.629	0.189	10	12.559	0.2494	ns	0.183673	0.210185	0.215519	0.237611	0.266904	0.330738	0.400778
	DB23	50	4.000	2.421	0.520	0.587	0.114	6	4.877	0.5596	ns	0.206531	0.230623	0.237396	0.264203	0.297490	0.364939	0.434879
	SR262	51	6.000	2.250	0.608	0.556	-0.094	15	15.594	0.4096	ns	0.250196	0.246436	0.254342	0.284841	0.321217	0.391369	0.461104
	BR6	51	4.000	2.666	0.725	0.625	-0.161	6	8.907	0.1789	ns	0.274510	0.214631	0.219924	0.241866	0.270994	0.334518	0.404234
	IR22	51	4.000	2.141	0.510	0.533	0.043	6	15.001	0.0203	*	0.290980	0.284338	0.291109	0.317703	0.350305	0.415207	0.481327
	7B	51	2.000	1.286	0.255	0.222	-0.146	1	1.088	0.2969	ns	0.612549	0.629373	0.635919	0.659935	0.686128	0.730298	0.768222
D.b. minor A (KwaZulu-Natal)	RH12	150	4.000	3.017	0.640	0.669	0.043	6	3.578	0.7335	ns	0.169664	0.180243	0.185020	0.205361	0.233353	0.296735	0.368290
	DB66	210	3.000	2.877	0.629	0.652	0.036	3	3.575	0.3111	ns	0.189702	0.195953	0.200660	0.220726	0.248366	0.310927	0.381437
	SR268	197	5.000	2.557	0.579	0.609	0.050	10	13.039	0.2215	ns	0.218999	0.230113	0.235403	0.257282	0.286224	0.349060	0.417723
	BlRh1C	211	5.000	2.516	0.673	0.602	-0.117	10	11.578	0.3143	ns	0.228346	0.213894	0.220832	0.248235	0.282174	0.350820	0.421993
	BR6	212	5.000	2.320	0.580	0.569	-0.020	10	7.695	0.6586	ns	0.233479	0.244138	0.251216	0.278986	0.312996	0.380757	0.450003
	DB23	196	2.000	2.000	0.480	0.500	0.041	1	0.327	0.5677	ns	0.362166	0.375000	0.377610	0.390057	0.409375	0.457143	0.513281
	SR63	212	4.000	2.011	0.495	0.503	0.015	6	2.079	0.9123	ns	0.362336	0.368933	0.371773	0.385005	0.405073	0.453845	0.510617
	DB52	212	3.000	1.901	0.415	0.474	0.124	3	3.990	0.2625	ns	0.364303	0.386813	0.390523	0.406553	0.428877	0.479335	0.535430
	7C	192	3.000	1.936	0.453	0.484	0.063	3	1.470	0.6892	ns	0.365456	0.381156	0.384555	0.399584	0.421094	0.470918	0.527205
	IR22	203	2.000	1.965	0.473	0.491	0.037	1	0.280	0.5966	ns	0.368288	0.379540	0.382483	0.396010	0.416224	0.464760	0.520817
	DB1	212	2.000	1.902	0.500	0.474	-0.054	1	0.619	0.4314	ns	0.397791	0.388801	0.392338	0.407786	0.429580	0.479413	0.535225
	SR281	211	2.000	1.749	0.422	0.428	0.015	1	0.046	0.8301	ns	0.414173	0.418687	0.423606	0.443474	0.468787	0.521113	0.575637
	BlRh37D	205	2.000	1.515	0.356	0.340	-0.048	1	0.465	0.4952	ns	0.491726	0.493493	0.500014	0.524763	0.553408	0.606084	0.655664
	BlRh1B	212	2.000	1.496	0.354	0.332	-0.067	1	0.940	0.3324	ns	0.499911	0.501647	0.508248	0.533214	0.561943	0.614358	0.663311

D.b. minor B (Zimbabwe)	BR6	178	8.000	5.453	0.787	0.817	0.037	28	40.428	0.0605	ns	0.055164	0.056582	0.061419	0.082069	0.110806	0.177580	0.255506
	DB66	178	7.000	3.636	0.708	0.725	0.024	21	21.952	0.4023	ns	0.104171	0.105026	0.111841	0.139087	0.173592	0.245809	0.323497
	7C	178	6.000	3.287	0.680	0.696	0.023	15	19.101	0.2092	ns	0.142576	0.142513	0.148374	0.172388	0.203837	0.271868	0.346576
	DB52	178	5.000	2.803	0.618	0.643	0.039	10	5.547	0.8518	ns	0.182695	0.192849	0.198475	0.221591	0.251927	0.317407	0.388857
	12F	178	4.000	2.470	0.635	0.595	-0.067	6	16.205	0.0127	*	0.224910	0.227956	0.234358	0.259939	0.292166	0.358569	0.428359
	SR63	178	4.000	2.371	0.590	0.578	-0.020	6	7.450	0.2812	ns	0.234622	0.236980	0.243911	0.271219	0.304891	0.372539	0.442172
	32A	169	5.000	2.219	0.538	0.549	0.020	10	20.884	0.0219	*	0.257678	0.246859	0.255182	0.287063	0.324650	0.396069	0.466127
	IR10	178	5.000	2.248	0.562	0.555	-0.012	10	6.139	0.8034	ns	0.261220	0.265355	0.271880	0.297735	0.329874	0.394954	0.462251
	SR262	178	5.000	1.928	0.528	0.481	-0.097	10	6.372	0.7831	ns	0.336190	0.327971	0.335391	0.363946	0.397825	0.462493	0.525901
	RH12	168	4.000	1.815	0.446	0.449	0.006	6	3.143	0.7907	ns	0.348090	0.345738	0.354329	0.386669	0.423655	0.490871	0.553799
	IR22	178	4.000	1.820	0.399	0.451	0.115	6	4.909	0.5555	ns	0.382467	0.398364	0.403015	0.422100	0.446960	0.499609	0.555525
	DB1	178	2.000	1.745	0.404	0.427	0.053	1	0.496	0.4814	ns	0.410588	0.419472	0.424419	0.444376	0.469758	0.522123	0.576606
	BlRh37D	178	2.000	1.725	0.399	0.420	0.051	1	0.469	0.4936	ns	0.416048	0.424268	0.429380	0.449858	0.475637	0.528223	0.582446
	BlRh1B	168	2.000	1.668	0.411	0.400	-0.026	1	0.113	0.7372	ns	0.438623	0.439720	0.445289	0.467196	0.494037	0.547096	0.600415
	DB23	175	2.000	1.582	0.406	0.368	-0.103	1	1.864	0.1721	ns	0.470411	0.467356	0.473517	0.497228	0.525301	0.578490	0.629984
	SR281	178	3.000	1.335	0.225	0.251	0.104	3	7.054	0.0702	ns	0.588078	0.576845	0.584762	0.613607	0.644682	0.696151	0.739518
D.b. minor C (Kruger National Park)	DB66	85	6.000	3.931	0.812	0.746	-0.089	15	23.080	0.0825	ns	0.107563	0.103201	0.108780	0.131914	0.162780	0.231117	0.307742
	7C	79	5.000	3.423	0.633	0.708	0.106	10	9.596	0.4766	ns	0.123012	0.135980	0.141483	0.164328	0.194794	0.261970	0.336782
	BR6	85	8.000	3.139	0.624	0.681	0.085	28	26.727	0.5332	ns	0.136695	0.140434	0.147429	0.175217	0.210017	0.281692	0.357555
	RH12	59	5.000	2.950	0.746	0.661	-0.128	10	4.372	0.9290	ns	0.165400	0.163262	0.169886	0.196395	0.229933	0.299670	0.373864
	BlRh1C	85	5.000	2.837	0.588	0.648	0.092	10	16.540	0.0852	ns	0.169468	0.167879	0.175082	0.203506	0.238704	0.310085	0.384511
	SR268	78	6.000	2.553	0.603	0.608	0.009	15	12.878	0.6117	ns	0.213120	0.211806	0.218489	0.245055	0.278277	0.346230	0.417329
	SR63	85	5.000	2.450	0.541	0.592	0.086	10	10.368	0.4088	ns	0.224650	0.247821	0.252995	0.274437	0.302851	0.364577	0.431960
	DB52	85	5.000	2.079	0.494	0.519	0.048	10	4.494	0.9223	ns	0.291317	0.315530	0.321057	0.343403	0.371982	0.431570	0.494420
	12F	83	4.000	2.033	0.530	0.508	-0.043	6	2.253	0.8951	ns	0.292389	0.301224	0.308577	0.337032	0.371101	0.436952	0.502320
	DB1	85	2.000	1.998	0.494	0.499	0.011	1	0.009	0.9226	ns	0.365266	0.375312	0.377946	0.390470	0.409853	0.457677	0.513811
	IR10	85	4.000	1.597	0.447	0.374	-0.196	6	7.044	0.3168	ns	0.391317	0.420187	0.429277	0.462885	0.500105	0.564590	0.621946
	IR22	82	2.000	1.993	0.549	0.498	-0.102	1	0.847	0.3573	ns	0.397471	0.375935	0.378616	0.391293	0.410804	0.458738	0.514862
	DB23	84	2.000	1.982	0.548	0.495	-0.105	1	0.931	0.3347	ns	0.399598	0.377298	0.380081	0.393087	0.412871	0.461040	0.517140
	32A	83	5.000	1.601	0.349	0.376	0.070	10	7.173	0.7090	ns	0.415222	0.414973	0.424319	0.458798	0.496834	0.562360	0.620309
	BlRh37D	85	2.000	1.738	0.471	0.425	-0.108	1	0.995	0.3184	ns	0.430252	0.421201	0.426209	0.446358	0.471887	0.524336	0.578727
	BlRh1B	84	2.000	1.615	0.321	0.381	0.156	1	2.047	0.1525	ns	0.446070	0.455843	0.461789	0.484849	0.512499	0.565731	0.618013
	DB44	85	3.000	1.386	0.282	0.278	-0.014	3	3.945	0.2674	ns	0.538095	0.540589	0.548789	0.578785	0.611343	0.665933	0.712616
	SR281	85	4.000	1.313	0.271	0.238	-0.136	6	2.081	0.9121	ns	0.583193	0.601194	0.608415	0.634824	0.663468	0.711387	0.752199

Table 8: Summary of number of individual animals from each species, subspecies/population included in this study, the number of loci evaluated in this analysis (loci/pm), mean number of alleles (Na), mean number of effective alleles (Ne), mean observed heterozygosity (Ho), mean expected heterozygosity (He), fixation index and inbreeding coefficient (F_{IS}), with the composite match probability (π) per species, subspecies/population.

Population	Animals	Na	Ne	Ho	He	F	F_{IS}	Loci/pm	π
C.s. simum	3085	2.478	1.807	0.349	0.386	0.084	0.097	13	1.05×10^{-6}
D.b. bicornis	*357*	3.348	2.054	0.406	0.438	0.059	0.0656	13	3.5×10^{-7}
D.b. michaeli	*51*	5.522	3.570	0.558	0.638	0.105	0.1236	17	1.04×10^{-16}
***D.b. minor* A**	212	2.913	1.809	0.360	0.382	0.057	0.0568	16	9.79×10^{-8}
***D.b. minor* B**	178	4.435	2.612	0.468	0.520	0.073	0.1003	16	4.93×10^{-10}
***D.b. minor* C**	85	4.043	2.194	0.461	0.484	0.027	0.0515	18	1.17×10^{-10}

4.3.1 Application of forensic data to specific forensic cases

The VGL has received over 100 000 specimens related to over 6000 rhinoceros criminal cases since 2010 and has submitted over 800 case reports that include more than 150 cases relating carcass material to evidence items (horn, tissue, blood stains and other confiscated materials). Table 9 summarizes nine illustrative cases relating to rhinoceros crimes for which samples were received and in which DNA matches were made. The table lists details and timeline for each case, the samples included, species identified and match probability calculated using the RhODIS® allele frequency database provided in Table 4.

Table 9: Summary of nine prosecuted cases of rhinoceros crimes for which samples were successfully matched using composite STR genotyping with cumulative match probability calculated using a conservative Theta (θ) of 0.1. Details of case with exhibits examined, matching evidence items, location of poaching incident, species and subspecies identified, sex of carcasses, cumulative match probability, status of the case, date of conviction and the nationalities of the accused are provided for 6 South African cases and single cases from Kenya, Namibia and Singapore. (KNP – Kruger National Park, SA – South Africa, ORTIA – OR Tambo International Airport, HiP – Hluhluwe-iMfolozi Park, OPC – Ol Pejeta Conservancy, ENP – Etosha National Park, F – Female, M – Male. [a] and [b] refer to match probability calculations for specific white and black rhinoceros.

Case Reference	Exhibits received	Match Result	Poaching Site	Species / subspecies	Sex	Match Probabilities	Status of Case	Convicted	Nationality
South Africa: State v Ali Nkuna and Gerson Khoza	3 horns and tissues from 2 carcasses	2 horns matched carcass 1 and 1 horn matched carcass 2	KNP, SA	White rhinoceros (*C.s. simum*)	F, M	4.20×10^{-9}, 2.03×10^{-10}	Sentenced: 29 years and 3 months and 29 years	2012/08/23	Mozambican
South Africa: State v Inaso Mkhabele and Jorudo Ngobeni	1 horn and tissues from a carcass	Horn matched carcass	Hoedspruit, SA	White rhinoceros (*C.s. simum*)	F	3.80×10^{-8}	Sentenced: 15 years each	2013/03/28	Mozambican and South African
South Africa: State v Rodgers Mukwene	3 horns and tissues from 2 carcasses	2 horns matched carcass 1 and 1 horn matched carcass 2	Waterberg, SA	White rhinoceros (*C.s. simum*)	F	1.96×10^{-8}, 1.35×10^{-8}	Sentenced: 10 years	2012/11/14	Zimbabwean
South Africa: State v Kenneth Ally Sibiya and Leonard Mhlongo	3 horns and tissues from 2 carcasses	2 horns matched carcass 1 and 1 horn matched carcass 2	KNP, SA	Black rhinoceros (*D.b. minor*)	F, M	4.18×10^{-12}, 1.03×10^{-12}	Sentenced: Leonard Mhlongo: 14 years, Kenneth Sibiya, escaped whilst on bail.	2013/08/15	Mozambican
South Africa: State vs Thomas Muyambo, Sam Sithole, Bornwise Jiyayo Mlambo	Clothing, axe and tissues from a carcass	The profile from clothing matched carcass	Limpopo, SA	White rhinoceros (*C.s. simum*)	M	1.19×10^{-8}	Sentenced: Thomas Muyambo: 8 years. Cases withdrawn against other accused	2015/02/24	Zimbabwean and Mozambican
South Africa: State v Lingrun Sheng	3 horns and tissues from 3 carcasses	3 horns matched 3 carcasses	ORTIA, SA HiP, SA	White rhinoceros (*C.s. simum*)	F	8.79×10^{-8}, 1.45×10^{-9a}, 8.08×10^{-8}	Sentenced: R800 000 fine or 6 years	2016/11/01	Chinese
Kenya: Nanyuki 2012	Horn and piece of carpet from vehicle	Horn matched blood on carpet	OPC, Kenya	Black rhinoceros (*D.b. michaeli*)	M	8.98×10^{-22}	Sentenced: 11 years	2017/05/12	Kenyan

Case Reference	Exhibits received	Match Result	Poaching Site	Species / subspecies	Sex	Match Probabilities	Status of Case	Convicted	Nationality
Namibia: Hosea Kutako Airport 2014	14 horns and tissues from a carcass	2 horns from a White rhinoceros and 12 horns from *D.b.bicornis* with 2 horns matched to a carcass	ENP, Namibia	Black rhinoceros (*D.b. bicornis*)	F	4.74×10^{-13b}	Sentenced: 14 years each	2016/10/30	Chinese
Singapore: Seizure 2014	6 horns	2 horns matched a carcass	KNP, SA	White rhinoceros (*C.s. simum*)	F	4.55×10^{-9}	Sentenced: 15 months	2014/01/16	Vietnamese

4.4 Discussion

The alarming, near exponential increase in illegal killing of rhinoceros in southern African countries since 2008 is driven by a recent increase in market value of rhinoceros horns, principally across eastern Asia. The demand has made exploitation of rhinoceros for their horns highly profitable and nearly risk-free due to difficulties in connecting confiscated horns to a specific crime scene and routine imposition of very light sentencing upon conviction (Milliken and Shaw, 2012). The increase in rhinoceros poaching has led to increased vigilance and resolve of wildlife law enforcement agencies. For these reasons we began collecting rhinoceros specimens from confiscated tissues and from crimes scenes, designed protocols for DNA extraction from horn and began compiling a database of STR genotypes. In this report we have updated and further characterized STR locus sets informative for robust genotype matching for White and Black rhinoceros and logged these in the RhODIS® database, which now contains some 50 000 rhinoceros acquisitions.

Population sub-structure can bias the computation of match probabilities in natural populations because of historic admixture, migration, demographic perturbations and inbreeding, which can distort genetic and linkage equilibrium (Weir, 1996). Three distinct population analyses (Figure 6a, b and c) identified four major groups: White rhinoceros (*C.s. simum*) and three subspecies of Black rhinoceros (*D.b. bicornis*, *D.b. michaeli* and *D.b. minor*) with some diffusion among the *D.b. minor* subspecies based on STRUCTURE (Figure 6c). The White rhinoceros species was markedly homogenous consistent with its recent founder effect when the population dropped to less than 50 individuals in the early 1900s (Player, 2013). The mean number of alleles per locus (2.478) was lowest in the White rhinoceros compared the three Black rhinoceros subspecies (Table 8) as might be expected given the early 20th century population bottleneck of White rhinoceros. The levels of observed heterozygosity (Ho) in the Black rhinoceros subspecies are lowest in the *D.b. minor* in KZN (0.360), similar to the White rhinoceros (0.349), confirming that this population also underwent a bottleneck in the early 20th century. *D.b. michaeli* has the highest Na (5.522) and highest Ho level of the Black rhinoceros subspecies (0.558). *D.b. bicornis* has a low Na (3.348) and a low Ho (0.406) which may be due to a smaller number of founders compared to the Zimbabwean *D.b. minor* and *D.b. michaeli* populations (Emslie and Brooks, 1999) (Table 8). A STRUCTURE population analysis affirmed the genetic distinctiveness between the three subspecies of Black rhinoceros and between the two *D.b. minor* groups (Figure 6c). STRUCTURE (at K=4) resolves the Zimbabwe, the KZN and the admixed KNP population as a third distinct but admixed population. The relationship between the groups is presented in a neighbor joining tree (Figure 6a). This tree shows that the Black rhinoceros, *D.b .michaeli* subspecies, is basal to the more recently diverged *D.b. minor* and *D.b. bicornis* sister

subspecies. Population differentiation (F_{ST}) values also show least differentiation between the *D.b .bicornis* and *D.b. minor* subspecies (Table 6).

The overall genetic uniformity and panmixia of the White rhinoceros (*C.s. simum*) (Figure 6a) which comprise over 90% of the criminal cases received by the VGL, would allow forensic application of the product rule for this species. One-locus match probabilities were constructed for STR loci for the White rhinoceros. Match probabilities ranged from (0.18) to (0.6), mean (0.36) (Table 7), in contrast to human (Butler, 2015) and outbred cats (Menotti-Raymond et al., 2005) that are generally around 0.1. The large dataset in this study confirmed the utility of monomorphic loci for species identification. The genotypic data allow the species/subspecies/population assignment of an unknown sample as Black or White rhinoceros. STRUCTURE analysis provides strong support for the classification of a sample into the three recognized Black rhinoceros subspecies (Figure 6c).

DNA profile matches are made using all amplified loci and comparing the DNA profile to all genotype data on the RhODIS® database using the Cervus identity match feature and confirmed manually and with electropherogram data. Match probabilities for White and Black rhinoceros matches can be done using the subspecies specific allele frequencies (Table 4) and a conservative Theta (θ) of 0.1 following The Second National Research Council report on forensic DNA evidence recommendation 4.2 for estimating random match probabilities in human populations (Weir, 1996). This provides the most conservative value for individual genotype matches.

Among populations within a species, genetic differentiation occurs due to various processes including migration, mutation and drift over time. If, for example migration between two sub-populations has occurred constantly, then very little differentiation will be observed and the statistic used to measure this degree of differentiation will be small. F_{ST}, as discussed previously, is this measure of differentiation. F_{ST} is also an allele frequency based statistic. F_{ST} can be used to identify areas of the genome under selective pressure since the F_{ST} at a locus will be large if an allele at that locus has been preferentially selected over time because it provides a selective advantage to the organism. F_{ST} statistics can be used in disease association mapping and forensics. As F_{ST} increases between sub-populations due to increasing differences between them, so the similarities within the sub-population increase making it more likely to observe a match between genotypes in the sub-population than would be expected by chance. This sub-population sub-structuring is incorporated into the calculation of MP by including an appropriate θ value (Butler, 2015).

This θ value is selected from the between population F_{ST} values to be a sufficiently high value to account for the degree of differentiation and thus infer an acceptable degree of conservativeness to

the MP calculation, also discussed previously. As an example, the degree of genetic differentiation between human populations is 5 to 10% based on an F_{ST} value of 0.05 when using microsatellite data to determine the F_{ST} value and 0.10 when using SNP data (Holsinger and Weir, 2009).

Table 7 includes the match probability values for the different loci for a range of theta values and are arranged from the lowest MP value to the highest MP value for a locus. These values are given for the panmictic White rhinoceros population and the individual Black rhinoceros sub-populations. Different loci are shown to be informative in white and black rhinoceros species and black rhinoceros subspecies or populations, indicating that the 23 STR loci used as the basic RhODIS® marker set are the minimum set of markers that should be used for DNA forensic investigations for African rhinoceros. The question arises then, for the calculation of MP in the Black rhinoceros, whether the overall Black rhinoceros allele frequency data can be used or whether a more conservative approach is required by using the specific Black rhinoceros sub-population allele frequency data to calculate the MP of a sample that clusters within that sub-population. This question becomes even more complex when calculating MP for samples from populations in which sub-population admixing has occurred such as the Kruger National Park population that has both the Zimbabwean *D.b. minor* and KwaZulu Natal *D.b. minor* groups. Gittelson et al. (2017) discuss the error caused by ignoring sub-population effects in human populations and that this error is in the order of a factor of 10, i.e. MP can vary by a factor of 10 if different databases are used for the calculation of the MP. If MP was 1 in 1 million using a specific database, one can accept that the MP could vary from 1 in 100 000 to 1 in 10 million if different databases are used. If a profile is compared to a sub-population from which it was derived then there is a greater likelihood of a matching profile being observed than if it were compared to another population or to the overall population if clear population sub-structuring occurs.

When genotypes are obtained from historic specimens, as can happen when museum horns are involved in criminal cases, these specimens do not cluster into the current sub-population groups and these historic samples often have unique and thus rare alleles. The MP for these profiles can, therefore, be expected to be extremely low and using the overall allele frequencies from the Black rhinoceros population to calculate the MP are also acceptable in these cases.

4.4.1 Policy Considerations

Regulations for marking of rhinoceros and rhinoceros horns, under the National Environmental Management: Biodiversity Act (10/2004) were published in the South African Government Gazette in April 2012 (Anonymous, 2012) . The regulations instruct that all rhinoceros should be sampled for DNA profiling when they are captured for identification, translocated or hunted and further that all

stored rhinoceros horn is sampled. Tissue specimens must be sampled in specific kits and the DNA genotypes are to be added to the RhODIS® database. Reports are issued for forensic cases in which horns or horn products are recovered and linked to a specific carcass or where weapons used in poaching incidents are recovered and associated blood traces linked to a carcass. The CITES (Convention on International Trade in Endangered Species of Fauna and Flora) Conference of Parties in Bangkok, Thailand, 3-14 March 2013, recommended (CoP16 Com II.24) that all CITES signatory countries should sample confiscated rhinoceros horn and submit this to an accredited forensic laboratory for DNA analysis. This imperative underpins the need to ensure that match probability estimations using a robust and uniform database are established in support of all international investigations.

CHAPTER 5

Historic subspecies admixture confirmed with pedigree verification in a population of black rhinoceros in South Africa

Abstract:

In 1961 and 1962 seven black rhinoceros animals of the *D.b. michaeli* subspecies were introduced to the Addo Elephant National Park (AENP) in the Eastern Cape Province of South Africa from Kenya. When the last adult bull in this population died in 1977 the African Rhino Specialist group (AfRSG) of the IUCN made the decision to introduce three bulls of the *D.b. minor* subspecies from KwaZulu-Natal to the population. This decision was reversed in 1981 and the *D.b. minor* bulls and their presumed offspring were removed so that the population would remain pure *D.b. michaeli*. The majority of the remaining *D.b. michaeli* animals with their offspring were translocated to a private game ranch between 1998 and 2003. Analysis of this population of *D.b. michaeli* revealed that a signature of the historic admixture between *D.b. michaeli* and *D.b. minor* remained in the population. This study confirms the presence of the admixture, verifies the pedigree of the population using STR marker data and presents a comparison between the STRUCTURE ancestry values of individuals in the population and the individual ancestry calculated using the pedigree information. The study further presents an assessment of the reproductive fitness of the population. The data provided is used to inform management decisions before a translocation exercise that moved some of these animals to a former black rhinoceros range where the species no longer occurred and demonstrates the value of these data to support the genetic management of black rhinoceros populations.

5.1 Introduction

The black rhinoceros (*Diceros bicornis bicornis*) occurred in the Addo area of the Eastern Cape Province of South Africa until 1863, when the last animal was apparently shot (Hall-Martin and Penzhorn, 1977). In 1961 and 1962, seven *Diceros bicornis michaeli* animals were introduced to the Addo Elephant National Park (AENP) from the Kiboko region in Kenya (Hall-Martin and Penzhorn, 1977). Only four animals from Kenya survived to form the founder population of *D.b. michaeli* in Addo and when the only remaining male animal died in 1977, three *Diceros bicornis minor* bulls from Zululand were translocated to AENP to supplement the population. These bulls and their presumed offspring were removed in 1981 following a recommendation by the African Rhino Specialist group of the IUCN in order to ensure that the AENP population remained pure *D.b. michaeli*. Between 1998 and 2003 the majority of these animals were translocated to the a private game ranch in South Africa to be replaced by *Diceros bicornis bicornis* animals that were translocated from Namibia, via the Augrabies and Vaalbos National Parks, in an attempt to return the subspecies to its historic range.

The population of *D.b. michaeli* translocated to a private game ranch represent an individual extra-limital population of this black rhinoceros subspecies in South Africa. A Structure analysis of the black rhinoceros data collected as part of the RhODIS® rhinoceros DNA database (Harper et al., 2018) provided support of the subdivision of the black rhinoceros population into 3 subspecies divisions, *D.b. bicornis, D.b. michaeli* and *D.b. minor*. This analysis further provided evidence of a *D.b. minor* signature in the translocated *D.b. michaeli* population that indicated that the historic admixture between the *D.b. minor* and *D.b. michaeli* populations had not been completely cleared, as had been reported (Hall-Martin, 1984).

This objective of this study was to identify the individual admixture in the population and identify the origin of the admixture by pedigree verification. The verification of the individual parentages further provided a comparison between the ancestry coefficients obtained from a Bayesian clustering program STRUCTURE to the admixture information obtained from the pedigree. The data available from historic, observed and validated breeding also allowed an assessment of the reproductive fitness of the population. The data provided by this study was used to inform management decisions before a translocation exercise that moved some of these animals to a former black rhinoceros range where the species no longer occurred.

5.2 Materials and methods

5.2.1 Samples

A total of 181 animals were included in the dataset. Samples were collected from the black rhinoceros at the private game ranch and submitted to the Veterinary Genetics Laboratory (VGL) of the University of Pretoria as part of the RhODIS® initiative. The majority of these samples were collected in RhODIS® sampling kits (Harper et al., 2018). These samples were submitted to the laboratory between 2011 and 2018 and a total of 116 samples were submitted that included blood, tissue and horn drilling samples. A background dataset of 518 samples was selected from the RhODIS® database that included 59 *D.b. bicornis* individuals from the Namibian population, 77 *D.b. michaeli* individuals from Kenya, 266 *D.b. minor* individuals including *D.b. minor* from KwaZulu-Natal (KZN) (100), Zimbabwe (ZIM) (100) and the Kruger National Park (KNP) (66) as well as the 116 samples from the private game ranch (*D.b. michaeli*-SA).

5.2.2 Genotyping and ancestry analysis

DNA was extracted from blood and tissue samples using the Prepfiler®kit (ThermoFisher Scientific) according to the manufacturer's instructions and from the horn drilling samples using the same kit with modifications described by Harper *et. al.* (Harper et al., 2013). PCR was performed in four multiplex reactions, using the 23 loci previously described (Harper et al., 2018) and the zinc finger (ZF) locus which was used to determine the gender of the animal from which the sample originated (Peppin et al., 2010). The number of alleles (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosities and fixation index (F) were determined for the populations from the private game ranch (*D.b. michaeli*-SA) (116), *D.b. bicornis* (59), *D.b. michaeli* (77), *D.b. minor* from KwaZulu-Natal (100), Zimbabwe (100) and the Kruger National Park (66) using GenAlEx 6.5 (Peakall and Smouse, 2012). The correlation coefficient (F_{ST}), indicating the between population differentiation was determined using GenAlEx 6.5 (Peakall and Smouse, 2012). For the private game ranch (*D.b. michaeli*-SA) population, individual inbreeding coefficients (*F*) were determined using the software program Coancestry V1.0.1.8 (Wang, 2010) and individual heterozygosity (Hi) using GenAlEx 6.5. Individual ancestry (Q_iStr) was calculating using the Bayesian clustering program STRUCTURE (van Wyk et al., 2013). Analyses were done using 18 loci that excluded locus 32F that is monomorphic in the black rhinoceros, loci IR12 and SR74 that are X-linked (Harper et al., 2018) and loci BlRh37D and SR268 in which null alleles occurred, from the 23 loci previously described (Harper et al., 2018). STRUCTURE analysis was run with a burn-in of 100 000 and 100 000 MCMC repeats, the admixture model with correlated allele frequencies, 10 repeats and the number of populations assumed was 3,

based on the analysis by Harper *et.al.* (Harper et al., 2018) that supported the clustering of the black rhinoceros into three distinct subspecies (*D.b. bicornis*, *D.b. michaeli* and *D.b. minor*). The background database was used to analyse the clustering of the Black rhinoceros and provide an individual admixture coefficient (Q_iStr) using the Bayesian clustering program STRUCTURE (van Wyk et al., 2013). The individual *D.b. michaeli* ancestry based on pedigree (Q_iPed) was calculated by adding the Q_iPed of each parent and dividing by 2.

5.2.3 Pedigree Analysis

Historic and anecdotal information was used to determine the origin and relationships of specific animals that were the founders of this population. When samples were available for the animals (116), the relationships and parentages were confirmed with the genotyping information. Age, environmental factors including gestation period, that could assist with confirming parentage were considered when assigning a parent to an offspring. The majority of cow-calf relationships were based on observational data and confirmed with genotyping. Assignment of the bull to a calf was done following confirmation of the cow by testing all possible bulls using Cervus 3.0.7 (Kalinowski et al., 2007). Parents were assigned based on no exclusions between the genotypes of the parents and offspring and between a trio if both parents were available and using the 18 loci used in the STRUCTURE analysis. A pedigree diagram was constructed using the software Pedigraph V2.4 (https://animalgene.umn.edu/download-pedigraph) (Garbe and Da, 2003).

5.3 Results

Figure 7 presents a STRUCTURE analysis showing the subdivision of the Black rhinoceros into three distinct subspecies, *D.b. bicornis, D.b. minor* and *D.b. michaeli.* The figure includes, as population 6, the current extralimital *D.b.michaeli* population on the private game ranch in South Africa, *D.b. michaeli* (SA) within the background dataset of 518 black rhinoceros samples. Population one is the *D.b. bicornis* from Namibia (NAM), population two is the *D.b. minor* from KwaZulu-Natal in South Africa (KZN), population three is the *D.b. minor* from Zimbabwe (ZIM), population four is the *D.b. minor* from the Kruger National Park in South Africa (KNP) and population 5 is the *D.b. michaeli* from Kenya (KEN).

Table 10 presents the animals, each individually identified (ID Code) and gives their date of birth and date of death, if this is available, their gender and the reported dam (RDID), confirmed dam (CDID) and confirmed sire (CSID). The Table also includes the individual admixture coefficients indicating the *D.b. michaeli* ancestry of the animal calculated from STRUCTURE (Q_iStr) as well as the calculated percentage *D.b. michaeli* based on its pedigree (Q_iPed). The ID Code M indicates that the animal was

not born on the private game ranch, but was introduced from the AENP or from another source population. N indicates that the animal is a Natal bull, T indicates that the animal was born on the private game ranch and Z indicates that the animal was introduced from a zoo population.

Table 11 relates the diversity of the *D.b. michaeli* (SA) population to a subset of the *D.b. bicornis* population in Namibia, *D.b. michaeli* population in Kenya and the *D.b. minor* populations in KwaZulu-Natal (KZN), Zimbabwe (ZIM) and the Kruger National Park (KNP) by comparing the number of alleles (Na), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosities and fixation index (F) in these populations. The Fixation index (F) in this table is calculated as (He-Ho)/He and represents the inbreeding coefficient of the population with values close to zero an indication of a randomly mating population. Observed (Ho) and expected (He) heterozygosities are similar within each subpopulation and the least diverse population is *D.b. minor* (KZN) as indicated by the lowest number of alleles (2.5) and lowest observed heterozygosity (0.37). The most diverse populations are *D.b. michaeli* (KEN) with the highest number of alleles (6.44) and *D.b. michaeli* (SA) with the highest observed heterozygosity (0.7).

Population differentiation (F_{ST}) between these populations is presented in Table 12. The *D.b. minor* populations in KwaZulu-Natal, Zimbabwe and the Kruger National Park are the least differentiated (0.092, 0.035 and 0.033), similar to the degree of differentiation between the *D.b. michaeli* in South Africa and the *D.b. michaeli* in Kenya (0.037). The most strongly differentiated populations are the *D.b. michaeli* in Kenya and the *D.b. bicornis* in Namibia (0.199) and *D.b. michaeli* in Kenya and *D.b. minor* in KwaZulu-Natal (0.221).

Tables 13 and 14 are a summary of the breeding statistics for the cows and bulls, respectively, in the AENP and the *D.b.michaeli* (SA) populations. In Table 13 the most prolific cows in terms of calf production, M024, M028, M029 and M034 have an average individual heterozygosity of 0.833 and are all admixed. These four cows have produced 49 calves out of a total of 155 (31%). The highest fecundity in the bulls, M037, M063 and M067, is not correlated with admixture or heterozygosity with an average individual heterozygosity for these three bulls of 0.667.

Figure 8 and Figure 9 present the complete seven generation pedigree of the AENP and the *D.b.michaeli* (SA) populations. In Figure 8 individuals for whom samples, and thus genotypes, were available, are shaded and in Figure 9 admixed individuals identified by pedigree verification are shaded.

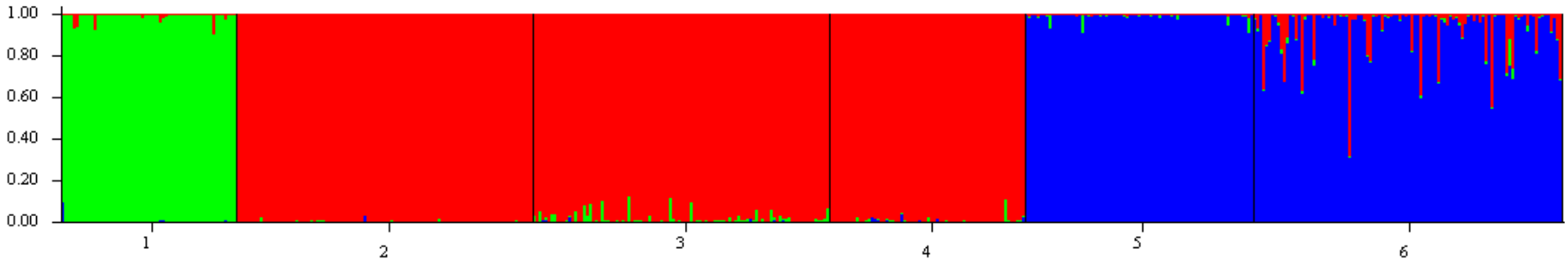


Figure 7: A STRUCTURE plot showing the Black rhinoceros subdivision with the three distinct population subdivisions; (1) *D.b. bicornis* (NAM), (2) *D.b. minor* (KZN), (3) *D.b. minor* (ZIM), (4) *D.b. minor* (KNP), (5) *D.b. michaeli* (KEN). Population 6, *D.b. michaeli* (SA) is an admixture of the *D.b. michaeli* and the *D.b. minor* groups.

Table 10: Summary of demographic data for all animals included in this study including the animal identification code (ID Code), gender, birth and death dates, *D.b. michaeli* ancestry coefficient derived from pedigree data (Q_iPed) and STRUCTURE analysis (Q_iStr), the reported dam identification code (RDID), the confirmed dam (CDID) and sire (CSID) identification codes.

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
M001	Male	30/06/1951	04/02/1972	100.0				
M002	Female	30/06/1953	31/12/1978	100.0				
M003	Male	30/06/1951	09/06/1977	100.0				
M004	Female	30/06/1951	01/09/1970	100.0				
M008	Female	06/09/1963	11/03/1996	100.0		M002		M001
M009	Female	26/03/1964	30/06/1981	100.0		M004		M003
M010	Male	01/12/1965	01/05/1974	100.0		M002		M001
M011	Female	27/01/1968	01/08/1968	100.0		M004		M003
M012	Female	01/08/1968	09/04/1996	100.0		M002		M001
M013	Male	20/01/1970	01/03/1970	100.0		M004		M003
M014	Male	07/02/1972	18/02/1998	100.0		M008		M001
M015	Female	16/03/1972	30/06/1998	100.0		M009		M003
M016	Male	29/10/1972	30/06/2004	100.0	99.5	M002		M001
M017	Female	28/12/1974	18/02/1998	100.0		M009		M003
M018	Male	02/11/1975	01/05/2006	100.0		M002		M003
M019	Female	21/05/1977	30/06/1985	100.0		M009		M003
M024	Female	01/12/1978		50.0	61.3	M012		N020
M025	Male	16/09/1979	20/12/1985	50.0		M015		N021
M026	Female	28/01/1980	28/07/1983	50.0		M017		N022
M027	Male	21/02/1980	28/07/1983	50.0		M009		N020
M028	Female	07/08/1981		50.0	54.7	M008		N021
M029	Female	01/12/1982		50.0	70.5	M012		N020
M030	Male	16/12/1982	15/11/2014	50.0	74	M015		N021
M031	Male	28/12/1982	12/06/2014	50.0	64.4	M017		N020

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
M032	Male	07/08/1984	22/06/1993	100.0		M008		
M033	Male	21/12/1984	30/06/1993	100.0		M009		
M034	Female	01/01/1985		75.0	96.6	M024	M024	M016
M035	Female	16/04/1985	30/06/1993	100.0		M015		
M036	Female	30/06/1987		75.0		M028		
M037	Male	15/01/1988		100.0	99.4			
M038	Male	15/03/1988	26/10/1996	100.0		M012		
M039	Female	30/06/1988		75.0	99.4	M024	M024	M016
M040	Male	30/06/1988	15/06/2011	50.0	91.2	M017	M024	M030
M041	Female	30/06/1988	21/03/1995	100.0		M015		
M042	Female	15/04/1989	30/06/1994	100.0		M008		
M043	Female	15/03/1990		75.0	99	M029	M029	
M044	Female	15/09/1990		100.0	99.6	M024		M016
M045	Female	15/09/1990		75.0		M028		
M046	Female	15/05/1991		100.0		M017		
M047	Female	15/01/1992		100.0		M012		
M048	Male	15/03/1992		87.5		M036		
M049	Female	15/04/1992		75.0		M029		
M050	Male	15/06/1992	07/09/1997	75.0		M028		
M051	Male	30/06/1992		100.0		M008		
M052	Male	15/01/1993	03/12/1999	100.0		M015		
M053	Male	15/01/1994		87.5		M034		
M054	Male	15/02/1994	07/02/2016	100.0	95	M017		
M055	Female	15/02/1994		100.0		M012		
M056	Female	15/04/1994		75.0		M029		
M057	Male	15/01/1995		50.0	52.9	M028	M028	M031
M058	Male	15/02/1995		100.0		M008		
M059	Male	15/12/1995	10/03/2001	50.0	76.1	M034	M029	M030

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
M060	Female	15/02/1996	20/02/2005	100.0		M015		
M061	Female	15/03/1996		87.5		M036		
M062	Female	15/03/1996		87.5	99.5	M039	M039	M016
M063	Male	05/04/1996		100.0	99.5			
M064	Female	15/10/1996		100.0	95.3	M017		
M065	Female	15/12/1996		75.0	91.9	M024	M024	M016
M066	Male	01/01/1997		62.5	93.3	M043	M043	M031
M067	Female	15/01/1997		75.0		M028		
M068	Male	31/01/1997		62.5	95.4	M039	M039	M030
M069	Male	15/03/1997		62.5	96.2	M029	M034	M031
M070	Male	15/01/1998		100.0		M015		
M071	Female	15/06/1998		75.0	82.4	M024	M024	M016
M072	Male	15/01/1994		50.0	68.4	M024	M024	M031
M073	Male	15/02/1999		50.0	64.8	M029	M029	M030
M074	Male	15/08/1999		100.0	99.5	M043	M044	M037
M075	Male	15/08/1999	15/08/2001	87.5		M043		
M076	Female	01/02/2001	08/04/2004	87.5		M065		
M077	Male	15/02/2001		100.0	93.1	M044		
M078	Female	15/10/2001		75.0	99.1	M029	M029	M016
N020	Male	30/06/1967	30/05/1981	0.0				
N021	Male	30/06/1967	30/05/1981	0.0				
N022	Male	30/06/1967	15/12/1983	0.0				
T001	Male	01/01/1999	12/09/2000	87.5		M034	M034	
T002	Male	01/05/2000		50.0	72.5	M024	M028	M031
T003	Male	01/02/2001		50.0	53.7		M024	M031
T004	Male	01/07/2001		87.5	99.5	M034	M043	M037
T005	Male	01/07/2001		68.8	98.4		M062	M072
T006	Female	01/08/2001	23/11/2001	100.0		M064		

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
T007	Unknown	10/10/2002	01/12/2002	75.0		M028		
T008	Female	01/06/2003		87.5	99.4	M039	M039	M037
T009	Male	01/07/2003		87.5	97.7	M064	M034	
T010	Male	01/10/2003		75.0	68.8		M024	M037
T011	Male	01/01/2004		100.0	99.5	M044	M044	M063
T012	Female	01/01/2004		87.5	99.3		M034	M037
T014	Male	01/05/2004		93.8	99.6	M062	M062	M037
T015	Male	01/02/2005		56.3	27.6	M028	M028	M069
T016	Male	01/04/2005		87.5	97.3	M065	M065	M037
T018	Female	01/08/2005		56.3	89	M024	M024	M069
T019	Male	01/11/2005		87.5	99	M039	M039	M037
T020	Male	01/12/2005		81.3	99.1		Z002	M066
T021	Female	01/02/2006		100.0	98.3		M064	M063
T022	Male	01/04/2006	04/12/2012	87.5	98.4		M034	M037
T023	Male	01/04/2006		87.5	98.6	M062	M062	M069
T024	Male	01/11/2006		93.8	99.1		Z001	M066
T026	Female	01/04/2007		87.5	99	M039	M039	M037
T027	Male	01/05/2007		75.0	98.4	M029	M029	M037
T029	Unknown	01/07/2007	01/08/2007	75.0		M024		
T030	Male	01/04/2008		81.3	97.8		Z002	M066
T031	Male	01/05/2008		81.3	96.4	M064	M064	M068
T032	Female	01/05/2008		68.8	98.3	M062	M062	T002
T033	Male	01/05/2008		68.8	98.7	M078	M078	M069
T034	Female	01/11/2008		87.5	99.1	M024	M039	M037
T035	Female	01/12/2008		87.5	99	M065	M065	M074
T036	Female	01/12/2008		81.3	97.5		Z001	M066
T037	Female	01/12/2008	29/03/2012	87.5	96.3		M034	M063
T038	Female	01/03/2009		75.0	99	M029	M029	M074

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
T039	Female	01/03/2009		75.0	98.1	M039	M024	M074
T041	Male	01/05/2009		68.8	99.1		M043	M068
T042	Female	01/06/2009		100.0	99.7	M044	M044	M063
T043	Male	01/10/2009		56.3	87.9	M028	M028	M069
T044	Female	01/05/2010		81.3	98.7		Z002	M066
T045	Female	01/12/2010		81.3	91.8	M064	M064	M068
T046	Female	01/01/2011		68.8	94.8	M078	M078	M069
T047	Female	01/02/2011		87.5	99.3	M034	M034	M063
T048	Male	01/02/2011		62.5	95.5	M039	M039	M073
T049	Male	01/03/2011		75.0	88.9	M024	M024	M037
T050	Male	01/05/2011		87.5	97.3	M065	M065	M037
T051	Female	01/05/2011		75.0	92.9	M028	M028	M037
T052	Male	01/07/2011		100.0	99.7	M044	M044	M063
T053	Female	01/12/2011		75.0	95	M029	M029	M074
T054	Female	01/02/2012		87.5	89.8	M043	M043	
T055	Male	01/03/2012		75.0	98.5	T008	T008	M069
T056	Male	01/11/2012		100.0	99.5		Z001	M063
T057	Female	01/11/2012		75.0	99.4	M062	M062	M069
T058	Female	01/02/2013		53.1	50.8	T018	T018	T003
T059	Male	01/02/2013		62.5	75		Z002	M072
T060	Female	01/03/2013		84.4	93.7	M064	M064	T005
T061	Female	01/04/2013		84.4	99	M034	M034	T014
T062	Male	01/04/2013		75.0	68.4	M039	M039	T010
T064	Female	01/08/2013		75.0	70.5	M024	M024	M074
T065	Female	01/11/2013	08/05/2016	75.0	64	M065	M065	T010
T066	Male	01/03/2014		75.0	97.8	M029	M029	M074
T067	Female	01/04/2014		87.5	97	M078	M078	
T068	Female	01/04/2014		93.8	98.8	M028	M044	T016

ID Code	Gender	Birth Date	Death Date	QiPed	QiStr	RDID	CDID	CSID
T069	Female	01/06/2014		75.0	82.1	T026	M028	
T070	Female	01/07/2014		93.8	89.9	M062	M062	
T073	Female	01/12/2014	09/10/2016	93.8	99.5	T034	T034	M074
T074	Female	01/12/2014		75.0	98.3	T008	T008	M069
T075	Female	01/01/2015		93.8	99.2	T042	T042	T016
T076	Male	01/02/2015		84.4	84.9	T032	T032	
T077	Female	01/04/2015		62.5	65.8	T039	T039	T003
T078	Female	01/04/2015		71.9	98.1	T018	T018	T019
T079	Female	01/05/2015		68.8	77.5		M024	T019
T080	Male	01/06/2015		96.9	99.4		Z002	T024
T081	Male	01/06/2015		87.5	98.4	T038	T038	M063
T084	Male	01/02/2016		100.0	74.4	M064	M064	
T085	Male	01/02/2016		62.5	72		M078	M031
T086	Male	01/02/2016		87.5	98.6	M065	M065	
T087	Female	01/02/2016		84.4	99.4	M034	M034	T014
T088	Male	01/05/2016		93.8	99.2	T026	T026	M074
T089	Male	01/11/2016		68.8	80.3	M028	M028	T016
T090	Male	01/06/2017		68.8	99.1		T035	T003
T091	Male	01/06/2017		75.0	94.8		M029	M063
T092	Male	01/06/2017		87.5	98.3		T047	T023
T093	Male	01/06/2017		87.5	99.6		M062	T019
T094	Male	01/06/2017		81.3	99.1		Z001	M066
T095	Male	01/06/2017		96.9	99.6		M044	T014
T096	Male	01/06/2017		87.5	97.7		T039	T011
T097	Male	01/06/2013		75.0	92.8		M029	M063
T098	Female	15/11/2015		75.0	78.9		M039	T010
Z001	Female	01/09/1998		100.0	99.4			M018
Z002	Female	01/12/1998		100.0	99.7	M046	M046	

Table 11: The number of samples (N), number of alleles (Na), number of STR loci (Loci), number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosities and fixation index (F) determined for the populations from *D.b. michaeli* (SA-South Africa) , *D.b. bicornis* (NAM-Namibia), *D.b. michaeli* (KEN-Kenya), *D.b. minor* (KZN-KwaZulu-Natal), *D.b. minor* (ZIM-Zimbabwe) and *D.b. minor* (KNP-Kruger National Park).

Population	N	Loci	Na	Ne	Ho	He	F
***D.b. bicornis* (NAM)**	59	18	3.278	2.093	0.439	0.439	0.017
***D.b. minor* (KZN)**	100	18	2.500	1.838	0.377	0.390	0.032
***D.b. minor* (ZIM)**	100	18	4.056	2.322	0.487	0.501	0.029
***D.b. minor* (KNP)**	66	18	4.000	2.237	0.496	0.492	-0.012
***D.b. michaeli* (KEN)**	77	18	6.444	3.755	0.644	0.686	0.057
***D.b. michaeli* (SA)**	116	18	5.722	3.404	0.705	0.681	-0.038

Table 12: Population differentiation (F_{ST}) between the populations from the private game ranch *D.b. michaeli* (SA) (116), *D.b. bicornis* (NAM) (59), *D.b. michaeli* (KEN) (77), *D.b. minor* from KwaZulu-Natal (KZN) (100), Zimbabwe (ZIM) (100) and the Kruger National Park (KNP) (66).

	***D.b. bicornis* (NAM)**	***D.b. minor* (KZN)**	***D.b. minor* (ZIM)**	***D.b. minor* (KNP)**	***D.b. michaeli* (KEN)**
***D.b. minor* (KZN)**	0.144				
***D.b. minor* (ZIM)**	0.124	0.092			
***D.b. minor* (KNP)**	0.118	0.035	0.033		
***D.b. michaeli* (KEN)**	0.199	0.221	0.179	0.176	
***D.b. michaeli* (SA)**	0.169	0.172	0.146	0.133	0.037

Table 13: Summary of breeding performance statistics of cows with total number of calves, age at first calving (AFC in years and months), inter-calving period (ICP in years), inbreeding coefficient (*F*), individual heterozygosity (H_i) and *D.b. michaeli* ancestry coefficient derived from pedigree data (Q_iPed).

ID Code	Birth Date	Calves	AFC	ICP	*F*	H_i	Q_iPed
M002	30/06/1953	6	5y 0m	3.5			100
M004	30/06/1951	3	12y 8m	2.9			100
M008	06/09/1963	6	8y 5m	4.6			100
M009	26/03/1964	5	7y 11m	3.2			100
M012	01/08/1968	5	10y 4m	3.8			100
M015	16/03/1972	7	7y 6m	3.1			100
M017	28/12/1974	5	5y 1m	4.2			100
M024	01/12/1978	14	6y 1m	2.3	-0.0973	0.889	50
M028	07/08/1981	13	5y 10m	2.4	-0.1015	0.889	50
M029	01/12/1982	12	7y 3m	2.5	-0.1225	0.944	50
M034	01/01/1985	10	9y 0m	2.4	0.0484	0.611	75
M036	30/06/1987	2	4y 8m	4.0			75
M039	30/06/1988	9	7y 8m	2.5	-0.0411	0.833	75
M043	15/03/1990	5	6y 9m	3.8	0.0435	0.833	75
M044	15/09/1990	7	8y 11m	3.0	0.0455	0.778	100
M046	15/05/1991	1	7y 6m				100
M062	15/03/1996	7	5y 3m	2.7	0.2366	0.647	87.5
M064	15/10/1996	6	4y 9m	2.9	-0.0163	0.889	100
M065	15/12/1996	6	4y 1m	3.0	-0.136	1	75
M078	15/10/2001	4	6y 6m	2.6	0.0386	0.778	75
T008	01/06/2003	2	8y 9m	2.8	0.1108	0.556	87.5
T018	01/08/2005	2	7y 6m	2.2	0.0307	0.722	56.25
T026	01/04/2007	1	9y 1m		-0.0108	0.722	87.5
T032	01/05/2008	1	6y 9m		0.0632	0.765	68.75
T034	01/11/2008	1	6y 1m		0.1339	0.611	87.5
T035	01/12/2008	1	8y 6m		0.0512	0.722	87.5
T038	01/03/2009	1	6y 3m		0.1927	0.667	75
T039	01/03/2009	2	6y 1m	2.2	0.0556	0.778	75
T042	01/06/2009	1	5y 7m		0.1347	0.611	100
T047	01/02/2011	1	6y 4m		0.0011	0.778	87.5
Z001	01/09/1998	4	8y 2m	3.5	0.1665	0.611	100
Z002	01/12/1998	5	7y 0m	2.4	0.3061	0.556	100

Table 14: Summary of breeding performance statistics of bulls with total number of calves, age at first calving (AFC in years and months), age at last calving (ALC in years and months), inbreeding coefficient (*F*), individual heterozygosity (H_i) and *D.b. michaeli* ancestry coefficient derived from pedigree data (Q_iPed).

ID Code	Birth Date	Calves	AFC	ALC	*F*	H_i	Q_iPed
M001	30/06/1951	5	12y 2m	21y 3m			100
M003	30/06/1951	7	12y 8m	25y 10m			100
M016	29/10/1972	7	12y 2m	28y 11m	0.2097	0.588	100
M018	02/11/1975	1	22y 9m	22y 9m			100
M030	16/12/1982	4	5y 6m	16y 1m	-0.1374	1.000	50
M031	28/12/1982	7	11y 0m	33y 1m	-0.1374	1.000	50
M037	15/01/1988	15	11y 7m	23y 3m	0.1915	0.556	100
M063	05/04/1996	10	7y 8m	21y 1m	0.1397	0.667	100
M066	01/01/1997	6	8y 11m	20y 5m	0.0421	0.722	62.5
M068	31/01/1997	3	11y 3m	13y 10m	-0.0192	0.788	62.5
M069	15/03/1997	9	7y 10m	17y 8m	-0.0291	0.778	62.5
M072	15/01/1994	2	7y 5m	19y 0m	-0.0274	0.778	50
M073	15/02/1999	1	11y 11m	11y 11m	0.1311	0.556	50
M074	15/08/1999	8	9y 3m	16y 8m	0.0193	0.778	100
T002	01/05/2000	1	8y 0m	8y 0m	-0.1267	0.944	50
T003	01/02/2001	3	12y 0m	16y 4m	-0.0406	0.778	50
T005	01/07/2001	1	11y 8m	11y 8m	0.1887	0.667	68.75
T010	01/10/2003	3	9y 6m	12y 1m	-0.0459	0.722	75
T011	01/01/2004	1	13y 5m	13y 5m	0.1263	0.722	100
T014	01/05/2004	3	8y 11m	13y 1m	0.0205	0.667	93.75
T016	01/04/2005	3	9y 0m	11y 7m	-0.0486	0.778	87.5
T019	01/11/2005	3	9y 5m	11y 7m	-0.0002	0.667	87.5
T023	01/04/2006	1	11y 2m	11y 2m	0.0713	0.706	87.5
T024	01/11/2006	1	8y 7m	8y 7m	0.1757	0.765	93.75

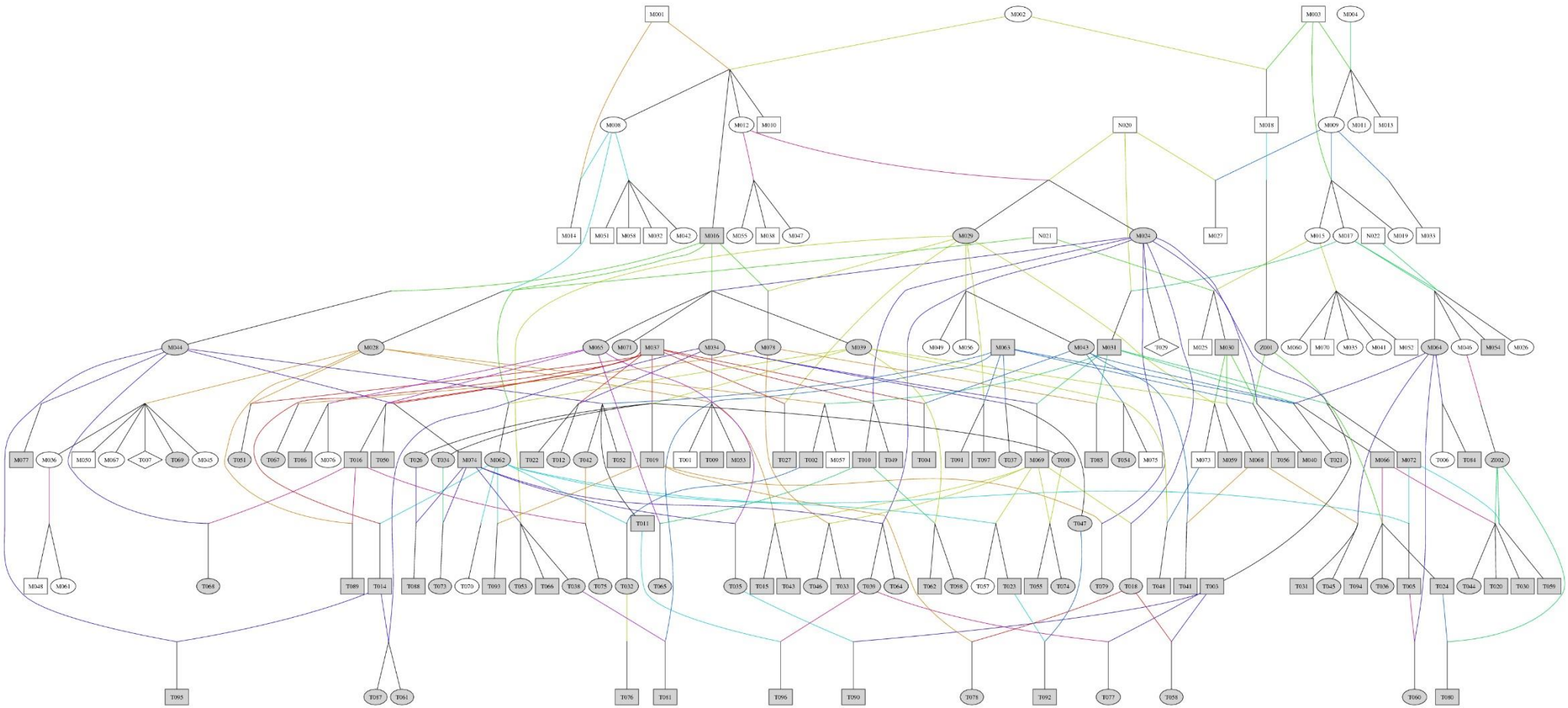


Figure 8: Pedigree of the AENP and *D.b. michaeli* (SA) populations with **sampled** individuals shaded. (Males are indicated by blocks and females by ovals, diamond shapes indicate that the gender is unknown)

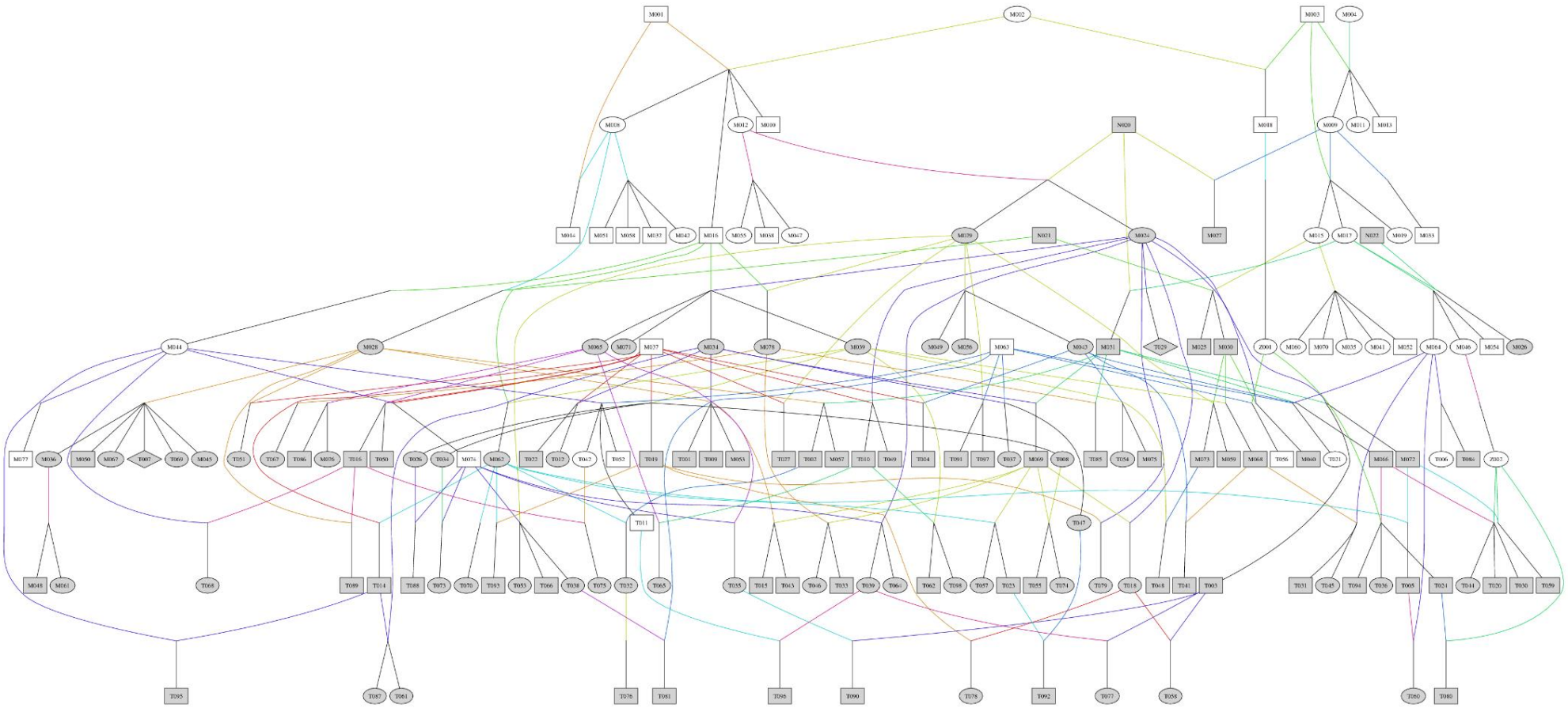


Figure 9: Pedigree of the AENP and *D.b. michaeli* (SA) populations with **admixed** individuals shaded. (Males are indicated by blocks and females by ovals, diamond shapes indicate that the gender is unknown)

5.4 Discussion

Historically the range of the black rhinoceros extended from the Cape, through southern Africa, Namibia, Angola, Botswana, Zimbabwe, Mozambique, Zambia, Malawi, Uganda, Tanzania, Kenya, Somalia, Ethiopia, Sudan, Chad, Central African Republic and North-Eastern Nigeria along the southern edge of the Sahara (Rookmaaker and Antoine, 2012) (Figure 2). The species included several subspecies classified according to skeletal differences and distribution ranges, of which the majority are now considered to be extinct (Groves and Grubb, 2011). The subspecies ranges most likely overlapped with individuals representing a gradient of phenotypic and adaptive characteristics occurring at the interface of geographically defined areas. The severe and rapid contraction of the black rhinoceros ranges created pockets of isolated populations where nominal to no natural exchange of genetic material occurred (Karsten et al., 2011, Kotzé et al., 2014, Muya et al., 2011, Van Coeverden de Groot et al., 2011).

In 1935 the black rhinoceros population in South Africa was less than 110 animals, all of the *D.b. minor* subspecies. These animals represented 0.1% of the African continental black rhinoceros population. By 2007 the number of black rhinoceros had increased to 1488 in South Africa representing 35% of the continental numbers and included all 3 the extant subspecies, *D.b. minor, D.b. bicornis* and *D.b. michaeli* (Knight and Kerley, 2009). The African Rhino Specialist Group (AfRSG) recognizes four subspecies of black rhinoceros based on geographical and ecological criteria; *D.b. minor* (Southern Central subspecies), *D.b. bicornis* (South Western subspecies), *D.b. michaeli* (Eastern subspecies) and *D.b. longipes* (Western subspecies), the latter declared extinct in 2011. Analyses of 518 black rhinoceros in this study and 883 black rhinoceros in Harper et al. (2018) supports this division of the extant *Diceros bicornis* species.

The *D.b. michaeli* animals that were introduced to the Addo Elephant National Park (AENP) in 1961 and 1962 included two animals that arrived in March 1961, a bull named JA (M001) and a cow named Brunni (M002) (Carter, 1965). The first observed mating between these animals occurred in August 1961 (Hall-Martin and Penzhorn, 1977). In January 1962 a further five animals were translocated to AENP, an adult bull named Darkie (M003), an adult cow named Ida (M004) with her subadult calf named Christopher and two subadult cows named Jenny and Marty. Marty was, in fact, the calf of Brunni and had been captured with her, but separated, until Marty's release with Brunni in February 1962. Marty was killed by Brunni shortly after release. Jenny and Christopher both died as a result of wounds obtained during fighting with Brunni and Ida, respectively. Thus, only four animals from Kenya survived to form the founder population of the *D.b. michaeli* in Addo, 2 males, JA and Darkie and 2 females, Brunni and Ida. Brunni produced 5 calves between 1963 and 1975 and Ida produced 3 calves

before she died in 1970. By 1977 there were 11 animals in AENP, 7 females, Brunni (M002) (~25 years old), Lucky Star (M008) (born in 1963 – 14 years), Doreen (M009) (born in 1964 – 13 years), Blom (M012) (born in 1968 – 9 years), Klein Ida (M015) (born in 1972 – 5 years), Slattery (M017) (born in 1974 – 3 years) and Vossie (M019) (born in 1977 – calf) and 4 males, Darkie (M003) (old bull), Jack (M016) (born in 1972 – 5 years), Rosenblum (M014) (born in 1972 – 5 years), and Gareth Edwards (M018) (born in 1975 – 2 years). The only remaining adult bull in the population, Darkie (M003), died in 1977 (Hall-Martin and Penzhorn, 1977). In June 1977, three *D.b. minor* bulls from Zululand were translocated to AENP to supplement the population. In 1980 the African Rhino Specialist group of the IUCN made the recommendation to remove the *D.b. minor* bulls and their offspring in order to ensure that the AENP population remained pure *D.b. michaeli*. Two bulls were removed in 1981. The third, which was castrated in 1979 due to the fact that it had 1 ear, remained in the AENP and was shot in 1984 after observations that it attempted to mate with the *D.b. michaeli* cows (Hall-Martin, 1984). In 1983 apparent offspring of the *D.b. minor* bulls were removed based on estimated date of birth and skin appearance. At that stage there were 8 calves born after the introduction of the *D.b. minor* bulls, 3 of which were conceived after their arrival. The 3 calves (identified as 1979Klein Ida (M025), 1980Doreen (M027) and 1980Slattery (M026) in Table 10) were moved to the National Zoological Gardens in Pretoria and a *D.b. michaeli* cow that originated in Kenya was translocated from the zoo to the AENP. This cow died within 3 months of arrival (Hall-Martin, 1984). By 1984, there were 15 animals in the AENP, which included the 1 castrated *D.b. minor* bull. In 1986, the population had increased to 17 (Hall-Martin, 1986). The majority of animals were translocated to the private game ranch (27 animals) between 1998 and 2003, 8 animals went to Mkomazi Game Reserve in Tanzania between 1997 and 2001 and 2 animals went to the Ngorogoro Crater in Tanzania, in 1998 (Knight and Kerley, 2009).

Indication of the historic admixture of the *D.b. michaeli* population translocated from Addo to the private game ranch, shown in Figure 7, suggest that not all presumed offspring of the *D.b. minor* bulls were removed. This admixture can be traced to specific individuals brought to the private game ranch from Addo. M024 (Q_iStr = 61.3), the female born in December 1978 to Blom (M012), was not identified as a potential offspring of a Natal bull, although her birth date was approximately 18 months after the arrival date of the 3 *D.b. minor* bulls in June 1977. From the STRUCTURE results it is clear that she is one of the animals that carried the *D.b. minor* signature to the private game ranch. M029, M030 and M031 were born in December 1982, apparently conceived after the removal of the 2 uncastrated *D.b. minor* bulls, however, they are admixed. M028, born in August 1981 managed to avoid identification as a Natal bull offspring and was also translocated to the private game ranch. The *D.b. minor* signature in the private game ranch population, therefore, originated from 5 animals, 3 females and 2 males.

Table 10 includes the reported dam (RDID) information and also the confirmed maternities (CDID) using genotyping information. Incorrect dam assignment, based on observational data occurred in 11 out of 101 cases (>10%). Both dam and sire were genetically confirmed for 95 offspring. The majority of individuals in the private game ranch pedigree have been sampled and parentage and admixture confirmed using the genotyping data (Figure 8). There is generally a large discrepancy between the individual ancestry coefficients (Q_iStr) calculated with STRUCTURE and the individual ancestry values calculated using the pedigree data (Q_iPed), particularly with increasing admixture. Individuals with lower admixture values (>68% *D.b. michaeli*), show a greater correlation between Q_iStr and Q_iPed, with STRUCTURE consistently overestimating the purity of the individual in all but 4 cases. Individuals with greater levels of admixture (between 50% and 68% *D.b. michaeli*) showed more variability in terms of the Q_iStr and Q_iPed values with no clear correlation, with STRUCTURE again overestimating the *D.b. michaeli* contribution in all but 2 cases (Table 10).

STRUCTURE provides a preliminary approach for identifying crude admixture in a population, but it is apparent from this data that it should not be used to guide decisions on management and removal of admixed or "hybrid" animals by utilising the Qi value as a so-called purity threshold. Verified parentage data provides a more consistent and accurate estimate of admixture particularly with increasing back-crosses over successive generations. This is particularly important in the absence of very large numbers of unlinked markers. Porras-Hurtado *et.al* (Porras-Hurtado et al., 2013) cautioned that Bayesian methods of population assignment are limited for small sample sizes and when a small number of genetic markers is used. Further that linkage disequilibrium between markers must be minimal, particularly with STRs. In 1997, Boecklen and Howard (Boecklen and Howard, 1997), also noted that one only needs a few markers to identify recent hybridization and F1 hybrids but that the number of markers required to identify advanced back-crosses and more specifically the level of purity of these back-crossed animals, increases significantly and may be in excess of 70 independent markers.

The private game ranch population also presents with greater genetic diversity than the three *D.b. minor* populations in Zimbabwe, KwaZulu-Natal and the Kruger National Park and the *D.b. bicornis* in Namibia. The number of alleles identified and observed heterozygosity of the population in the private game ranch (5.722 and 0.705) is similar to that of the *D.b. michaeli* population in Kenya (6.444 and 0.644) and markedly greater than the number of alleles identified and observed heterozygosity of the black rhinoceros population in KwaZulu-Natal (2.500 and 0.377) (Table 11).

The between population differentiation (F_{ST}) values in Table 12 show that there is very little differentiation between the private game ranch population and the *D.b. michaeli* in Kenya (0.037) and

that the greatest divergence exists between the *D.b. michaeli* and *D.b. minor* in KwaZulu-Natal (0.221). In discussing the concept of genetic rescue, Whiteley et al. (2015) indicated that the aim of improving genetic diversity through admixture, was to avoid inbreeding depression. From Table 12 it is clear that the 2 subspecies that are most divergent in this study still have very low divergence values and performed exceptionally well in terms of reproductive success following an admixture event.

The extensive sampling of this population permitted the verification of the pedigree over 5 generations of black rhinoceros using the RhODIS® DNA profiling data and identify the origin of the admixture detected by the Bayesian clustering program STRUCTURE. The verified pedigree information also provided accurate ancestry coefficients with limited STR marker information compared to the STRUCTURE values to support future selection of individuals for translocation based on their ancestry estimates. The stringency level applied to the classification of individuals can vary depending on factors including how endangered the species is. A very stringent threshold is considered to be more than 0.95 (van Wyk et al., 2013). In May 2017, 18 black rhinoceros were translocated from the private game ranch to Akagera National Park in Rwanda, returning the species to the area following an absence of 10 years. Taking into account the critically endangered status of the species and subspecies, the IUCN African Rhino Specialist Group recommended selecting animals with a Q_iStr of 0.85 and higher *D.b. michaeli* ancestry to provide a selection of founders for a newly translocated population with sufficient variability (Richard Emslie, personal communication, 2018). The translocation has proven to be very successful with the first calf born within 4 months of arrival, in August 2017 (https://www.africanparks.org/newsroom/press-releases/first-wild-rhino-born-rwanda-over-decade). The success of the translocation would support the view that the admixture did not negatively affect the adaptability and reproductive potential of these animals.

The breeding performance of the female animals on the private game ranch has been noteworthy and is shown in Table 13. The 3 female animals that were sired by the *D.b. minor* bulls in the AENP have produced a total of 39 calves between them with an average inter-calving period of 2.4 years. On the private game ranch, 5 bulls have produced the majority of calves, showing that these data should be utilized to manage bull selection and removal, to avoid inbreeding and maintain genetic diversity in the population. DNA samples of all the translocated animals have been stored and will be used to support pedigree verification, monitor breeding performance and support future management decisions once the established population reaches carrying capacity and can be used for further range expansion.

This study affirms the value of the RhODIS® program to verify a pedigree and identify subspecies admixture in a black rhinoceros population and provide the ancestry coefficients from a Bayesian

clustering analysis and directly from the pedigree. The verified pedigree information enabled an assessment of the reproductive fitness of the population and these data have informed management decisions relating to the successful establishment of a new black rhinoceros population in a former range state. The RhODIS® data, therefore, provides an effective management tool to support rhinoceros breeding programs as the focus on the species shifts towards increasing the reproductive success and population growth rates to counteract the progressive population decline caused by an escalation in rhinoceros poaching across the African continent.

CHAPTER 6

6. General conclusion

Rhinoceros poaching has increased steadily since 2008 with more than 8000 rhinoceros poached across the African continent between 2006 and 2017. Rhinoceros are killed for their horns which are trafficked through the various levels of organized crime syndicates to the final destination of the horn in, mainly Asian countries, where the horn is used in Traditional Chinese Medicine (TCM) and more recently, carved into high value items or sold as status symbols. Forensic tools, including DNA, have been used extensively to support the investigation of crime and increasingly in the investigation of wildlife crime. Prior to 2009 (Harper et al., 2013), no method successfully obtained nuclear DNA from rhinoceros horn in order to link the horn to an animal by individual DNA profiling. The development of this method, therefore, proved to be a game changing tool in the protection of rhinoceros, by providing forensic evidence that could link poachers and traffickers directly to specific crime scenes. The method has been shown to be robust and repeatable, providing complete DNA profiles from as little as 0.1 mg of horn powder. The selection of a set of 23 STR markers that co-amplified in a multiplex PCR test with a gender marker provides good quality DNA profiling data that not only uniquely identifies rhinoceros products, but also identifies the species and sex of the animal from which the sample has been collected.

RhODIS® (The Rhino DNA Index System) was established in 2010 and stores DNA profile information from rhinoceros on a secure database. The RhODIS® database has expanded to include more than 50 000 individual items, including samples from live rhinoceros, rhinoceros horns and items related to poaching incidents submitted from all the African rhinoceros range states. A DNA profile match can only be considered proof of the identity of an individual when the relative rarity of the profile can be established using a representative population DNA database of the species in question. The size and scope of the RhODIS® database, including samples from various rhinoceros populations across Africa and representing all three extant black rhinoceros subspecies and the southern white rhinoceros subspecies, provides a powerful statistical support for matches between items in rhinoceros criminal cases. RhODIS® data has been used in more than 150 rhinoceros cases and assisted in successful prosecutions and in support of aggravation of sentencing, not only in South Africa, but also in other African range states including Namibia, Kenya and Swaziland. Over 6000 rhinoceros criminal cases have been received by the VGL since 2010. Several of these cases included DNA matches and aided successful prosecution and sentencing of criminals. Nine of these cases are presented in this manuscript to illustrate the application of the tool and acceptance by the courts not only in South Africa but in other African countries.

The RhODIS® program has included the development and field application of specialized forensic sampling kits used to collect samples from rhinoceros carcasses, live rhinoceros animals and rhinoceros horns stored in stockpiles. The RhODIS® program also launched an electronic data collection app to be used in conjunction with the sample collection kits to effectively collect, handle and store data from field sampling. The app was developed as part of the program.

The inclusion in national legislation of the requirement to sample all live rhinoceros, killed rhinoceros and rhinoceros horn in South Africa for DNA profiling and inclusion onto the RhODIS® database endorsed the tool as an effective method to ensure traceability of rhinoceros and rhinoceros products. A further recommendation by CITES that samples must be collected from international seizures also supported the usefulness of the RhODIS® program at an international level.

A large sample set of 3085 white rhinoceros and 883 black rhinoceros from three recognized subspecies were genotyped using the 23 STR loci and the loci characterized to identify sex linkage, linkage disequilibrium, monomorphic loci and other characteristics of the markers that could affect their suitability as forensic markers. These data were collected for each of the African rhinoceros species and subspecies. The data was analyzed using a Bayesian clustering method to confirm the subdivision of the black rhinoceros into three subspecies *D.b. bicornis*, *D.b. michaeli* and *D.b. minor* with an additional partition evident between the *D.b. minor* subspecies originating in Kwazulu-Natal (South Africa), Zimbabwe, and a third group that are an admixture of these two groups in the Kruger National Park, South Africa. The classification of unknown samples of black rhinoceros into a subspecies based on this analyses has assisted in tracing the origin of the unknown sample when the specific match was not available.

The RhODIS® data has not only been used a forensic tool but has become a management tool for individual rhinoceros population management. RhODIS® data was used to identify historic admixture in a single extra-limital population of *D.b.michaeli* in South Africa, to verify the pedigree of this population and trace the origin and introgression of the admixture through 5 generations.

The RhODIS® sample bank, DNA profile database and test methodologies provide the opportunity to support a number of future research projects, including the development of additional marker systems for forensic and population management purposes, genome sequencing and re-sequencing and mitochondrial DNA studies to investigate black rhinoceros subspecies and individuals carrying unique and valuable genetic types. These data can be used as a meta-population management tool to inform translocation decisions and the establishment of new rhinoceros populations and to support a comparative study to investigate the relationship between different rhinoceros populations in Africa

to provide a review of the current understanding of the rhinoceros species and subspecies classification. Additional opportunities exist to investigate the use and value of the RhODIS® data as forensic evidence in rhinoceros cases by following cases from sampling through reporting to conclusion in court. The project has grown into a self-sustaining program and an inventory of African rhinoceros and rhinoceros horns throughout the continent.

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APPENDIX 1: Animal sampling kit form

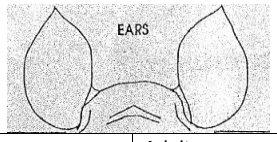
Veterinary Genetics Laboratory

RhODIS Animal Sample Collection Form

University of Pretoria

RhODIS Kit number	VGLA044792	Date Collected	
		CAS Number (Poaching)	Police Station: CAS

Animal Information

Animal ID / Name	**Ear Notches (from front)**	**Microchip nr 1 (Body)**	Placement
			
		Microchip nr 2 (Body)	Placement

Age	Old	Adult	Subadult	Juvenile	Calf
Sex	Male	Female			
Species	White	Black	**Subspecies**		

Horn Information (If horns are removed – please complete a separate horn form for each horn and follow the instructions provided on that form for handling each horn)

Front Horn	**Microchip Number**			Placement		Sample collected	
	Circumference (cm)		Length front (cm)		Length Back (cm)		
Back Horn	**Microchip Number**			Placement		Sample collected	
	Circumference (cm)		Length front (cm)		Length Back (cm)		

Sample Information

Type	**Number of each sample type**		**Ear Tag Nr**
Blood		Other sample/s	
Hair			
Tissue		**Translocated**	
Horn		**From:**	**To:**

	Outer Bag seal intact		**Hunted**	
Outer Bag number Kit	**VGLA044792**		**Poaching** case	
Inner Bag number Kit	**VGLB044792**		**Live** rhino	
Permit number			**Dehorned**	

Owner Information

Owner	Farm	Tel number	Cell number	Email address

Area Information

Area / Town	**Coordinates**		**Province**
	Lat (S)	long (E)	
Additional information			**Additional Information**
Veterinarian name (the veterinarian signature is not required unless he/she is the person taking / responsible for the samples – in that case the veterinarian must act as authorized person and sign accordingly)			
Authorized Person name (if not veterinarian)	Authorized Person signature	Date	

APPENDIX 2: Horn sampling kit form

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Rhinoceros Horn sample collection Form

University of Pretoria

Horn ID		Date	
		Permit Number	
Seal Bag Number			

Horn Information							
Front Horn		Dehorned		Confiscated		Stockpile	
		Trophy		Mounted		Unmounted	

Length front (cm)	Length Back (cm)	Circumference base (cm)	Base front to back Base left to right (cm)	Weight (kg)
Image taken (L) - left	Image taken (R) - right	Image taken (C) - base	Image taken (F) – front	Image taken (B) - back

Microchip Number		
Microchip Placement Position	Horn sample collected for DNA	RhODIS Kit number animal information DEHORNING / Microchip number of animal if previously sampled

Back Horn		Dehorned		Confiscated		Stockpile	
		Trophy		Mounted		Unmounted	

Length front (cm)	Length Back (cm)	Circumference base (cm)	Base front to back Base left to right (cm)	Weight (kg)
Image taken (L) - left	Image taken (R) - right	Image taken (C) - base	Image taken (F) - front	Image taken (B) - back

Microchip Number		
Microchip Placement Position	Horn sample collected for DNA	RhODIS Kit number animal information DEHORNING / Microchip number of animal if previously sampled

Owner Information				
Owner / Investigating officer (designation)	Area and Province	Tel number	Cell number	Email address

Additional information Information		
How was the horn obtained	Approximate age of the horn at sampling (if known	
Taxidermist Name	Taxidermist contact details	
Sample collectors name and designation	Sample collectors signature	Date

APPENDIX 3: Animal sampling kit instructions

RhODIS Animal Kit: DNA Sample Collection Guidelines

Post Mortem Sample collection:

1. Take a photograph of the sealed bag next to the carcass before opening the bag.
2. Use one sampling kit per animal. Open sealed bag and spread it on the ground to provide a clean surface to work on.
3. Remove the white bucket in kits that include the white bucket. Open the bucket and remove the contents placing them on the opened bag. In the case of kits that do not contain the white bucket, place contents of the kit on the opened bag.
4. Put on the gloves provided.
5. Collect a full thickness skin sample from the ear or alternatively any skin as far from the internal organs as possible and not contaminated with insect, bird or other environmental products. Best is to use a thin skin section such as ear or tip of the tail, facial skin or skin folds or on the limbs not more than 1cm thick. Collect a piece 2cm² using the disposable scalpel and blade provided.
6. Place in the screwcap sample container with coarse salt.
7. A second skin sample can also be collected as back-up.
8. If a carcass is a few days old and rotting always select skin that has not been exposed to extensive enzymatic degradation including areas that feel soapy and spongy. Rather collect from dry areas.
9. If a carcass is a few weeks old and only dry skin is available, select a thinner piece 2cm² - it may be necessary to use a hacksaw to collect these samples. This is acceptable as long as the blade is exposed to cleaning with bleach for at least a minute before it is used on a next carcass.
10. If only bones remain at a very old carcass, these may be collected – a rib or mandible, long bone head and short piece of the bone shaft or vertebrae. Please ensure that the piece of bone fits into the ziplock bag provided and do not submit whole bones or skulls to the laboratory. Please note that this sample may not provide a complete DNA profile in every case. If a toenail is available at an old scene, this is the best sample to collect and provides better results than bone. Collect the whole toenail in the ziplock bag provided.
11. DO NOT collect blood from a carcass and DO NOT collect hair from a carcass. Avoid soapy and spongy muscle or other tissue in a rotting carcass.
12. Each sample container is pre-labelled with the RhODIS Kit number.
13. Take a photograph of each sample with the kit number visible next to the place of collection.
14. Ensure that all sample bottles are closed and replaced in the white bucket and reseal the bucket with the new lid provided. Or if the kit does not contain the bucket, place all samples, the receipt bag and used equipment, including the gloves directly into the new forensic bag supplied.
15. Seal the new outer bag and take a photograph next to the carcass.
16. Complete the sampling form in full and place in the pouch on the new outer bag. The pouch can be sealed immediately or sealed later if a copy or additional information is needed but must be sealed before shipment to the laboratory. If there is more than one carcass at a scene PLEASE ensure that each is clearly identified on the bag as A, B or C to ensure that a profile is linked to a specific carcass. Please write on the bag the required case information with permanent marker.
17. Make sure that the bag is kept cool after collection and refrigerated if kept for less than a week before shipment to the laboratory or frozen at -20°C if kept for longer.

Live Sample collection:

1. Use one sampling kit per animal. Open sealed bag and spread it on the ground to provide a clean surface to work on.
2. Collect one blood sample in the labelled tube provided. Collect a tissue sample if the animal is being notched in the container with coarse salt. If notching is not taking place then this sample can be omitted. Collect tail hairs by plucking to ensure that hair roots are collected. Collect at least 10 hairs and confirm the presence of roots before placing in the long plastic tube provided.
3. Ensure that all sample bottles are closed and replaced in the new outer bag and seal the bag next to the animal.
4. Complete the sampling form in full and place in the pouch on the new outer bag. The pouch can be sealed immediately or sealed later if a copy or additional information is needed but must be sealed before shipment to the laboratory.
5. Make sure that the bag is kept cool after collection and refrigerated if kept for less than a week before shipment to the laboratory or frozen at -20°C if kept for longer.
6. If an animal has been sampled previously and is being dehorned, please add the animal microchip number to the horn identification form when the horn is sampled and identified. It is not necessary to resample the animal.
7. If an animal is being translocated and has been sampled previously please confirm the microchip number and add to the form. Collect only a hair sample in these cases and CLEARLY identify the bag with “RESAMPLE” so that the laboratory does not re-test the same animal multiple times.
8. If an animal is being resampled and the microchip cannot be found or has failed then collect samples as usual and add the new microchip number to the form.

Contact details for further information:

Cindy Harper / Claudette van Zyl / Chantelle Schutte / Amy Clarke - Veterinary Genetics Laboratory, Faculty of Veterinary Science, University of Pretoria, Onderstepoort.
Email: vgl@up.ac.za ; Contact number: 012 529 8240
Courier to: Veterinary Genetics Laboratory; Faculty of Veterinary Science; University of Pretoria; Soutpan Road; Onderstepoort; 0110 **Or delivered by hand.** Proof of delivery and receipt by a VGL staff member is required.

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Animal Ethics Committee

PROJECT TITLE	**Development and implementation of a DNA index system for the forensic analysis of African rhinoceros related crimes**
PROJECT NUMBER	**V054-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. C K Harper**

STUDENT NUMBER (where applicable)	**UP_2519305**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

CONDITIONS

1) The Lab needs to have an assigned information officer in compliance with the Promotion of Access to Information Act 2 of 2000 (PROATIA).

2) Use of the data for research purposes needs to be delinked from any identifying information as the above act.

ANIMAL SPECIES	**Bio bank samples**	
NUMBER OF ANIMALS	**To be reported**	
Approval period to use animals for research/testing purposes		**May 2016 –May 2017**
SUPERVISOR	**Prof. P Thompson**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 15 June 2016
CHAIRMAN: UP Animal Ethics Committee	Signature