Parentage analysis in a free-ranging, closed population of southern white rhinoceros: genetics, pedigrees and management

by

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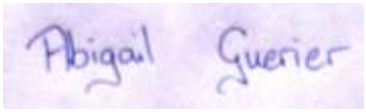
I, ……  ……., declare that the thesis/dissertation, which I hereby submit for the degree ……**Master of Science**……at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Long may the free rhino live!

SUMMARY

Small populations of animals are vulnerable to the consequences of breeding within a closed group – inbreeding depression and genetic drift lead to reductions in genetic variability, which in turn can give rise to the amplification of deleterious traits. Traditionally, managers attempt to minimise these effects by controlling the genetic structure via a manipulation of the paternal line, in the case of rhinos usually by translocation of breeding and sub-adult bulls. This strategy depends on having access to detailed pedigree data, and, in particular, some knowledge of the parentage of the offspring within the population. This information is particularly difficult to obtain in long-lived, free-ranging populations (particularly for rare and endangered species) where the identity of the father must be inferred from behavioural observations, and often the identity of the mother cannot be determined once juveniles have dispersed.

In this thesis I present the results of a study to determine the parentage within a free-ranging, enclosed population of southern white rhinoceros (*Ceratotherium simum simum*) located on Ongava Game Reserve (OGR) in the north of Namibia. I used genetic techniques to obtain a genotype for each animal in the population, and then used detailed reserve management records from the period 1993-2009 to constrain the statistical process of parentage assignment. Using these different methods, I was able to assign both parents for 22 of 23 offspring with 80% confidence (16 of 23 at 95% confidence, mother only in 1 of 23), making this study the first to successfully complete a comprehensive parentage analysis in a free-ranging population of southern white rhinoceros. The key to the success of this study was a combination of accurate pedigree data and a complete set of genotype data.

The parentage assignments allowed me to construct a complete lineage diagram for each of the founder matrilines, and further analyse the status and reproductive success of the population. OGR's southern white rhinoceros population is expanding at close to 14%, well over the expected maximum growth rate for rhinoceros metapopulations (9%). The mean inter-calf interval is about 2.2 years and average age at first parturition is 6 years, indicating good fecundity. Conception is strongly seasonal, occurring mainly (89%) during the rainy season. The current management practice is to replace dominant bulls after their breeding tenure, and also to remove all young bulls from the population before they reach breeding age (with the aim to restrict potential inbreeding). Only one calf of seven in the F_2 generation is

inbred. My analysis indicates that, at least in the 2006 cohort of eight calves, founder females bred only with the founder male, while offspring females (F_1 generation) bred only with introduced males. This suggests some form of mate selection leading to inbreeding avoidance within the population. Finally, there is some evidence that certain matrilines exhibit/experience different reproductive potential (daughters in one matriline exhibit longer inter-calving intervals and male bias in calf birth sex ratios). These details indicate that genetic data provide valuable information for management.

When reviewing management decisions to date, I found that one of the founder bulls was the more successful in terms of calves sired (10 of 13). Management had, however, selected the other founder bull for removal by sale based on the assumption that he was behaviourally dominant and territorial and therefore likely to have been more successful at breeding. I also found that introduced bulls were breeding successfully before they appeared to have established territories. Thus these findings challenge the assumption that male white rhinoceros reproductive success is related to dominant, territorial behaviour.

I conclude that in order to optimally manage small, free-ranging enclosed populations of southern white rhinoceros it is essential to have reliable and accurate pedigree data (this includes a methodology for identifying individual rhino), as well as genetic data for the entire population. I recommend that conservation management programmes for rhinoceros populations incorporate both genetic and demographic data. This will allow for the development of white rhinoceros population management strategies that attempt to optimize genetic diversity and population health, and benefit the establishment of new, robust populations. Translocations of animals are an important aspect of meta-population management of rhinoceros and data which provides accurate insight into the true mating system and reproductive success within a population allows for the correct selection of individuals for this process.

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CHAPTER 1: GENERAL INTRODUCTION

The rhinoceros is one of the most well-known endangered mammals and is considered a flagship species for conservation world-wide (Emslie & Brooks, 1999). There are five recognized, extant species of rhino: the white rhinoceros (*Ceratotherium simum*), the black rhinoceros (*Diceros bicornis*), the Sumatran (*Dicerorhinus sumatrensis*), the Indian rhinoceros (*Rhinoceros unicornis*) and the Javan rhinoceros (*Rhinoceros sondaicus*) (Skinner & Chimimba, 2005). All rhinoceros taxa are under threat and all but 1 taxon (*Ceratotherium simum simum*) are endangered (Emslie & Brooks, 1999; Emslie, 2004).

The white rhinoceros formerly inhabited large parts of southern, central, east and west Africa (See Figure 1.1). At present white rhinoceros only occur in small, fragmented and protected parts of southern and east Africa (International Rhino Foundation, 2010). This drastic reduction in numbers was a direct result of excessive hunting and/or poaching for rhinoceros horn as well as severe habitat destruction caused by increasing numbers of settlers and human encroachment (Emslie & Brooks, 1999; Skinner & Chimimba, 2005). There are two recognized sub-species of the white rhinoceros; the southern *Ceratotherium simum simum* and northern *Ceratotherium simum cottoni.* Recent evidence, however, advocates the elevation of *C.s. cottoni* to a full species (Groves et al. 2010) although this has not yet been implemented and is still under review (IUCN Red List, 2012). The northern white rhino is thought to be extinct in the wild (with the last live sighting in the Democratic Republic of Congo in 2006, Emslie, 2011) and is currently classified as Critically Endangered in the IUCN Red List of Threatened Species. The last four potentially breeding northern white rhinoceros were moved from the Dvur Kralove Zoo in the Czech Republic to a private sanctuary in Kenya in 2009 in a final attempt to encourage these animals to breed (Emslie, 2011). This small effective founder number essentially means that the continuation of this subspecies is highly unlikely to be viable and as a result in an attempt to preserve as many northern white rhinoceros genes as possible these animals will likely be bred/inter-crossed with southern white rhinoceros (Emslie, 2011).

The southern white rhinoceros is a renowned conservation success story, having literally been brought back from the brink of extinction nearly a century ago. In the late 1880's there was a

remaining, surviving population of less than fifty animals in Natal, South Africa (Emslie & Brooks, 1999; Skinner & Chimimba, 2005). The proclamation of the Natal Reserves in 1895 (also referred to as Hluhluwe and Umfolozi Game Reserves) and subsequent protection afforded this population the chance of recovery and prevented the extinction of this species (Rookmaaker, 2000; Skinner & Chimimba, 2005). The advancement of rhinoceros capture techniques led to the translocation of animals from the Natal Reserves to form new populations elsewhere (Skinner & Chimimba, 2005). The majority of animals were translocated to other areas within South Africa and in addition small groups were re-introduced into Botswana, Namibia, Mozambique, Swaziland and Zimbabwe. Animals were also translocated into areas outside their historical range such as Zambia, Uganda and Kenya (Emslie & Brooks, 1999; Skinner & Chimimba, 2005) although subfossil evidence suggests that rhinoceros formerly did occur in Kenya (IUCN, 2012). Today, thanks to extreme protection and conservation efforts, there are approximately 20,000 animals in Africa (IUCN, 2012) and this rhinoceros has been re-classified as 'Near Threatened' in the IUCN Red List of Threatened Species (from 'Lower risk / conservation dependent' in 1996). The majority of white rhino currently occur in only four countries – South Africa, Namibia, Zimbabwe and Kenya with South Africa as the major stronghold for this species (IUCN data, 2012).

Namibia holds, amongst others, one 'Key 2' and three 'Important' populations of southern white rhinoceros (rated using AfRSG (African Rhinoceros Specialist Group) criteria, Emslie & Brooks, 1999; Emslie, 2008). White rhinoceros may be privately owned in Namibia and there are several private reserves and sanctuaries, as well as government parks, which host and maintain small populations of southern white rhino. As a result, Namibia now holds 2% of the remaining southern white rhinoceros population (Emslie & Brooks, 1999). Namibia has endorsed one of the management policies that the Southern African Rhinoceros Management Group has recommended, which is the establishment of smaller, supervised populations of rhinoceros across a number of reserves and parks, effectively creating a metapopulation. This is intended to minimize the risk of a catastrophic event eradicating an entire segment of the larger population, to maintain genetic diversity across subspecies and to support range expansion for this species (Emslie & du Toit, 2006). A definitive aspect of metapopulation management is the exchange of genetic material between subpopulations which are geographically separated, thereby ensuring that collectively they amount to a larger, single, genetically viable population (du Toit, 2006).

Maintaining genetic diversity is a key concern of conservation biologists, particularly in small isolated groups of animals, as is experienced by the current distribution of rhino (Emslie & Brooks, 1999; Frankham et al. 2002; Heller et al. 2010). According to Van Dyke (2003), the five principal genetic threats facing small populations are inbreeding depression, loss of genetic variation and reduction in heterozygosity, accumulation of harmful mutations, introgression and hybridisation with larger populations, and outbreeding depression. Inbreeding depression refers to the loss of fitness (reduction in fecundity or survival) as a result of inbreeding within the population (Frankham et al. 2002; Van Dyke, 2003). The loss of genetic variation refers to the decrease in the number and range of alleles found in a population which can result in a reduced ability for/of? that population to adapt to changing environments thus potentially compromising their survival (Frankham et al. 2002; Van Dyke, 2003). The accumulation of harmful mutations refers to the increased fixation of deleterious mutations in a population which in turn lead to a loss of fitness (Frankham et al. 2002; Van Dyke, 2003). Introgression refers to the incorporation of genetic material from one species into that of another (Frankham et al. 2002; Van Dyke, 2003). It has the potential to compromise the genetic integrity of the species affected. Outbreeding depression is the decline in fitness that can/may occur when individuals breed with unrelated or only distantly-related individuals (Van Dyke 2003). As genetic diversity is an essential component for adaptive evolution, its loss via genetic drift and/or selection decreases the probability of any population persisting through time (Frankham, 2005). Small populations that exhibit random mating are particularly susceptible to the loss of diversity via enhanced genetic drift as well as potential inbreeding; in turn this increases the risk of further losses of both adaptive and neutral genetic diversity (Frankham et al. 2002; Spielman et al. 2004). At the individual level, loss of genetic diversity via inbreeding is conclusively linked to reduced reproductive fitness; mating between related individuals or individuals with similar genetic background leads to short-term inbreeding depression in virtually all species studied thus far, both in captivity and in the wild (cf. Keller & Waller, 2002; Charlesworth & Willis, 2009). Inbreeding depression reflects the deleterious consequences of increased homozygosity at loci that affect fitness, either by permitting the expression of recessive, deleterious alleles or by reducing heterozygote advantage (Frankham et al. 2002). Numerous examples of correlations between heterozygosity and fitness are available in the literature (see Szulkin et al. 2010). However, there is a lack of comparable data in the rhinoceros taxa (particularly *in situ*, Linklater, 2003), due to their characteristic long generation times, limiting the feasibility of multigenerational studies, coupled with the difficulty of collecting long-term life-history data from free-ranging

populations. Inbreeding is considered an inevitable consequence of small population size (Frankham et al. 2002). In addition, a number of recent studies highlight the central role of drift in isolated populations of large mammals (Haag et al. 2010; Heller et al. 2010). Thus in species where dispersal is no longer possible and the effective population size is minimal, management informed by the breeding structure of each population is therefore critical. Minimizing founder effects, inbreeding and further loss of genetic diversity in isolated populations, and in particular those descended from small founder numbers, is a major challenge for conservation managers.

Genetic diversity varies amongst extant rhinoceros species, with southern white rhinoceros exhibiting particularly low levels of variability within the group in terms of both heterozygosity and allelic richness (Kellner et al. 2001; Florescu et al. 2003; Scott, 2008; Coutts, 2009). It has been suggested that these low levels of diversity are not the result of recent bottlenecks (Scott, 2008; Coutts, 2009) but are more likely the consequence of evolutionary events. Irrespective of the contributing factors (effects of bottleneck, small founder effects, drift and inbreeding associated with small populations), this low genetic diversity needs to be considered when developing future management and conservation strategies for this species. Successful conservation strategies need to incorporate both demographic and genetic factors (Spielman et al. 2004; du Toit, 2006). The meta-population management strategy currently recommended for African rhinoceros species (Emslie & Brooks, 1999; du Toit, 2006) incorporates translocations of animals as a means of establishing new populations, supplementing or re-stocking existing populations and exchange of genetic material to prevent localised inbreeding depressions. In addition, Linklater et al (2012) suggest that achieving successful translocation of rhinoceros is not as complicated as formerly believed and that they are likely to be robust to ecological challenges on introduction and as such are suitable candidates for crisis management. Therefore, the use of translocation as an active management tool for rhinoceros populations is likely to continue. And therefore accurate data that contributes to the best selection of these animals is essential. To date, the selection of rhinoceros (individuals or groups) for translocation has been performed with minimal genetic consideration or concern regarding meta-population structure (Swart & Ferguson, 1997; Linklater, 2003; Braude & Templeton, 2009; Muya et al. 2011). This may be because appropriate data are not available for many populations or that anthropogenic and demographic threats may often be of more immediate concern and translocation decisions are dictated by crisis situations (Braude & Templeton, 2009).

The translocation of animals has the potential to disrupt the social structure of a population which could ultimately lead to decreased productivity and growth (for example through home range shifts and resulting conflict between animals; as seen for black rhinoceros in Reid et al. 2007, Patton et al. 2008 in Weladji & Laflamme-Mayer, 2011). Thus, existing population structure and knowledge of social systems should be considered before translocations occur. In addition to the anthropogenic threats and genetic challenges that face small populations of rhinoceros, they are also particularly vulnerable to demographic and environmental stochasticity (Emslie & Brooks, 1999; Frankham et al. 2002). The metapopulation management approach requires reliable demographic data on the performance and status in these populations which can be used for biological decision making (Emslie & Brooks, 1999; Emslie & du Toit, 2006).

Ongava Game Reserve (OGR, located in Northern Namibia, See Fig. 2) is home to a successful breeding, free-ranging, enclosed population of southern white rhinoceros. This population (rated 'Important 1' using AfRSG criteria) was founded in 1993 and was already producing second-generation calves by 2004, thus providing an ideal study group to assess the value of breeding structure data in the management of small populations. Traditionally, populations of white rhinoceros such as these have been managed by controlling the sex ratio of adult individuals (keeping a limited amount of breeding males) and employing an exchange (or sales) programme for dominant breeding males (i.e. at such time when a bull could mate with its own offspring he will be exchanged for a bull from a different reserve or part of the country) (Bothma, 2002). Mating success and identification of breeding bulls is commonly determined through behavioural observations and monitoring of the population in the field.

However, molecular analysis of parentage in an increasing number of species has revealed that the observed social mating system in natural populations is not always supported by genetic parentage assignment (as reviewed in Bishop et al. 2004). It appears that in many instances the so-called 'dominant' or expected male is not always the father, with one (or a number) of the other males having successfully mated with a number of females (cf Avise et al. 2002; Westneat & Stewart, 2003). This has been found to be the case for a wide range of species (birds, rodents, primates, reviewed in Bishop et al. 2004), and is likely to be exacerbated in species such as the rhinoceros where males establish and defend well-defined home ranges. Owen-Smith (1971) observed that white rhinoceros exhibit a social system

based on a clearly delineated mosaic of exclusive adult male territories, with bulls occupying territories for several years and these territorial males exhibit specialised techniques of defecation and urination (dung scattering and spray urination) to define their territories (cows, non-territorial males and immature animals do not exhibit these characteristics). Solitary adult males (males without their own territories – either previously deposed bulls or young males which have not yet been able to establish their own territories) may reside within dominant male territories (Shrader & Owen-Smith, 2002). While these subsidiary bulls are tolerated (often amicably) by the territorial bull, Owen-Smith (1971) reports that a strict dominance-subordinance relationship exists between the two. The author concludes that the 'territoriality of the white rhinoceros may thus be described as a system for ordering specifically reproductive competition among males' (i.e. a bull without a territory forgoes the opportunity to reproduce).

In white rhinoceros, field-based studies suggest that sexually receptive females associate primarily with dominant territorial males (Rachlow et al. 1999). However females have overlapping home ranges that can include the territories of a number of mature males, potentially leading to numerous mating opportunities (Owen-Smith 1988; Rachlow et al. 1999). When a territorial bull encounters a cow in oestrous he enters into a consort relationship with her which may last up to 3 weeks and during which time he attempts to keep her within his territory, but he is not always successful at this (Owen-Smith, 1971; Owen-Smith, 1988). Subsidiary bulls do not associate with cows for any length of time and while they may remain nearby when a cow is being mated by the territorial bull they do not interfere (Owen-Smith, 1971). As it is very difficult to observe the actual mating events for any one female (and in any case, a female may mate with more than one male) it is not possible to confidently ascribe successful breeding to a particular individual male. A final complication for attempting to assign parentage using behavioural observations for rhinoceros is that the historical records for the populations are often not explicit about the maternity assignments either – typically rhinoceros calves mature and disperse from their mothers before having their identifying marks applied, and subsequently become 'anonymous'.

Hence unambiguous information on parentage is essential in order to verify lineages used in genetic management and to study the impact of inbreeding (Frankham et al. 2002). As a result, molecular data are required to evaluate both maternal and paternal parentage within the population. A reliable method for obtaining this information is through the analyses of genetic

profiles for individual animals. Microsatellites, which are a variable number of short tandem repeats found in DNA, are rapidly becoming the preferred marker for population studies such as this (Bruford & Wayne, 1993; Frankham et al. 2002; Jones & Ardren, 2003). They have advantages over other markers such as RAPDs (Random Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism) or SNPs (Single Nucleotide Polymorphism) as they provide much higher levels of polymorphism per locus and hence greater resolution (Wiseman et al. 2000; Frankham et al. 2002; Van Dyke, 2003). Microsatellite markers are routinely being used to investigate the genetic and population structuring of natural populations (Balloux & Lugon-Moullin, 2002). Numerous studies have successfully employed microsatellites to investigate a range of questions in natural populations including paternity and maternity queries, mating systems and social structure, inbreeding and hybridisation, relatedness and reproductive skews (Swart & Ferguson, 1997; Alderson et al. 1999; Banks et al. 2003; Piggott & Taylor, 2003; Bishop et al. 2004; Hampton et al. 2004; Lorenzen & Siegismund, 2004 but see Dakin & Avise, 2004). Studies such as these have been performed across a wide range of different species from fish and birds to various mammals such as grey seals - ***Halichoerus grypus*****,** mole rats - ***Cryptomys hottentotus*****,** African wild dogs - ***Lycaon pictus***, grey wolves – *Canis lupus*, bears - *Ursus maritimus*, elephant – *Loxodonta africana* and rhinoceros (Paetkau et al. 1995; Alderson et al. 1999; Ellegren, 1999; Norris et al. 2000; Garnier et al. 2001; Comstock et al. 2002; Gautschi et al. 2003; Bishop et al. 2004; Twiss et al. 2006; Moueix, 2006; Coutts, 2009).

In this study I present a complete parentage analysis of the white rhinoceros population on OGR. I use genetic information from 11 microsatellite loci to derive genotypes for 31 individual rhinoceros, and combine this information with comprehensive pedigree data construct an accurate family tree for the 6 founders, 2 introduced bulls and their 23 offspring. In addition, I use this data to analyse inbreeding and mate selection within the population and evaluate population performance indicators. Finally, I present an interpretation of the status of the population from a management perspective.

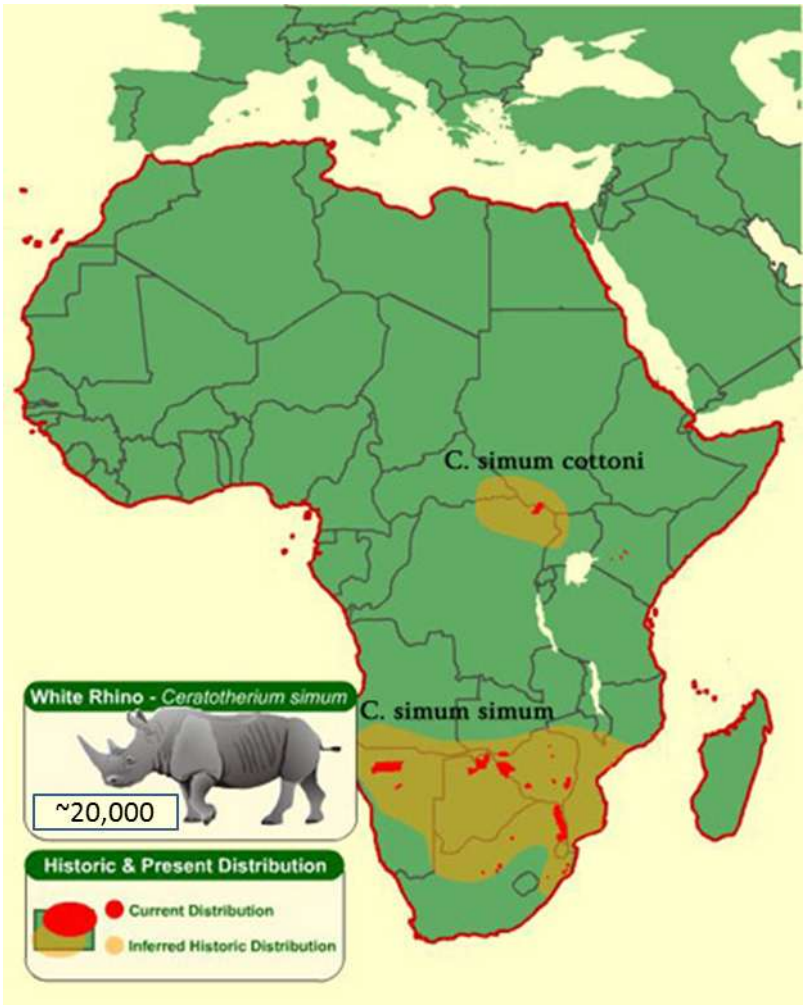

Figure 1.1 *White rhinoceros distribution* – Map showing historic and present distribution of white rhino in Africa. Current number of white rhino indicated is approximately 20 000 (IUCN Red List, 2012).Original image courtesy of International Rhino Foundation.

Study Site

This study was conducted on Ongava Game Reserve (OGR) in northern Namibia. The reserve is located immediately to the south of Etosha National Park (ENP), with a common boundary on OGR's north side and covers an area of approximately 300km^2 in size (see Figure 1.2). The boundary between OGR and ENP is permeable to carnivores and small animals such as warthog, but not medium and large-sized herbivores. All other fences are game-proof and electrified and border on commercial farmland to the east and west. The southern boundary is shared between commercial farmland and another wildlife reserve.

OGR was established in 1990 following the purchase of four adjacent farms previously used for commercial livestock farming. Internal fencing was removed between (and within) the

farms and the area was steadily restocked with wildlife species in order to convert it into a wildlife reserve for use as a tourism destination for photographic safaris. There had been extensive grazing on the farms and the veld is still in recovery, as evidenced by a preponderance of primary and intermediate grasses. The habitat is termed Karstveld, with vegetation primarily (up to 70%) *Colophospermum mopane* shrub and woodland, with some areas savannah-like (about 35%). A ridge of dolomite hills runs east – west close to the north boundary, with *Terminalia* and *Acacia* species providing some differentiation of habitat (Berry & Loutit, 2002). Mostly the substrate of the reserve is composed of loams and dolomite sands; there are, however, some smaller areas of the reserve which have a red, sandy soil as their substrate (Mendelsohn et al. 2000). There are several natural water dams on the reserve, although most of these only contain water during the rainy season (December to April). Most of the water supplies are via artificial waterholes, which are opened in rotation to encourage grazing and browsing across the reserve. The weather zone for the reserve is typical for semi-arid northern Namibia, with an average annual rainfall of 380mm. At the end of this study period in 2009, OGR had a population of 34 southern white rhinoceros, consisting of 2 adult bulls, 13 adult females, of which 11 have calves, 2 sub-adult females, and 6 sub-adult males. The population of white rhinoceros on OGR was established with the introduction of 6 animals (2 adult bulls and 4 adult females) from the Hluhluwe and Umfolozi Game Reserves in 1993.

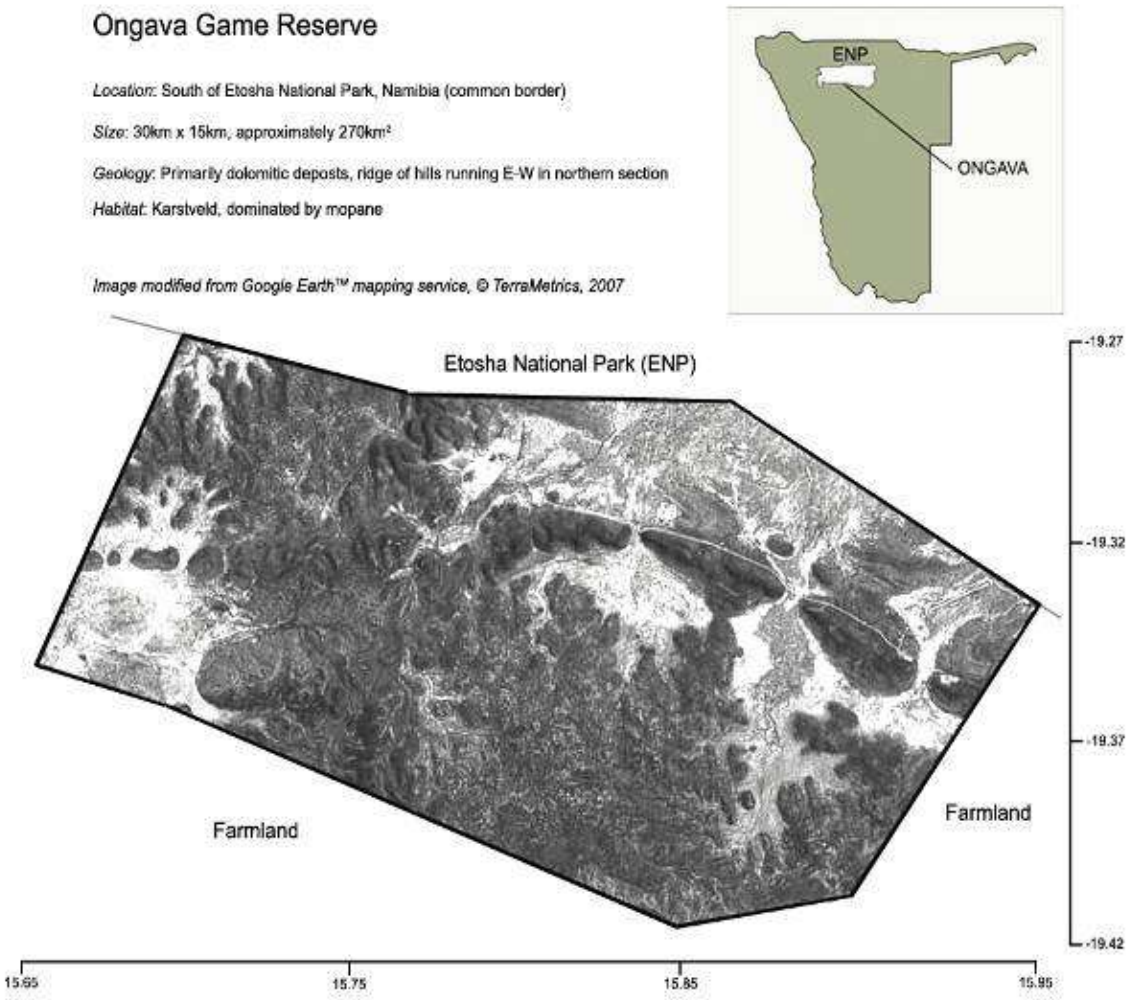

Figure 1.2 *Study Site* - Modified Google Earth image of Ongava Game Reserve (OGR). Lighter areas indicate plains-like habitat, hills are shown in dark relief. Fences to farmland (east, south and west) are electrified, boundary with Etosha National Park (ENP) is permeable to small herbivores and carnivores but not to rhinoceros. *Insert* – Location of OGR in Namibia, immediately to the south of ENP.

CHAPTER 2: GENETICS

2.1. INTRODUCTION

Research in conservation and evolutionary biology has been aided by the development of high-resolution molecular techniques for population genetic analyses (Ellegren, 1999; Frankham et al. 2002; Selkoe & Toonen, 2006). Some methods reveal higher levels of genetic diversity and provide greater accuracy for use in conservation biology (Frankham et al. 2002), such that choosing the appropriate molecular technique for a specific research project is important. The mode of inheritance is a significant determinant of the utility of a technique (Frankham et al. 2002). For example, in parentage studies, methods using co-dominant inheritance markers are particularly valuable.

Microsatellite markers/loci are non-coding, tandem repeats of short DNA segments typically less than five bases in length (Sutherland, 2000; Frankham et al. 2002; Florescu et al. 2003) present in abundance in the nuclear genomes of eukaryotes (Müller et al. 1999; Florescu et al. 2003). Microsatellites are characterized by higher mutation rates than other neutral regions of DNA (Levinson & Gutman, 1987; Blouin et al. 1996; Selkoe & Toonen, 2006). Polymerase slippage during DNA replication causes these variations in repeat sequence length within the microsatellites which can then be amplified using Polymerase Chain Reaction (PCR) and thus used for DNA profiling (Schlötterer & Pemberton, 1998). Microsatellite markers reveal much higher levels of genetic diversity per locus than allozymes (Frankham et al. 2002) and are co-dominantly inherited (Bruford & Wayne, 1993; Frankham et al. 2002) The co-dominant inheritance, high levels of polymorphism (Li et al. 2002), potential for cross-species application and relative ease with which suitable microsatellites can be obtained, make them a preferred technique for use in parentage studies (Frankham et al. 2002; Florescu et al. 2003; Scott, 2008; Jones et al. 2010). Microsatellites have many applications and have been used in a range of disciplines including (but not limited to) conservation genetics/biology, evolutionary biology, population genetics, genetic mapping, medical genetics and forensics (Goldstein & Schlötterer, 1999). The use of microsatellite analysis has been extensively applied in the field of animal genetics including studies on parentage (Goosens et al. 1998; Schnabel et al. 2000; Garnier et al. 2001; Bishop et al. 2004), population structure (Goldstein et al. 1999), inbreeding and relatedness (Blouin et al. 1996; Ellegren, 1999; Norris et al. 2000;

Gautschi et al. 2003), and genetic diversity and differentiation studies (Paetkau et al. 1995; Scott, 2008; Haag et al. 2010).

Another advantage is that as PCR based analyses are very sensitive they can be performed using minute sample quantities thus allowing for non-invasive or minimally invasive sampling methods, an important consideration when dealing with endangered species (Bruford & Wayne, 1993; Frankham et al. 2002; Fernando et al. 2003) and also allows for the use of forensic or ancient (such as museum specimens) samples (Bruford & Wayne, 1993; Selkoe & Toonen, 2006). Sampling considerations are important to take into account when studying endangered or elusive or cryptic species (Creel et al. 2003) and samples that are non-invasively or minimally invasively collected are preferred. The three sample collection methods used by field biologists as described in Taberlet et al (1999) are 'destructive sampling' where the animal is killed in order to obtain tissues/samples, 'non-destructive sampling' where typically the animal is captured and a blood or tissue sample is obtained invasively (some methods do not require the animal to be captured and a sample can be obtained remotely, i.e. through the use of a biopsy dart. I will refer to this method as minimally invasive) and the third method is 'non-invasive sampling' which is where the source of the DNA is left behind by the animal and can be collected without catching or disturbing the animal (for example faeces). Whilst non-invasive samples are ideal there are potential limitations to their uses; they are more sensitive to contamination than other samples (such as blood or tissue), these samples can result in low quality and/or low quantity DNA or poor extract quality (presence of PCR inhibitors) which can result in genotyping errors (Taberlet et al. 1999). Genotyping errors can include allelic dropout, null alleles and incorrect scoring, therefore approaches such as pilot studies, rigorous methodology and duplication as well as software programmes used to quantify and detect errors should be employed to ensure confidence in results obtained from non-invasive samples (Taberlet et al. 1999; Jones et al. 2010).

Microsatellite primers are developed either by direct isolation of species-specific markers through the use of a genomic library or by the optimization of markers originally isolated from related species (Florescu et al. 2003; Moueix, 2006). Microsatellite markers have been developed and/or optimized for both African and Asian rhinoceros species (Brown & Houlden, 1999; Cunningham et al. 1999; Florescu et al. 2003; Zschokke et al. 2003; Scott et al. 2004; Nielsen et al. 2008). Limited studies in a particular (or across) rhinoceros taxa have

been conducted which have made use of microsatellite analysis specifically and include mainly genetic variability and differentiation studies as well as investigations on effects of bottlenecks on populations, evaluation of evolutionary relationships between taxa, parentage and reproductive system investigations, population estimates and a reported case of hydbridisation between species (Cunningham et al. 2001; O'Ryan et al. 2001; Steyn & Stalmans, 2004; Harley et al. 2005; Robinson et al. 2005; Scott 2008; Coutts 2009; Kim, 2009; Van Coeverden de Groot, 2011). Research such as this makes a vital contribution to our understanding of endangered rhinoceros biology which allows *in situ* and *ex situ* conservation efforts to be improved upon and directed accordingly.

In this chapter I aim to collect a complete set of samples for all individuals in a free-ranging population of southern white rhinoceros using both non-invasive and minimally invasive methods. I then aim to create individual genotype profiles for each of the samples collected using 11 microsatellite loci which can then be used for parentage analysis.

2.2. METHODS

2.2.1. Sample collection

Faecal Samples

The collection of faecal samples involved several steps; the detection of rhinoceros tracks/spoor, the tracking of tracks/spoor until the rhinoceros was located, the identification of the individual animals located, if a sample was required from the identified rhinoceros then that animal was followed until defecation was observed and the final step was then the collection of the faecal sample provided that no local contamination was evident (see below).

All tracking was performed under the supervision and guidance of OGR's Anti-Poaching Unit (APU). Tracking was undertaken in areas where we expected to find white rhinoceros (i.e. waterholes, or areas which were known to be frequented by white rhino). Typically tracks would be found at waterholes or crossing roads and if these were fresh they would be followed until rhinoceros were found. Identification of rhinoceros was through a pattern of ear notches which indicate the assigned individual number. Regular rhinoceros tracking expeditions were undertaken in order to obtain samples. Tracking of rhinoceros occurred over a period of 31 months, beginning in October 2004 through to May 2007. Typically I would

accompany the APU out on patrols twice a day, 3 to 4 times a week. This frequency depended on circumstances such as weather, availability on patrols, etc. At times it was as often 6 days a week whilst during other times it was only once or twice a week. Rhinoceros tracking expeditions varied in their format but generally we would start tracking fresh spoor until we located the rhinoceros (or group) upon which we would record all details (ID, location, time, group, etc.) and attempt to obtain photographs. On most trips we successfully found rhinoceros but not necessarily a candidate rhinoceros (i.e. one for which we still required a sample). If there were candidate rhinoceros in the group or if a lone candidate rhinoceros had been located then we would follow and remain (at a distance) with the rhinoceros until defecation occurred or, more typically, until the rhinoceros caught scent of us and moved off. If the rhinoceros moved off in a sedate manner we would continue following the animals, whereas if they were clearly disturbed we would no longer continue tracking them and tracking was abandoned. Upon leaving a sighting we would continue to look for fresh spoor elsewhere and the process would be repeated. Tracking a single individual typically took between 0.5-3 hours. We spent between 1 and 4 hours with a candidate rhinoceros waiting for samples. In some cases if fresh undisturbed dung was encountered, I took samples and then we back-tracked to locate the rhinoceros. These samples were retained only if we could assign an unambiguous ID to the animal.

Dung which had been scraped by the rhinoceros or distributed across or close to a midden was not considered suitable for collection. Samples were only collected when the dung pile was estimated at being less than 6 hours old and had not yet begun to dry. Samples were obtained by peeling off (using separate gloves for individual dung heaps) small portions of the outer layers of each dung bolus. These collections were combined to fill a 50ml tube and then covered with 90% ethanol. Samples were kept at ambient temperature in the field and then subsequently taken back to the lab and refrigerated at 4-6 ºC until DNA extractions could be performed.

Tissue Samples - Ear notch samples

Collection of ear notch tissue samples occurred during the annually scheduled earmarking and micro-chipping procedures of individual rhinoceros for identification purposes. The rhinoceros were located by the OGR manager and darted by an attending veterinarian from a helicopter. The drug regime used during darting consisted of a mixture of Etorphine (also known as M99) and Azaperone in order to anaesthetise the animal. Once the animal was

safely immobilized the vet proceeded to insert the microchips into the horns and cut out the specific notches from the ears. These ear parts where then directly collected for tissue samples. Once all procedures (ear notching, microchipping and body measurements taken) were completed the rhinoceros was revived. The reversal (antidote) drug regime consisted of a mixture of Diprenorphine (also known as M5050) and Naltrexone injected sub-cutaneously. Tissue samples were placed in 50ml tubes and covered with 90% ethanol. Tissue samples were kept at ambient temperature in field before being transferred back to the lab and stored in the refrigerator at 4-6 ºC until DNA extraction could be performed.

Tissue samples from ear notches from a total of 14 rhinoceros were collected during OGR's annual rhinoceros marking / notching programme in the period 2005 - 2009

Tissue Samples - Biopsy samples

For the rhinoceros for which ear notch tissues samples were not be available (either because they were not scheduled for earmarking or had been earmarked previously elsewhere), biopsy darting was used to obtain tissue samples. I explored a number of methods to obtain such samples in the least invasive manner. I found that this required the development of new methods of biopsy darting using a CO_2 – powered dart gun. (Conventionally, biopsy darting of thick-skinned animals, such as elephant and rhino, is performed by using a Cap-chur or Pneudart rifle - a modified shotgun with small charge - which I considered too disruptive for these animals).

I used a Dan-inject rifle (model JM Special) with telescopic sight, loaded with 20mm biopsy needles attached to 3ml darts. When I used the biopsy darts and needles without any modification, I found that on occasion the dart penetrated the rhinoceros but fell out without removing a core of tissue. I modified the systems by inserting a dental barb (Maillefer 21mm coarse barbed broach) into the biopsy needle. The darts were also filled with water to weigh them down to both allow them to fall out easily after penetration, and also make them more aerodynamic. Needles were attached onto darts using superglue to prevent any dislodging from the dart during the darting process. Once a rhinoceros had been sighted, darts and needles were sterilized by rinsing with 99% ethanol shortly before loading into the rifle.

Rhinos were darted at night at a number of the artificial waterholes on the reserve. I waited at waterholes between 7pm and 11pm since that is the time at which rhinoceros most often drink

(pers. comm. OGR manager). Darting typically occurred at a distance of about 10 – 25m. Animals were darted in either the front or hind legs only, since I found that the flexible skin in these positions allows the dart to fall as soon as the limb is moved. After the dart had penetrated and fallen out of the animal, I waited until the rhinoceros had moved off on its own accord (i.e. finished drinking) before collecting the dart and obtaining the sample. The biopsy needle was removed from the dart and the entire needle was placed in a tube containing 90% ethanol. This tube was stored at ambient temperature before being transferred back to the lab. At the lab the biopsy sample was carefully removed from the needle and barb and placed in a 50ml tube containing 90% ethanol and then stored in the refrigerator at 4-6 ºC until DNA extraction could be performed.

I collected a total of 17 biopsy samples over a period of 5 months from August 2007 to January 2008. After this set had been obtained, one individual needed to be re-sampled. This individual, Jeff, was particularly shy and difficult to locate. This 18th sample was only successfully obtained in October 2008. In four cases I was able to obtain a biopsy sample from a calf while it was still with its mother (i.e. before being pushed off) and then also later obtain an ear tissue sample for this same animal.

Tissue Samples - Trophy mount sample

One of the founder male rhinoceros (Bob) was sold from the Reserve in 2004. This individual was subsequently shot by trophy hunters, and a trophy was prepared from the head. I was able to contact the owner of the trophy, who agreed to supply a small piece if skin from the mount. This sample had been chemically preserved and dried as part of the treatment process, and was subsequently stored at room temperature in a paper envelope.

2.2.2. DNA extraction

DNA extraction from faecal samples

DNA from faecal samples was extracted using the Qiagen QIAamp DNA Stool Mini Kit (Cat. No. 51504). Typically, 3g of dung sample was lysed overnight using 30ml of Lysis Buffer ASL. The rest of the extraction was routinely carried out following the kit Protocol for DNA Isolation from Larger Amounts of Stool. Eluted DNA was stored in Storage Buffer AE under refrigeration of 4-6ºC. DNA was eluted in 200µl aliquots (and was later concentrated down to 100µl or 50µl where necessary).

DNA extraction from tissue samples

DNA from tissue samples (both ear notch and biopsy samples) was extracted using the Qiagen DNeasy Tissue Kit (Cat. No. 69504) following the Protocol for Purification of Total DNA from Animal Tissues. The recommended sample size (25 mg) was typically lysed overnight using double the quantities of Lysis Buffer ATL and Proteinase K solutions in order to achieve complete lysis of tissue. Consequently double quantities of Buffer AL and ethanol were used in subsequent processing. The rest of the protocol was carried out routinely. Eluted DNA was stored in Storage Buffer AE under refrigeration of 4-6ºC. DNA was eluted in 2 batches of 200µl aliquots which were either combined or stored separately.

DNA extraction from trophy mount sample

DNA from the sample obtained from the trophy mount was extracted in the scat laboratory at the Cheetah Conservation Fund (CCF) under sterile conditions. Preserved samples are expected to yield degraded DNA and thus this DNA would be particularly sensitive. This was the first rhinoceros sample to be processed in that laboratory, therefore I was able to minimise the possibility of cross-contamination. DNA from the trophy mount sample was extracted using the Qiagen DNeasy Tissue Kit (Cat. No. 69504). I attempted several modifications to the protocols in an attempt to maximize the chances of recovering DNA. Three extractions were performed in parallel to increase chances of success, and one extraction blank was included in order to test for contamination that may have happened during the extraction. A first extraction was performed following the protocol; the concentration of the DNA aliquot was increased through evaporation. A second extraction was performed with the following modifications: an additional wash step with Buffer AW1 in order to eliminate salts present in preserved skin (according to a recommendation from Qiagen customer service) and the DNA of the 3 extractions performed in parallel was eluted with the same elution buffer aliquot, thereby collecting the majority of DNA from 3 extractions performed in parallel in a total of 100µl. This step was repeated with a second aliquot of 100µl in order to obtain a maximum yield of DNA, and both elutions were used for PCR.

2.2.3. DNA visualization and assessment

The presence of DNA in extracted samples was established by submitting samples to gel electrophoresis (Gel XL Ultra V2, Labnet) through a 1.5% agarose gel in TBE buffer at 70V

for approximately 2 hours. DNA was visualized by adding ethidium bromide (which fluoresces under UV light) to sample extracts before running on gel. Gel was then viewed using UV light (UVIview UV Transilluminator, UVItec BXT 20M) to give a rough indication of quality and quantity of DNA present.

At the University of Cape Town (UCT) I used a 'nanodrop' spectrophotometer to obtain DNA concentration values. These were subsequently used for estimating amounts of template used in PCR. Concentration values for the DNA extracted from trophy sample 'Bob' were obtained from the spectrophotometer (Beckman DU 650) at CCF, no gel electrophoresis was performed due to the insufficient concentration of DNA; a test spectrophotometer run performed on the second extraction confirmed this as no DNA was detectable using this method.

2.2.4. Transportation of DNA samples

Samples which were taken to UCT were dried down before transportation. This was achieved by heating the tubes on a hot block set to a temperature of 60°C. Lids of tubes were left open to allow for evaporation. Each sample was dried until no liquid could be seen in the tube and then removed. Upon arrival at UCT these samples were re-suspended using purified, DNA-free water and then refrigerated.

Samples which were taken to CCF were not dried down, and were simply placed in a cooler box for transportation. Upon arrival samples were stored in refrigerators at 4-6ºC.

2.2.5 Polymerase Chain Reactions

The microsatellite primers used for this study were selected in collaboration with Drs. O'Ryan and Bishop at UCT, based on work being undertaken in their laboratory by Natalie Coutts (reported in Coutts, 2009). The primer details are shown in Table 2.1. At UCT, primers were provided by the Evolutionary Genetics Laboratory, while those we used at CCF were manufactured to specification by the Synthetic DNA Laboratory at UCT.

Locus	Repeat motif/microsatellite motif	Primer pair sequence (5'-3')	Reference
7B	$(TG)_{16}$	F: CCTCTGTGATTAAGCAAGGC R: ATGAACAGGAAGGAAGACGC	Florescu et al (2003)
7C	$(CT)_{14}(AT)_{11}$	F: TGAACTCTGATGGAAATGAG R: AACAGGTCTTGATTAGTGC	Florescu et al (2003) adapted by Coutts (2009)
DB1	$(CA)_{14}$	F: AGATAATAATAGGACCCTGCTCCC R: GAGGGTTTATTGTGAATGAGGC	Brown & Houlden (1999)
DB44	$(CA)_4G(CA)_{16}$	F: GGTGGAATGTCAAGTAGCGG R: CTTGTTGCCCCATCCCTG	Brown & Houlden (1999)
DB49	$(CA)_{14}$	F: GTCAGGCATTGGCAGGAAG R: CAGGGTAAGTGGGGGTGC	Brown & Houlden (1999)
BR6	$(CA)_{15}$	F: TCATTTCTTTGTTCCCCATAGCAC R: AGCAATATCCACGATATGTGAAGG	Cunningham et al (1999)
Rh7	$(TG)_{17}$	F: CCGTCACATATGACAGTGTGC R: GGGCAGCTTATGCTCAAGTC	Zschokke et al *(*2003)
Rh8	$(TG)_{22}(AG)_2ANACA(GA)_{28}CA(GA)_3CA(GA)_9CG(TA)_5$	F: ACACACCTTTATAAACAATATGGTCAC R: AGTCTCTAGTCAAAAGGGATTGC	Zschokke et al *(*2003)
Rh9	$(TG)_4TT(TG)_{17}TA\ (TG)_5$	F: TCTGGTACCACCAAATGTAGC R: ACGATTACGTCTTTCAGTTGC	Zschokke et al *(*2003)
WR1 / AY138542	$(AC)_{18}$	F: GGCAAAACTAAGAGAACTTG R: GATACCAAACTGGAAATGG	Nielsen et al (2008) redesigned Florescu et al. (2003)
WR2 / AY138545	$(TA)_8(CA)_4$	F: ACAGCTAGAATCACCAAAAC R: TCCTGCTGCATAAATCTC	Nielsen et al (2008) redesigned from Florescu et al (2003)

Table 2.1 *Primer details* – Specification for each of the 11 microsatellite loci used in this study.

'F' refers to the sequence of the forward primer while 'R' refers to the sequence of the reverse primer.

University of Cape Town

PCR amplification of samples was performed in thin-walled 0.2ml PCR tubes and contained the following reagents in a 20µl reaction volume: 10 to 50 ng genomic DNA, 0.25 units of HotStarTaq^{TM} Flexi DNA polymerase (Promega), 1× reaction buffer (final concentration: 16mM $[NH_4]_2SO_4$; 67mM Tris-HCl pH 8.8; 0.01% Tween-20), 0.3µM fluorescently labelled (FAM™ or HEX™ fluorescent dye) forward and unlabelled reverse primers, 2mM $MgCl_2$ and 0.2mM dNTPs. The PCR protocol for eight of the loci (7B, 7C, DB1, DB44, DB49, BR6, WR1 and WR2) consisted of one denaturing step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 seconds, the specific annealing temperature for 30 seconds (following Coutts, 2009), 72°C for 45 seconds, and followed by a final extension step of 72°C for 10 minutes.

The PCR reaction for the three loci isolated in Indian rhinoceros (Rh7, Rh8, Rh9) contained just 0.05µM forward primer with a M13 sequence appended to the 5' end (5'-TGTAAAACGACGGCCAGT-forward primer-3') and 0.2µM reverse primer and M13 primer labelled with either FAM™ or HEX™ fluorescent dye. The PCR protocol consisted of one denaturing step at 94°C for 2 min, followed by 30 cycles of 94°C for 30 seconds, annealing temperature of 55°C or 57°C for 30 seconds, 72°C for 45 seconds, and then eight cycles of 94°C for 30 seconds, 53°C for 30 seconds for M13 attachment, 72°C for 45 seconds and then followed by a final extension step of 72°C for 10 minutes.

Unsuccessful PCR amplifications were repeated using a range of variations in conditions in an attempt to optimise PCR conditions. Conditions such as different annealing temperatures and lengths of cycles, using more template per PCR tube, using a new sample of HotStar Taq, adding BSA to the mixtures, etc. were applied. The PCR products were stained with ethidium bromide and then subjected to electrophoresis on a 2% agarose gel and then viewed under UV light. PCR product was stored by refrigeration. I made several visits to UCT to repeat samples which were giving difficulties and to add new samples to the set as they became available.

Cheetah Conservation Foundation

PCR amplification of samples was performed in thin-walled 0.2ml PCR tubes and contained the following reagents in a 10µl reaction volume: 10 to 50 ng genomic DNA, 5ul of AmpliTaq Gold ® MasterMix, 0.4µM fluorescently labelled (FAM™ or HEX™ fluorescent

dye) forward and unlabelled reverse primers and 2µg of Bovine Serum Albumine (BSA). The PCR protocol for all loci except WR2, consisted of one initial step at 95 °C for 10 minutes, followed by 10 touchdown cycles, for which annealing temperature is reduced by 1 °C at each cycle: 95 °C for 15 seconds, 60 °C - 51 °C annealing temperature for 30 seconds, 72 °C for 45 seconds, followed by 30 cycles consisting of one denaturing step at 95 °C for 15 seconds, 50 °C for 30 seconds, 72 °C for 45 seconds, and followed by a final extension step of 72 °C for 30 minutes. For microsatellite locus WR2, 12 touchdown cycles were performed with annealing temperature varying from 60 °C to 49°C and an annealing temperature of 48 °C for the 30 remaining cycles.

Trophy sample

For the trophy sample (Bob) a modified touchdown PCR program was used: The PCR protocol consisted of one initial step at 95 °C for 10 minutes, followed by 10 touchdown cycles, for which annealing temperature is reduced by 1 °C at each cycle: 95 °C for 15 seconds, 60 °C - 51 °C annealing temperature for 30 seconds, 72 °C for 45 seconds, an additional 10 cycles consisting of 95 °C for 15 seconds, 50 °C for 30 seconds, 72 °C for 45 seconds, followed by 30 cycles consisting of 95 °C for 15 seconds, 48 °C for 30 seconds, 72 °C for 45 seconds and followed by a final extension step of 72 °C for 30 minutes.

2.2.6. Genotyping

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For all dung samples processed the results obtained indicated insufficient product and were not reproducible and therefore no dung samples were taken to the genotyping phase.

Amplified PCR products obtained from DNA tissue samples were visualised by automated fragment size analysis on an ABI Prism™ 373 Genetic Sequencer (Applied Biosystems) by means of electrophoresis on a 6% polyacrylamide gel according to the specifications of the manufacturer.

The resultant fragment size data was collected and analysed using GENESCAN™ 672 Data Collection Software version 1.2.1 (Applied Biosystems). Microsatellite alleles were sized manually in relation to a fluorescent-labelled internal size standard (GeneScan™ Rox-350, Applied Biosystems). A positive control of known size, to ensure consistency of allele

fragment size scoring between gels, and a negative control, to ensure that PCR amplification was free of DNA contaminants, was included on every gel. Samples which provided undetermined results were reanalysed.

On my last visit to UCT the Genetic Sequencer malfunctioned and could no longer be used. As a result I sent my remaining samples to the Genome Facility at the Human Genetics Division of the UCT Medical School to be analysed at their facilities. The resultant microsatellite allele sizes were adjusted in order that the baseline matched with the calibration from the first sequencer. This allows allele sizes to be directly compared.

Cheetah Conservation Foundation

Amplified PCR products were visualised by automated fragment size analysis on an ABI Prism™ 310 Genetic Analyser (Applied Biosystems) by means of electrophoresis in a capillary. The resultant fragment size data was collected using Data Collection software version 3.1 (Applied Biosystems) and analysed using Genemapper software version 4.0 (Applied Biosystems). Relevant Microsatellite peaks were identified manually by their shape and location; Microsatellite allele sizes were obtained in relation to a fluorescent-labelled internal size standard (GeneScan™ Liz-500, Applied Biosystems). For each microsatellite locus an individual with known genotype was run in order to calibrate the results to those obtained previously, the allele sizes were then re-adjusted accordingly so that they too were comparable with the initial genotype data obtained at UCT.

The presence of null alleles, stutter errors or short allele dominance was determined using the program MICROCHECKER version 2.2.3 (Van Oosterhout et al. 2004). Observed and expected heterozygosity, together with the polymorphic information content (PIC) and tests for deviation from Hardy-Weinberg expectations were calculated using Cervus 3.0 (Kalinowski et al. 2007).

2.3. RESULTS

2.3.1. Faecal Analysis

Samples

In the 31 month sampling period I collected a total of 25 of white rhinoceros dung samples. These 25 samples came from 19 individual animals. An additional 8 dung samples were obtained as part of notching operations which occurred during study period. These samples were obtained via direct anal sampling whilst the animal was sedated. These samples were preserved for later comparison with tissue samples from the same individual.

DNA was extracted from the dung samples on site in the ORC laboratory. The DNA elutes obtained were visualized by staining with ethidium bromide and running on agarose gels and viewing under UV light. The concentration values for several of these DNA extracts were also measured using a Nanodrop spectrophotometer at UCT (See Table 2.2). The mean concentration of DNA derived from dung was 11.4ng/µl. The DNA obtained from the dung samples failed to successfully and/or consistently amplify during PCR processing for the 11 microsatellite loci. As no useful PCR product was obtained these samples were not taken to the genotyping phase.

Sample	Sample Type	DNA (ng/µl) in 200µl
D1	Dung	7.78
D2	Dung	8.23
D3	Dung	8.55
D4	Dung	8.05
D5	Dung	12.80
D6	Dung	5.30
D7	Dung	13.45
D8	Dung	7.85
D9	Dung	8.90
D10	Dung	37.88
D11	Dung	13.10
D12	Dung	16.05
D13	Dung	5.05
D14	Dung	16.98
D15	Dung	12.43
D16	Dung	4.63
D17	Dung	7.19
Average =		11.42

Table 2.2 *DNA extraction from dung* - Nanodrop readings for DNA extracted from dung samples

2.3.2. Tissue Analysis

Tissue collection

The combination of ear notches and biopsy methods provided a total of 32 tissue samples. These represented all rhinoceros aged at least 2 years old on the Reserve at the end of 2008, plus 3 recently deceased animals, as well as animals sold during 2004 - 2009. DNA from all tissue samples was extracted on site at the ORC laboratory. The extracts were stained with ethidium bromide and visualized by running on agarose gel and viewing under UV light. DNA concentration readings were taken for a portion of the samples using the nanodrop spectrophotometer at UCT (See Table 2.3). The mean concentration of DNA derived from tissue samples was 146.5 ng/µl, more than 10 times that for DNA derived from dung. DNA from the trophy mount sample was obtained and extracted using the Qiagen DNeasy Tissue Kit (Cat. No. 69504). The treatment of skin to stabilize it in order to obtain a trophy mount is expected to degrade DNA, therefore the DNA obtained from a trophy mount sample is expected to be of low quantity and of bad quality, making the sample highly sensitive to contamination. In order to avoid the risk of contamination, extractions were performed at CCF, as this laboratory had not handled rhinoceros samples in the past.

Sample	Sample type	DNA (ng/µl) in 200 µl
A81T	Tissue	165.2
A82T	Tissue	90.0
A83T	Tissue	151.6
A84T	Tissue	103.6
A85T	Tissue	114.8
A86T	Tissue	100.0
A87T	Tissue	110.4
A88T	Tissue	145.6
A89T	Tissue	76.0
A90T	Tissue	143.2
A91T	Tissue	128.8
A92T	Tissue	95.6
A93T	Tissue	80.0
A94T	Tissue	45.2
A95T	Tissue	133.2
A97T	Tissue	116.0
A98T	Tissue	65.6
A75T	Tissue	401.2
A99T	Tissue	194.9
A100T	Tissue	281.2
A101T	Tissue	216.4
A102T	Tissue	202.4
A103T	Tissue	219.7
A104T	Tissue	224.6
A106T	Tissue	57.7
Average =		146.5

Table 2.3 *DNA extraction from tissue* - Nanodrop readings for DNA extracted from tissue samples

2.3.3. Genotypes

University of Cape Town

I took the total set of 32 DNA samples (including 4 duplicates, thus the total set represented 28 individuals) to UCT for further analysis between November 2007 and November 2008. These samples were taken in batches at different times as they became available. Tissue DNA samples were subjected to PCR and amplified for all 11 loci. PCR's were repeated with variations in conditions if amplification failed. Samples were processed over several visits to UCT. In each visit I attempted to reprocess failed PCRs where possible, and also add a new batch of samples. Genotyping was then undertaken for all 32 samples. These results are shown in Table 2.4. In 31 of 352 cases it was not possible to determine a reliable genotype for an individual at a particular locus. 15 of the 32 samples (47%) produced a genotype at every locus and no one sample had more than 3 missing genotypes.

Duplicate and additional samples

The data set contains genotypes from 4 calves which were sampled via biopsy dart when with their mothers in 2006 and 2007 - hence I am certain about the identity of the mother of these calves. In 2007 there were a total of 8 'pushed-off' calves (now separated from their mothers and thus un-identifiable) from which we obtained ear notch tissue samples. The genotype profiles of the 4 darted individuals would be expected to be able to be perfectly matched within this set of 8 calves – 4 of the 8 should be identical to the 4 'known' calves. On review of the results (see Table 2.4) only 2 of the calf genotype profiles could be matched, suggesting that errors had occurred during the PCR / genotyping process. In order to investigate this anomaly further we decided to run repeat PCRs for all duplicate samples. . In addition, during 2009 I added 2 extra samples that had become available in the interim via the latest ear notching programme. I also had available the trophy mount sample of one of the initial founder male rhinoceros.

This provided a full data set of 35 samples, representing 31 individuals. In 2009 the Cheetah Conservation Fund (CCF) in Namibia opened a new genetics facility (Applied Biosystems Genetic Conservation Laboratory), and therefore I undertook all subsequent PCR and genotyping in collaboration with their geneticist, Dr. Anne Schmidt-Kuntzel using their facilities.

Sample		7B (FAM)		7C (HEX)		WR1 (FAM)		WR2 (HEX)		BR6 (HEX)		DB1 (FAM)		DB44 (FAM)		DB49 (FAM)		Rh7 (M13)		Rh8 (M13)		Rh9 (M13)	
ONG01	Jeff	266	266	0	0	186	174	239	239	155	155	0	0	183	183	0	0	199	199	162	162	143	141
ONG02	Long Horn	268	266	253	249	186	174	225	225	0	0	0	0	183	177	174	170	201	201	162	162	143	143
ONG03	Au Kooi	268	266	255	253	186	174	239	225	155	135	0	0	183	183	174	170	0	0	162	162	143	141
ONG04	Tony	266	262	255	255	186	174	239	225	143	135	0	0	183	183	172	172	199	199	162	162	151	141
ONG05	Sharon	266	266	255	255	186	178	225	225	135	135	130	130	183	183	174	174	199	199	162	160	143	141
ONG06	Anne	266	266	255	255	178	174	239	239	135	135	130	130	183	177	174	174	199	199	164	162	143	141
ONG07	Short Horn	266	262	255	253	178	174	225	225	0	0	130	130	183	183	170	170	199	199	0	0	143	141
ONG08	New calf of LH *	268	266	253	249	174	174	239	225	0	0	130	130	183	183	174	170	0	0	162	162	143	141
ONG09	New calf of Sharon	266	266	255	255	186	186	239	225	135	135	130	130	183	183	174	174	199	199	162	162	141	141
ONG10	Janine	266	266	255	253	186	174	225	225	155	137	132	130	183	183	174	170	201	199	162	162	143	143
ONG11	New calf of AK *	266	266	255	255	186	174	239	239	0	0	132	130	183	177	174	174	199	199	162	162	143	141
ONG12	Ivan	266	266	255	253	186	174	239	225	135	135	132	130	183	183	174	170	199	199	162	162	143	141
ONG13	Diane	268	266	255	255	178	174	239	225	135	135	130	130	183	177	174	174	199	199	162	162	143	141
ONG14	Lisa	266	266	255	253	178	174	239	239	155	135	132	130	183	183	174	172	199	199	162	162	143	141
ONG15	#20	266	266	255	253	174	174	0	0	135	135	132	130	183	177	174	170	201	199	160	160	141	141
ONG16	#21	268	268	253	253	174	174	0	0	155	135	130	130	183	177	174	174	201	199	160	160	143	141
ONG17	James	268	266	255	253	174	174	239	239	135	135	130	130	183	183	174	174	199	199	162	162	141	141
ONG18	#23	268	266	255	253	178	174	239	239	135	135	132	130	183	183	174	170	199	199	162	162	141	141
ONG19	#24	268	268	255	253	178	174	225	225	155	135	132	130	183	183	174	174	199	199	160	160	141	141
ONG20	New calf of SH *	268	266	0	0	178	174	239	225	155	135	132	130	183	183	172	172	199	199	160	160	141	141
ONG21	Rene	268	262	255	253	178	174	239	225	155	135	132	130	183	177	172	170	199	199	162	160	141	141
ONG22	Derek	268	266	255	253	174	174	239	239	135	135	130	130	183	177	174	170	201	199	162	162	141	141
ONG23	New calf of Rene *	266	262	255	253	186	178	239	239	0	0	132	130	183	177	174	174	199	199	162	162	141	141
ONG24	Sonja	268	268	255	253	174	174	239	225	143	135	130	130	183	177	174	174	201	199	162	162	141	141
ONG25	One Horn	266	266	255	255	178	178	239	225	135	135	130	130	183	183	174	174	199	199	164	162	143	141
ONG26	John	266	266	255	255	178	174	239	239	135	135	130	130	183	177	172	172	199	199	164	164	143	141
ONG27	#6	266	262	255	253	174	174	0	0	155	143	130	130	0	0	174	172	199	199	162	162	141	141
ONG28	#7	268	266	0	0	178	174	0	0	155	135	132	130	183	183	174	172	199	199	160	160	143	141
ONG29	#15	266	262	0	0	186	174	0	0	155	135	130	130	183	183	174	172	199	199	162	162	141	141
ONG30	#16	266	266	0	0	0	0	0	0	135	135	132	130	183	177	174	174	199	199	162	162	143	141
ONG31	#1	266	262	253	253	0	0	0	0	155	155	132	130	183	177	174	174	199	199	0	0	143	141
ONG32	#5	268	266	253	249	0	0	0	0	135	135	130	130	183	183	174	170	201	199	162	162	143	141

Table 2.4 *Genotype Results from University of Cape Town* – Each pair of numbers represents the genotype as determined for each locus (11 columns) for each of the 32 samples. Four of these samples (marked *) are duplicates of the series of samples ONG27-ONG32 (biopsy sample from calf with mother, then tissue sample from ear notch). It should be possible to locate an exact match to each of those calf samples in the tissue sample set.

Trophy sample

DNA was successfully obtained from the trophy mount sample through a series of combined extraction elutions. Although the quantity of DNA obtained was insufficient to allow for visualisation, successful PCR was achieved and genotypes were obtained for seven of the eleven loci (See Table 2.5).

Additional samples

Upon the completion of processing of the trophy mount sample I took the other 34 rhinoceros DNA samples to CCF. PCR product was obtained successfully for the 2 additional samples at all loci except for 7C. Repeat PCRs with variations were attempted for this locus for the 2 new samples as well as for other DNA samples (samples which had previously amplified at this locus). Despite numerous variations in PCR conditions, no product was achieved for any of the samples and thus I was unable to obtain genotypes for this locus in the second phase of my analysis.

Missing data and duplicate data mismatches

I then ran a series of repeated 'processing cycles' (PCR and genotyping) in an attempt to fill in missing data gaps in the genotype table obtained with results from UCT. I also attempted to investigate the mismatches which were located in the duplicate data set of calves. I was able to determine an additional 26 genotypes (21 from repeats and 5 of which were determined by independently reviewing size data collected at UCT). Using new results obtained for the areas of mismatch in the duplicate data set I was able to update the genotype profiles of these animals. Comparison of these new genotype profiles allowed for all 4 calves to be perfectly matched within the set of 8 calves.

Incorporating pedigree data and error rates

I compiled a new genotype table incorporating all of the data obtained above. I then performed preliminary manual parentage assignments using this table. A preliminary review of this genotype table in light of known pedigree information allowed me to identify possible errors occurring in the data table. These 'alerts' included occurrences such as known calf-mother mismatches and instances where calves could not be assigned to any of the candidate fathers in the population. In order to investigate these anomalies further and evaluate error rates in the data set, I performed repeat PCR's and genotyping for a sub-set of 128 samples across targeted and random loci and individuals. The results of these PCR's gave rise to 29

new or altered genotypes including 17 results for previous 'blanks' and 12 corrections (3.75%) to previous incorrect results. Results that were altered were repeated again to confirm the new results. A total of 154 genotypes were obtained from PCR's performed at CCF. Corrected genotypes were incorporated into the final data set (Table 2.5).

The analysis undertaken at CCF resulted in a final genotype profile data set consisting of of 376 genotypes (35 samples for 31 individuals across 11 microsatellite loci). For the total dataset, nine genotype gaps remained (2.3%), of which six occurred at locus 7C and a further three occurred in the trophy sample (See Table 2.5). Of the 35 samples, 29 (83%) produced a genotype at every locus and with exception of the trophy sample no one sample had more than one missing genotype.

Allelic dropout

The DNA sample for 'Bob' was obtained from treated skin from a trophy mount of the animal which was several years old. This sample was therefore expected to provide low quality and quantity DNA and thus repeat extractions and multiple PCR's were performed on the sample. Two of the loci gave one homozygous reading for three repeats and a different homozygous reading for the third repeat thereby alerting us to the presence of allelic dropout. For four of the loci (7B, 7C, DB49 and WR2), no results were obtained for Bob.

Sample	7B (FAM)		7C (HEX)		BR6 (HEX)		DB1 (FAM)		DB44 (FAM)		DB49 (FAM)		Rh7 (M13)		Rh8 (M13)		Rh9 (M13)		WR1 (FAM)		WR2 (HEX)	
ONG01 Jeff	266	266	0	0	155	155	130	130	183	183	174	174	199	199	162	162	143	141	186	174	239	239
ONG02 Long Horn	268	266	253	249	155	143	130	130	183	177	174	170	201	201	162	162	143	143	186	174	225	225
ONG03 Au Kooi	268	266	255	253	155	135	132	130	183	183	174	170	201	199	162	162	143	141	186	174	239	225
ONG04 Tony	266	262	255	255	143	135	130	130	183	183	172	172	199	199	162	162	151	141	186	174	239	225
ONG05 Sharon	266	266	255	255	135	135	130	130	183	183	174	174	199	199	164	162	143	141	186	178	225	225
ONG06 Anne	266	266	255	255	135	135	130	130	183	177	174	174	199	199	164	162	143	141	178	174	239	239
ONG07 Short Horn	266	262	255	253	135	155	132	130	183	183	172	170	199	199	160	162	143	141	178	174	225	225
ONG08 Calf of LH	268	266	253	249	155	135	130	130	183	183	174	170	201	199	162	162	143	141	174	174	239	225
ONG09 Calf of Sharon	266	266	255	255	135	135	130	130	183	183	172	174	199	199	162	162	141	141	186	186	239	225
ONG10 Janine	266	266	255	253	155	137	132	130	183	183	174	170	201	199	162	162	143	143	186	174	225	225
ONG11 Calf of AK	266	266	255	255	135	135	132	130	183	177	174	174	199	199	162	162	143	141	186	174	239	239
ONG12 Ivan	266	266	255	253	135	135	132	130	183	183	174	170	199	199	162	162	143	141	186	174	239	225
ONG13 Diane	268	266	255	255	135	135	130	130	183	177	174	174	199	199	162	162	141	141	178	174	239	225
ONG14 Lisa	266	266	255	253	155	135	132	130	183	183	174	172	199	199	162	162	143	141	178	174	225	239
ONG15 #20	266	266	255	253	135	135	132	130	183	177	174	170	201	199	162	162	141	141	174	174	239	239
ONG16 #21	268	268	253	253	155	135	130	130	183	177	174	174	201	199	162	162	143	141	174	174	225	239
ONG17 James	268	266	255	253	135	135	130	130	183	183	174	174	199	199	162	162	141	141	174	174	239	239
ONG18 #23	268	266	255	253	135	135	132	130	183	183	174	170	199	199	162	162	141	141	178	174	239	225
ONG19 #24	268	268	255	253	155	135	132	130	183	183	174	174	199	199	160	160	141	141	178	174	239	239
ONG20 Calf of SH	268	266	0	0	155	135	132	130	183	183	174	172	199	199	160	160	141	141	178	174	239	225
ONG21 Rene	268	262	255	253	155	135	132	130	183	177	174	170	199	199	162	160	141	141	178	174	239	225
ONG22 Derek	268	266	255	253	135	135	130	130	183	177	174	174	199	199	162	162	141	141	174	174	239	239
ONG23 Calf of Renee	266	262	255	253	155	155	132	130	183	177	174	174	199	199	162	162	141	141	186	178	239	239
ONG24 Sonja	268	268	255	253	143	135	130	130	183	177	174	174	201	199	162	162	143	141	174	174	239	225
ONG25 One Horn	266	266	255	255	135	135	130	130	183	183	174	174	199	199	164	162	143	141	178	178	239	225
ONG26 John	266	266	255	255	135	135	130	130	183	177	174	174	199	199	164	164	143	141	178	174	239	239
ONG27 #6	266	262	255	253	155	143	130	130	183	183	174	172	199	199	162	162	143	141	174	174	225	225
ONG28 #7	268	266	255	255	155	135	132	130	183	183	174	172	199	199	160	160	141	141	178	174	239	225
ONG29 #15	266	262	0	0	155	135	130	130	183	183	174	172	199	199	162	162	141	141	186	174	225	225
ONG30 #16	266	266	255	255	135	135	132	130	183	177	174	174	199	199	162	162	143	141	186	174	239	239
ONG31 #1	266	262	253	253	155	155	132	130	183	177	174	174	199	199	162	162	141	141	186	178	239	239
ONG32 #5	268	266	249	253	155	135	130	130	183	183	174	170	201	199	162	162	143	141	174	174	239	225
ONG33 Tom	266	266	0	0	155	135	130	130	183	183	174	174	199	199	164	164	143	141	178	174	239	225
ONG34 Max	268	262	0	0	143	135	130	130	183	177	174	172	199	199	162	162	143	141	174	174	225	225
ONG35 Bob	0	0	0	0	135	137	130	130	183	183	0	0	199	199	160	162	143	141	186	178	0	0

Table 2.5. *Final Genotype Results* – The full set of 35 genotypes (including 4 duplicates) after processing at CCF. Data were obtained for all but 9 of 385 genotype profiles (2.3%).

Null Allele

For three samples (namely Calf of Sharon, John and Tom) I found that none of the genotypes for candidate fathers matched the genotype of the offspring. In particular these mismatches occurred at locus Rh8. As it was confirmed from pedigree data that the one of the candidate fathers had to be the correct father, these samples were reprocessed. None of the genotypes changed after repeated amplification and analysis (and a high number of homozygous genotypes were noted) therefore I suspected that a null allele occurred at Rh8. The presence of a null allele at Rh8 was confirmed statistically using both Cervus (see Table 2.6) and MICROCHECKER.

Genotype summary

I used Cervus 3.0 to produce summary statistics for each of the microsatellite loci used in this study (see Table 2.6).

Locus	k	N	HObs	HExp	PIC	HW	F(Null)	
7B (FAM)	3	34	0.529	0.543	0.469	ND	0.0055	
7C (HEX)	3	29	0.586	0.532	0.430	ND	-0.0471	
BR6 (HEX)	4	35	0.543	0.552	0.474	NS	0.0079	
DB1 (FAM)	2	35	0.429	0.342	0.280	ND	-0.1189	
DB44 (FAM)	2	35	0.400	0.325	0.269	ND	-0.1097	
DB49 (FAM)	3	34	0.500	0.465	0.414	ND	-0.0534	
Rh7 (M13)	2	35	0.200	0.227	0.199	ND	0.0568	
Rh8 (M13)	3	35	0.171	0.384	0.346	ND	0.3679	*
Rh9 (M13)	3	35	0.571	0.467	0.367	ND	-0.1128	
WR1 (FAM)	3	35	0.686	0.609	0.533	NS	-0.0675	
WR2 (HEX)	2	34	0.471	0.500	0.372	NS	0.0233	

Table 2.6 *Summary Statistics for Each Locus* – k: Number of Alleles, N: Sample count, HObs – Observed heterozygosity, HExp, Expected heterozygosity under Hardy-Weinberg (H-W) equilibrium, PIC – Polymorphic Information Content, HW: Test for H-W equilibrium (ND not done, NS not significant). F(Null): The F-score for the null hypothesis that the locus is in H-W equilibrium. * Locus Rh8 deviates significantly from H-W

2.4. DISCUSSION

This study was able to confidently assign genotypes for an entire free-ranging, closed population of southern white rhinoceros (n = 31 individuals). For genetic analyses (and in particular parentage analyses) it is preferable but not often possible to obtain samples for an entire study population, particularly for endangered species and for species which are elusive such as the rhinoceros (Taberlet et al. 1999; Creel et al. 2003; Scott, 2008; Jones, et al. 2010) thus making this data set particularly valuable. Data which can contribute to the understanding of the genetic structure of rhinoceros populations is an important contribution to aid in the conservation efforts of these endangered animals.

2.4.1. DNA from Faecal Samples

Nielsen et al (2008) conducted a study to investigate whether or not rhinoceros (both black and white) faecal samples could provide good quality DNA for use in genetic studies. Their results showed that not all of the faecal samples revealed comparable patterns with the blood samples (amplified faecal samples showed high background without a main product peak which suggested low quality DNA or contamination). Thus they concluded that faecal DNA from neither white nor black rhinoceros is an adequate source of DNA for genetic analyses. However, black rhinoceros dung has provided adequate DNA for other studies (Cunningham et al. 1999; Garnier et al. 2001; Harley et al. 2005). I attempted to use white rhinoceros faecal matter as a DNA source and also found that it was not suitable. DNA extracted from the rhinoceros faecal samples I collected, failed to amplify during PCR despite numerous variations at different loci. In the white rhinoceros several reasons may exist for this, including the possibility that the DNA obtained was particularly degraded, the presence of inhibitors , or perhaps more simply that there is insufficient rhinoceros DNA present in the samples. Other collections and storage methods (e.g. combining a short period of storage of the sample in ethanol with subsequent desiccation using silica, per Nsubuga et al. 2004) might yield higher concentrations of DNA. To my knowledge no other studies have been successful in obtaining reliable DNA samples from white rhinoceros dung. Further investigation into alternative techniques for obtaining suitable DNA from white rhinoceros faecal samples is certainly warranted.

2.4.2. Population Genetics

Low levels of genetic diversity typically appear to characterize southern white rhinoceros populations (Cunningham et al. 1999; Kellner et al. 2000; Seror et al. 2002; Florescu et al. 2003; Nielsen et al. 2008, Scott 2008). Scott (2008) showed that amongst the rhinoceros taxa the southern white and Indian species had the lowest level of microsatellite genetic variability while one of the black subspecies (*Diceros bicornis michaeli*) showed the highest. My findings are consistent with other studies performed using microsatellites in southern white rhinoceros populations. Table 2.7 shows a comparison of mean values obtained in this study with other microsatellite genetic studies which included southern white rhinoceros populations.

There does appear to be increased genetic diversity in the white rhinoceros population studied by Florescu et al (2003), however the authors selected for polymorphic loci, and this may have elevated the measured diversity in their data. Nielsen et al (2008) also reported a higher number of alleles than any of the other studies, this one included. A comparison of parameters of genetic diversity obtained at each locus for various studies that included southern white rhinoceros is given in Appendix 2A. There are two loci where my study had noticeably different results, namely locus DB49 where our results were consistently lower than the four populations studied by Coutts (2009) and at locus DB1 where my results were consistently higher than three of the four populations studied by Coutts (2009) (the fourth population - also Namibian - had similar results to mine). At locus WR2 Nielsen et al (2008) obtained significantly higher results for the parameters observed than in any of the other populations which may be due to sample size. These data suggest that comparisons across populations can be complicated and that consistency in primer specification is essential where population measures are used to measure differences in genetic variability.

Source/Reference	k	H_{Obs}	H_{Exp}	PIC	# of Microsatellites	n
This study	2.73	0.456	0.447	0.375	11	31
Coutts, 2009	2.60	0.440	-	0.370	13	144
Nielsen et al. 2008	3.31	0.436	0.420	0.368	16	22
Florescu et al.2003	2.80	0.597	0.578	0.481	5	unknown
Scott, 2008	2.70	0.342	0.388	-	24	59

Table 2.7 ***Genetic diversity across different studies of southern white rhinoceros*** **- k: mean number of alleles obtained, H_{Obs}: mean observed heterozygosity, H_{Exp}: mean expected heterozygosity, PIC: mean polymorphic informative content, # of Microsatellites: number of microsatellite loci tested for each study, n: number of individuals sampled.**

2.4.3. Methodological and Sampling Considerations

A number of methodological and sampling considerations arose during the course of this study. I present them in point form in no particular order or significance:

- The circumstances which prevailed at UCT (i.e. several people sharing the same laboratory facilities, plus a malfunction of the sequencing machine) meant that analysis could not be completed successfully there and other facilities were required. This eventually led to the use of three different sequencing machines for the analysis and genotyping. These transitions were challenging (primarily due to calibration) and increased the margin for errors to occur.

- The development of primers for microsatellites from genome maps can be an expensive and time-consuming process. For a novel species, geneticists will examine many putative sequences before they arrive at a battery of polymorphic loci (Zane et al. 2002). In this study, several of the microsatellite loci that I used were developed for other rhinoceros species (see Table 2.1), and hence the possibility existed that they might not provide the same levels of individual variation. Locus Rh8 is an example of a marker developed for Indian rhinoceros (Zschokke et al. 2003) that appears to have a null allele in white rhinoceros (see also Coutts, 2009). In addition locus Rh7 showed low polymorphic information content and is also from Indian rhino. Null alleles result in heterozygous individuals appearing to be homozygous at that particular locus due to the lack of amplification of one of the alleles. Dakin and Avise (2004) show that null

alleles can introduce substantial errors when they lead to false exclusion of parents during parentage assignments

- Details for Locus 7C are incorrectly provided in the original paper by Florescu et al (2003). See Table 2.1 for correct sequence.

- Obtaining DNA from the sample obtained from the trophy mount (Bob) required a significant effort in both logistics and laboratory time. This included an environment free of any rhinoceros DNA, repeated attempts at amplification under different conditions, etc. However this effort did prove to be worthwhile. During the preliminary review of pedigree information for parentage assignments there were a number of instances where it was not possible to exclude the other founder bull (Derek) from being the father, yet Bob's genotype, even at only 6 of the 11 loci, was sufficient to exclude him. This emphasizes the importance of obtaining samples from all animals in the population, and in particular all the candidate parents (concurs with Jones et al. 2010).

- The presence of duplicate samples in the data set alerted me to the occurrence of errors in the genotype data and was thus especially useful as it allowed me to identify which areas needed further investigation in that regard and direct my efforts accordingly as well as verify the accuracy of results. I recommend including a subset of anonymous samples within the data set for any similar future studies as a means of control along with the more typical methods such as multiple DNA extractions from each sample, repeat PCRs per sample, calculation of error rates, etc. (see reviews in Bonin et al. 2004; Selkoe & Toonen, 2006; Scott, 2008).

- It is worth noting that the sample collection element of this study required considerable effort and time to complete – I estimate at least 65% of the total project effort. In particular the dung collection required a substantial amount of time spent tracking particular animals and then waiting to obtain a suitable, viable sample. Significant amounts of time and effort were also spent tracking spoor which lead to animals which were not candidate animals (or from which samples were not needed) which meant that the process needed to be repeated until the required animals were

located. Even upon locating the required animals this did not necessarily provide opportunity to obtain samples and thus the entire process was repeated on a different occasion until faecal samples were successfully obtained. For studies such as this (parentage or kinship investigations) it is absolutely essential that the sample obtained is accurately matched to a positively identified animal thus this makes the collection of faecal samples particularly challenging.

- The collection of the tissue samples was slightly less demanding as it did not involve tracking many animals. The ear tissue samples were routinely collected during the annual ear-notching programme for marking individual rhino. I found that the best method for obtaining the biopsy samples was to dart the rhinoceros at waterholes and thus this required some time spent waiting at appropriate waterholes waiting for the animals to come in to drink. Initially, when there are several animals to be darted the process is faster but towards the end when there are only one or two specific individuals remaining it can require many nights of vigilance before the individuals are encountered and samples obtained. Overall, the rate of collection of samples was significantly higher for the biopsy darting method than for faecal sample collection. Future studies of a similar kind should take this into consideration when planning their collection methods.

Appendix 2A

A comparison of parameters of genetic diversity for each microsatellite locus. Number of individuals sampled (n), observed number of alleles (k), observed (H_{Obs}) and expected (H_{Exp}) heterozygosity, and polymorphic informative content (PIC).

Locus	Source/Reference	Population	k	n	HObs	HExp	PIC
7B	This study	OGR	3	30	0.500	0.544	0.469
	Florescu et al. 2003		3		0.533	0.582	0.494
	Coutts, PhD 2009	Waterberg Park	3		0.670	0.500	0.420
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		0.640	0.530	0.470
	Coutts, PhD 2009	Welgevonden Reserve	3		0.590	0.590	0.520
	Coutts, PhD 2009	Matobo National Park	3		0.610	0.550	0.480
7C	This study		3	26	0.577	0.520	0.413
	Florescu et al. 2003		3		0.462	0.542	0.450
	Coutts, PhD 2009	Waterberg Park	2		**0.280**	**0.320**	**0.260**
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		0.430	0.480	0.410
	Coutts, PhD 2009	Welgevonden Reserve	3		0.470	0.510	0.430
	Coutts, PhD 2009	Matobo National Park	3		0.600	0.560	0.460
WR1	This study		3	31	0.677	0.607	0.530
	Nielsen et al. 2008		4		0.727	0.646	0.558
	Coutts, PhD 2009	Waterberg Park	3		0.670	0.510	0.490
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		0.680	0.660	0.570
	Coutts, PhD 2009	Welgevonden Reserve	3		0.570	0.610	0.520
	Coutts, PhD 2009	Matobo National Park	3		0.730	0.650	0.570
WR2	This study		2	30	0.467	0.506	0.374
	Nielsen et al. 2008		4		**0.857**	**0.677**	**0.601**
	Coutts, PhD 2009	Waterberg Park	2		0.440	0.510	0.370
	Coutts, PhD 2009	Hluhluwe Imfolozi	2		0.550	0.480	0.360
	Coutts, PhD 2009	Welgevonden Reserve	2		0.470	0.500	0.370
	Coutts, PhD 2009	Matobo National Park	2		0.460	0.420	0.330
BR6	This study	OGR	4	31	0.548	0.551	0.478
	Coutts, PhD 2009	Waterberg Park	4		0.560	0.570	0.470
	Coutts, PhD 2009	Hluhluwe Imfolozi	4		0.710	0.610	0.550
	Coutts, PhD 2009	Welgevonden Reserve	4		0.370	0.470	0.430
	Coutts, PhD 2009	Matobo National Park	3		**0.180**	**0.170**	**0.160**
DB1	This study	OGR	2	31	0.387	0.317	0.263
	Coutts, PhD 2009	Waterberg Park	2		0.440	0.360	0.290
	Coutts, PhD 2009	Hluhluwe Imfolozi	2		**0.140**	**0.190**	**0.170**
	Coutts, PhD 2009	Welgevonden Reserve	2		**0.200**	**0.250**	**0.220**
	Coutts, PhD 2009	Matobo National Park	2		**0.100**	**0.100**	**0.090**
DB44	This study	OGR	2	31	0.387	0.317	0.263
	Coutts, PhD 2009	Waterberg Park	2		**0.560**	**0.460**	**0.350**
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		0.320	0.280	0.260
	Coutts, PhD 2009	Welgevonden Reserve	3		0.220	0.260	0.230
	Coutts, PhD 2009	Matobo National Park	3		**0.550**	**0.490**	**0.420**
DB49	This study	OGR	3	30	0.500	0.473	0.420
	Coutts, PhD 2009	Waterberg Park	3		**0.720**	**0.680**	**0.580**
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		**0.670**	**0.670**	**0.580**
	Coutts, PhD 2009	Welgevonden Reserve	3		**0.630**	**0.660**	**0.580**
	Coutts, PhD 2009	Matobo National Park	3		**0.650**	**0.640**	**0.560**
Rh7	This study	OGR	2	31	0.194	0.228	0.200
	Coutts, PhD 2009	Waterberg Park	2		**0.500**	**0.510**	**0.370**
	Coutts, PhD 2009	Hluhluwe Imfolozi	2		**0.430**	**0.380**	**0.310**
	Coutts, PhD 2009	Welgevonden Reserve	2		0.200	0.280	0.240
	Coutts, PhD 2009	Matobo National Park	2		**0.570**	**0.450**	**0.340**

Rh8	This study	OGR	3	31	0.194	0.381	0.344
	Coutts, PhD 2009	Waterberg Park	1		**0.000**	**0.000**	**0.000**
	Coutts, PhD 2009	Hluhluwe Imfolozi	3		0.210	0.400	0.350
	Coutts, PhD 2009	Welgevonden Reserve	3		0.160	0.150	0.150
	Coutts, PhD 2009	Matobo National Park	3		0.270	0.280	0.260
Rh9	This study	OGR	3	31	0.581	0.476	0.373
	Coutts, PhD 2009	Waterberg Park	2		0.720	0.510	0.370
	Coutts, PhD 2009	Hluhluwe Imfolozi	4		0.640	0.570	0.460
	Coutts, PhD 2009	Welgevonden Reserve	4		0.550	0.540	0.430
	Coutts, PhD 2009	Matobo National Park	2		**0.260**	**0.290**	**0.250**

CHAPTER 3: PARENTAGE ANALYSIS

3.1. INTRODUCTION

The advances in the ability to infer genealogical relationships through the use of molecular data and statistical methods have been of particular importance to areas of research in evolution and behavioural ecology as well conservation (Blouin, 2003; Jones & Ardren, 2003). These methods are critical for understanding the behaviour and evolution of social animals and allow patterns in these fields to be determined which would otherwise be difficult to establish through the use of behavioural observations alone (Archie & Chiyo, 2012). Genetic parentage analyses, in particular, play a major role since not only is the determination of parentage in natural populations in itself important but it also has relevance to other aspects of population biology (Chakraborty et al. 1988; Blouin, 2003). Such aspects include clarification on mating systems and behaviour, reproductive skews, social organisation, genetic dispersal and gene flow, (Morin et al. 1994; Goossens et al. 1998; Garnier et al. 2001; Burton, 2002; Bishop et al. 2004; Mouiex, 2006). Parentage analyses also provide vital information used to study the impact and occurrence of inbreeding which is important in the management of threatened species (Frankham et al. 2002).

Parentage analysis methods are typically categorized into either exclusion or likelihood based approaches (Jones & Ardren, 2003; Honda et al. 2009). The exclusion method (the earliest and most simple technique) is based on classic Mendelian rules of inheritance and rejects putative parentage propositions based on genotype incompatibilities between the parents and offspring. Likelihood-based methods assign offspring to non-excluded parents based on likelihood scores derived from their genotypes (putative parents with the highest likelihood ratio are assigned parentage) (Jones & Ardren, 2003; Honda et al. 2009). This is a statistically based method which is useful when multiple candidates are unable to be excluded (Marshall et al. 1998).

The strict exclusion based method does have potential disadvantages in that genotyping error, null alleles and mutations may give rise to false exclusions (Jones & Ardren, 2003). This approach is most useful for studies with few candidate parents and highly polymorphic genetic markers (as the potential for genotyping errors or mutations to occur increases as the dataset is increased) but has shown to be unreliable for paternity analysis in natural populations (Chakraborty et al. 1988).

The development of likelihood based methods, originating with Thompson (1975, 1976a,b) and Meagher (1986 - in Marshall et al. 1998), and then developed further by others such as Marshall et al (1998) has provided alternatives to exclusionary models for parentage analysis (Chakraborty et al. 1988; Jones & Ardren, 2003). Likelihood techniques assign offspring to parents either categorically or fractionally (Jones & Ardren, 2003). The categorical approach assigns an offspring to a particular male whereas the fractional approach divides the allocation of an offspring among all compatible males (Jones & Ardren, 2003; Marshall et al. 1998). Thus the categorical approach is most useful for parentage studies whereas the fractional approach can be used for studies involving mating system parameters, such as comparing reproductive success of males, or for producing less biased estimates for offspring proportion in a population parented by each of the adults (as referred to in Jones & Ardren, 2003), or for population-level patterns of paternity to be assessed (Marshall et al. 1998). Most likelihood based methods are assessed by simulations assuming randomly mating populations under the Hardy-Weinberg equilibrium (HWE) and as such are suitable for free-ranging/natural populations (Honda et al. 2009). Likelihood based methods can be used in populations with low levels of genetic diversity or when not all parental candidates can be sampled (Marshall et al. 1998).

The categorical likelihood based technique selects the most likely parent from a pool of non-excluded parents by calculating a logarithm of the likelihood ratio (LOD score, calculated by determining the likelihood of select individuals being the parents (or parent) of a given offspring divided by the likelihood of these individuals being unrelated (Marshall et al. 1998; Jones & Ardren, 2003). Offspring are thus assigned to parent (or parents) with the highest LOD score.

To date there have been limited parentage studies performed for any of the rhinoceros taxa; for wild populations of southern white rhino only Coutts (2009) and Kellner et al (2001) have reported findings while Seror et al (2002) investigated paternity for a group of captive born white rhinoceros at the Zoological Centre in Israel.

Having successfully obtained a comprehensive set of genotypes for this rhinoceros population, I aim to complete a parentage analysis for the entire population using different methods. I establish whether it is possible to complete parentage for a population with reportedly low levels of genetic variation and also use the parentage results to examine inbreeding and mate choice in the population.

3.2. METHODS

3.2.1. Rhinoceros Pedigree Data Collection

OGR has resident Anti-Poaching Units (APU) which patrol the reserve throughout the year to track and monitor the rhinoceros. 'Rhino Monitoring' includes daily foot and vehicle patrols as well waterhole monitoring (normally carried out at full moon during winter months). I accompanied these foot patrols in order to collect samples and data. For each rhinoceros sighting a range of information was collected such as: number & name, date, sex, age class, GPS location, area name, time, group composition, condition of rhinoceros and, if possible, photos are taken of each individual. Additional data from observations and sightings made by researchers and other members of staff on OGR are also recorded. All other information regarding the rhinoceros population (such as, births, deaths, disease, translocations, sales, etc.) is documented by the Reserve Management.

I also had access to sightings information from Management's historic (from 1993) and current records. These records in addition to my own sightings data were compiled for a period of 6 years (2004 to 2009) and these data were used to create individual rhinoceros profiles. Further information from the management records was also collated in order to provide data on sex and age structure of the population as well as any groupings or associations noted between individuals. Age classes were defined as calves: up to 2 years of age, sub-adults: 2 – 4 years of age and adults as 5 years and older. A database of all this data was then established in a spreadsheet.

Using these records I created a unique profile for each rhinoceros which details all information. These profiles were used to create a database and I used this information database to construct the initial family-tree diagram for the population. I then also used this data and diagram later to identify pedigree constraints which are applied to the parentage analysis stage.

3.2.2. Analysis

I investigated individual parentage using a manual assignment approach based on exclusion criteria together with computational assignments calculated within a likelihood framework.

Manual parentage analysis

Initially, I calculated the number of possible parent pairs in the candidate data set based on all males and females in the total population; this was done using the in-house software (available on request K. Stratford). I then compared each locus across the entire genotype profile for an offspring with the genotype profile for all possible males and females. I used strict exclusion criteria to eliminate non-matching parents. These results of all possible parent-offspring combinations were recorded. I then refined the process by constraining the numbers of candidate parents based on the information we derived from the historic records database, including date of birth for each animal, sex of individuals, tenure times on the reserve (for bulls) and groups of calves which were all born in the same year. Thus the next step was to narrow the possible parents selection down to only those male and female candidates which would have been of breeding age and present on OGR at the time of conception for all the offspring. For this step each offspring's genotype was compared to the afore-mentioned parent candidates' genotypes and individuals were discarded based on mismatches at one (or more) locus. The influence that the presence of a possible null allele at Rh8 was also investigated by repeating step 2 and removing Rh8 from all the genotypes. The fourth and final step in refining the parentage assignments was to narrow the candidate parent options down according to the calf's date of birth (i.e. only mothers which gave birth in that particular year available as candidates) and also to exclude mothers which had been previously unequivocally assigned in step 2 and 3 (e.g. if three calves are born in a particular year, and for one of these calves a single mother has been assigned in step 2 and 3, then that mother is excluded as a candidate for the remaining two calves). The results from this analysis were recorded as the set of possible parent pairs for each offspring.

Parentage analysis using software

I used two independent computational methods to determine parentage in my data set: Cervus 3.0 (Kalinowski et al. 2007) and Colony 2.0.1.3 (Jones & Wang, 2009); both programs implement likelihood methods to determine parentage using multi-locus genotype data.

Analysis using Cervus

Through simulation, Cervus generates criteria for parentage assignment at a given level of confidence for all offspring analysed. As for the manual analysis, initially I allowed Cervus to select candidate parents from all males and all females in the population to allow for unrestricted assignments based solely on genotypes. Subsequently the number of candidate parents was constrained using the historic records. I then provided only candidate parents to the program based on their breeding age and residency on the Reserve relative to the times of conception. Next I excluded Rh8 as per the manual assignments process. Finally, I further refined the candidates from step 2 by narrowing that candidate selection down to only provide those parents available based on the calf's date of birth. When running simulations the following parameters were used: 100,000 offspring, 1% mistyped loci and 96.9% typed loci (determined by Cervus from our data set). In all simulations 100% of candidate mothers and fathers were represented in the data set. Confidence was calculated using LOD (Likelihood-of-Difference) scores at both 95% (strict) and 80% (relaxed) confidence levels. Each analysis required a minimum of six genotyped loci and possible parent pairs were ranked based on their joint LOD score.

Because relatively small samples sizes together with a large number of highly related individuals can significantly bias parentage assignment, I tested whether the use of my samples as the reference population influenced parentage assignment in Cervus. To do this I repeated the analysis using genotype data from a sub-set (n=49) of individuals sampled from the Hluhluwe-Umfolozi Game Reserve, SA as the reference population (Coutts et al. unpublished data).

Analysis using Colony

Colony 2.0.1.3 (Jones & Wang, 2009) was also used to determine parentage for each calf in my data set. As in Cervus, I first ran the analysis with unconstrained parents and then excluded mothers and fathers accordingly based on historic records and the pedigree data as mentioned previously. I implemented the full-likelihood method and a polygamous mating system without inbreeding; in the final analysis I used medium precision and medium run lengths, as tests with higher precision and longer runs did not make a significant difference to the assignments. I also tested a number of genotyping error rates (0.001, 0.01) for type I and II errors and found that, as expected, with higher error rates assignment probabilities were reduced; nonetheless, I did not see a difference in assignment order. In the final analysis I

used an error rate of 0.001. Due to the fact that I have a closed data set i.e. all individuals were sampled, I set the probability that both the father and the mother were amongst the candidate male and female parents to one. Unlike Cervus, Colony asks for an exclusion list of candidate parents. For the constrained runs, each offspring was therefore given a list of individuals that could be neither the father nor the mother. I also replicated the date-of-birth refinement process by sequentially removing mothers who had unambiguous assignments to calves with the same year of birth.

Analysis of mismatches

Because of the low levels of genetic variation observed in this species, I excluded possible parent pairs based on genotype mismatches at one or more loci. To explore the possibility that I erroneously excluded candidate parents due to incorrect genotypes at a particular locus, I analysed the distribution of mismatches across the loci (using in-house software, available on request K. Stratford) used in this study to assess whether any one particular locus accounts for a disproportionate number of single locus mismatches.

Inbreeding and shared allele distance

I calculated inbreeding coefficients for both individuals and the population using methods described in Frankham et al (2002), and used χ^2 statistics when determining whether observed data departed from random. Finally, to explore the degree to which individual genetic similarity may influence the assignment of parentage and possibly male mating success among OGR rhinoceros, I examined the shared allele distance ds (Chakraborty & Jin, 1993) between all individuals in the population (using in-house software, K. Stratford). Where relevant, results are reported as Mean ± Standard Error.

3.3. RESULTS

3.3.1 Rhinoceros Pedigree Data

All data collected for the rhinoceros population was collated and Figure 3.1 (a and b) was created as an initial family tree for this population. This lineage diagram is based on the historic and observation records only.

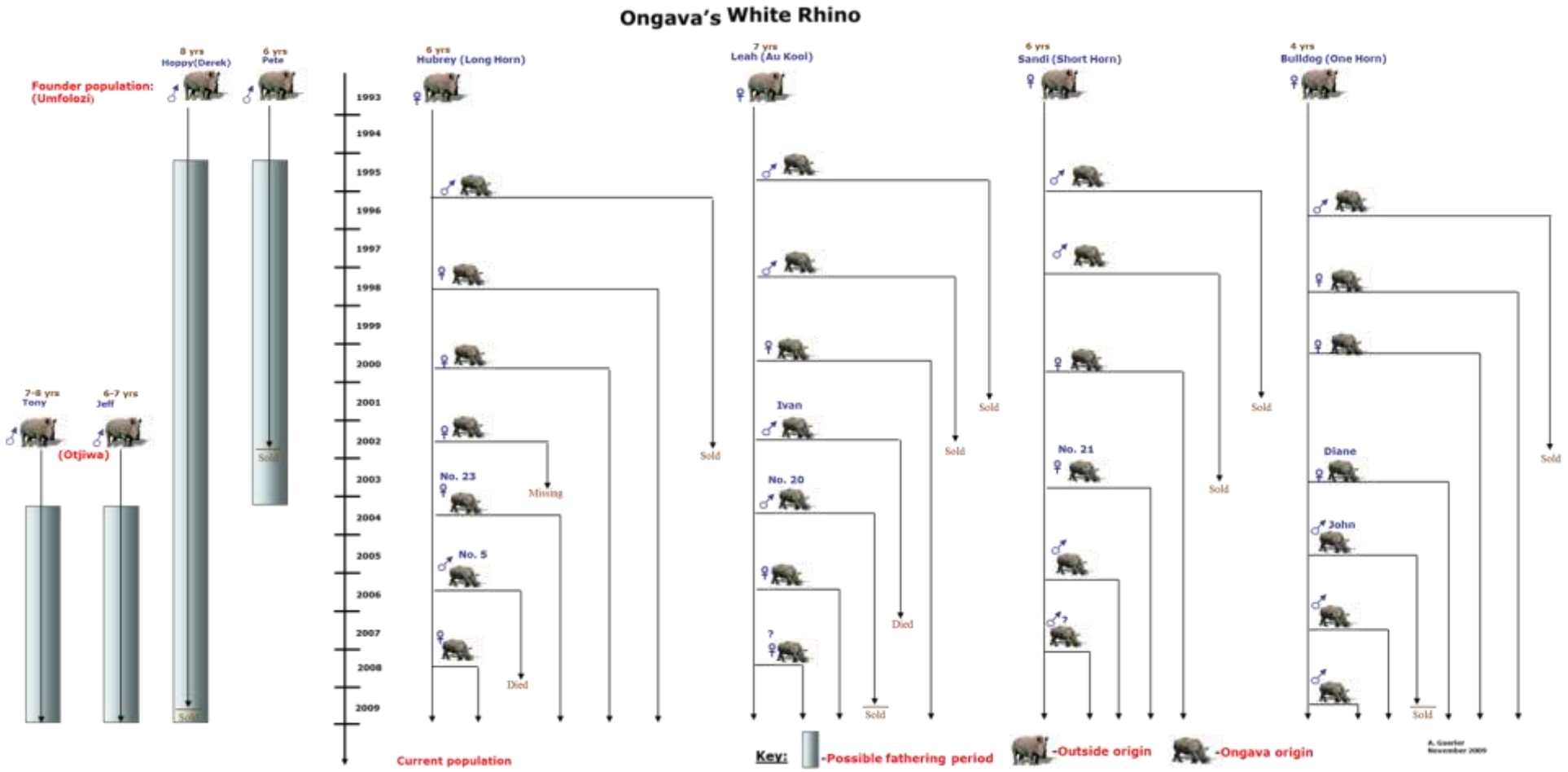

Figure 3.1a ***Preliminary Lineage Diagram for Ongava's Southern White Rhinoceros Population, 1994-2009*** **– Detailed pedigree information was derived from the historic records and used to create a template for lineage information. Candidate fathers are shown on the left-hand side, with the blue bars showing the time for which they were breeding candidates. The top line shows the founder population (2 males, 4 females) with each branch reflecting birth. Note that none of the calves born before 2001 are identified in the diagram, since I do not know to which matriline they belong. These animals and their subsequent offspring are shown in Figure 3.1b. The '?' indicates unknown sex whereas the ♂? And ♀? symbols indicate suspected male or female sex respectively but sex is not yet confirmed.**

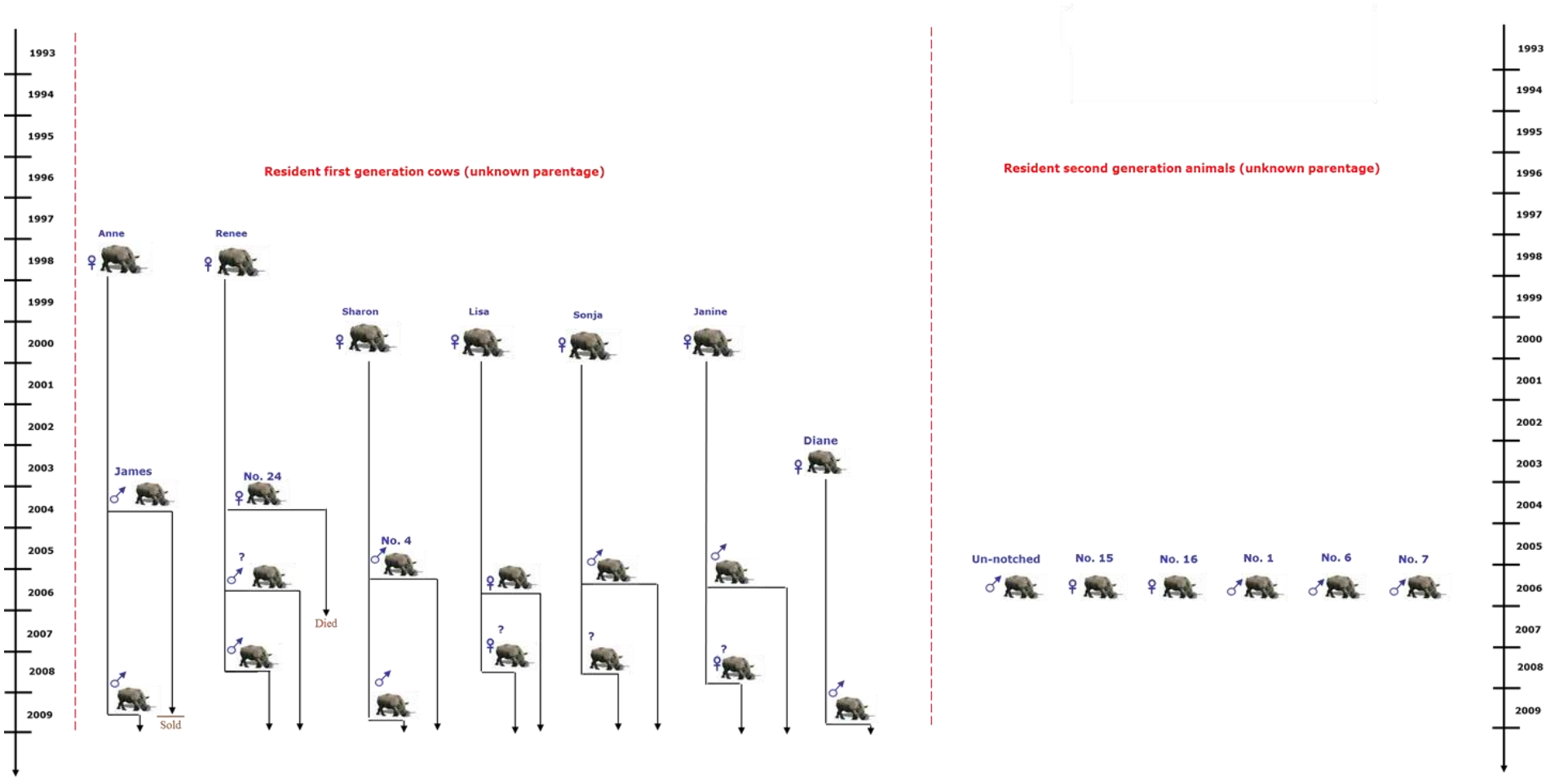

Figure 3.1b ***Preliminary Lineage Diagram for Ongava's Southern White Rhinoceros Population, 1994-2009, Unassigned Individuals*** **– Detailed pedigree information was derived from the historic records and used to create a template for lineage information. This figure shows details of the F_1 and F_2 populations. The positions of these individuals in the matrilines shown in Figure 3.1a are not known, and therefore they are shown separately. For example, the calves born in 1998 (Anne and Renee) appear in Figure 2A as two unknown females calves born to Long Horn and One Horn. Only once a mother has been assigned will it be possible to insert these individuals and their offspring in to the final lineage diagram.**

3.3.2. Parentage Analysis

In order to explore the range of solutions that different assumptions make, I performed parentage analysis for the 23 offspring in the sample set using both manual and software techniques under the following conditions:

a) Unconstrained - Allow all possible parent combinations for each offspring given the sexes for all individuals
b) Constrained – Specify a set of candidate parents for each offspring based on pedigree information
c) Constrained without Rh8 – As for b), but with all genotype data for the locus with the null allele, Rh8, removed before analysis
d) Constrained in combination with date of birth – as for b) but with the added parameter of the dates of birth of all offspring included to further narrow the possibilities.

For all constrained manual assignments the sample for Bob was not excluded from any potential assignments based on a homozygous genotype (i.e. he was not considered incompatible with the offspring in question based on a homozygous locus since due to allelic dropout occurrence I could not rule out the possibility of a heterozygote as the true data). The summary of this analysis is shown in Table 3.1.

Manual assignments

In 16 of 23 cases it was possible to uniquely assign parentage to one set of parents. For 5 of the 23 cases there were 2 candidate parent pairs possible and for the remaining 2 of the 23 cases there were 3 possible candidate parent pairs. In the manual assignments adding pedigree constraints significantly reduced the number of possible parent pair combinations. The unconstrained analysis was undertaken with the UCT output data to demonstrate that in some case several combinations were possible, but in others (7 of 23) no possible parent pairs were present in the genotype data set. This confirms the presence of genotyping errors. See Appendix 3A for the full set of results for this method.

Cervus assignments

Unconstrained assignments using Cervus resulted in 18 of 23 ‘most-likely’ parent pair combinations that were in fact not possible given pedigree information for the population (thus only five parent pair combinations produced were possible). Once the number of

candidate parents had been constrained according to the pedigree data; then for 18 of 23 'most-likely' Cervus assignments, parent pairs were assigned at the 95% confidence level. For the remaining offspring, 4 were assigned parent pairs at the 80% confidence level and in only one instance was the parent pair assignment not statistically significant. In the latter case, the mother (Short Horn) could be assigned with high confidence, but the software could not discriminate between Derek and Jeff as the true father. See Appendix 3B for the full set of results using this method. When the Cervus analysis was run using a different reference population from Hluhluwe-Umfolozi, the top ranked pairs assigned were identical to those assigned when using the Ongava sample set as the reference population.

Colony assignments

Similarly to Cervus, in the unconstrained case, Colony assigned parents that were not possible given the pedigree information for the population. Once the candidate parent pairs were constrained by pedigree, Colony assigned exactly the same fathers as Cervus for all offspring (i.e. the statistically most-likely father was the same for both software analyses). In 3 of 23 cases, the top-ranked mother assigned by Colony did not agree with that assigned by Cervus, but in all three cases that assignment was included in the possible parent pair combination derived from the manual analysis. See Appendix 3C for the full set of results from this method.

Comparison between manual, Cervus and Colony assignments

A comparison between the different modes of parentage assignments showed that in all instances where there was an unambiguous manual assignment (i.e. only one parent pair possible), both the Cervus and Colony 'most-likely' assignments were the same parent pair. For the remaining seven manual assignments, both the Cervus and the Colony 'most-likely' assignments based on a pedigree-constrained dataset were always one of the manual assignment possibilities. See Table 3.1 for a summary of parentage assignment for each of the analysis methods.

		MANUAL		*CERVUS*		*COLONY*	
Calf ID	DOB	*Mother*	*Father*	*Mother*	*Father*	*Mother*	*Father*
Anne	1998	One Horn	Derek	One Horn	Derek*	One Horn	Derek
Renee	1998	Au Kooi Short Horn Short Horn	Bob Bob Derek	Short Horn	Derek*	Short Horn	Derek
Sharon	2000	One Horn	Bob	One Horn	Bob*	One Horn	Bob
Sonja	2000	Long Horn	Derek	Long Horn	Derek*	Long Horn	Derek
Janine	2000	Au Kooi Short Horn	Bob Bob	Au Kooi	Bob*	Au Kooi	Bob
Lisa	2000	Au Kooi Short Horn Short Horn	Bob Bob Derek	Short Horn	Derek*	Short Horn	Derek
Ivan	2002	Au Kooi Au Kooi	Bob Derek	Au Kooi	Derek+	Au Kooi	Derek
Diane	2003	One Horn	Derek	One Horn	Derek*	One Horn	Derek
#20	2004	Au Kooi	Derek	Au Kooi	Derek*	Au Kooi	Derek
#21	2004	Long Horn	Derek	Long Horn	Derek*	Long Horn	Derek
#22	2004	Anne	Derek	Anne	Derek*	Au Kooi	Derek
#23	2004	Short Horn Short Horn	Bob Derek	Short Horn	Derek+	Short Horn	Derek
#24	2004	Renee Renee	Bob Derek	Renee	Bob*	Renee	Bob
John	2005	One Horn	Derek	One Horn	Derek*	One Horn	Derek
Long Horn calf[1]	2006	Long Horn	Derek	Long Horn	Derek*	Long Horn	Derek
Au Kooi calf[2]	2006	Au Kooi	Derek	Au Kooi	Derek*	Au Kooi	Derek
Short Horn calf[3]	2006	Short Horn	Derek	Short Horn	Derek^	Short Horn	Derek
Renee calf[4]	2006	Renee	Jeff	Renee	Jeff*	Renee	Jeff
Sharon calf / Zac	2006	Sharon	Tony	Sharon	Tony*	Sharon	Tony
#1[4]	2006	Renee	Jeff	Renee	Jeff*	Renee	Jeff
#5[1]	2006	Long Horn	Derek	Long Horn	Derek*	Long Horn	Derek
#6	2006	Janine	Tony	Janine	Tony+	Au Kooi	Tony
#7[3]	2006	Short Horn Short Horn	Derek Jeff	Short Horn	Derek^	Short Horn	Derek
#15	2006	Lisa	Tony	Lisa	Tony+	Au Kooi	Tony
#16[2]	2006	Au Kooi	Derek	Au Kooi	Derek*	Au Kooi	Derek
Max	2006	Sonja	Tony	Sonja	Tony*	Sonja	Tony
Tom	2007	One Horn	Jeff	One Horn	Jeff*	One Horn	Jeff

Table 3.1 *Summary of results from the different parentage assignment methods used* - For each of the assignment methods - Manual, Cervus and Colony - the final parent pairs selected are shown. These assignments incorporate all the pedigree constraints: animal gender, tenureship, restricted parent candidates, parameters due to Date-of-Birth (DOB) of calves. Cervus and Colony provide only the most-likely candidate parent pairs while the manual assignments show all possible parent pairs available given the constraints applied. Note: calves #1, #5, #7 and #16 are duplicate samples collected after ear-notching for known-mother calves; the superscript number indicates the corresponding pre-dispersal biopsy dart sample determined after parentage assignment. Cervus assignments: *strict confidence (95%), +relaxed confidence (80%), ^indicates most-likely parent pair assigned at <80% confidence.

Analysis of genotype mismatches

I also explored the potential problem of 'false' parent pair exclusions due to genotype mismatches arising from genotyping errors. For both the unconstrained case (all possible parent pair matches) and the constrained case (candidate parents restricted according to prior pedigree information) the frequency distribution of locus mismatches (see Figure 3.2 below which shows this distribution for the constrained case) was computed (using in-house software, available K. Stratford). In the constrained case, possible matches were obtained in 9.6% of pairings. In summary, relatively few parent-pair exclusions occurred as the result of single locus mismatches (13%) while approximately three-quarters (77.4%) of candidate parent pairs were excluded based on genotype mismatches at more than one locus. For those parent pairs excluded based on single locus mismatches a difference in the distribution of mismatches over the 11 loci was observed, however in the context of the low error rate in the full study this is unlikely to have led to a significant number of erroneous exclusions.

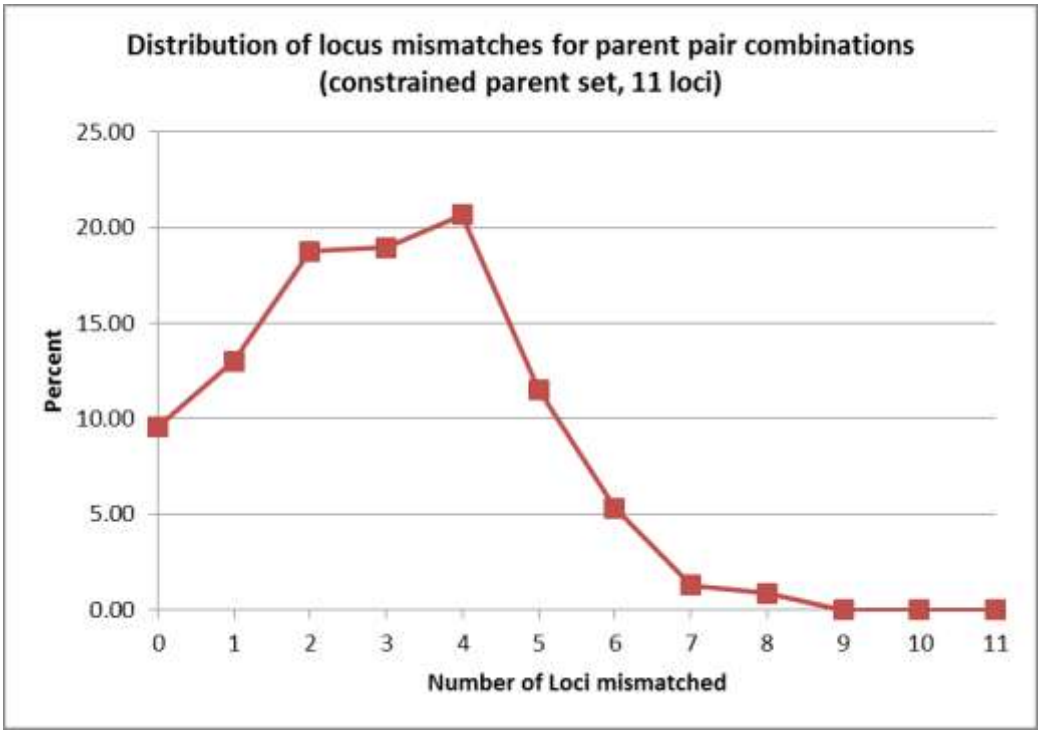

Figure 3.2 *Locus mismatches* – Distribution of locus mismatches for the constrained case of candidate parent pairs

I then further analysed the distribution of loci for those parent pairs excluded based on single locus mismatch. Figure 3.3 shows the frequency distribution of these single locus exclusions by locus. Locus WR1 (the most variable locus, PIC=0.53) contributed most instances of these exclusions (20 of 61), with locus Rh8 (a locus with a null-allele) contributing 15 of 61

mismatches. Exclusions based on single locus mismatches for these loci combined led to about 7% of the total number of exclusions for the constrained parent set. Given the low genotype error rate, in the context of the full study I do not think that any erroneous exclusions at these loci will have significantly biased the assignments.

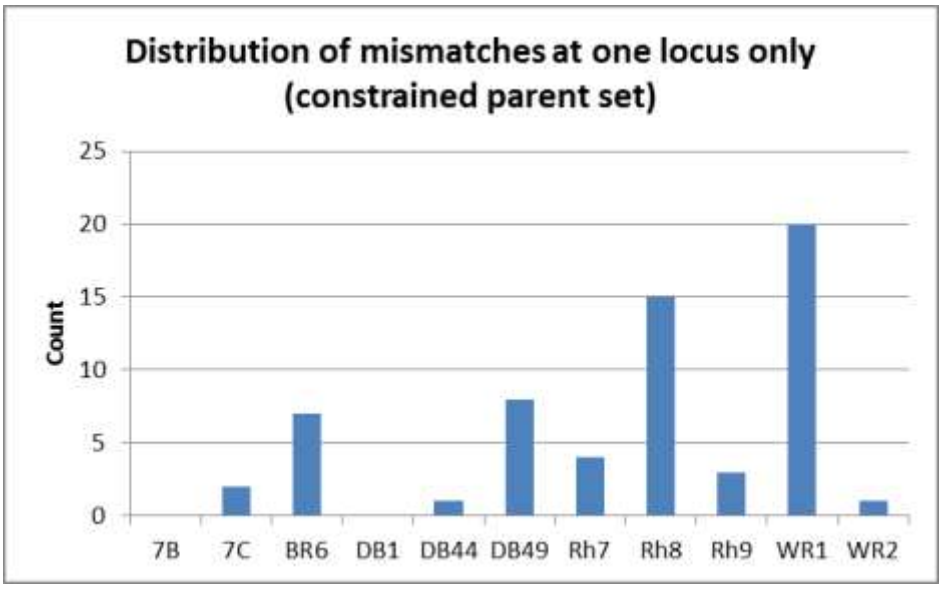

Figure 3.3 *Single locus mismatches* - Distribution by locus of single locus mismatches for the constrained case of candidate parent pairs

The complete set of parentage assignments allowed me to resolve the ambiguities shown in the lineage diagram (Figure 3.1) and produce a full lineage diagram, arranged by matrilines, for the current white rhinoceros population on OGR, as shown below in Figure 3.4.

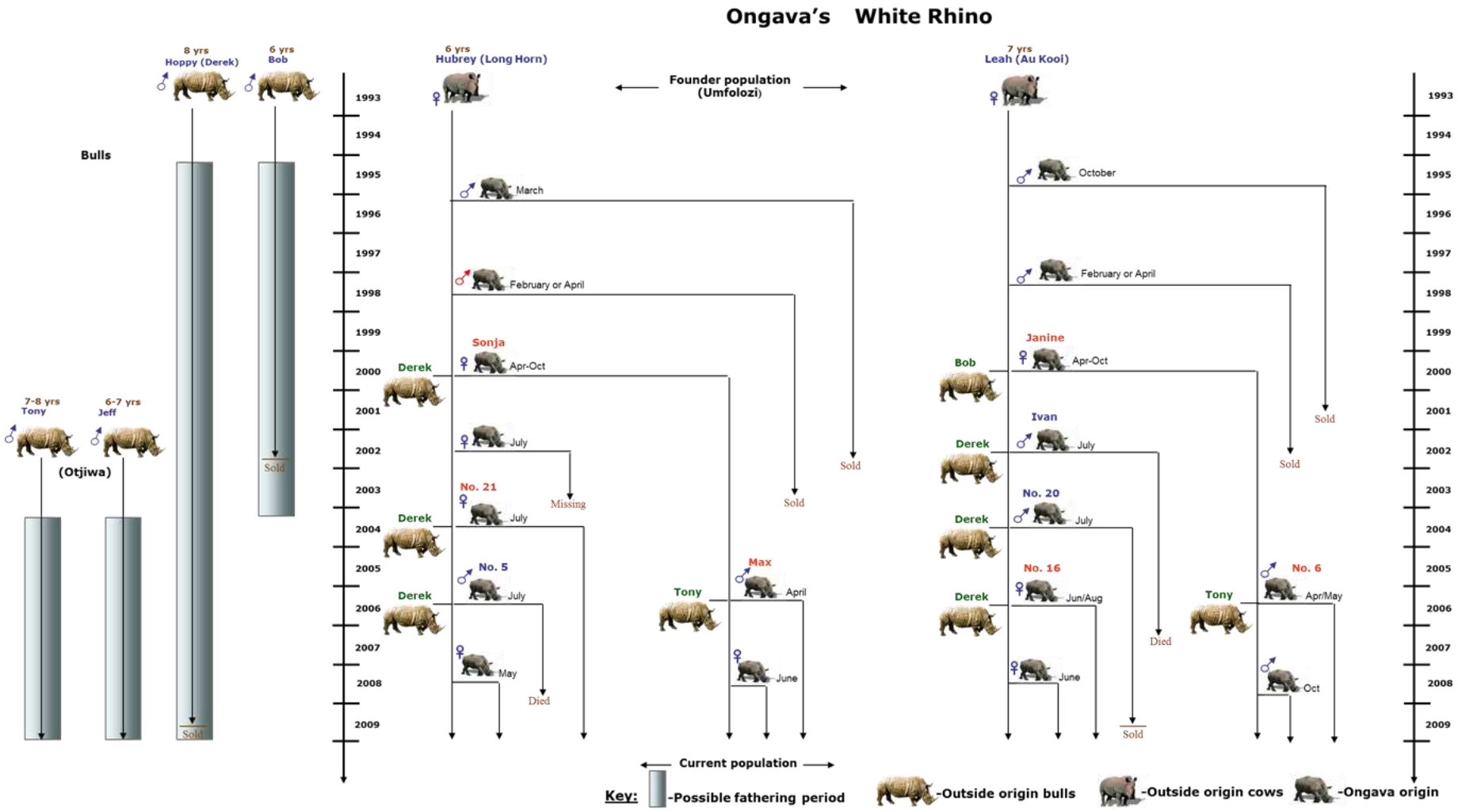

Figure 3.4a Final Lineage Diagram for OGR's Southern White Rhinoceros Population – Long Horn and Au Kooi Matrilines

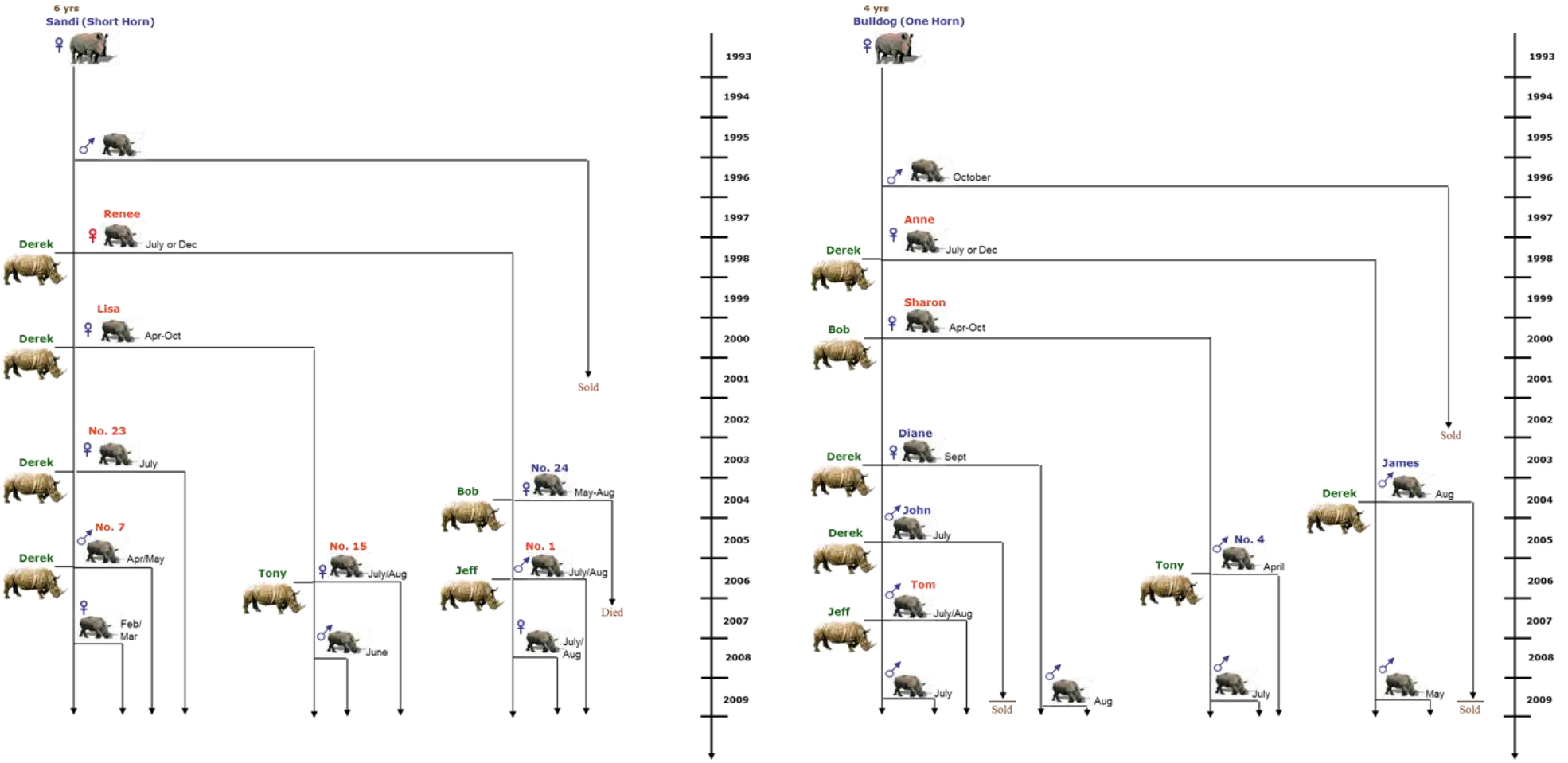

Figure 3.4b *Final Lineage Diagram for Ongava's Southern White Rhinoceros Population* – Short Horn and One Horn Matrilines

Pairwise genetic distance estimates and parentage assignment

Pairwise distance estimates were provided by simulations assessing genotyping frequencies across all loci which delivers scores ranging from 0 (full relatedness) to 1 (no relatedness). See Appendix 3. D for the full set of results.

Adult breeding males were, on average, as genetically similar to each other as they were to the females with which they sired offspring (mean pairwise male ds=0.40 ±S.E=0.01; mean parental pair ds=0.43±0.02). The most successful male, Derek (13 successful sires in the study period), was on average as genetically similar to females with which he successfully sired calves (ds=0.42±0.02; range 0.22-0.50) as compared to Tony (ds=0.40±0.03; range 0.31-0.50) or Jeff (ds=0.48±0.06; range 0.36-0.50), suggesting that male mating success at OGR is not influenced by genetic similarity among mated pairs. The mean shared allele distance among founder breeding females was ds= 0.42±0.02. These results together suggest that assignments to potential sires are not biased to a particular candidate father by chance genetic similarity among individuals. Indeed, the mean overall pairwise ds (0.37±0.004) at OGR was similar to that for the alternative reference population sample (Hluhluwe-Umfolozi Park, ds= 0.401±0.01; n=49).

3.3.3. Population Genetics

In order to assess population parameters across generations, I re-arranged the lineage diagram (Figure 3.4) by sequential maternal generations, see Figure 3.5. This shows an F_0:F_1:F_2 population structure of 4:16:7 (excluding breeding males). The descriptive statistics for each of the microsatellite loci grouped by generation are given in Table 3.2

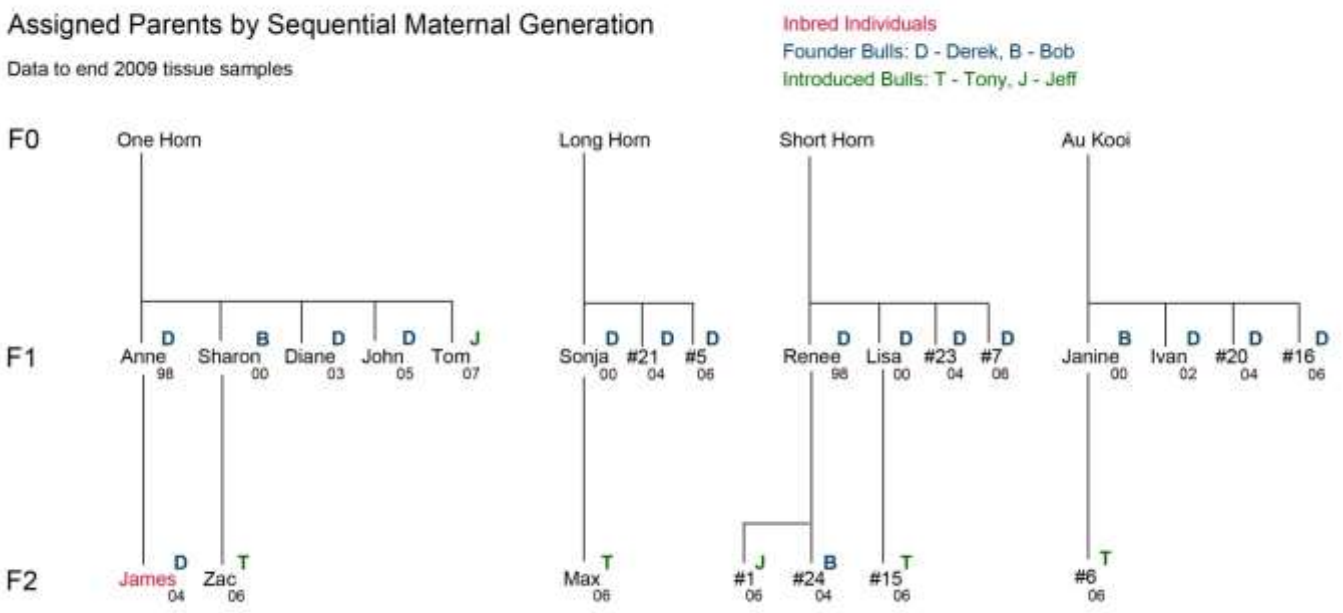

Figure 3.5 ***Assigned Parents by Maternal Generation*** **– Generation chart showing each matriline by generation. For each offspring the assigned father is shown by the superscript initial (D, B are founder bulls Derek and Bob in blue; T, J are introduced bulls Tony and Jeff in green). The subscript number refers to the year of birth. The single inbred individual (James) is shown in red, and arose from Derek breeding with Anne, his daughter by One Horn.**

	F_0		F_1		F_2	
Locus	H_{Obs}	H_{Exp}	H_{Obs}	H_{Exp}	H_{Obs}	H_{Exp}
7B	0.714	0.560	0.313	0.462	0.714	0.703
7C	0.667	0.591	0.533	0.503	0.600	0.556
BR6	0.625	0.675	0.500	0.448	0.571	0.648
DB1	0.250	0.233	0.500	0.387	0.286	0.264
DB44	0.250	0.233	0.500	0.387	0.286	0.264
DB49	0.429	0.626	0.500	0.411	0.571	0.440
Rh7	0.125	0.325	0.313	0.272	0.000	0.000
Rh8	0.375	0.342	0.188	0.454	0.000	0.264
Rh9	0.750	0.592	0.625	0.484	0.286	0.264
WR1	0.750	0.692	0.750	0.571	0.429	0.615
WR2	0.429	0.538	0.625	0.508	0.143	0.538
Mean	0.488	0.492	0.486	0.444	0.353	0.414
Mean (no Rh7, Rh8)	0.540	0.527	0.538	0.462	0.432	0.477

Table 3.2 ***Heterozygosity for each Locus by Generation*** **– H_{obs}: Observed, H_{exp}: Expected**

The mean heterozygosity across all 31 individuals, H_{pop}, is 0.447, and the polymorphic information content (PIC) is 0.357. Heterozygosity appears to be reduced in the F_2 generation, but the sample size is small (n = 7 individuals).

3.3.4. Inbreeding

By Pedigree

All offspring males were removed from the population before they had the opportunity to become candidate fathers, and the F_2 females were too young to have produced calves within this study period. Therefore at the pedigree level, instances of inbreeding in this white rhinoceros population will only be evident when an F_1 female has produced an F_2 offspring with her father. Examination of Figure 3.3 shows that father-daughter inbreeding has occurred just once; Anne's father is founder bull Derek, and he is also the father of her calf, #22. Hence the inbreeding coefficient for this individual is $F_{\#22}$=0.25. All other 6 F2 offspring have differing fathers and grandfathers. This value for F assumes that there are no pedigree contributions from the founder animals. Thus from the parentage assignment data there are 1 of 7 possible calves inbred.

By Genes

To assess at the genetic level whether the population shows signs of progressive inbreeding, I used Cervus to calculate heterozygosity across all loci, H, for each generation (See Table 3.2).
I then estimated the effective inbreeding coefficient, F_e, for the F2 generation (from Frankham et al. 2002, equation 11.7), for both observed and expected values of H.

From H_{obs}

$$F_{e,F2} = 1 - (H_2 / H_0) = 0.276$$

From H_{exp}

$$F_{e,F2} = 1 - (H_2 / H_0) = 0.157$$

3.3.5. Mate Selection

In order to determine whether any biased selection of fathers was present in the population, I examined the fathers of offspring by maternal generation (see Table 3.3 and Figure 3.5).

Father	Tenure	F_1 Generation	F_2 Generation
Bob (founder)	1993-2003	2	1
Derek (founder)	1993-2009	13	1
Tony (introduced)	2002-present	0	4
Jeff (introduced)	2002-present	1	1
Total		16	7

Table 3.3 ***Assigned fathers by generation***

Founder bull Derek was the dominant breeding bull (13 of 16 calves) for the F1 generation (i.e. with founder cows) but not with the F2 generation (1 of 7 calves).

These numbers are confounded since one founder bull (Bob) was removed from the reserve and replaced by two introduced bulls (Tony and Jeff) during this study period. In order to examine the distribution of fathers more closely, I further analysed the largest yearly birth cohort, the calves born in 2006 (n=8). At this time, 3 breeding bulls were present on the reserve (founder bull Derek and the two introduced bulls Tony and Jeff). The distribution of mothers and fathers for this sub-set of offspring is shown in Table 3.4 (numbers derived from Figure 3.5). Interestingly, founder mothers only produced offspring with founder fathers, and offspring mothers (all of whom were themselves fathered by founder bulls) only produced offspring with the introduced bulls, which was significantly difference from a random distribution ($\chi^2 = 7.95$, df = 1, $P<0.01$).

	Founder Mother	Offspring Mother
Founder Father	3	0
Introduced Father	0	5

Table 3.4 ***Distribution of assigned mothers and fathers by generation for 2006 offspring***

3.4. DISCUSSION

To my knowledge this is the first study in which it has been possible to confidently assign a complete set of parents to all offspring in a free-ranging, closed population of white rhinoceros and thereby be able to produce a conclusive family tree for such a population.

The two major factors contributing to the success of this study were that I was able to obtain DNA via a tissue sample from every individual in the population, and that I had comprehensive pedigree information for each rhinoceros which allowed me to constrain the number of candidate parents for any one offspring.

3.4.1. Techniques for Parentage Analysis

In this analysis, I used a sequential approach to integrate pedigree information with genetic data. I collected both sets of data independently, first determined the genotype for each individual at each locus, and then refined the parentage assignment by restricting the number and identity of candidate parents based on pedigree information. I performed this in an iterative manner, using first parental presence (i.e. the only candidates were those breeding males and females on the reserve at the time) and then calf dates of birth (mothers only produce a single offspring). This approach has been used in the investigation of kinship and parentage across a number of species (Jones & Ardren, 2003) and options for restricting candidate parents exist in parentage analysis software (e.g.: Cervus 3.0, Kalinowski et al. 2007 and Colony 2.0, Jones & Wang, 2009).

According to the criteria suggested in Jones et al (2010) – I have samples for all candidate parents, and our family size is small – the exclusion and categorical (as recommended in Cervus) methods I have used are most appropriate for my data set. There is however an alternative approach to the issue of integrating pedigree and genetic data, which is to simultaneously estimate parentage and the population-level parameters in which one is interested, termed full probability modelling. Hadfield et al (2006), for example, present a Bayesian framework for this process, which they state provides a parentage assignment that may be less biased. The primary basis for implementing this rigorous statistical methodology is to compensate for genetic data sets that may have high rates of genotyping errors, or pedigrees with limited information (see for example the software suggested by Walling et al. 2010). While my final set of genotypes does contain a negligible error rate (as shown by the genotype repeatability of 26 random samples), and my baseline pedigree information is both accurate and comprehensive, I chose to repeat my analysis using the alternative modelling framework provided by

Colony 2.0 (Jones & Wang, 2009). The computational methods both gave very similar results, suggesting that my assignments were not biased by any one particular statistical methodology.

The presence of duplicate samples in the data set allowed me to verify of the accuracy of the assignment results. I recommend including a subset of anonymous samples within the data set for any similar future studies to act as internal controls along with the more typical methods such as multiple DNA extractions from each sample, repeat PCRs per sample, calculation of error rates, etc. (see recommendations in Bonin et al. 2004; Hoffman & Amos, 2005; Selkoe & Toonen, 2006; Scott, 2008).

3.4.2. Parentage Analysis

To my knowledge, there have been few parentage studies in free-ranging white rhinoceros populations, or indeed other rhinoceros species. Success in those attempts at parentage assignment has been limited.

Coutts (2009) attempted to confirm maternity and assign paternity in two populations of southern white rhinoceros. Both populations had 10 calves with 12 candidate fathers. Her analyses showed that very few paternities could be assigned with statistical confidence and even with the removal of the non-informative loci the resolving power did not improve. Her paternity assignment rate at the 95% confidence level was only 20% and 10% for each of the populations. At the 80% confidence level only 40% of calves could be assigned fathers. An attempt was made to ascertain parentage in both populations for these calves (i.e. both parents to be determined) but at the 95% level no assignments could be made for any of the calves and at the 80% confidence level only 20% and 30% of the calves in each population could be assigned parent pairs. In this study I was able to assign 22 of 23 (96%) of parent pairs unambiguously.

Steyn and Stalmans (2004) report on their plans to apply genetic profiling (by means of microsatellites) to create an individual genetic ‘fingerprint’ for each of the southern white rhinoceros in the various populations in protected areas in Mpumalanga. They indicate via an example how parentage assignment might work, but have not followed this report with any population data.

In other rhinoceros taxa, to date, only Garnier et al (2001) have been able to unequivocally assign parentage to all 19 offspring in a small (n=33), wild population of black rhinoceros in Zimbabwe (*Diceros bicornis*). The authors used DNA extracted from faecal samples to derive genotypes across

10 microsatellite loci (on average 3 alleles per locus, range 2-7) and were successful in determining 100% of genotypes (here I determined 97.7%). In their analysis, most mothers were first assigned based on behavioural observations and paternity was then tested using Cervus. Up to 10 males were candidate fathers (in comparison with this study, where either 2 or 3 males were candidates), however they had DNA samples from 9 of these 10. Their study period was not long enough to assess inbreeding or mate selection.

Kim (2009) assessed populations of black rhinoceros (*Diceros bicornis bicornis*) in Namibia using microsatellite markers and also conducted parentage studies in two small game reserves with limited or no pedigree information. His investigations revealed that even though the number of candidate fathers was small (five and two respectively in each of the reserves/farms) not all calves could be assigned at the strict 95% confidence level. In one of his study populations there were 7 calf-cow dyads and 2 candidate bulls. Five calves could be assigned paternity at the 95% confidence level while 2 remained unassigned. Just one calf-cow-bull relationship was determined to be correctly assigned with significance. By relaxing confidence levels to 80%, the calves could all be assigned paternity to either of the 2 bulls. In his second population there were 6 calf-cow dyads and 5 candidate bulls. For that population only 2 calves could explicitly be assigned to candidate fathers with strict 95% confidence.

Given the additional concern that parentage assignment in species with low genetic diversity might be compromised, I undertook several additional analyses. I found that the manual assignments were not biased by an excessive number of exclusions of possible parent pairs due to a mismatch at just one locus. I also calculated the genetic distance between individuals in the population and found that distances were similar across the breeding elements of the population, suggesting that assignments to potential sires were not biased to a particular candidate father based on levels of individual genetic similarity. I am therefore confident that the pedigree results presented in this study are accurate and robust.

It is likely that higher levels of variability found in microsatellite studies in black rhinoceros may allow for more successful parentage analysis using genetic data alone (without complementary pedigree data, mate histories or other population structure data. Cunningham et al. 1999; Garnier et al. 2001; Harley et al. 2005). Research on other species supports this theory. For example, Tokarska et al (2009) found they were unable to use microsatellites for parentage assignment in the severely bottlenecked European Bison - *Bison bonasus*, where all surviving members of this species are thought to have descended from just two individuals; this is reflected in very low heterozygosity

(H_{Exp}<0.3 across 17 microsatellites) and high levels of inbreeding (F_{bar}>0.5). Conversely, Archie et al (2007) were able to assign parentage in a high proportion of elephant calves (152 of 183) using faecal samples typed across 11 microsatellites – this species shows relatively high heterozygosity (mean H_{Obs}=0.77 across 20 populations in 16 microsatellites, Comstock et al. 2002).

My study shows that pedigree and genetic information are complementary to each other and necessary for assigning parentage with significant levels of confidence, particularly in species such as white rhinoceros where genetic variation is low. Here I was able to successfully assign parentage to an entire population of free-ranging white rhinoceros with significant levels of confidence (16 of 23 offspring at the strict 95% confidence level, 6 at 80% confidence).

Although microsatellites are considered more informative (Frankham et al. 2002) alternative types of molecular markers have also been used in previous rhinoceros parentage studies. Seror et al (2002) used the Random Amplified Polymorphic DNA (RAPD) technique to reveal the paternity of rhinoceros born at the Zoological Center in Israel. For a total of 8 offspring, they could assign paternity for 5 out of 7 calves (2 remained unassigned). The 8th calf could not be analysed as its mother's DNA was not available and thus could not be excluded from the RAPD allocation. A technique using Amplified Fragment Length Polymorphic (AFLP) DNA was applied by Kellner et al (2001) in an attempt to study the breeding patterns in a small population of southern white rhinoceros on a game farm in South Africa. They showed that for 14 calves (with known mothers and 5 possible candidate sires) the most probable father could be determined for 12 of the offspring, while the remaining 2 offspring showed two possible father choices. This latter study reports similar success rates to ours, however they had certain knowledge of the mother-calf assignments – since RAPD and AFLP both have dominant modes of inheritance they may work where one parent is known. For parent-pair studies microsatellites, which are co-dominantly inherited, are preferred (Frankham et al. 2002).

3.4.3. Inbreeding

The current white rhinoceros population has produced one inbred F_2 individual, (14%). Based on random mixing between males and females, as is the case on OGR, I would expect at least 33% inbreeding, or even more if founder males are dominant over introduced males. Thus, based on a very small samples size, this population shows signs of possible inbreeding avoidance. Yet effective inbreeding coefficient Fe for the F2 generation suggests that genetic diversity is being reduced. This

may be an anomaly and needs more data over the next generations to be calculated properly with confidence.

While many species demonstrate deleterious effects from inbreeding depression (Frankham et al. 2002), in both managed and natural populations of free-ranging, long-lived species these effects are very difficult to measure. Krummenacher and Zschokke (2007) measured mortality rates of captive white rhinoceros and found a small but not significant increase in inbred individuals. The average juvenile mortality rate of non-inbred white rhinoceros individuals was 14.7%, whereas the average juvenile mortality rate of inbred individuals was 18.7%. However they conclude that their sample size was too small to be able to draw any conclusions. In the one analysis to date, free-ranging white rhinoceros did not display reduced genetic diversity in seeded populations and also showed no evidence of inbreeding depression (Coutts, 2009). In addition, given that all white rhinoceros are descended from just a few individuals (<100 in the late 1800's; Emslie & Brooks, 1999; Skinner & Chimimba, 2005) it could be argued that inbreeding is not a significant factor in reducing fitness, since genetic diversity is already very low. Nevertheless, unchecked inbred matings are likely to lead to further loss of genetic variation and some form of inbreeding depression in the future, and should be avoided (Edmands, 2007). It is worth noting here that a lack of evidence for inbreeding depression (e.g. juvenile survival) does not support the conclusion that there is no inbreeding depression for other factors (e.g. fecundity) or indeed that diverse fitness components will not interact to reduce overall fitness in the future (Hedrick & Kalinowski, 2000; Edmands, 2007).

There are no studies of inbreeding based on shared ancestry in combination with pedigree data in wild rhinoceros populations, since they require explicit parentage assignment. This study is the first to be able to determine accurate inbreeding numbers. As the database of genetic information builds up over the coming generations of breeding on OGR, it will allow for a better assessment of how inbreeding impacts on the fitness of individuals and the population.

3.4.4. Mate Selection and Inbreeding Avoidance

The data show that, at least for later calves, some form of mate selection is occurring in the population. For the 8 calves born in 2006, founder bull Derek fathered all three that were born to founder mothers, while the five calves born to F_1-generation mothers (i.e. mothers born on OGR) were all fathered by the introduced bulls Tony and Jeff. Thus, none of these F_1-generation mothers was impregnated by Derek, effectively avoiding the possibility of inbreeding. Given that all of these

females had access to each of the bulls (overlapping home ranges), it is possible that disassortative mating' (the mating of individuals with traits more dissimilar than likely in random mating) occurred.

In a number of species, and in particular those where dispersal is not a mechanism that is available to inhibit inbreeding (Pusey & Wolf, 1996), individuals show a preference for mating with non-related others (for example, Hoffman et al. 2007 – fur seals - *Arctocephalus gazella*; see also Anderson & Iwasa, 1996). The sexual selection literature has long debated how this happens (Clutton-Brock, 2009). In a closed system, to avoid breeding with a potential mate that is related, the individual has to be able to identify that potential mate, a process termed 'kin recognition' (Penn & Frommen, 2010). Research in this area has focused around the Major Histocompatibility Complex (MHC), which plays a central role in controlling resistance to parasites and disease, as well as immunological self/non-self recognition (Klein & O'Huigin, 1994). These genes are all generally highly variable in mammals, even those that have come through recent bottlenecks (Penn & Potts, 1999). In a number of species, mates select for differences in MHC genes (for example, Setchjell et al. 2010 – mandrill - *Mandrillus sphinx*). By extension, it is thought that individuals may be able to discriminate between mates on the basis of smell since the more different the smell, the less related (Charpentier et al. 2008 – lemurs – *Lemur catta*, see also Hurst & Beynon, 2010). For example, in mice - *Mus musculus musculus*, females prefer the scent of outbred males (Ilmonen et al. 2009). Thus the hypothesis is that MHC-disassortative mating preferences will further drive MHC diversity, which in turn confers greater resistance to parasites and disease (Penn & Potts, 1999). However, Paterson and Pemberton (1997) found no evidence for MHC-dependent mating patterns in free-living Soay sheep - *Ovis aries.* The mechanisms for kin recognition in white rhinoceros are unknown at this stage. Dr. Bishop's laboratory at UCT has tested 4 loci in the MHC gene for southern white rhinoceros, and to date found that all have low sequence divergence (Coutts, 2009; pers. comm.) – essentially they have been unable to detect any differences in the MHC gene that might lead, for example, to inheritance-dependent differences in odour.

In long-lived mammals, mate selection effects are difficult to measure at the pedigree level. In African elephants, sex-biased dispersal does not lead to the complete separation of male and female relatives, and so individuals may engage in mate selection to recognize kin and avoid inbreeding. In Amboseli National Park, Kenya, males engaged in proportionally fewer sexual behaviours and sired proportionally fewer offspring with females that were natal family members or close genetic relatives (both maternal and paternal) than they did with non-kin (Archie et al. 2007). Yet Keane et al (1996) found no evidence for inbreeding avoidance in the dwarf mongoose - *Helogale parvula*, a social carnivore that also shows limited or incomplete dispersal.

The observation of disassortative mating in this population will need to be re-assessed once more genotypes for calves born in 2008 and beyond can be obtained to see if the trend continues. A possible alternative explanation might be that individuals prefer mates of similar ages, or that the new bulls were not sufficiently experienced for the senior females. Archie et al (2007) suggest that elephant males may follow simple rules such as 'avoid the daughters of females you mated with', but this would require some form of kin recognition – olfactory cues (and hence MHC genes) are a strong candidate in elephants. On the other hand, Frere et al (2010) found in a highly inbred population of dolphins - *Tursiops truncatus* that female's earlier calves are more likely to be inbred, suggesting that experience may play a role in mate selection – perhaps unlikely in this study population since it appears to be the younger females that are able to avoid breeding with the founder bull. Finally, if there are intrinsic mechanisms that rhinoceros use for mate selection, the population may require outbred males from which to select. It seems likely that however white rhinoceros choose their mates, their strategies may be species and perhaps even population specific.

APPENDIX 3.A

Parent assignment results using manual method. These assignments incorporate all the pedigree constraints applied in 4 sequential steps. For the unconstrained analysis only the number of possible pair parent combinations are given. For the constrained steps all possible parent pairs available are shown. Note: Table divided over two pages.

Animal ID	**Manual Assignments**						
	1. Unconstrained UCT output (Gender Known)	*2. Constrained (Candidate Parents)*		*3. Constrained (Rh8 removed - null allele)*		*4. Constrained (Offspring's DOB)*	
	# of possible mother/father combinations	*Mother*	*Father*	*Mother*	*Father*	*Mother*	*Father*
Anne	1	One Horn	Derek	One Horn	Derek	One Horn	Derek
				Au Kooi	Bob		
Renee	0	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
		Au Kooi	Bob	Au Kooi	Bob	Au Kooi	Bob
		Short Horn	Bob	Short Horn	Bob	Short Horn	Bob
Sharon	1	One Horn	Bob	Au Kooi	Bob	One Horn	Bob
				One Horn	Bob		
Sonja	0	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
Janine	0	Long Horn	Bob	Long Horn	Bob	Au Kooi	Bob
		Au Kooi	Bob	Au Kooi	Bob	Short Horn	Bob
		Short Horn	Bob	Short Horn	Bob		
Lisa	3	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
		Au Kooi	Bob	Au Kooi	Bob	Au Kooi	Bob
		Short Horn	Bob	Short Horn	Bob	Short Horn	Bob
Ivan	7	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
		Au Kooi	Bob	Au Kooi	Bob	Au Kooi	Bob
		Short Horn	Bob	Short Horn	Bob		
Diane	4	One Horn	Derek	One Horn	Derek	One Horn	Derek
		Au Kooi	Bob	Au Kooi	Bob		
#20	0	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
#21	0	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
		Au Kooi	Derek	Au Kooi	Derek		
#22	16	Au Kooi	Derek	Au Kooi	Derek	Anne	Derek
		Anne	Derek	Anne	Derek		
		Renee	Derek	Renee	Derek		
#23	9	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
		Renee	Derek	Renee	Derek	Short Horn	Bob
		Short Horn	Bob	Short Horn	Bob		
		Au Kooi	Bob	Au Kooi	Bob		
		Anne	Bob	Anne	Bob		
		Renee	Bob	Renee	Bob		
#24	0	Renee	Derek	Renee	Derek	Renee	Derek
		Renee	Bob	Renee	Bob	Renee	Bob
				Au Kooi	Bob		
LH calf	-	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek

Sharon calf/Zac	4	Sharon	Tony	Sharon	Tony	Sharon	Tony
AK calf	-	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek*
SH calf	-	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
Renee calf	-	Renee	Jeff	Renee	Jeff	Renee	Jeff
John	0	One Horn Anne	Derek Derek	One Horn Anne Lisa	Derek Derek Derek	One Horn	Derek*
#1	3	Renee	Jeff	Renee	Jeff	Renee	Jeff
#5	4	Long Horn Au Kooi	Derek Jeff	Long Horn Au Kooi	Derek Jeff	Long Horn	Derek
#6	2	Au Kooi Janine Lisa	Tony Tony Tony	Au Kooi Janine Lisa	Tony Tony Tony	Janine	Tony
#7	1	Short Horn Short Horn Renee	Derek Jeff Tony	Short Horn Lisa Renee	Derek Derek Tony	Short Horn Short Horn	Derek Jeff
#15	10	Au Kooi Renee Lisa	Tony Tony Tony	Au Kooi Renee Lisa	Tony Tony Tony	Lisa	Tony
#16	3	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
Tom	-	One Horn Sharon	Jeff Jeff	Lisa One Horn Lisa Sharon	Derek Jeff Jeff Jeff	One Horn	Jeff
Max	-	Sonja	Tony	Sonja	Tony	Sonja	Tony

APPENDIX 3.B

Parent assignment results using Cervus method. These assignments incorporate all the pedigree constraints applied in 4 sequential steps. Only 'most-likely' candidate parent pair assignments are shown. Trio LOD score (Trio likelihood of difference significance): *=95% significance, + = 80% significance, - = <80% confidence. Note: Table divided over two pages.

Animal ID	**Cervus Assignment - Most Likely Parents**											
	1. Unconstrained (Gender Known)			*2. Constrained (Candidate Parents)*			*3. Constrained (Candidate Parents, Rh8 removed)*			*4. Constrained (Offspring's DOB)*		
	Mother	*Father*	*Trio LOD score*	*Mother*	*Father*	*Trio LOD score*	*Mother*	*Father*	*Trio LOD score*	*Mother*	*Father*	*Trio LOD score*
Anne	One Horn	Derek	+	One Horn	Derek	*	One Horn	Derek	*	One Horn	Derek	*
Renee	Short Horn	#7	-	Short Horn	Derek	*	Short Horn	Derek	*	Short Horn	Derek	*
Sharon	One Horn	Zac	+	One Horn	Bob	*	One Horn	Bob	*	One Horn	Bob	*
Sonja	Long Horn	James	-	Long Horn	Derek	*	Long Horn	Derek	*	Long Horn	Derek	*
Janine	Long Horn	Ivan	-	Au Kooi	Bob	*	Au Kooi	Bob	*	Au Kooi	Bob	*
Lisa	#23	Jeff	+	Short Horn	Derek	*	Short Horn	Derek	+	Short Horn	Derek	*
Ivan	Au Kooi	Zac	-	Au Kooi	Derek	+	Au Kooi	Derek	-	Au Kooi	Derek	+
Diane	One Horn	Derek	+	One Horn	Derek	*	One Horn	Derek	*	One Horn	Derek	*
#20	#16	Derek	-	Au Kooi	Derek	*	Au Kooi	Derek	*	Au Kooi	Derek	*
#21	Sonja	#7	-	Long Horn	Derek	*	Long Horn	Derek	*	Long Horn	Derek	*
#22	#23	Derek	-	Anne	Derek	*	Anne	Derek	*	Anne	Derek	*
#23	Renee	James	-	Short Horn	Derek	+	Short Horn	Derek	+	Short Horn	Derek	+
#24	#21	#7	+	Rene	Bob	*	Rene	Derek	*	Rene	Bob	*
LH calf				Long Horn	Derek	*	Long Horn	Derek	*	Long Horn	Derek	*

Sharon calf/Zac	Sharon	Ivan	+	Sharon	Tony	*	Sharon	Tony	*	Sharon	Tony	*
AK calf				Au Kooi	Derek	*	Au Kooi	Derek	+	Au Kooi	Derek	*
SH calf				Short Horn	Derek		Short Horn	Derek	*	Short Horn	Derek	
Renee calf				Rene	Jeff	*	Rene	Jeff	*	Rene	Jeff	*
John	Anne	James	-	Anne	Derek	-	Anne	Derek	-	One Horn	Derek	*
#1	Janine	#6	-	Rene	Jeff	*	Rene	Jeff	*	Rene	Jeff	*
#5	Long Horn	James	+	Long Horn	Derek	*	Long Horn	Derek	*	Long Horn	Derek	*
#6	#15	Tony	+	Janine	Tony	+	Janine	Tony	+	Janine	Tony	+
#7	Renee	#1	-	Short Horn	Derek		Short Horn	Derek	-	Short Horn	Derek	
#15	Sharon	#6	+	Lisa	Tony	+	Lisa	Tony	+	Lisa	Tony	+
#16	Anne	Ivan	-	Au Kooi	Derek	*	Au Kooi	Derek	+	Au Kooi	Derek	*
Tom	-	-		One Horn	Jeff	-	One Horn	Jeff	-	One Horn	Jeff	*
Max	-	-		Sonja	Tony	*	Sonja	Tony	*	Sonja	Tony	*

APPENDIX 3.C

Parent assignment results using Colony method. These assignments incorporate all the pedigree constraints applied in 4 sequential steps. Only 'most-likely' candidate parent pair assignments are shown.

Animal ID	**Colony Assignment - Most Likely Parents**							
	1. Unconstrained (Gender Known)		*2. Constrained (Candidate Parents)*		*3. Constrained (Candidate Parents, Rh8 removed)*		*4. Constrained (Offspring's DOB)*	
	Mother	*Father*	*Mother*	*Father*	*Mother*	*Father*	*Mother*	*Father*
Anne	One Horn	Derek	One Horn	Derek	One Horn	Derek	One Horn	Derek
Renee	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
Sharon	One Horn	Bob	One Horn	Bob	One Horn	Bob	One Horn	Bob
Sonja	Long Horn	James	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
Janine	Au Kooi	Bob	Au Kooi	Bob	Au Kooi	Bob	Au Kooi	Bob
Lisa	Short Horn	Jeff	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
Ivan	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
Diane	One Horn	Derek	One Horn	Derek	One Horn	Derek	One Horn	Derek
#20	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
#21	Long Horn	James	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
#22	Lisa	Derek	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
#23	Short Horn	James	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
#24	Renee	#7	Renee	Bob	Renee	Jeff	Renee	Bob
LH calf	Long Horn	James	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
Sharon calf/Zac	#16	Tony	Au Kooi	Tony	Sharon	Tony	Sharon	Tony
AK calf	#16	James	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
SH calf	Renee	#7	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
Renee calf	Renee	Jeff	Renee	Jeff	Renee	Jeff	Renee	Jeff
John	One Horn	Derek	One Horn	Derek	One Horn	Derek	One Horn	Derek
#1	Renee	Jeff	Renee	Jeff	Renee	Jeff	Renee	Jeff
#5	Long Horn	James	Long Horn	Derek	Long Horn	Derek	Long Horn	Derek
#6	Lisa	Tony	Au Kooi	Tony	Au Kooi	Tony	Au Kooi	Tony
#7	Short Horn	Bob	Short Horn	Derek	Short Horn	Derek	Short Horn	Derek
#15	Lisa	Tony	Au Kooi	Tony	Au Kooi	Tony	Au Kooi	Tony
#16	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek	Au Kooi	Derek
Tom	One Horn	Jeff	One Horn	Jeff	One Horn	Jeff	One Horn	Jeff
Max	Sonja	Tony	Sonja	Tony	Sonja	Tony	Sonja	Tony

Appendix 3D

Genetic distance estimates using shared allele proportion for all individual rhinoceros in the population.

	Bob	Derek	Au Kooi	Long Horn	One Horn	Short Horn	Tony	Jeff	Anne	Renee	Sharon	Sonja	Janine	Lisa	Ivan	Diane	#20	#21	James	#23	#24	LHcalf	Shcalf	AKcalf	SHcalf	Renee Calf	John	#1	#5	#6	#7	#15	#16	Tom	Max
Bob																																			
Derek	0.864																																		
Au Kooi	0.773	0.364																																	
Long Horn	0.864	0.500	0.318																																
One Horn	0.773	0.364	0.409	0.636																															
Short Horn	0.727	0.500	0.273	0.500	0.409																														
Tony	0.818	0.409	0.364	0.545	0.364	0.364																													
Jeff	0.727	0.364	0.364	0.500	0.364	0.500	0.409																												
Anne	0.773	0.227	0.455	0.591	0.136	0.455	0.409	0.318																											
Renee	0.773	0.318	0.273	0.500	0.455	0.227	0.455	0.545	0.409																										
Sharon	0.727	0.409	0.409	0.545	0.091	0.364	0.364	0.364	0.182	0.500																									
Sonja	0.818	0.227	0.273	0.364	0.409	0.500	0.409	0.455	0.364	0.364	0.455																								
Janine	0.818	0.545	0.182	0.273	0.500	0.318	0.500	0.409	0.545	0.455	0.409	0.455																							
Lisa	0.773	0.364	0.182	0.500	0.273	0.182	0.318	0.318	0.318	0.273	0.318	0.364	0.273																						
Ivan	0.773	0.318	0.136	0.455	0.273	0.273	0.318	0.318	0.318	0.318	0.273	0.364	0.227	0.136																					
Diane	0.818	0.136	0.364	0.500	0.227	0.455	0.318	0.409	0.182	0.273	0.273	0.273	0.545	0.318	0.318																				
#20	0.864	0.182	0.273	0.500	0.455	0.455	0.500	0.455	0.318	0.318	0.500	0.318	0.364	0.318	0.227	0.318																			
#21	0.818	0.273	0.273	0.364	0.455	0.500	0.500	0.409	0.409	0.364	0.500	0.091	0.455	0.364	0.409	0.318	0.364																		
James	0.864	0.045	0.318	0.545	0.318	0.455	0.364	0.318	0.273	0.364	0.364	0.273	0.500	0.318	0.273	0.182	0.227	0.318																	
#23	0.818	0.227	0.182	0.500	0.318	0.273	0.364	0.455	0.364	0.182	0.364	0.364	0.364	0.182	0.136	0.182	0.227	0.409	0.182																
#24	0.818	0.318	0.364	0.682	0.455	0.364	0.545	0.455	0.409	0.227	0.500	0.409	0.545	0.318	0.409	0.364	0.409	0.409	0.273	0.273															
LHcalf	0.818	0.318	0.136	0.273	0.409	0.364	0.409	0.364	0.455	0.364	0.455	0.227	0.318	0.273	0.273	0.364	0.318	0.182	0.273	0.273	0.455														
Shcalf	0.818	0.318	0.364	0.591	0.227	0.455	0.227	0.364	0.318	0.455	0.227	0.455	0.455	0.273	0.227	0.227	0.364	0.500	0.273	0.273	0.500	0.409													
AKcalf	0.773	0.227	0.318	0.545	0.273	0.455	0.364	0.273	0.136	0.409	0.273	0.364	0.409	0.273	0.182	0.227	0.227	0.409	0.273	0.318	0.409	0.455	0.273												
SHcalf	0.727	0.455	0.364	0.636	0.455	0.318	0.455	0.409	0.500	0.273	0.500	0.545	0.545	0.273	0.409	0.364	0.500	0.500	0.409	0.273	0.227	0.409	0.409	0.500											
Renee Calf	0.818	0.273	0.364	0.545	0.455	0.409	0.455	0.318	0.364	0.273	0.455	0.455	0.455	0.318	0.364	0.318	0.318	0.455	0.318	0.318	0.318	0.500	0.409	0.273	0.455										
John	0.818	0.273	0.500	0.636	0.182	0.500	0.455	0.364	0.045	0.455	0.227	0.409	0.591	0.364	0.364	0.227	0.364	0.455	0.318	0.409	0.409	0.500	0.364	0.182	0.500	0.409									
#1	0.818	0.318	0.409	0.545	0.500	0.455	0.500	0.318	0.409	0.318	0.500	0.500	0.500	0.364	0.409	0.364	0.364	0.409	0.364	0.364	0.364	0.500	0.455	0.318	0.455	0.045	0.455								
#5	0.818	0.318	0.136	0.273	0.409	0.364	0.409	0.364	0.455	0.364	0.455	0.227	0.318	0.273	0.273	0.364	0.318	0.182	0.273	0.273	0.455	.	0.409	0.455	0.409	0.500	0.500	0.500							
#6	0.864	0.364	0.318	0.364	0.409	0.227	0.273	0.364	0.455	0.409	0.364	0.318	0.318	0.227	0.318	0.409	0.455	0.364	0.318	0.364	0.500	0.273	0.364	0.455	0.455	0.409	0.500	0.455	0.273						
#7	0.818	0.409	0.318	0.636	0.364	0.273	0.364	0.500	0.409	0.227	0.409	0.500	0.500	0.227	0.364	0.273	0.455	0.500	0.364	0.227	0.182	0.409	0.318	0.409	0.091	0.409	0.409	0.455	0.409	0.409					
#15	0.727	0.409	0.364	0.455	0.455	0.318	0.273	0.273	0.500	0.409	0.364	0.500	0.409	0.318	0.364	0.364	0.500	0.455	0.364	0.364	0.500	0.364	0.273	0.455	0.273	0.409	0.545	0.409	0.364	0.227	0.364				
#16	0.773	0.227	0.318	0.545	0.273	0.455	0.364	0.273	0.136	0.409	0.273	0.364	0.409	0.273	0.182	0.227	0.227	0.409	0.273	0.318	0.409	0.455	0.273	.	0.500	0.273	0.182	0.318	0.455	0.455	0.409	0.455			
Tom	0.727	0.455	0.409	0.591	0.227	0.409	0.455	0.227	0.273	0.455	0.273	0.455	0.500	0.273	0.364	0.364	0.545	0.409	0.409	0.409	0.409	0.364	0.409	0.409	0.273	0.500	0.227	0.500	0.364	0.409	0.364	0.318	0.409		
Max	0.727	0.364	0.455	0.409	0.500	0.409	0.364	0.409	0.455	0.409	0.455	0.273	0.545	0.409	0.455	0.364	0.500	0.318	0.409	0.455	0.591	0.364	0.455	0.455	0.409	0.545	0.500	0.545	0.364	0.227	0.500	0.227	0.455	0.409	

CHAPTER 4: POPULATION DYNAMICS AND PERFORMANCE

4.1. INTRODUCTION

The monitoring and data collection performed for OGR's rhinoceros population provides demographic data for the construction of pedigrees and genealogical structures for the population as well as vital data which contributes towards the overall assessment of the status of the population and thus provides useful information on its performance. In addition, genealogical data obtained can sometimes highlight/provide supplementary insight into traits determined for population performance (i.e. specific matrilines exhibiting differing reproductive potential). Data such as this is particularly important for the management of small populations and allows managers to direct efforts aimed at conservation accordingly (Emslie & Brooks, 1999; Ferreira et al. 2011). Sound biological management of rhinoceros populations requires reliable performance indicator data which can be used to contrast the performance of different populations and contribute to the making of informed decisions with a view to increasing overall meta-population growth rates (Emslie & Brooks 1999; Patton et al. 2008).

In this chapter I estimate several population performance parameters to provide indicators of the current status of this population of white rhinoceros using the data obtained. Furthermore I examine whether variation in sex ratio at birth is influenced by rainfall/environmental conditions at time of conception.

Standard population performance indicators (many of which are related) assessed include average annual growth rates and density estimates. Growth rates are considered a suitable overall indicator of population performance and can be compared across populations (Ouma, 2004). However, growth rates require accurate population census data which can be difficult to obtain for elusive species such as rhinoceros (Morellet et al. 2007). In addition, they can become less meaningful in small populations where individual deaths or births have the potential to significantly impact growth rates (Ouma, 2004). Density estimates influence carrying capacities for areas as well as behaviour of a population. For rhinoceros, high densities imply greater competition and limited resource availability and this may impact negatively on the demography of a population (Rachlow et al. 1999). Rachlow and Berger (1998) reported that as density in a population of white rhinoceros increased, the rate of

population growth and recruitment of calves subsequently decreased. In addition, as density increased, age at first reproduction for females increased significantly and loss of body condition was also reported (which in turn also affects female reproductive status). Females that breed earlier should gain a genetic advantage over those that delay reproduction (in Rachlow & Berger, 1998). Thus, both inter-calving intervals and age of female at first parturition provide good measures of reproductive success and are useful in small populations, but do also require detailed and continuous dates of birth data for calves (Ouma, 2004). White et al (2007) reported longer inter-calving intervals for white rhinoceros after the birth of sons rather than daughters, although differences only approached significance. The authors thus suggest costs to future reproduction as a consequence of increased investment in sons and that this cost would likely be greater for mothers in poor condition.

Age and sex ratio demographics within populations also have the potential to significantly influence growth rates and reproductive success (Knight, 2001). However, these estimates should be interpreted with caution as they in turn are strongly affected by factors such as adult female survival and calf recruitment rates (deCesare et al. 2011). Biases in sex ratios can have significant effects on population performance with male biased skews increasing generation time as fewer females are available to breed (Law & Linklater, 2007). For polygynous animals, such as rhinoceros, females are considered the limiting sex with regards to population growth as they can produce only a single offspring per successful breeding event whereas male rhinoceros can breed several times during that same time period (Berkeley, 2011). However, the number of males and in particular the age structure of available males should still be taken into consideration as these factors will also contribute to breeding/mating success (Owen-Smith, 1988; Ginsberg & Milner-Gulland, 1994). While the estimated long term maximum growth rate for rhino is 9-9.4% per annum, populations with skewed sex ratios in favour of females may grow at a faster rate (Owen-Smith, 1988). In addition, reproductive skews in small populations also have the potential to reduce genetic diversity (Berkeley, 2011). In particular, biases in birth sex ratios can have profound effects on population growth by reducing effective population size especially in small and fragmented populations (Wedekind, 2002) and as such provide significant implications for wildlife managers to consider (Weladji & Laflamme-Mayer, 2011). Berkely (2011) reports that increased variation in birth sex ratios can increase probability of extinction rates, especially in rhinoceros populations with less than 50 animals. In previous studies on black rhinoceros populations it was demonstrated that environmentally-cued birth sex allocation occurs

(Hrabar & du Toit, 2005; Berkely & Linklater, 2010; Weladji & Laflamme-Mayer, 2011). Hrabar and du Toit (2005) reported that the percentage of male calves born increased with increasing rainfall while Berkely and Linklater (2010) observed that females were more likely to be observed with male calves if they conceived during the wet season. In addition, the authors also reported that females were more likely to raise male calves if they conceived during wet years as opposed to dry years. These results support the theory that maternal energy balance and body condition around the time of conception influences birth sex allocation (Cameron et al. 1999; Cameron & Linklater, 2007). For most large ungulates, especially in savannah environments, it can be assumed that maternal body condition will correlate with seasonal and annual rainfall (and hence resulting vegetation conditions) (Owen-Smith, 1988). These results appear to follow the Trivers and Willard (1973) theory that natural selection will favour parental ability to adjust the sex ratio of offspring produced according to their ability to invest, and that as maternal condition declines females produce a lower ratio of male to female offspring. For polygynous species, this may mean that when conditions are good, mothers can produce high quality male offspring which stand a better chance of outcompeting rivals for breeding access to females (thus investing in the sex that provides the greatest reproductive benefit) as seen in wild horses – *Equus caballus* (Cameron et al. 1999). Berkely and Linklater (2010) and Hrabar and du Toit (2005) also suggest that for species with long periods of parental investment, parents in better condition would be expected to show bias toward male offspring. Thus we can expect male biased birth sex ratios for rhinoceros populations with females in good condition.

In this chapter I review population and demographic data for the study population and use the genetic data obtained to explore performance trends. This study allows for the unique opportunity to concurrently consider population performance parameters and genetic information for a population of free-ranging white rhinoceros.

4.2. METHODS

OGR rhino monitoring data (historic and current) and my own observation data were collated for a total period of 6 years (2004 to 2009) (see ‘Rhinoceros Pedigree Data Collection’ in Methods 3.2.1 section of Chapter 3 for full collection details). Even though rhinoceros are an elusive and difficult-to-monitor species, due to the long, continuous period of monitoring performed relative confidence could be assigned to data collected such as birth dates and

dates of mortality occurrences. Age classes for this population were defined as – calves: up to two years of age, sub-adults: two to four years of age and adults as five years and older. The data were used to estimate the following population performance indicators:

Overall population growth rate was calculated as below.

Population growth was calculated assuming simple exponential rate based on the equation:

$$X_t = X_0(1+r)^t$$

Where X_0 is the initial population size and X_t is the predicted size in interval t. And where r is the growth rate expressed as a ratio.

For application to this study I calculated annual growth rate as:

$$X_{y+1} = X_y(1+r)$$

Where y refers to a specific year and y +1 refers to the subsequent year.

This is the increase (or decrease) of a population's size expressed as a percentage of the population's size at the start of a year (biological growth rate, Emslie & Brooks, 1999) and includes all births and deaths occurring in the population but does not include any translocations (introductions and removals of animals).

Density. Rhino densities are expressed as numbers of rhinos per km^2.

Inter-calving intervals (ICI). ICI refers to the period from the birth of one calf to the next for individual females. Mean ICIs are expressed as an average (± SD) in months. The average gestation period for white rhino females in natural populations is 16 months (Owen-Smith, 1988).

Female age at first reproduction. This refers to the age at first parturition for female rhinoceros and is expressed as a mean (± SD) across the population.

Age ratios. Age ratios are estimated by the proportion of adults, sub-adults and calves in the total population. Estimates are expressed as a percentage of the total population.

Sex ratios. Sex ratios are derived by determining the proportion of males to females in the population. This is expressed as a ratio and is calculated for adults and calves in the population. In addition, I compared birth sex ratios, conception dates and annual rainfall to investigate if these factors experienced any correlation. The rainy season (termed wet season) is considered to be from December to April with the remainder of the year termed the dry

season. Average annual rainfall is 380mm. Each year was classified as either a 'Wet' year if annual rainfall was above average or a 'Dry' year if annual rainfall was below the average. Accurate rainfall data for OGR was only available from 1997 onwards. For classification of rainfall years prior to 1997 estimates of rainfall from the neighbouring Etosha National Park records were used.

4.3. RESULTS

Table 4.1 presents the data recorded for annual population numbers by age class and rainfall data where available.

Year	Rainfall (mm)	Rain Year Class	Population numbers	Physical Density (rhino/ km²)	Total calves in population	Calves 'on foot'	Total calves born	# of male calves born	# of female calves born	Total calves conceived	# Male calves conceived	# Female calves conceived	Total adults	# of adult males	# of adult females	Total sub-adults	# of sub-adult males	# of sub-adult females
1993	350	WET	6	0.020	0	0	0	0	0	0	0	0	6	2	4	0	0	0
1994	180	DRY	6	0.020	0	0	0	0	0	3	3	0	6	2	4	0	0	0
1995	250	DRY	7	0.023	1	0	1	1	0	1	1	0	6	2	4	0	0	0
1996	180	DRY	10	0.033	4	1	3	3	0	2	2	0	6	2	4	0	0	0
1997	350	WET	10	0.033	4	4	0	0	0	2	0	2	6	2	4	0	0	0
1998	209	DRY	14	0.047	4	0	4	2	2	0	0	0	6	2	4	4	4	0
1999	184	DRY	14	0.047	4	4	0	0	0	4	0	4	6	2	4	4	4	0
2000	354	WET	18	0.060	4	0	4	0	4	0	0	0	6	2	4	8	6	2
2001	286	DRY	16	0.053	4	4	0	0	0	2	1	1	8	4	4	4	2	2
2002	345	WET	16	0.053	2	0	2	1	1	2	0	2	7	3	4	7	1	6
2003	320	DRY	16	0.053	3	1	2	0	2	4	2	2	9	3	6	4	0	4
2004	328	DRY	20	0.067	6	2	4	2	2	4	4	0	9	3	6	5	1	4
2005	380	WET	21	0.070	5	4	1	1	0	5	3	2	13	3	10	3	1	2
2006	545	WET	30	0.100	10	2	8	6	2	2	1	1	13	3	10	7	3	4
2007	290	DRY	28	0.093	9	8	1	1	0	6	2	4	13	3	10	6	3	3
2008	430	WET	35	0.117	8	1	7	2	5	4	4	0	15	3	12	12	9	3
2009	352	WET	34	0.113	11	7	4	4	0	0	0	0	15	2	13	8	6	2

Table 4.1 *Population data* – Rainfall is indicated in mm per year and rain year class refers to 'Wet' years (above average rainfall) and 'Dry' years (below average rainfall). Population numbers are the total number of rhino per year while physical density refers to the number of rhino per km² (for both these categories numbers given are the actual number of rhino on the Reserve and include all translocations). Total calves in population indicate the actual number of calves in the population per year while calves 'on foot' refer to calves born the previous year which are still walking with their mothers. Total calves born refer to the number of calves born per year and this is further represented as the number of males born and number of females born. Total calves conceived refers to the number of calves conceived in that particular year, also indicated as number of males and/or females conceived. For the adult and sub-adult age classes, the total number of animals, number of males and number of females is shown.

4.3.1. Population parameters

4.3.1.1 Growth rate

At the end of this study period in 2009 OGR had a population of 34 white rhinoceros, consisting of 2 adult bulls, 13 adult females, of which 11 have calves, 2 sub-adult females, and 6 sub-adult males.

To the end of 2009, the founder population and their offspring have produced a total of 41 calves, giving a total population size of 47. This population shows an annual growth rate of 13.9% per annum (see Figure 4.1) from the date of establishment.

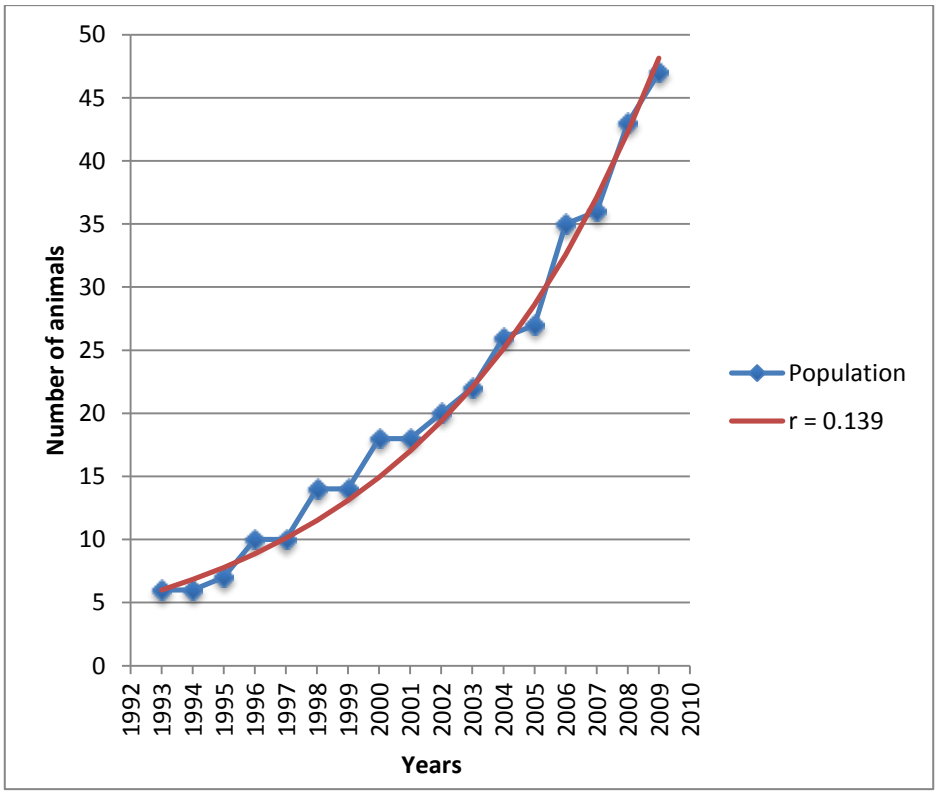

Figure 4.1 Population *Growth Curve* – Blue trace is the total annual population data, in this case with all translocations removed. Red trace represents the overall growth rate (13.9%).

4.3.1.2. Density

The rhino population density on OGR at the end of 2009 was 0.11 rhino/km^2. Figure 4.2 shows the change in population density per year.

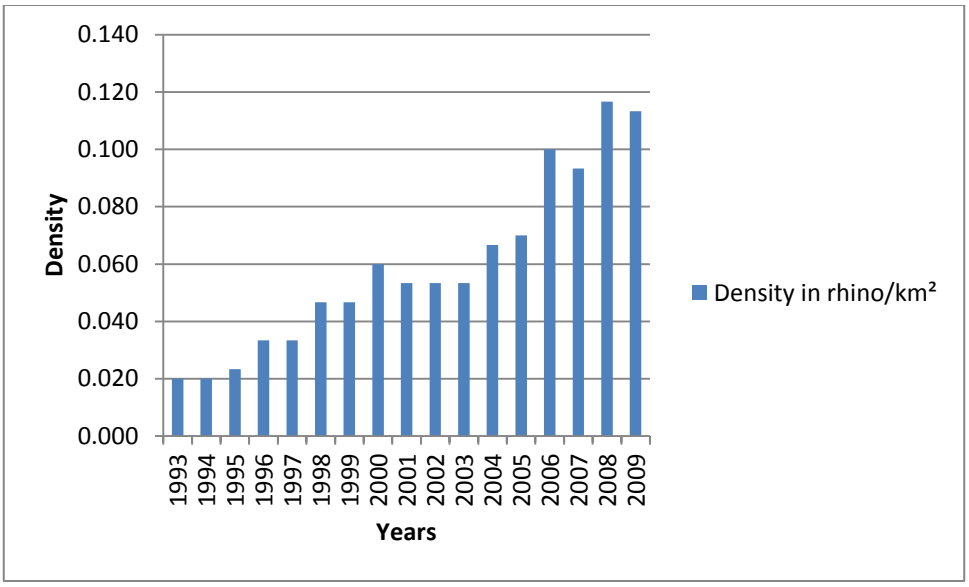

Figure 4.2 *Population density* – Population density per year for entire group (includes translocations)

4.3.1.3. Age ratios

At the end of the study period the OGR population consisted of 44% adult animals, 24% sub-adults and 32% calves. The age structure of the population for each year is shown in Figure 4.3.

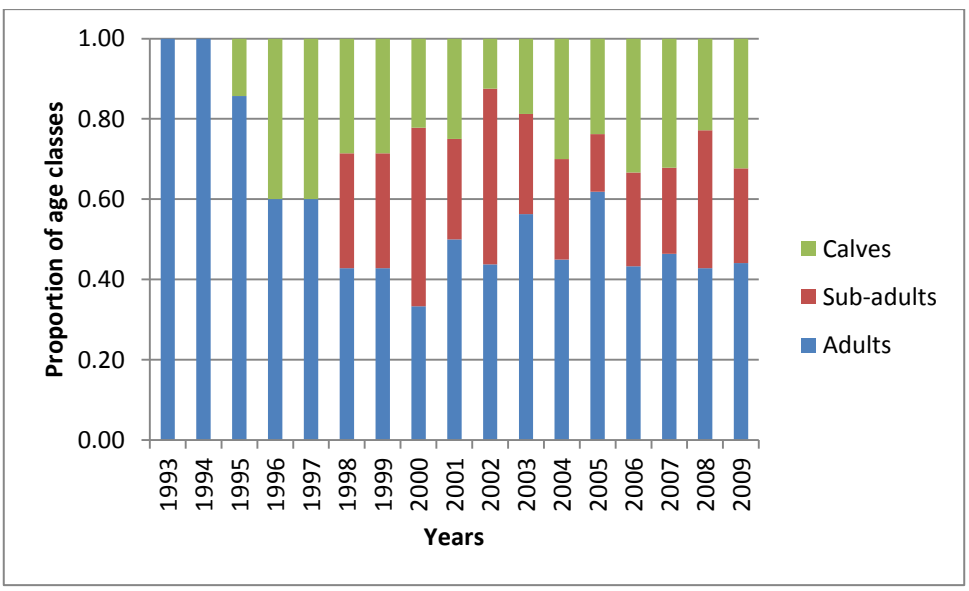

Figure 4.3 *Population age structure* – Proportion in each age class by year across the population

4.3.1.4. Sex ratios

The sex ratio for adult male to female rhinoceros on OGR shifted from a founding ratio of 1 : 6.5 (1993) to 1 : 2 at the end of the study period (2009).

The sex ratio for all rhinoceros calves born during the study period was 1: 0.8 (males:females).

The proportional changes in sex ratio across the three age classes for the population are shown in Figure 4.4 below.

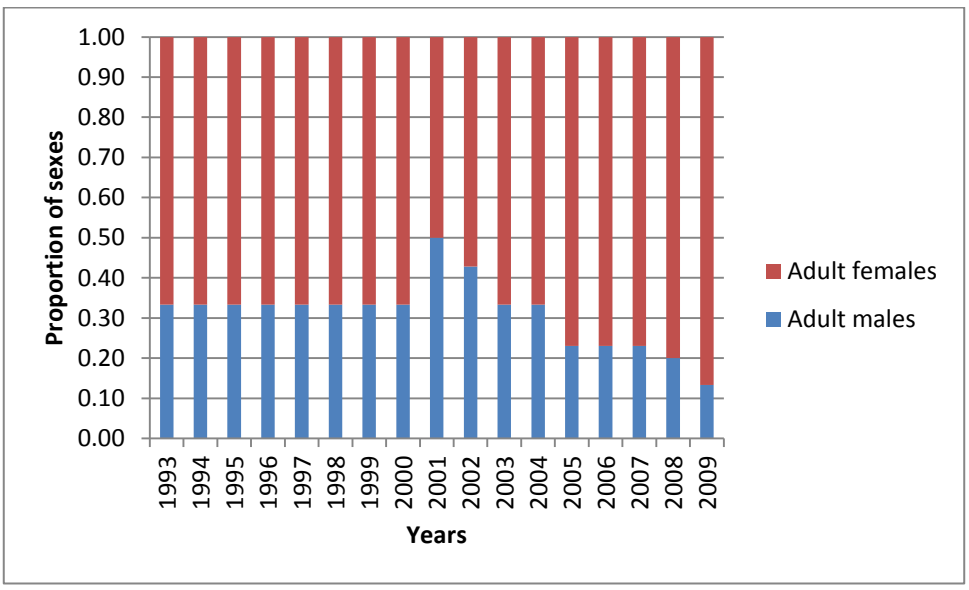

Figure 4.4a *Adult sex ratios*

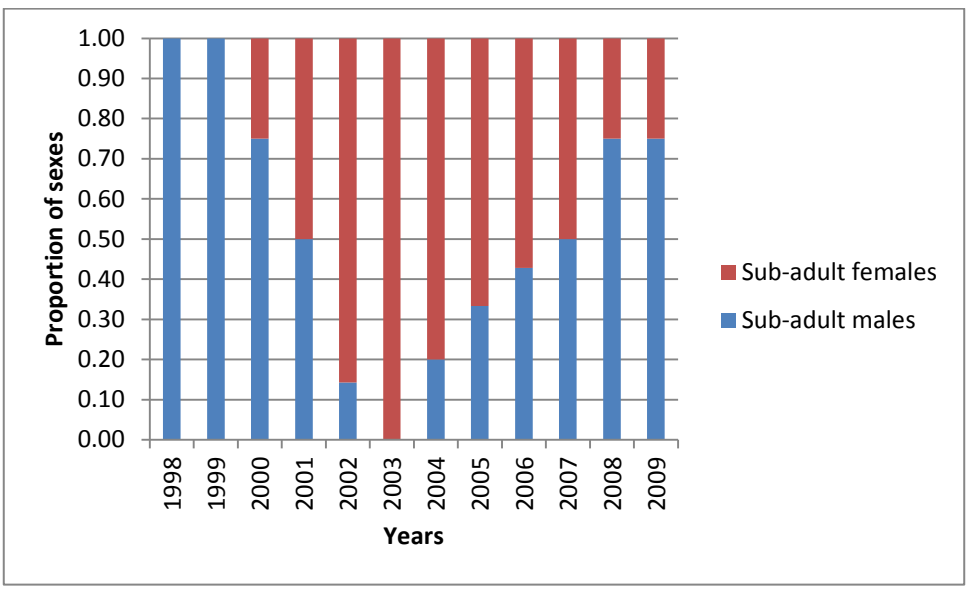

Figure 4.4b *Sub-adult sex ratios*

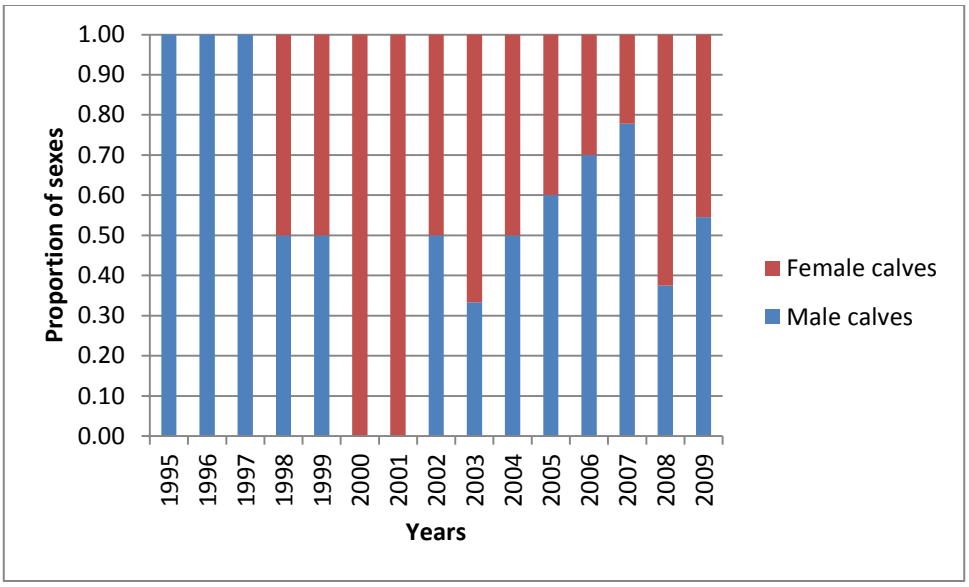

Figure 4.4c *Calf sex ratios*

Figure 4.4 *Population sex ratios* - Proportion of sex ratios for each age class across the population shown by year. Fig. 4.4a shows ratios for the adult animals, Fig. 4.4b represents the sub-adult animals and Fig. 4.4c represents the calves.

4.3.2. Reproductive parameters

4.3.2.1. Female age at first reproduction

A total of eight females gave birth for the first time during the study period; each of these females was aged 6 years at age of first parturition. The majority (75%) of these first born calves were male (6 out of 8).

4.3.2.2. Inter-calving interval (ICI)

The average inter-calving interval for all breeding females in the population at the end of the study period was 26.9 ± 7.7 months. Table 4.2 shows the individual ICIs for all breeding females in the population. In addition, using the gestation period of 16 months (Owen-Smith, 1988) this ICI obtained shows that, on average, females are conceiving within less than a year (~11 months) after giving birth.

Female rhino name	Inter-calving interval in months between each calf					
	Calf 1 to 2	Calf 2 to 3	Calf 3 to 4	Calf 4 to 5	Calf 5 to 6	Calf 6 to 7
Long Horn	28	25	23	24	24	22
Au Kooi	18	27	24	24	24	23
Short Horn	25	32	24	27	22	
One Horn	39	16	40	22	24	24
Anne	57					
Renee	27	24				
Sharon	39					
Sonja	26					
Janine	29					
Lisa	23					
Mean	31.1	24.8	27.75	24.25	23.5	23
SD	10.6	5.2	7.1	1.8	0.9	0.8

Table 4.2 *Inter-calving intervals* – ICI in months for all breeding females in population per calf.

There is a decrease in ICI as females produce more calves (shown in Figure 4.5), but this is not statistically significant (correlation coefficient, $r = -0.823$, $p>0.05$).

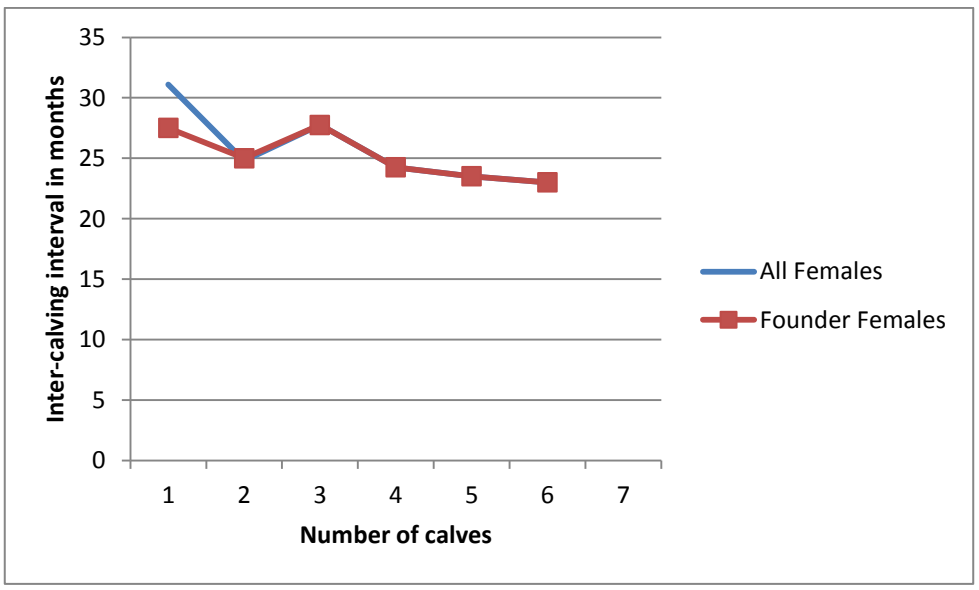

Figure 4.5 *Average inter-calf interval* – average ICI according to calf number for all females

In addition, the length of ICI did not alter depending on the sex of the preceding calf. Average ICI following a male calf was 26.5 ± 10.8 months whilst the average ICI following a female calf was 25.3 ± 5.5 months. The length of ICI was therefore not significantly different

following births of calves of different sex (t-test for difference between means, t=1.05, DF=22, p=0.847).

Table 4.3 shows the ICI determined for females in each matriline in the population. The matriline #4 (founder is female One Horn) shows a longer ICI than the other three matrilines. Due to the small sample size, this difference is however not statistically significant (t-test for difference between mean ICI for One Horn (matriline 4) and the mean ICI for Short Horn (matriline 3), t=-1.805, DF=7, p=0.083). The t-test for difference between mean ICI for One Horn (matriline 4) and Au Kooi (matriline 2) is t=-1.988, DF=6, p=0.065. While the t-test for difference between mean ICI for One Horn (matriline 4) and Au Kooi (matriline 2) shows t=-2.098, DF=6, p=0.056.

Matriline 1		Matriline 2		Matriline 3		Matriline 4	
Name	ICI	Name	ICI	Name	ICI	Name	ICI
Long Horn	28	Au kooi	18	Short Horn	25	One Horn	39
Long Horn	25	Au kooi	27	Short Horn	32	One Horn	16
Long Horn	23	Au kooi	24	Short Horn	24	One Horn	40
Long Horn	24	Au kooi	24	Short Horn	27	One Horn	22
Long Horn	24	Au kooi	24	Short Horn	22	One Horn	24
Long Horn	22	Au kooi	23	Renee	27	One Horn	24
Sonja	26	Janine	29	Renee	24	Anne	57
				Lisa	23	Sharon	39
Mean	24.6		24.1		25.5		32.6
SD	1.8		3.2		3.0		12.6

Table 4.3 *Matriline inter-calving intervals* – ICI in months for females in each matriline across the population

4.3.2.3 Calf birth sex

The majority of calves born during the study period were males. For a total of 41 births, 23 were male (56%). Figure 4.6 represents the total number of calves born annually throughout the study period with respect to numbers of males and females.

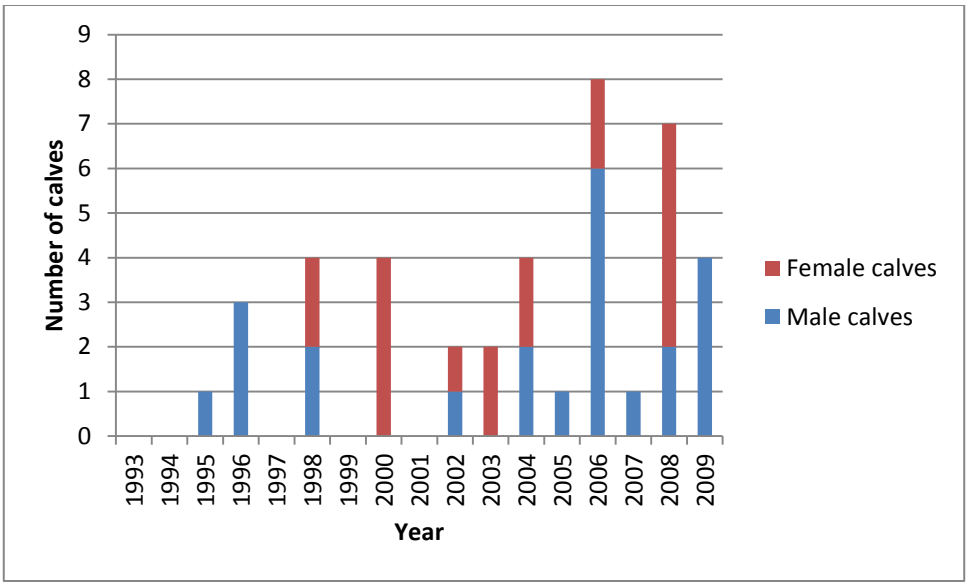

Figure 4.6 *Calf births* – Total number of calves born per year represented by number of males and females born

Calf sex investigated in light of matrilines revealed male biased calf births for certain matrilines (See Table 4.4).

Matriline 1		Matriline 2		Matriline 3		Matriline 4	
Mother name	Calf sex	Mother name	Calf sex	Mother name	Calf sex	Mother name	Calf sex
Long Horn	m	Au Kooi	m	Short Horn	m	One Horn	m
	m		m		f		f
	f		f		f		f
	f		m		f		f
	f		m		m		m
	m		f		f		m
	f		f	Renee	f		m
Sonja	m	Janine	m		m	Anne	m
	f		m		f		m
				Lisa	f	Sharon	m
					m		m
						Diane	m
Total calves	9		9		11		12
Male ratio	0.44		0.67		0.36		0.75

Table 4.4 *Calf birth sex by matriline* – Calf birth sexes for females in each matriline in the population. m indicates male calves and f indicates female calves born. Total calves represents the total number of calves born to a particular matriline. Male ratio indicates the proportion of male calves born to a particular matriline.

4.3.2.4 Conception data

For the comparison of conception dates and calf birth sex against rainfall, only data from 1997 could be considered due to incomplete rainfall records in earlier years. A total of 35 calves were conceived during the period 1997 to 2009. The majority of calves (89 %, 31 of 35) were conceived during the rainy season. Of these, 52% were male (16 out of 31). Of the 4 (11% of total) animals conceived during the dry season, only 1 was male (25%). (See Figure 4.7). There was no significant difference in calf sex based on conception rainfall season (Chi-squared=0.7693, DF=1, p = 0.31).

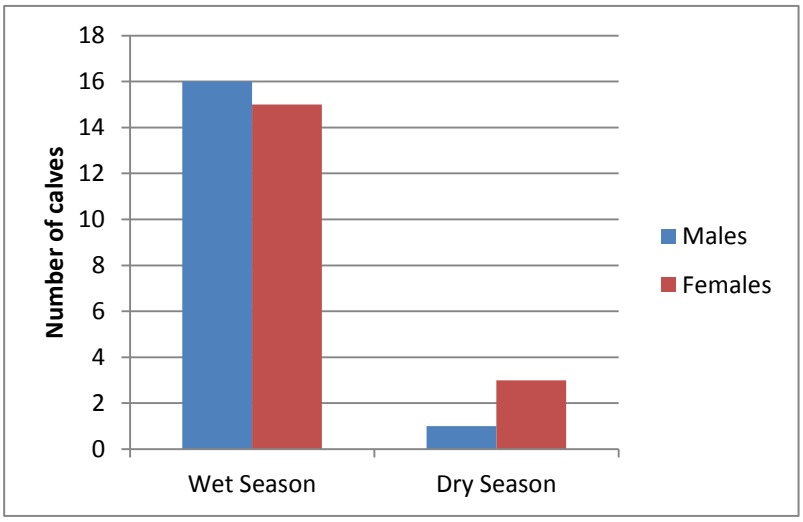

Figure 4.7 *Calf birth sexes by season*- Conceptions occurring for period 1997 – 2009 where Series 1 represents conceptions occurring during the Dry season and Series 2 represents the Wet season.

Out of the total of 35 conceptions, 15 (43%) occurred during 'Wet' years and 20 (57%) occurred during 'Dry' years. A total of 8 males calves (53%) were conceived during the wet years. In the dry years, 9 male calves (45%) were conceived (See Figure 4.8). There was no significant difference in calf birth sex based on conception rainfall class (Chi-squared=0.316, DF=1, p=0.61).

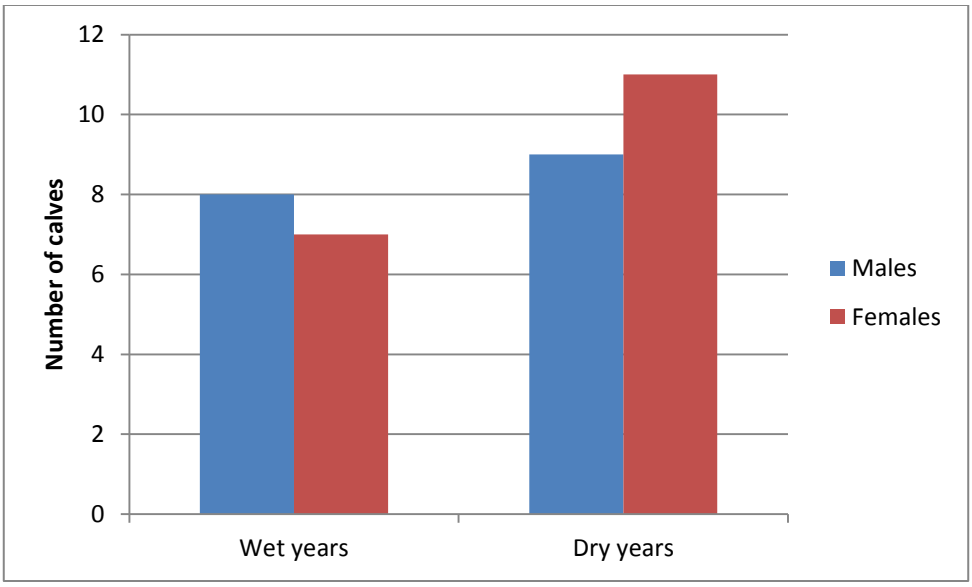

Figure 4.8 *Calf birth sexes by rainfall class* - Conceptions occurring for period 1997 – 2009 where Series 1 represents conceptions occurring during Wet years and Series 2 represents Dry years.

In addition, calf birth sex does not appear to be influenced by population density at time of conception for this study (See Fig. 4.9).

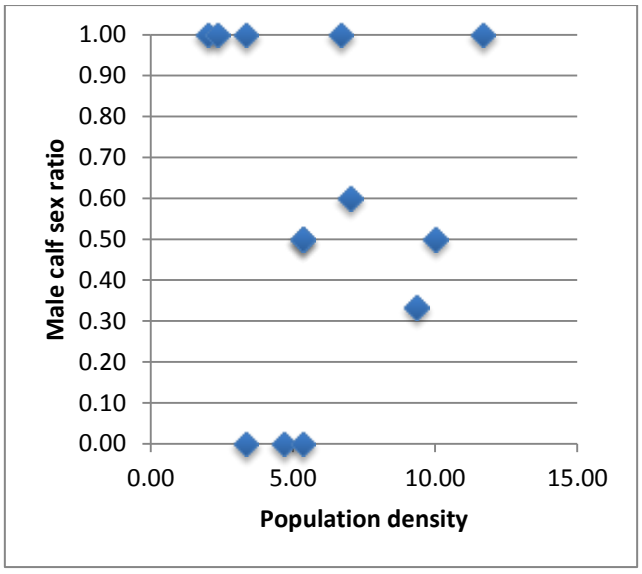

Figure 4.9 *Calf birth sexes by density* – Ratio of male calves born versus population density (rhino/km^2) at time of conception

4.4. DISCUSSION

I was able to determine important population performance indicators for this study population and also evaluate several of them in light of the previously determined genetic data in order to investigate if trends observed could be explained through accurate lineage data.

Population growth

The Southern African Development Community Regional Programme for Rhinoceros Conservation (SADC RPRC) which assists in (amongst others) developing rhinoceros conservation strategy plans for the various regions, proposes a minimum underlying target growth rate of 5% per annum per population in order to achieve sustained meta-population growth and minimise possible genetic drift. This figure represents an achievable target which is lower than the estimated intrinsic maximum rate (9%), which is characteristically supported by rhinoceros populations with a typical age and sex structure (du Toit, 2006). According to Emslie and du Toit (2006), rhinoceros areas which are stocked well below habitat carrying capacity, have female biased sex ratios and low mortality rates, can sometimes attain average population growth rates as high as 15% per annum. OGR's rhinoceros population has an average overall population growth rate of almost 14% to date which is amongst the highest reported for white rhinoceros. Kretzschmar (2002) reported the highest growth rate of 15% for a population in the Limpopo region of South Africa while Rachlow and Berger (1998) reported a growth rate of 10.4% after introduction for rhinoceros in Matobo National Park (although this figure decreased to 6.6% for a later period as density increased). Growth rates for white rhinoceros populations can vary (see Table 4.5) however the IUCN Species Survival Commission (SSC) African Rhinoceros Specialist Group (AfRSG) report of January 2007 states that in the period 1995-2005 the annual growth rate for white rhinoceros as a species was 6.8%. As laid out in the Guidelines for SADC Rhinoceros Conservation Strategies (du Toit, 2006) this growth rate of 6.8% indicates 'moderate to good' performance (5-7.5%). The results for our study (13.9%) fall in the category which indicate 'good to excellent' performance (>7.5%).

Annual Growth Rate	Population/Location	Reference
15.00%	Limpopo, S.A.	Kretzschmar, 2002
13.90%	OGR, Namibia	This study
10.40%	Matobo NP, Zimbabwe (early period 1967-1974)	Rachlow & Berger, 1998
9.70%	Garamba National Park, Zaire	Data cited in Rachlow & Berger, 1998
8.60%	Hluhluwe-iMfolozi Park, S.A.	Owen-Smith, 1988
8.40%	Kruger National Park, S.A.	Pienaar, 1994
6.60%	Matobo NP, Zimbabwe (later period 1987-1994)	Rachlow & Berger, 1998
6.00%	Ndumu Game Reserve, S.A.	Conway & Goodman, 1989

Table 4.5 *Growth Rate* - Overall annual population growth rates for white rhinoceros populations (note the data for Garamba refers to a northern white rhinoceros population)

Population density

The densities of these various populations (see Table 4.6) differ as well and this may influence the growth rates (Rachlow & Berger, 1998). Higher densities imply greater competition for resources and this may influence the demography and behaviour of a K-selected species (such as rhinoceros) negatively (Rachlow & Berger, 1998). The density estimate obtained in this study is the lowest in the range of reported values from other studies. While population density estimates cannot always be compared explicitly as they are influenced by other factors including habitat carrying capacity or resource availability, size of area/reserve and population structure (Owen-Smith, 1988), they do provide useful indicators when evaluating different populations. For example, our study site area is comparable with that of Kretzschmar (2002) in the Limpopo as both are approximately 300km^2 in size, yet our density estimate is approximately half that for the Limpopo population. This may suggest a young, increasing population in our study and/or more abundant resources / suitable habitat in the Limpopo area. One also needs to consider the concept of 'effective density' which would reflect the density across only the areas which might be considered suitable white rhinoceros habitat (in OGR's case, about 35% of the reserve) and thus density comparisons need to be interpreted with caution. The only other Namibian rhinoceros population for which data is available is Otjiwa (Mraz et al. 2001), which has a similar habitat to this study population, with a reserve size approximately one-third that of OGR (100km^2). Their population density estimate is more than double that obtained in my study. This may also support the theory that the OGR population is still growing and could possibly support much higher densities.

Density (rhino/km^2)	Population/Location	Reference
0.11	OGR, Namibia	This study
0.23	Limpopo, S.A.	Kretzschmar, 2002
0.26	Otjiwa, Namibia	Mraz et al. 2001
0.30	Sum on private land, S.A	Castley & Hall-Martin, 2003
0.50	Hluhluwe, S.A.	Owen-Smith, 1988
0.70	Kyle Game Park, Zimbabwe	Data cited in Kretzschmar, 2003
0.23 - 0.83	Matobo NP, Zimbabwe	Rachlow & Berger, 1998
0.5 - 1.4	Kruger National Park, S.A.	Pienaar et al. 1993b
0.6 - 1.75	Ndumu Game Reserve, S.A.	Conway & Goodman, 1989
2.5 - 5.7	Umfolozi, S.A.	Owen-Smith, 1988

Table 4.6 *Population Density* - Average or estimated densities for southern white rhinoceros populations

Inter-calving intervals

Together with growth rate, other population performance indicators include average inter-calving intervals (ICI) and average age of cow at first calving. du Toit (2006) provides classifications of ICI for rhinoceros as follows; >3.5 years indicates poor to very poor fecundity, 3.1-3.5 years indicates moderately poor to poor fecundity, 2.5-3.0 years indicates good to moderate fecundity and <2.5 years indicates good to excellent fecundity. The average ICI obtained in this study was 26.9 months (= 2.2 years) which indicates good fecundity. This value is the lowest obtained when compared to others reported for free-ranging white rhinoceros populations (see Table 4.7). It appeared that certain matrilines (One Horn matriline) exhibited longer average ICIs than others but not with any significance. This may however suggest that certain matrilines give rise to individuals with different reproductive potential. In addition, Anne (Offspring of One Horn) who had the longest ICI of 57 months for the entire group was also the only animal to inbreed (her calf's father was also her father as determined by the parentage analyses). The paternity for her second calf (after the long ICI) has not yet been determined. Interestingly the matriline founded by One Horn also experienced the birth of more male calves than the other matrilines. Therefore, it is possible that calf birth sex may be genetically influenced, but further research is required.

ICI (years)	Population/Location	Reference
2.20	OGR, Namibia	This study
2.30	Limpopo, S.A.	Kretzschmar, 2001
2.50	Otjiwa, Namibia	Mraz et al. 2001
2.60	Umfolozi, S.A.	Owen-Smith, 1988
2.70	Kruger National Park, S.A.	Data cited in Owen-Smith 1988
2.85	Matopos Game Park, Zimbabwe	Data cited in Owen-Smith 1988
2.90	Matobo NP, Zimbabwe (early period 1967-1974)	Rachlow & Berger, 1998
3.30	Matobo NP, Zimbabwe (later period 1987-1994)	Rachlow & Berger, 1998
3.45	Kyle Game Park, Zimbabwe	Data cited in Owen-Smith 1988

Table 4.7 *Inter Calving Intervals* - Average inter-calving intervals for free-ranging southern white rhinoceros populations

Age at first parturition

Female age at first reproduction is also a useful gauge of breeding performance (Rachlow & Berger 1998; du Toit, 2006). White rhinoceros females become sexually mature between 4-5.5 years of age (Owen-Smith, 1988; Bothma, 2002) and age at first parturition is typically from 6.5 years onwards (Owen-Smith, 1988; Rachlow & Berger, 1998). Rachlow and Berger (1998) showed that, in African rhino, age at first calving is particularly sensitive to population density. For their white rhinoceros populations, the mean age at first birth was 7.4 years in the low density group and this increased to 10.1 years in the high density group. Age at first parturition in this study was estimated to be 6 years (n = 6) and this may indicate a rapidly growing population (du Toit, 2006). This consistency in birth timing resulted in females in a particular matriline being synchronized in years of calf production (i.e. Long Horn and her daughters all produce calves at two yearly intervals on even year numbers (2002, 2004, 2006, etc.) while One Horn and her offspring produce calves on odd year numbers (2003, 2005, 2007, etc.).

Age and sex ratios

Age and sex ratios can have profound effects on growth rates of populations. At the end of 2009 this study population consisted of 2 adult males, 13 adult females - of which 11 have calves, 2 sub-adult females and 6 sub-adult males. Our population composition is as follows; 44% adult animals, 24% sub-adults and 32% calves. This is comparable to the numbers obtained by Owen-Smith (1988) which had an overall composition for white rhinoceros in

the Umfolozi-Hluhluwe complex of 46% adults, 32% sub-adults and 22% calves. Kretzschmar (2001) showed an unbalanced population which had an over-representation of adult animals with 55% adults, 25% sub-adult and 20% calves, as did Conway and Goodman (1989) with only 21% calves. The sex ratio of adult males to females in this study population was 1:6.5 at the end of 2009 (at time of founding it was 1:2). Sex ratios in this population are kept in female bias in an attempt to maximize reproductive success. Owen-Smith (1988) reported an adult sex ratio of 1:1.3 (male:female) for the entire Umfolozi-Hluhluwe complex, while Kretzschmar (2001) had ratios of 1:2.5 initially and then a large increase to 1:8 later in the study. Kretzschmar (2001) suggests that the sex ratio should not become more skewed than this in favour of females as a potential limiting factor to breeding success is the length of the consort phase before mating for white rhinoceros (i.e. low numbers of males will not be able to successfully accompany and copulate with all breeding females if numbers are too skewed). A survey of white rhinoceros on private land in South Africa undertaken by Castley and Hall-Martin (2003) showed that the mean ratio of adult males to females across the survey was 1:1.8, well below a ratio that would maximize breeding potential. This figure however may not comparable with other populations as 70% of the properties surveyed supported fewer than 10 animals.

The observed sex ratio for all calves born in this study was 1:0.8, (23 males : 18 females). This is lower than that reported for white rhinoceros in Madlozi in Umfolozi (Owen-Smith, 1988) which had a calf sex ratio of 1:1.5 but higher than values obtained by Kretzschmar (2001) of 1:0.4. Calf sex may be influenced by various factors, such as body mass, stress and blood glucose levels of the mother at time of conception (Rachlow & Berger, 1998; Cameron, 2004; Linklater, 2006) and Owen-Smith (1988) showed that younger females produced more male calves than old females and had shorter ICI. This was supported by Kretzschmar (2001) who shows that cows that gave birth to females experienced in increased ICI and subsequently produced male calves which may suggest that the higher costs involved in raising female calves will cause a shift in the sex ratio. Contrary to these findings, White et al (2007) showed that in a population of wild white rhinoceros over a period of 3 years, male calves received increased investment compared to female calves and that cows had longer ICI after the birth of male calves. There was no apparent trend observed in this study to support either hypothesis, and ICI did not alter depending on birth sex. I did however note that the majority of first born calves were male (82%). This may be due to the fact that many of the cows were young (supporting per Owen-Smith, 1988) or it might be that the animals in this population are considered to be in good condition and as a result the females produce more

male calves (Cameron & Linklater, 2002). Generally, all the indicators point towards this population being a healthy, rapidly increasing population that is not yet exhibiting signs of resource competition or density dependence factors. In addition, for this study no correlation was observed between amounts of rainfall (assumed to influence resource abundance) at time of conception and calf birth sexes. While the majority of calves were conceived during the rainy season, no resultant bias in calf sex was observed. There was also no resultant bias in calf birth sex depending on their conception in wet years or dry years. However, the number of calves was relatively small (n = 35) and as the population increases and more calves are produced this may be worth further investigation.

It has been demonstrated that many complex and interrelated factors have the ability to influence birth sex ratio including factors such as body condition of the mother (both at time of conception and during gestation), stress and resulting hormone levels in the mother (also both at time of conception and throughout gestation), glucose levels in mother and external resource conditions (typically rainfall related influencing food availability) (Owen-smith, 1988; Cameron et al. 1999; Cameron, 2004; Linklater, 2007; Berkeley & Linklater, 2010). While the mechanisms involved in these factors are beyond the scope and data set for this study it is worth noting their potential ability to bias birth sex ratios which may subsequently influence the demographics of a population. For example, Berkeley (2011) surmises that an area previously void of rhino may have an excessive amount of preferred forage and if a group of rhino were subsequently introduced this would result in a temporary increase in body condition and as such may result in a pulse of male calf births. This pulse could have long-term consequences for population growth rates. Linklater (2007) demonstrated that translocations have the ability to influence birth sex depending on their timing during gestation and in addition to that, captivity of rhinoceros induced a strong male bias for birth sex ratios for conceptions occurring after confinement. Consequences such as these can have considerable effect and should be considered during the establishment of *in situ* populations.

CHAPTER 5: GENERAL DISCUSSION AND MANAGEMENT

This study has provided significant new insight into the breeding strategies/mating system and reproductive success of southern white rhinoceros and will hopefully contribute to the development of appropriate biological management of small populations of free roaming rhinoceros.

Genetic analyses are proving to be increasingly valuable tools in conservation management of social species (Frankham et al. 2002; Archie & Chiyo, 2012). In particular, they are able to provide precise data for biological systems that conventional behavioural methods are not always able to do with accuracy (as demonstrated in for example in Bishop et al. 2004; Moueix, 2006; Twiss et al. 2006; Archie & Chiyo, 2012). However, they cannot stand alone and optimal meta-population management will rely on a combination of genetics and pedigree data.

Review of OGR Population Management

A complete parentage analysis allowed me to review population management decisions that have been taken to date from a genetic perspective, as well as make recommendations for future management strategies that will both satisfy the micro-management objectives for OGR's population and consider implications for the continued conservation of the southern white rhinoceros.

Since the OGR population was founded in 1993 through the introduction of six animals from Hluhluwe-Umfolozi Park in South Africa, management decisions have been based on the traditional bull translocation strategies used to manage most stock herds (Bothma, 2002). In theory, the dominant territorial bull is the most successful breeder (Owen-Smith, 1971; Rachlow et al. 1998). Indeed Owen-Smith (1977) says that among polygynous ungulates, territorial behaviour is believed to function primarily as a reproductive strategy to secure mates. He hypothesized that territorial white rhinoceros bulls achieved greater reproductive success than non-territorial bulls. Among white rhinoceros, dominant males occupy clearly demarcated breeding territories and defend them from other dominant males (Owen-Smith, 1971; Pienaar, 1994; Rachlow et al. 1998). These bulls also engage in mate-guarding behaviour (Owen-Smith, 1971; Hutchins & Kreger, 2006). Thus typically, management strategies identify male rhinoceros exhibiting dominance and territorial traits (such as dung scraping, spray urination and mate guarding, described in Owen-Smith, 1971) as the

successful breeder and will translocate those individuals at a time when their tenure would mean the possibility of breeding with their own offspring. Until the end of 2009, OGR's management of their white rhinoceros population comprised of the sale of the founder bulls, the introduction of new adult males to the population as well as the sale of sub-adult males born on the reserve.

The first sale of one of the founder bulls occurred in 2002. Up until then a total of fourteen offspring had been fathered by the two founder bulls (Bob and Derek). Based on behavioural observations and home range data, management at that point decided that Bob was the dominant territorial breeding bull, and sold him to minimise potential risk of inbreeding occurring within the population. The sale of the second founder bull (Derek) occurred in 2009. According to management records, Derek began to exhibit territorial behaviour after the sale of the other founder male (Bob) and indeed moved into and occupied Bob's vacated territory. Thus in theory he should have been able to successfully breed from that time on. In addition, two new adult males were introduced in 2002. A total of 27 offspring were fathered in the period of 2003 to 2009. Male offspring born on the reserve were sold periodically as they approached adulthood (normally at almost 5 years of age). These males are sold to generate income and also to prevent them breeding with their kin on the reserve once they reach maturity. This also helps to minimise conflict between bulls, since injuries due to translocation related conflict are a major cause of rhinoceros deaths in meta-populations, in particular for sub-adult males (Owen-Smith, 1988; Berger, 1994; Rachlow et al. 1998; Pitlagano, 2007)

The results of this study show that although, according to OGR records, the bull Bob clearly exhibited dominant territorial behaviours and occupied a defined territory, he was not the most successful breeding bull. Indeed, the bull Derek who did not appear to occupy or defend a territory and was not seen to display territorial or dominant behaviour was the more successful breeder of the two (fathering 76% of the offspring sired during their joint tenure time and for which we had samples). Thus if the aim had been to avoid potential inbreeding within the population, this may not have been attained by the selection of Bob as the male to remove in order to achieve this. Therefore, the assumption that more successful breeders are dominant territorial bulls may not be correct, since in this study the more successful breeding male was non-territorial. In addition, when two new adult males were introduced to the population they were successfully breeding before they had established territories of their own or exhibited dominant behaviour. Thus it does not appear that in order for male white

rhinoceros to breed successfully, that they need to either be dominant or defend their own territories.

Derek continued to breed successfully during the latter part of his tenure, while he was also exhibiting dominant territorial behaviour. The introduced bulls, Tony and Jeff, also bred successfully (although to a lesser degree) both before they had territories and after they had established themselves on the reserve. A hypothesis for Bob's less successful reproduction success was the consideration of the high costs involved in defending a territory (Owen-Smith, 1977; Rachlow et al. 1998) but this does not appear to have affected the success rates with the other males. Tony has been more successful at breeding than Jeff at this stage (4 calves / 2 calves), and with Derek removed from the population in 2009 the only remaining mate options for the cows are Tony and Jeff.

Findings such as these have considerable impact for conservation managers of other small populations and for the future selection of males for translocation as breeding animals. This area of research certainly warrants further investigation. In particular it will be of significant interest to obtain detailed information of bull home ranges/territories and dominance behaviour so that the relationship between these and successful breeding can be evaluated further. The factors determining male rhinoceros reproductive success need to be determined across several populations in order to determine which factors have significance and if there are any that can be monitored. Identify what the determinants there are (if any) for male reproductive success.

Mate selection

In 2006 there appeared to be evidence for mate selection which resulted in effective inbreeding avoidance; eight calves were born in 2006, of which three were to Derek and founder cows, whereas the remaining five were born to F_1 cows with either Tony (4) or Jeff (1) as the fathers. This suggests that if there are different bulls available, active mate selection may occur. In addition, the remaining founder bull which was sold in 2009 had the opportunity to breed with his offspring (by that stage all the F_1 cows were of breeding age) for a period of four years. However, my data shows that only one inbred calf was produced in this time despite the potential for several more to have occurred. This suggests that a form of natural inbreeding avoidance may occur within the population. Rachlow et al (1998) suggested that while mechanisms for female mate choice among white rhinoceros were not clear and may be influenced by choice for high-quality resources within territories (it

appeared that territorial males spent more time with females with high reproductive potential). This suggests that relative mating success is higher for males who adopt a territorial strategy as opposed to younger non-territorial males. There are relatively few behavioural inbreeding avoidance studies for long-lived mammals (Keane et al. 1996) but Archie et al (2007) demonstrated that such behaviour exists for elephants. The authors were able to determine that male elephants avoided engaging in sexual behaviour with their close genetic relatives even at the cost of missed mating opportunities. The mechanisms for any mate selection occurrence were not clear in this study but the sample was small and as future/next generations are born this will be worth further investigation. The seven calves born in 2008 and the four born in 2009 (See Figure 3.5 in Chapter 3) will have their genotypes determined thus allowing for a larger sample size and further data.

Genealogical traits

The results of the parentage analysis allowed me to investigate if certain traits in the reproductive parameters observed for this population could be interpreted in light of genetic information. Although not significant, certain matrilines exhibited different reproductive potential. The matriline founded by the female One Horn in particular displayed longer inter-calving intervals and a higher ratio of male calves born than the other three matrilines. The potential for certain family lines to have alternative reproductive potential has important implications for managers. In particular, sex ratios can have a considerable effect on growth rates for rhinoceros populations (Owen-Smith, 1988; Berkely, 2011; Weladji & Laflamme-Mayer, 2011) and as such, if certain matrilines appear to have the potential for skewed calf birth sex ratios this needs to be considered by managers. Accurate data with regards to the fecundity of females in the population would allow managers to consider sales of females. I would again recommend further research into this area in order to investigate if any factors exist that can be determined to influence the reproductive potential for certain matrilines. It is generally assumed that females in most mammals determine the sex of their offspring, but it has been shown that male pygmy hippopotamus - *Choeropsis liberiensis*, have the ability to influence offspring sex ratio by biasing the ratio of X and Y chromosome-bearing spermatozoa in their ejaculates (Saragusty et al. 2012). It would be worth investigating if such mechanisms exist or can be determined for male rhinoceros, in particular for males that appear to sire sex biased calves. If this were the case this too would have significant implications for translocations of bulls between populations. Kretzschmar et al (2004) demonstrated that male white rhinoceros showed elevated androgen metabolite concentrations (reflecting testicular activity) during months with high rainfall compared to

months of little or no rainfall. This suggested a seasonal trend in reproduction (confirmed by a higher frequency of conceptions within the first 4 months of increased androgen metabolite concentrations). It was also apparent that males accompanying a receptive female had higher faecal androgen metabolite concentrations (compared to lone males) and that the elevated levels were thus likely to be induced by female presence. This trend of strongly seasonal conception was also demonstrated by Berkely and Linklater (2010) who found that for a population of black rhinoceros most (73.6%) conceptions occurred during the rainy season. The results from this study also suggested a seasonal trend in conception, with higher rates of conception occurring during the rainy season. Taking this into consideration, a possible feature to investigate would be the effect climate change may have on conception rates and the possibility that such consequences may result in births at non-optimal times for calf rearing.

Population status

OGR's southern white rhinoceros population is expanding at almost 14% per annum, well over the expected maximum growth rate for rhinoceros meta-populations (9%) (Emslie et al. 2009). The other population performance indicators evaluated also indicated a healthy population exhibiting signs of good growth and fecundity. At this stage the population does not seem to be exhibiting any signs of population density dependence effects; increased inter-calving intervals, increased age at first parturition (Rachlow & Berger, 1998; Hrabar & du Toit, 2005). However at the current rate of population increase, the effective density will approach the higher end relative to other comparable populations (as reviewed in Chapter 4) within the next four years. Carrying capacity will then become a consideration for the management of this population if it does begin to exhibit signs of density dependence.

Furthermore it appears that the current F_2 generation in this population has lower heterozygosity in the observed markers than the F_1 generation but further data is needed in order to establish whether this is significant and whether this trend continues or develops in the future.

Management Conclusions

In order to optimally manage a small, free-ranging enclosed population of southern white rhinoceros it is essential to have the following data; reliable and accurate pedigree data (this includes a methodology for identifying individual rhino) and genetic data for as many animals as possible (preferably all potentially breeding animals). The availability of accurate

data with regards to the existing reproductive strategies occurring in the population is essential.

In particular, this kind of detailed information allows management to avoid the possible consequences of inbred populations and supports metapopulation management. In theory any rhinoceros population could be managed without detailed data by simply removing and replacing all adult bulls every five to six years. This should effectively prevent the possibility of inbreeding but it may not allow for maximum breeding success rates as the population will need time to settle after such disruptions. Furthermore the high costs and logistics involved for such a strategy are prohibitive. Information such as this will also reduce conflict in the population by managing translocations (Rachlow et al. 1998; Pitlagano, 2007) as the most frequent cause of death in a population of translocated white rhinoceros is due to injuries sustained during fighting.

This information can also be used to optimize genetic diversity and population health; this includes the selling matched pairs of unrelated males and females to provide founder animals for other populations. Metapopulation management (as recommended for rhinoceros, Emslie, 1999; du Toit, 2006; Emslie et al. 2009) can only be enhanced by the establishment of new subpopulations of rhinoceros through non-related founders. The organisation of conservation programmes has to be directed towards the mating system exhibited by the species and to the correct selection and identification breeding individuals (Caballero & Toro, 2002).

The data provided by the estimated shared allele distances (as determined in Chapter 3) for individuals may also provide another useful tool when selecting animals to establish new populations. In theory animals could be selected based on maximising their shared allele distance when establishing new populations. Or such data could be used when selecting which animals to remove from a population (i.e. one with similar allele distances could preferably be translocated). Management approaches such as this are common practice in *ex situ* facilities such as zoos (Leus et al. 2011).

In addition, information of this kind will enable the selling individuals that are not peak breeding animals (such as bulls not attaining reproductive success, or females that do not produce calves at regular intervals) in order to maintain optimum growth rates. Also it allows managers to monitor the diversity of the offspring by generation with a view to minimizing genetic drift and subsequent problems associated with small populations.

Furthermore, it appears that some form of mate selection does occur in white rhinoceros populations thus it is important to ensure that suitable alternative male candidates are available for this to occur naturally in the population.

Considering the array of genetic, demographic and increasing anthropogenic threats that face white rhinoceros, data that contributes to the establishment of robust populations are essential to the continuation of this species.

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